HEALTH EFFECTS OF SEASONAL CONSUMPTION OF LOCAL PHENOLIC-RICH FRUITS

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Doctoral Thesis

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FEM CONSTAR que aquest treball titulat "Health effects of seasonal consumption of local phenolic-rich fruits" que presenta Álvaro Javier Cruz Carrión per a l'obtenció del títol de doctorat ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira I Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HACEMOS CONSTAR que este trabajo titulado "Health effects of seasonal consumption of local phenolic-rich fruits" que presenta Álvaro Javier Cruz Carrión per a la obtención del título de doctorado ha estado realizado bajo unestra dirección en el Departament de Bioquímica y Biotecnologia de la Universitat Rovira i Virgili y que cumple con los requisitos para la obtención de la Mención Internacional de Doctorado.

WE STATE that the present study entitled "Health effects of seasonal consumption of local phenolic-rich fruits" presented by Álvaro Javier Cruz Carrión for the award of the degree of Doctor has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

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NUTRISALT

PER A UNA ALIMENTACIÓ INNOVADORA, SALUDABLE, SEGURA I SOSTENIBLE

Erasmus+

Sociedad Española de Genética

A Dios por darme la vida,

A la vida por privilegiarme con mis padres,

A mis padres por regalarme una familia,

A mi familia y amigos por brindarme su amor y apoyo,

A su amor y apoyo por permitirme desarrollar inteligencia,

A la inteligencia por dotarme de conocimiento y sabiduría,

Al conocimiento y la sabiduría por permitirme plasmarlo en esta tesis.

DESDE MI CORAZÓN

racias a la vida que me ha dado tanto…

Familia, amigos y compañeros un "*Dios le pague*" por formar parte de esta aventura llamada tesis y hacer de este camino una experiencia inolvidable. A todos y a cada uno de ustedes, mi gratitud y estima.

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"La raíz de todo bien reposa en la tierra de la gratitud"

Dalai Lama

"One child, one teacher, one book, one pen can change the world"

Malala Yousafzai
Peace Prize laureate 2014

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SUMMARY

Nutrition-related factors are the main determinants of noncommunicable diseases, which are currently the leading cause of mortality and morbidity in the world. As part of a healthy dietary pattern, World Health Organization recommends a minimum of 400 g of fruits and vegetables per day for the prevention of chronic diseases. Fruits and vegetables are known to be rich in phytochemicals, such as phenolic compounds, which promotes health effects. In this sense, xenohormesis theory postulates that when heterotrophs consume the phytochemicals synthesized by stressed plants, these compounds act as signals of the external conditions in which the plants were grown and allow them to adapt to changes in the environment. Therefore, each plant contains a characteristic phenolic composition providing information about environmental conditions.

In this respect, the objective of this thesis was to evaluate whether geographical origin of cultivation, farming systems and seasonal consumption can condition the bioactivity and bioavailability of phenolic compounds from plant-based foods. To meet this objective, it was determined whether the cultivation system conditions the concentration of phenolic compounds and the antioxidant capacity of local plant-based food grown in the Camp de Tarragona, Spain. Subsequently, we evaluated whether the geographical origin of tomatoes and sweet cherries cultivation and their seasonal consumption influences their bioactivity and phenolic bioavailability. Firstly, it was shown that the cultivation system affects the phenolic composition and antioxidant capacity according to the species and cultivar studied, with no prevailing farming system. Secondly, it was found that tomatoes and sweet cherries show different antioxidant responses depending on where they were grown, *i.e.*, local, or non-local, and when they were consumed, *i.e.*, in-season or out-of-season; the responses that could be associated with their phenolic composition. Finally, it was revealed that the bioavailability of tomato-derived phenolic compounds is affected by the above-mentioned fruit-related factors. These results show that in-season consumption of local fruits contributes to achieving optimal health status.

RESUMEN

Los factores relacionados con la nutrición son los principales determinantes de las enfermedades no transmisibles, que actualmente son la principal causa de mortalidad y morbilidad en el mundo. Como parte de un patrón dietético saludable, la Organización Mundial de la Salud recomienda un mínimo de 400 g de frutas y verduras al día para la prevención de enfermedades crónicas. Se sabe que las frutas y las verduras son ricas en fitoquímicos, como compuestos fenólicos, que promueven efectos saludables. En este sentido, la teoría de la xenohormesis postula que cuando los heterótrofos consumen los fitoquímicos sintetizados por las plantas estresadas, estos compuestos actúan como señales de las condiciones externas en las que se cultivaron las plantas y les permiten adaptarse a los cambios del entorno. Por lo tanto, cada planta contiene una composición fenólica característica que proporciona información sobre las condiciones ambientales.

En este sentido, el objetivo de esta tesis fue evaluar si el origen geográfico del cultivo, los sistemas de cultivo y el consumo estacional pueden condicionar la bioactividad y biodisponibilidad de los compuestos fenólicos de los alimentos de origen vegetal. Para cumplir este objetivo, se determinó si el sistema de cultivo condiciona la concentración de compuestos fenólicos y la capacidad antioxidante de los alimentos cultivados en el Camp de Tarragona, España. Posteriormente, se evaluó si el origen geográfico del cultivo del tomate y la cereza dulce y su consumo estacional influyen en su bioactividad y biodisponibilidad fenólica. Se demostró que el sistema de cultivo afecta a la composición fenólica y a la capacidad antioxidante según la especie y el cultivar estudiado, sin que prevalezca ningún sistema de cultivo. Además, se comprobó que los tomates y las cerezas dulces muestran diferentes respuestas antioxidantes en función del lugar de cultivo, es decir, local o no local, y del momento de consumo, es decir, en temporada o fuera de temporada; respuestas que podrían estar asociadas a su composición fenólica. Por último, se reveló que la biodisponibilidad fenólica del tomate se ve afectada por los factores antes mencionados. Estos resultados

muestran que el consumo de frutas locales en temporada puede contribuir a alcanzar un estado de salud óptimo.

RESUM

Els factors relacionats amb la nutrició són els principals factors determinants de les malalties no transmissibles, que actualment són la principal causa de mortalitat i morbiditat en el món. Com a part d'un patró dietètic, l'Organització Mundial de la Salut recomana un mínim de 400 g de fruites i verdures per dia per a la prevenció de malalties cròniques. Se sap que les fruites i les verdures són riques en fitoquímics, com els compostos fenòlics, que promouen els efectes de la salut. En aquest sentit, la teoria de la xenohormesis postula que quan els heterotrofs consumeixen els fitoquímics sintetitzats per les plantes estressades, aquests compostos actuen com a senyals de les condicions externes en les quals les plantes eren cultivades i els permeten adaptar-se als canvis en l'ambient. Per tant, cada planta conté una composició fenòlica característica que proporciona informació sobre les condicions ambientals.

En aquest sentit, l'objectiu d'aquesta tesi era avaluar si l'origen geogràfic del cultiu, els sistemes agrícoles i el consum estacional poden condicionar la bioactivitat i biodisponibilitat dels compostos fenòlics procedents d'aliments basats en plantes. Per tal d'aconseguir aquest objectiu, es va determinar si el sistema de cultiu condiciona la concentració de compostos fenòlics i la capacitat antioxidant dels aliments de planta local conreats al Camp de Tarragona. Posteriorment, vam avaluar si l'origen geogràfic del cultiu de tomàquet i cirera dolça i el seu consum estacional influeix en la seva bioactivitat i biodisponibilitat fenòlica. En primer lloc, es va demostrar que el sistema de cultiu afecta la composició fenòlica i la capacitat antioxidant segons l'espècie i el cultivar estudiat, sense sistema agrícola predominant. En segon lloc, es va descobrir que els tomàquets i les cireres dolces mostren diferents respostes antioxidants depenent d'on fossin conreades, és a dir, locals o no, i quan eren consumits, és a dir, en temporada o fora de temporada; les respostes que podrien estar associades amb la seva composició fenòlica. Finalment, es va revelar que la biodisponibilitat dels compostos fenòlics derivats de tomàquets es veu afectada pels factors relacionats amb la fruita abans esmentats. Aquests resultats mostren que el consum de fruita local durant la temporada contribueix a aconseguir un estat de salut òptim.

LIST OF ABBREVATIONS USED

ADME	Absorption, distribution, metabolism, and excretion
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area-under-the-curve
BAT	Brown adipose tissue
Bw	Body weight
CAD	Cinnamic acid derivatives
C_{max}	Maximum serum concentration
COMTs	Catechol-*O*-methyltransferases
CVD	Cardiovascular diseases
Cy3R	Cyanidin-3-*O*-rutinoside
DH	Daylight hours
DIO2	Iodothyronine deiodinase type II
EWAT	Epididymal white adipose tissue
EYA3	Eyes absent 3
F344	Fischer 344 rats
FRAP	Ferric reducing antioxidant power
Fw	Fresh weight
GA	Gallic acid
GSH	Reduced glutathione
IWAT	Inguinal white adipose tissue
L12	12 h light/day, standard photoperiod, which simulated the spring/autumn daylight hours
L18	18 h light/day, long photoperiod, which simulated the summer daylight hours
L6	6 h light/day, short photoperiod, which simulated the winter daylight hours
LC	Local sweet cherries
LD	Long-day
LPH	Lactase phlorizin hydrolase
LT	Local tomatoes
MDA	Malondialdehyde

MRT	Mean residence time
MWAT	Mesenteric white adipose tissue
NLC	Non-local sweet cherries
NLT	Non-local tomatoes
NORG	Non-organic farming system
ORAC	Oxygen radical absorbance capacity
ORG	Organic farming system
P	Photoperiod effect
P×T	Photoperiod×treatment interaction effect
PAD	Phenylpropanoic acid derivatives
PCA	Principal component analysis
PCC	Phenolic compounds content
PT	Pars tuberalis
ROS	Reactive oxygen species
SCN	Suprachiasmatic nucleus
SD	Short-day
SULTs	Sulfotransferases
T	Treatment effect
T3	Triiodothyronine
T4	Thyroxine
TAC	Total anthocyanins content
TBARS	Thiobarbituric acid-reactive substances
TDF	Total dietary fiber
TFaC	Total flavan-3-ols content
TFoC	Total flavonols content
TH	Thyroid hormones
T_{max}	Time of peak serum concentration
TPC	Total phenolic content
TSHβ	Thyroid-stimulating hormone beta
UGTs	Uridine-5′-diphosphate glucuronosyltransferases
uHPLC-MS^n	Ultra-high performance liquid chromatography coupled to multistage mass spectrometry
VH	Vehicle

PREFACE

The food system is constantly evolving as consumer tastes, practices and preferences change over time. In parallel, health challenges are shifting, and non-communicable diseases dominate the future of health in the rapidly changing society of the European region. Indeed, two of the main threats to global health are climate change and non-communicable diseases, both inextricably associated with diet. In this context, non-communicable diseases are currently the leading cause of mortality and morbidity in both developed and developing countries, with nutrition-related factors being one of the main determinants. Dietary patterns directly influence health outcomes through the relationship between nutrition and chronic disease, and indirectly influence health through the social, economic, and environmental consequences of food production systems. In this line, the environmental impacts of current food production and consumption patterns are substantial and threaten the future availability of natural resources such as land, healthy soil, and clean water. In total, the agricultural sector is estimated to have accounted for 11% of global greenhouse gas emissions over the past decade, while the broader food system, which includes agricultural input manufacturing, food processing and transportation, has been responsible for up to 37% of global emissions. Therefore, the World Health Organization suggests that to face the future, changes in food systems will not only have to address the increase in obesity and other diet-related health problems but will also have to promote a shift towards a sustainable diet in the quest for a healthy planet. In fact, changing to more sustainable food patterns is a key strategy to satisfy present and future food needs. As defined by the Food and Agriculture Organization, sustainable diets are those "having low environmental impact and contributing to food and nutrition security and healthy life for present and future generations. Sustainable diets are protective and respectful of biodiversity and ecosystems; culturally acceptable; accessible; economically fair and affordable; nutritionally adequate, safe, and healthy; and optimize natural and human resources". The sustainability of diets is influenced by both the foods that make up the diet and the way in which food is produced. In this context, emphasis has

been placed on the need for sustainable diets, with local and seasonal low-input agroecological food production, as well as short-distance production-consumption networks for fair trade.

In industrialized countries, agroecological food production systems, generally called "organic" and supported in Europe by the Commission, already represent 10% or even more of the agricultural sector and are proving to be effective in providing quality food with reasonable yields while respecting the environment. According to the Council of the European Union, organic production is a global system of agricultural management and food production that combines the best environmental practices, a high level of biodiversity, the preservation of natural resources and a production method in line with the preference of certain consumers for products made with natural substances and processes. In this sense, more than half of the organic area in the European Union is concentrated in four countries: Spain, Italy, France, and Germany. In fact, Spain has the largest area devoted to organic fruit (39% of the organic fruit area). In this regard, it is well known that, among other factors, farming management systems influence the biosynthesis and accumulation of phenolic compounds in crop plants.

In this connection, "locally" is synonymous with "seasonally" harvested food. But the interpretation of seasonal foods can differ depending on who is using it and the context in which it is used. In this framework, the Department for Environment, Food and Rural Affairs (DEFRA) proposed two definitions of seasonal foods, the first based on where the food is produced, and the second based on where it is produced and consumed: 1) Produced in-season (global seasonality): Food that is outdoor grown or produced during the natural growing/production period for the country or region where it is produced, although it is not necessary to consume them where they were grown. For example, apples grown and harvested during the growing season in New Zealand but eaten in Europe during the spring and summer seasons. 2) Produced and consumed in-season (local seasonality): Food that is produced and consumed in the same climatic zone without high-energy use for climate modification or storage. This refers to foods that are harvested and eaten locally

during the natural growing season. For instance, apples grown and harvested during autumn, and eaten in October, in Europe. In this sense, each type of fruit and vegetable has its own specific set of conditions for ideal growth. That is why each fruit and vegetable is grown and harvested in different locations and in different seasons. For instance, oranges are climate-sensitive plants and grow best in places with hot, dry summers, including Spain, Italy, and Greece.

In reference to the above, this thesis is part of the Projecte d'Especialització i Competitivitat Territorial (PECT) de Reus i el Camp de Tarragona per una alimentació innovadora, segura, saludable i sostenible per al benestar de la població i l'impuls del territori (NUTRISALT). PECT-NUTRISALT is an initiative promoted by the City Council of Reus, with the complicit of the rest of the local administrations of the Camp de Tarragona, with a decisive involvement of the main decisive involvement of the main R+D+I agents, including the Universitat Rovira i Virgili. PECT-NUTRISALT project aims to ensure the welfare of the citizens of Reus and Camp de Tarragona through the stimulation and strengthening of one of its main economic engines: the food sector, from plant and animal production to the food industry and associated services. To meet this objective, the Universitat Rovira i Virgili, through the Nutrigenomic Research Group, has conducted an in-depth research to generate scientific evidence on the consumption of local products. Thus, the results obtained in this thesis contribute to and strengthen this initiative.

I.

INTRODUCTION

1. Phenolic compounds and health effects

Phenolic compounds are secondary metabolites produced by plants, commonly implicated in the defense against stress factors [1]. Their structure is very varied, and more than 8000 structures have been described [2], and many of them are only present in a limited number of species. They are characterized by one or more hydroxyl groups on least one aromatic ring.

1.1. Classification

According to their structure, phenolic compounds can be classified in two major families: flavonoids and non-flavonoids [3].

1.1.1. Flavonoids

Flavonoids are 15-carbon phenolic compounds with two aromatic rings joined by a three-carbon bridge (C6-C3-C6) and may themselves be divided into 6 subclasses, based on the connection position of the B and C rings as well as the degree of saturation, oxidation, and hydroxylation of the C ring, as anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols and isoflavones (Figure 1) [3,4]. There are also other subclasses of flavonoids that are more minor dietary components, including aurones, chalcones, coumarins, dihydrochalcones, dihydroflavonols, and flavan-3,4-diols. The basic structure of flavonoids can have several substituents. Most flavonoids occur naturally as glycosides and not as aglycones [1,3,4].

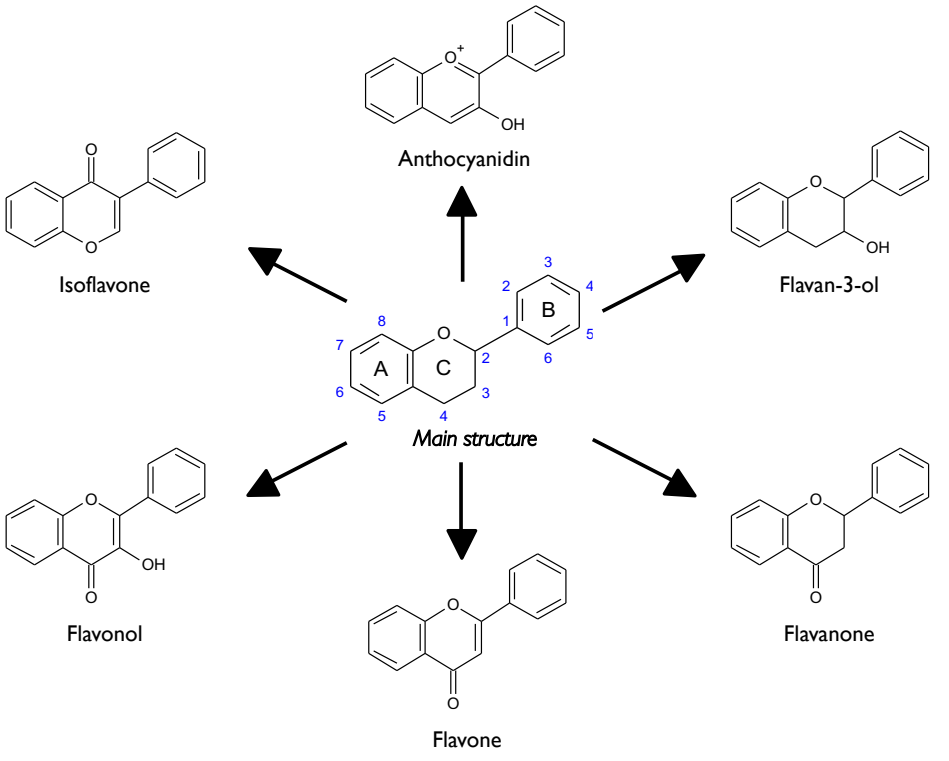


Figure 1. Flavonoid families and their basic chemical structures.

- Anthocyanidins

Anthocyanidins are water-soluble pigments that give red, blue and purple colors to plant tissues [5]. The most widespread anthocyanidin aglycones are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin, which are conjugated with sugars and organic acids to generate a multitude of anthocyanins which are pigments dissolved in the vacuole sap of the epidermal tissues of the flowers and fruits, giving them a pink, red, blue or purple color [1,3]. They exist in different chemical forms and, depending on the pH, they can be colored or uncolored. In addition, these compounds are stabilized by forming complexes with other flavonoids (co-pigmentation) [1]. In the human diet, anthocyanins are found abundantly in red grapes, red wine, cherries, berries and certain leafy and root vegetables [1,6].

- Flavan-3-ols

Flavan-3-ols are the most complex subclass of flavonoids, in fact, they exist both in monomeric form (catechins) and in oligomeric and polymeric form (proanthocyanidins), the latter are also known as condensed tannins and can occur as polymers of up to 50 units. Proanthocyanidins consisting exclusively of (epi)catechin units are called procyanidins, and are the most abundant type of proanthocyanidins in plants [1,3]. Among the different flavan-3-ol representatives, (+)-catechin and (-)-epicatechin are the most widespread in nature [3]. Unlike other classes of flavonoids, flavan-3-ols are not glycosylated in foods [1]. However, conjugation with gallic acid is much more common. These forms are called gallate flavan-3-ols, and include epicatechin gallate, catechin gallate, epigallocatechin gallate and gallocatechin gallate [3]. Flavan-3-ols are found in many types of fruit, such as apricots, apples and grapes; they are also present in red wine, but green tea and chocolate are by far the richest sources [1].

- Flavanones

Flavanones encompasses a wide range of compounds with O and/or C substitutions on the A or B ring (**Figure 1**) [7]. These compounds have a saturated C-ring and are generally glycosylated by a disaccharide at position 7: either a neohesperidose, which imparts a bitter taste (such as to naringin in grapefruit), or a rutinose, which is flavorless [1,3]. Flavanones are widely distributed in about 42 higher plant families [7]. Among flavanones, the naringenin and hesperidin aglycones and their glycosides are highly prevalent in foods [7]. Indeed, in human food, flavanones are found in tomatoes and certain aromatic plants such as mint, and in citrus fruits they can be found in high concentrations [1].

- Flavones

Flavones, including apigenin, baicalein, luteolin and wogonin are structurally akin to flavonols, except that they are not oxygenated at C-3 [3]. Many flavones occur as 7-*O*-glycosides. Moreover, flavones are far less prevalent than flavonols in fruits and vegetables [1]. In most of the plant kingdom, flavones occur is small amounts compared with other flavonoids, although substantial amounts have been

detected in celery, parsley, and some herbs. In addition, polymethoxylated flavones, such as nobiletin and tangerine occur in citrus species [1,3].

- Flavonols

Flavonols are the most widespread of the flavonoids, as they are present throughout the plant kingdom, with the exception of fungi and algae [3,8]. The main dietary flavonols, kaempferol, quercetin, isorhamnetin and myricetin are most commonly found as *O*-glycosides with conjugation occurring at the 3-position of the C-ring but substitutions can also occur at the 5-, 7-, 4′-, 3′- and 5′-carbons [3,8]. Though the aglycones are limited in number, there are more than 200 kaempferol sugar conjugates [8,9]. The richest sources are onions, broccoli, curly kale, leeks, tomatoes, apricots, blueberries, and cherries. Red wine and tea also contain notable amounts of these compounds [1,3,8].

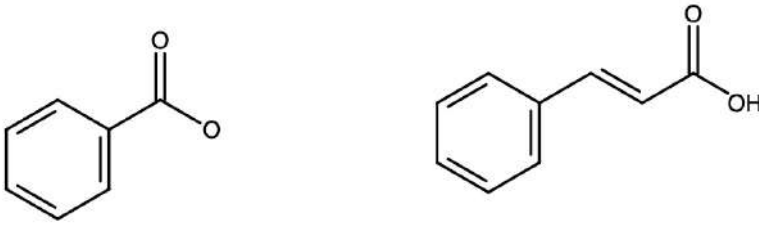
- Isoflavones

Isoflavones are biologically active compounds with estrogenic properties and are often classified as phytoestrogen [3,10]. Isoflavones have the B-ring attached at C-3 rather than at the C-2 position [3,8]. They have hydroxyl groups at the 7′ and 4′ positions in a configuration analogous to that of the hydroxyl groups of the estradiol molecule [1]. The main representative components are genistein, daidzein, biochanin A, and glycitein [10]. They are found almost exclusively in leguminous plants with substantial quantities of daidzein and genistein occurring in soybean [3,8]. Other sources such as apple, apricot, black currant, cherry, cabbage, sweet potato, plum, date, onion, wheat and pineapple have been reported [10].

1.1.2. Non-flavonoids

The main non-flavonoids of dietary significance are the C6–C1 phenolic acids [3,10] and are characterized by a carboxyl group linked to benzene ring [10]. Dietary sources of phenolic acids include fruits such as apricots, cherries and peaches, as well as other vegetal sources such as eggplant, cereals and spinaches [1,11]. Two classes of phenolic acids can be distinguished: benzoic acid derivatives and cinnamic acid derivatives (**Figure 2**) [1]. The hydroxybenzoic acids are constituents of complex structures like hydrolysable tannins [1] and their main representatives are gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids [11]. Indeed,

gallic acid is the commonest phenolic acid, and occurs widely as complex sugar esters in gallotannins [3]. Moreover, the hydroxybenzoic acid concentration of edible plants is usually very low, except for some red fruits, black radish and onions where they are especially abundant [1]. On the other hand, the C6–C3 hydroxycinnamates occur mainly as conjugates and collectively are referred to as chlorogenic acids [3]. The hydroxycinnamic acids are more widespread than hydroxybenzoic acids and mainly include *p*-coumaric, caffeic, ferulic and synaptic acids, with caffeic acid dominating [1,8]. Furthermore, the hydroxycinnamic acids are abundant in fruits such as apples, apricots, blueberries, cherries, kiwis, plums, as well as coffee [1].



Benzoic acid Cinnamic acid

Figure 2. Two main classes of phenolic acids and their basic chemical structures.

Lignans, stilbenes, tannins and xanthones also belong to non-flavonoids phenolic compounds [10]. They are compounds with at least two aromatic rings, whereas only tannins have more aromatic rings [10]. In detail, lignans are vascular plant secondary metabolites, with widespread occurrence in the plant kingdom [10]. In fact, lignans can be found in oilseeds, whole-grain cereals, legumes, various vegetables, and fruits as well as beverages, such as coffee, tea, and wine. The most important dietary lignans are lariciresinol, matairesinol, medioresinol, pinoresinol, secoisolariciresinol and syringaresinol [10]. Moreover, stilbenes are phytoalexins synthesized by plants in reaction to disease, injury, and stress, and have a C6-C2-C6 structure [3]. The main stilbene is resveratrol, which occurs in the form of cis- and trans-isomers as well as conjugated derivatives [3]. Significant sources of stilbenes include grapes, almond, bean, blueberries, bilberries, peanuts, grapevine, cranberries, mulberries, plum, and wine [10]. Furthermore, tannins

(commonly referred to as tannic acid) are water-soluble phenolic compounds. Tannins are reported to be constituent of legumes such as beans, fruits particularly berries and nuts [10]. And finally, xanthones are very stable molecules; they comprise a family of *O*-heterocycle symmetrical compounds with a dibenzo-γ-pyrone scaffold and are known as xanthone, xanthene-9-one, or dibenzo-γ-pyrone [12]. The principal natural sources of xanthones are mangosteen and mango fruits [13].

1.2. Factors affecting phenolic compounds composition

Phenolic compounds are naturally synthesized both during the regular plant growth and development. Certainly, phenolic compounds synthesis and accumulation are generally stimulated in response to biotic and abiotic stresses [1]. In fact, exposure to environmental stress can lead to the stimulation of a secondary metabolism, which results in an increased synthesis of phenolic compounds, notably by modulating the phenylalanine ammonia-lyase (PAL) activity [14]. In this sense, it has been shown that PAL is stimulated differently depending on the plant species, due to genetic changes or even the particular function that phenolic compounds perform in the metabolism and physiology of plants [15]. In addition, some authors have also suggested that phenolic compounds content in plants depends on different intrinsic and extrinsic factors such as plant genetics and cultivar, soil composition and growing conditions, maturity state and postharvest conditions, among others [14], and may be present at different levels in the same plant, thus, every plant food contains a unique combination of several hundreds of phenolic compounds [16].

Genetic factors of the cultivars are crucial. For example, Nonpareil, Carmel, Butte, Sonora, Fritz, Mission and Monterey almond cultivars were shown to have a differential impact on individual phenol synthesis [17]. In this line, the effect of cultivar on phenolic content of 23 randomly selected cultivars was evaluated, resulting in a significant effect of both factors on phenolic content of plum cultivars [18]. Similarly, differences among lettuce cultivars appeared to have a larger impact on phenolic profiles, and, genetic material generated distinctions in total phenolic content of white asparagus spears cultivars [19]. Likewise, this trend was

observed in three genotypes of murtilla (*Ugni molina*e Turcz), were a significant effect of genotype on phenolic compound content was observed [20].

Among environmental factors affecting phenolic composition include the following: temperature, salinity, water stress, drought, light intensity, soil nutrient content, geographical origin of cultivation, farming systems, growing period, preharvest and postharvest practices and other environmental conditions [18,21,22], these environmental stresses may trigger oxidative stress in plants, generating the formation of reactive oxygen species, leading to cellular damage, metabolic disorders, and senescence processes [21]. Indeed, in a cultivation experiment 18 different Amaranthus genotypes were cultivated in parallel in Argentina, Mexico, Spain and two different locations in the Czech Republic, resulting in the flavonoid rutin exhibiting large variations with varying environmental conditions, whereas the flavonoid, nicotiflorin, was less affected. In addition, the variations between location/environmental condition were primarily reflected by changes in the content of *p*-coumaric acid and protocatechuic acid [23].

1.2.1. Farming systems

As previously mentioned, the farming systems in which the plants are grown can modulate their phenolic composition. However, the effect of farming system (biodynamic, conventional, organic or sustainable) is not easily predictable, and the explanation for different behaviors is neither univocal nor understandable on the basis of a biochemical pathway [22]. In the comparison of farming systems, considerable emphasis should be placed on different yields and experimental growing conditions [22]. In fact, as mentioned above, environmental conditions affect the phenolic compounds composition and, therefore, the differences should not be attributed exclusively to the method of farming [22]. Table 1 lists studies of fruits and vegetables, in which it is compared their phenolic content under different farming systems. In this regard, Faller and Fialho [14] suggested that the type of farming system applied could have different effects over the type of phenolic compound synthesized according to the plant species analyzed.

Table 1. Fruits and vegetables, growing location and results in the determination of phenolic compounds.

Sample	Growing location	Results achieved	Reference
Apple	Different farms	No differences	24
Blueberry	Different farms in a surrounding area	Higher in organic than in conventional	25
Cherry	Different farms	Dependent on year and cultivars	26
Grapes	Neighboring parcels	Higher in conventional than in organic	27
Orange	Different farms	Higher in organic than in conventional	28
Orange	Different farms	Higher in conventional than in organic	29
Peach	Same orchard	Higher in organic than in conventional	30
Strawberry	Same farm	Higher in organic and sustainable than conventional production	31
Strawberry	Same pedoclimatic area	Higher in biodynamic than in conventional production	32
Yellow plums	Same farm	Higher in conventional than in organic production	33
Onion	Same farm	No differences between organic and conventional	34
Pepper	Different farms	No differences between organic, integrated, and soilless	35
Pepper	Near fields	Higher in organic than in conventional	36
Tomato	Fields under same conditions	Higher in organic than in conventional	37
Tomato	Same farm	Dependent on year and compound	38
Tomato	Next greenhouses	No differences between organic and conventional	39

Adapted from Heimler and colleagues [22].

Importantly, organic farming is a very strict cultivation system, supported by law. In this system, only natural kinds of fertilizers are allowed and natural plant protection methods [26]. As for agronomic practice of fertilization, a decrease in phenol content is associated to a higher nitrogen supply. Indeed, nitrogen supply can come from inorganic and/or organic soil fertilization, and depletion is theoretically predictable based on the biochemical pathway leading to phenolic compounds synthesis [22].

1.2.2. Geographical origin of cultivation

As noted above, environmental conditions which are intrinsically related to the geographical origin of cultivation such as temperature, wind, water erosion, salinity, water stress, drought, light intensity, or soil nutrient content, can strongly impact the biosynthesis of phenolic compounds. In fact, under conditions of environmental stress, the biosynthesis and release of phenolic compounds (stress molecules) is stimulated, as phenolic compounds are known to transmit resistance to abiotic stress conditions [40]. In this sense, a recent study reported consistent differences on the phenolic composition of tomatoes grown in two locations of Spain; here, higher concentrations of caffeic acid, ferulic acid, *p*-coumaric acid and total phenolic content were observed in tomatoes grown in Barbastro. In contrast, tomatoes grown in Montañana showed significantly higher concentrations of chlorogenic acid. Suggesting that tomatoes grown in open fields exhibit substantially higher concentrations some phenolic acids when grown in traditional areas [41]. These results are in agreement with the study carried out with eggplant fruits, where it was found that the environment exerts a key role in determining the phenolic composition of these vegetables, and that there can be large differences in composition between seasons, even with the same cultivation system (open field or greenhouse) [42]. Moreover, in a cultivation experiment 18 different Amaranthus genotypes were cultivated in parallel in Argentina, Mexico, Spain and two different locations in the Czech Republic, resulting in large variations of rutin content under different environmental conditions, whereas nicotiflorin was affected less. In fact, the variations between location/environmental condition were mainly defined by the variations in the content of *p*-coumaric acid and protocatechuic acid in the seed samples [23]. In

addition, a wide range of concentrations of total phenolics of sweet cherry that varies depending on the crop area was reported [43]. For instance, Usenik and colleagues [44] informed that content of total phenolics ranged from 44.3 (Lapins) to 87.9 mg/100 g (Ferprime) for cultivars grown in northern Europe. Nonetheless, other authors found that the concentration of total phenols varied between 84.96 (Napoleona Grappolo) to 192 mg/100 g (Sonata) for cultivars grown in a southern area of Europe [45]. This difference reflects the influence of agronomic and environmental conditions on concentrations of these bioactive compounds.

1.2.3. Postharvest practices

The food industry uses postharvest practices to extend the shelf life of fruits and vegetables and allow their commercialization. Among these practices outstand the following: transportation and storage conditions, postharvest irradiation with UV-B light, thermal and freezing processes, technological treatments, handling and packaging technologies [46]. In fact, phenolic content of fruits and vegetables, can be strongly affected by the postharvest practices to which fresh fruit and vegetables are submitted during their postharvest life until their consumption [47]. In this sense, different patterns of phenolic compound accumulation have been reported when fruits and vegetables are subjected to these postharvest practices [48]. In one such study by Castagna and colleagues [49], tomatoes were subjected to postharvest irradiation with UV-B light. The treatment was generally effective in increasing phenolic, flavonoid and flavonol concentration in both peel and flesh of tomato. In addition, with few exceptions, UV-B irradiation also induced a higher accumulation of individual flavonoids both in the peel and in the flesh of fruits independently from harvesting stage. Likewise, postharvest ripening of cherry tomatoes at increasing temperatures (4, 12 and 20 °C, for 1-3 weeks) led to a strong reduction in chalconnaringenin content [50]. In addition, it has been reported that storage of tomato products (pulp, puree, paste) at 30, 40 and 50 °C for 3 months resulted in a decrease in total phenolics at > 40 °C [51]. In another study about the influence of postharvest storage of oranges and mandarins on phenolic compounds, it was observed that postharvest storage of mandarin at 12 °C for 5 weeks resulted in a considerable rise in phenolic compounds, as well as an

enhancement of their bioaccessibility [52]. In another such study on evaluation of the effects of postharvest storage on the phenolic composition of sweet cherries, it was reported that phenolic acids decreased with storage at 1-2 °C and increased with storage at 15 ± 5 °C, while anthocyanins increased at both storage temperatures, moreover, flavonols and flavan-3-ols remained quite constant [53]. In this line, another research on the effect of postharvest storage temperature on sweet cherry phenols reported that the total anthocyanin concentration was significantly lower in fruits stored at 1 °C compared to the initial fruits at harvest, while no significant differences were detected in the concentration of total phenolics, these results suggest that cold storage of sweet cherries at low temperature slows down the accumulation of anthocyanins [54]. Conversely, there are several reports in which the anthocyanins of fruits and vegetables increased during cold storage [47,53]. Finally, a review that analyzed 325 publications on the effects of storage and postharvest processing on the phenolic acid and flavonoid content of foods concluded that the effect of food storage and processing on phenolic compounds content is negligible compared to the differences between different plant varieties. And that variety dependence must always be taken into account for all classes of compounds [55].

1.3. Bioactivity and health effects of phenolic compounds

Over the last years, phenol-rich foods and phenolic compounds have received great attention due to their potential beneficial effects toward human health [3]. In fact, human intervention trials have provided evidence for protective effects of various phenol-rich foods against overall and cardiovascular diseases (CVDs)-related mortality [56], certain cancers [57], anthropometric measures [58], neurogenerative disorders [59] and mood disorders [60]. Notably, several reviews summarized the available evidence concerning phenolic compounds and polyphenol-rich foods and their association with non-communicable diseases related to oxidative stress and inflammation, with special reference to CVD, diabetes, hypertension, obesity, certain cancers, and neurodegenerative diseases [1,3,10,61,62]. More recently research has been focused on the impact of phenolic compounds on healthy aging and/or age-related diseases [63]. The beneficial effects of such diets have often been attributed to the phenolic compounds they contain

[3]. Indeed, there have been numerous studies with animals and humans, indicating associations between the dietary intake of phenolic compounds and reductions in the risk of chronic diseases. For example, regarding coffee polyphenols and CVD risk factors, data from the "Health Survey of São Paulo (ISA-Capital)" showed that among 557 individuals, in São Paulo, Brazil, coffee intake of 1-3 cups/day and its polyphenols were associated with lower odds of elevated systolic blood pressure, diastolic blood pressure, and hyperhomocysteinemia [64]. In another study flavonoids were linked to ventilator function. Specifically, from a stratified random study, in which 55000 adults aged 15 to 75 answered a questionnaire on respiratory symptoms, it was concluded a positive association of total flavonoid intake and pro-anthocyanidins and ventilatory function, and a negative association with spirometric restriction in European adults [65].

Various molecular mechanisms have been suggested to explain the health effects of phenolic compounds, nevertheless, one of the most studied phenolic compounds bioactivities is their antioxidant activity [66]. In this respect, two main mechanisms have been suggested to explain the phenolic compounds bioactivity including basic biochemical mechanisms and epigenetic mechanisms [67]. In detail, the basic biochemical mechanisms are associated with inflammation and lipid and energy metabolism [68], focusing on the ability of phenolic compounds to bind strongly to proteins. In fact, this type of interaction can generate biological effects determined by the function of the proteins involved, including modification of enzymatic activities, binding of receptors and ligands, and binding of transcription factors to their specific sites on DNA [67]. For instance, it was demonstrated the capacity of proanthocyanidins to increase the expression and/or activity of several antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase [69]. On the other hand, the epigenetic mechanisms include histone modifications, DNA methylation and modulation of microRNAs [67]. Finally, phenolic compounds through both mechanisms can modulate enzymatic activities, cell signaling cascades and gene expression, resulting in modulation of cellular functionality [67].

On the other hand, currently, a growing body of research show phenolic compounds supplementation alter gut microbes in promoting human health by

stimulating the abundance of probiotic gut microbiota to benefit gut health [70,71]. Especially, phenol-supplementation boosted the abundance of health-promoting *Lactobacillus* and *Bifidobacterium* species, but inhibited the abundance of pathogenic *Clostridium* species in the intestinal microbiota of human subjects [71]. The modes of action could be through modulation of intestinal barrier function, innate and adaptive immune response, signaling pathways, as well as the ability to modify the composition of the intestinal microbiota [72]. Taken together, phenolic compounds intake modifies gut microbiota species, which contributes to human health benefits [71,72].

1.3.1. Phenolic compounds on oxidative stress

Oxidative stress means excessive production of reactive oxygen species (ROS) in cells and tissues and the antioxidant system may not be able to neutralize them [73]. Phenolic compounds are the main class of antioxidants capable of neutralizing oxidative stress [73]. Antioxidant capacity of phenolic compounds depends on the structure of their functional groups [74]. In fact, the number of hydroxyl groups strongly impacts various mechanisms of antioxidant activity, including radical scavenging and metal ion chelating capacity [74]. For example, flavonols such as quercetin that contain the 3-hydroxy group have demonstrated relatively greater antioxidant activity than those that do not in neutralizing free radicals [74]. The antioxidant mechanisms of the phenolic compounds include the suppression of ROS formation, either by inhibition of the enzymes involved in their formation, the scavenging of ROS, or the enhancement or protection of antioxidant defenses [75]. Phenolic compounds can downregulate the catalytic activity of enzymes implicated in the formation of ROS [76]. These compounds can protect against oxidative damage through several mechanisms [73,76]. In this sense, human intervention trials have provided evidence for antioxidant effects of various phenolic compounds to reduce the oxidative damages of lipids, proteins, enzymes, carbohydrates, and DNA in living cells and tissues [77]. In addition, many toxic products, such as malondialdehyde, 4-hydroxynonenal and several 2-alkalines are formed due to lipid peroxidation [78]. The formation of ROS has been informed to increase free metal ions through the reduction of hydrogen peroxidase with the formation of the highly reactive hydroxyl radical [79]. In this

regard, extensive studies have examined closely the molecular mechanisms underlying the protective effects of phenolic compounds, including their antioxidant capacity, which can be referred to the regulation of redox enzymes by reducing the production of ROS and modulating the phase II enzymes responsible for the cellular oxidative response [80–83], in other words, phenolic compounds have the ability to act as reaction chain breakers and scavengers of damaging free radicals and ROS, which are produced in excess under oxidative stress conditions [84]. The lower redox potentials of phenolic compounds are thermodynamically capable of reducing highly oxidizing free radicals due to their ability to chelate metal ions and free radicals [79].

Phenolic compounds are terminators of free radicals as well as chelators of metal ions that are capable to catalyze oxidative reactions [85]. In fact, phenolic compounds can cross-react in the plasma membrane with nonpolar compounds in the hydrophobic layer of the inner membrane; these changes in the membrane can alter the rate of oxidation of lipids or proteins [86]. Indeed, some flavonoids in the hydrophobic core of the membrane can impede oxidant access and preserve membrane structure and function [86]. Interaction of phenolic compounds with nitric oxide synthases activity may condition the production of nitric oxide [73]. Concerning the inhibition of the enzymes involved in oxidation, research has shown that phenolic compounds modify the activity of arachidonic acid metabolizing enzymes, including cyclooxygenase, lipoxygenase and nitric oxide synthases [87].

Talking of anti-oxidative stress activities, an integrated cellular redox enzyme system consisting of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and peroxiredoxins, is a cellular defense mechanism that maintains oxidative balance [88]. Humans have various mechanisms to counteract oxidative stress, either by producing antioxidants from endogenous antioxidant systems or externally through exogenous antioxidants [78].

Specifically, flavonoids can prevent injury caused by free radicals by scavenging of ROS, activation of antioxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases, and mitigation of oxidative stress caused by nitric oxide, increase in uric acid levels, and increase in antioxidant

properties of low-molecular antioxidants. Flavonoids also act as prooxidants and promoting the oxidation of other compounds [89]. In addition, resveratrol is able to stimulate endothelial production of nitric oxide, reduce oxidative stress, inhibit vascular inflammation and prevent platelet aggregation [77].

1.4. Bioavailability and metabolism of phenolic compounds

The most common phenolic compounds in the human diet are not necessarily the most active within the body, either because they have less intrinsic activity or because they are poorly absorbed in the intestine, are highly metabolized or are rapidly eliminated [1]. Several authors point out that the real bioactive molecules are dietary phenolics metabolites rather than the natural phenolic compounds of the plant-based foods [90–92]. So, as a first step towards elucidating the potential connections between phenolic compounds and health benefits, understanding their metabolism and bioavailability is key [4]. Actually, the bioavailability profile does not directly enhance with the intake of a high content of phenolic compounds [93]. The chemical structure of phenolic compounds determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma [94].

In general terms, bioavailability can be described as the portion of a nutrient or non-nutrient that is uptaken and metabolized for physiological functions and/or storage through regular pathways [95]. The concept of bioavailability therefore incorporates: availability for absorption or bioaccessibility, absorption, tissue distribution, and bioactivity [96]. In particular, the bioavailability of phenolic compounds involves the following digestive process: release of phenolic compounds from the food matrix, chemical modifications during gastric or intestinal digestion, cellular uptake of aglycones and some conjugated phenolics by enterocytes, microbiological fermentation of unabsorbed or re-excreted phenolic compounds through the bile or pancreas to produce additional metabolites, phase I/II enzymatic modifications that occur in the small intestine/colon after absorption, transport into the bloodstream and subsequent tissue redistribution, and excretion *via* the kidney or re-excretion in the intestine *via* bile and pancreatic juices [95]. The bioavailability of phenolic compounds varies among the different classes and ranks as follows: phenolic acids > isoflavones >

flavonols > catechins > flavanones, proanthocyanidins > anthocyanins, confirming data from previous pharmacokinetic studies [97].

1.4.1. Digestion, absorption, distribution, metabolism, and excretion of phenolic compounds

Extensive knowledge of the absorption, distribution, metabolism, and excretion (ADME) of phenolic compounds is thus essential if their health effects are to be understood [1,3]. The ADME of phenolic compounds in humans is schematically illustrated in **Figure 3**. Digestion is initiated in the oral cavity *via* metabolism reactions [95]. In the transformation of food, mechanical action, *i.e.*, mastication, plays an essential role in the transformation of food components, which releases the compounds [95]. After ingestion, polyphenolic glycosides can be modified in the oral cavity by the hydrolyzing activity of saliva, immediately as the come in contact with bacterial glycosidase enzymes, with amylase being the predominant enzyme [3,95]. The impact of enzymatic digestion on phenolic compounds release is supposed to be low due to the short interaction time [98]. Moreover, the reduction of food matrix particles that takes place in the oral cavity allows better enzymatic accessibility at other stages of digestion [95].

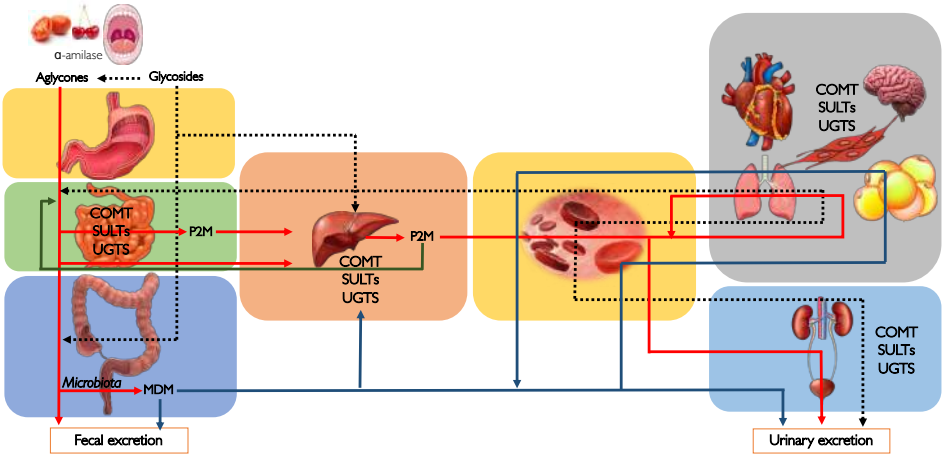


Figure 3. Overview of the digestion, absorption, distribution, metabolism, and excretion of phenolic compounds. Abbreviations: COMT, catechol-*O*-methyltransferases; MDM, microbial-derived metabolites; P2M, phase-II metabolites; SULTs, sulfotransferases; UGTs, uridine-5′-diphosphate glucuronosyltransferases. Adapted from Iglesias-Carres [99].

After acute ingestion, absorption of most components into the circulatory system occurs in the small intestine [3]. In fact, the intestine is the main site of absorption of phenolic compounds [4], although some phenolic families can be absorbed at the stomach level, including anthocyanins and phenolic acids such as caffeic acid [93]. However, the majority of phenolic compounds occur in food in the form of esters, glycosides or polymers that cannot be absorbed in their native form and must be hydrolyzed by intestinal enzymes or colonic microflora before they can be absorbed [1]. Within the gastrointestinal tract, their absorption is linked to the hydrolyzing activity of a cascade of enzymes [4]. In the small intestine the pH increases to 7, which enables the activation of pancreatic and biliary enzymes [95]. This pH increase is mainly important for anthocyanins, which change to pseudobasic chalcone structures [100]. Most phenolic compounds, apart from aglycones and some glucosides, need to be cleaved from their sugar moiety by brush border enzymes to be absorbed. Usually, phenolic compounds are only absorbed as glycosides when pharmacological doses are reached [95]. Nevertheless, some phenolic compounds can be absorbed in their native from, for instance, hesperidin [3]. In addition, anthocyanidins can also be absorbed *via* the stomach to reach the bloodstream [93]. On the other hand, the absorption of flavonoid glycosides is associated with cleavage and release of the aglycone as a consequence of the action of lactase phlorizin hydrolase (LPH) in the brush border of epithelial cells [3,4,8]. LPH displays a broad substrate specificity for flavonoids-*O*-β-D-glucosides, and the released aglycone can enter the epithelial cells by passive diffusion due to its increased lipophilicity and its proximity to the cellular membrane [101]. It is important to note that the absorption of phenolic compounds can be *via* facilitated transport, active transport or passive diffusion [95,101].

Once absorbed, before passage into the bloodstream, the phenolic compounds are recognized as xenobiotics and follow the common metabolic pathway of exogenous organic substances undergo phase II enzymatic metabolism [3,4,8]. The aglycones undergo some degree of phase II metabolism and subsequently are conjugated into their sulfated, glucuronidated, and/or methylated forms through the corresponding action of sulfotransferases (SULs), uridine-5′-diphosphate

glucuronosyltransferases (UGT) and catechol-*O*-methyltransferases (COMT) [3]. The small intestine is the first organ where phenolic compounds undergo these detoxifying reactions [4]. The principal phase-II reaction occurring in the small intestine is glucuronidation. In this regard, the small intestine has been identified as a primary source of glucuronidation for catechin and other flavonoids including kaempferol, quercetin, hesperitin and pelargonidin [1]. Nevertheless, the ability to metabolize phenolic compounds in the intestine is not unlimited, so high phenolic doses can lead to lack of glucuronidation, methylation and sulfation, this effect arises from phase-II enzymatic systems saturation [102].

Once in the portal bloodstream, the metabolites through the portal vein rapidly reach the liver, where they can undergo phase II metabolism with further conversions before entering the systemic circulation and eventually undergoing renal excretion [4]. It is possible that enterohepatic recirculation may lead to some recycling back to the small intestine through bile excretion [103], this rout can contribute to phenolic compound bioavailability and metabolism [104]. The liver is a major organ in the phase II metabolism of phenolic compounds. In fact, the liver generates a wide variety of phase II enzymes in high quantities [4,105]. After being metabolized by the liver, the metabolites reach the systemic circulation. Various phase-II metabolites can exist together in the plasma, such as singly-conjugated metabolites like catechin-glucuronide, and multiply-conjugated metabolites like methylquercetin diglucuronide [106,107]. However, the major phase II metabolite appearing in plasma differs from one flavonoid family to another, for instance, quercetin-3′-sulfate is the major plasmatic phase II metabolite of quercetin in humans [106] while catechin-glucuronide and epicatechin-glucuronide have been described as the main plasmatic phase-II metabolites of catechin and epicatechin in humans [108] and rats [109,110]. In general, peak concentrations of phase II metabolites in human plasma are reached between 1 and 4 hours after ingestion of phenolic compounds [106].

Once in the plasma, circulating phenolic metabolites can reach diverse tissues and organs, such as aorta, brain, lungs, muscles, perirenal adipose tissue, spleen, and testicles, among others [102,109]. Several research results have shown that the metabolite profile found in plasma does not correspond to that observed in

other tissues [111]. In fact, deglucuronidation may occur at a vascular level, liberating free flavonoids into the target tissues [112]. Furthermore, a number of organs and tissues can continue to metabolize phenolic compounds as their express several phase II enzymes [1,96].

The phenolic compounds not absorbed in the small intestine to any degree can be absorbed in the large intestine where they are exposed to the action of the resident microflora [3]. In this regard, it has been reported that 90-95% of phenolic compounds in the diet are not absorbed in the small intestine and therefore reach the colon [104]. The colonic microflora cleave the conjugating moieties and subject the resultant aglycones to ring fission, resulting in the production of smaller molecules, such as phenolic acids and hydroxycinnamates [8]. In fact, it has been demonstrated that colonic microflora is the main metabolic site for the release of free hydroxycinnamic acids and flavonoid aglycones from their conjugated forms after cleavage of ester or glycoside bonds [105]. The microbial metabolites can be absorbed and can undergo metabolism in the liver before being excreted in the urine in substantial quantities that, in most cases, are much higher than the metabolites that enter the circulatory system *via* the small intestine [113,114]. Furthermore, more than half of the carbon in the glycosides and the A-ring of the flavonoids can be further metabolized to short-chain fatty acids and thus be available for host energy metabolism and ultimately exhaled as CO_2 [115]. In addition, gut bacteria can hydrolyze amides, esters, glucuronides, glycosides, lactones and sulfates, and the flavonoid skeleton undergoes ring fission, the products of which can then be subjected to reduction, decarboxylation, demethylation and dihydroxylation reactions [116]. Importantly, depending on the structure of the phenolic compound different microbial metabolites can be generated [117]. The diversity of the host microbiota also has a significant role in the type and amount of microbial-derived metabolites [118]. For instance, healthy and obese animals, which have differences in their microbiota composition and diversity [119], have shown different microbial-derived metabolite's profile after the ingestion of grape seed polyphenols [110]. The metabolites generated can be absorbed in situ and reach the liver through the portal vein [104]. Subsequently, microbial metabolites can reach various organs and tissues including aorta, brain

and plasma [109,120]. Although unlike phase II metabolites, microbial-derived metabolites occur in the plasma at later times [107]. For instance, peak concentrations of hippuric acid are detected in rats plasma 7 hours after consumption of grape seed flavanols [120]. In fact, *in vitro* studies assessing microbial transformation of phenolic compounds indicate extended times for the phenolic acids metabolites generation [121]. Moreover, the final health effects of dietary phenolic metabolites depend on the gut microbiota composition [118].

The phase II metabolites as well as those microbial-derived reach the kidneys [102,122] and are subsequently eliminated *via* the urine [4,108]. The plasma clearance of phenolic metabolites is rapid. Particularly, within 24 hours, the plasma is generally clear of phase II metabolites, while microbial-derived metabolites may still be found in the plasma [106,110,120]. Particularly, studies of chronic phenolic compounds supplementation show a lack of tissue accumulation of phenolic metabolites [123].

Perhaps the subclasses of flavonoids in which most progress has been made in the understanding of their ADME in the last years are the flavan-3-ols and the anthocyanins [4].

1.4.2. Factors affecting phenolic compounds bioavailability and metabolism

Bioavailability studies are not easy to perform, since several potentially affecting factors exist [83], as illustrated in **Table 2.** These factors can affect bioavailability either directly or by decreasing the phenolic content from foods [83]. The bioaccessibility and bioavailability of phenolic compounds vary depending on the physical condition of an individual, including digestive, absorptive, metabolic response capability and effective dose [95]. In this sense, external factors such as processing methods and interaction with various food matrices also play a vital role on the bioavailability of phenolics after ingestion [83,95]. Overall, there are numerous factors affecting bioavailability and metabolism of phenolic compounds, including environmental factors, food processing, food-related factors, interactions between food components, chemical properties, host-related factors such as intestinal factors and systemic factors [83,95].

External or environmental factors may condition the phenolic content in plants and their subsequent bioavailability [83,97,124]. Moreover, the degree of ripeness

influences the concentrations and proportions of the various phenolics in different ways [125]. Thermal treatment, cooking and the methods of culinary preparations, and also storage influence the concentration and, as a result, the amount of absorbed phenolic compounds in various manners [83]. For instance, processing of tomatoes into different end products may result in changes in bioavailability of tomato antioxidants [126], specifically, the addition of fat enhances the bioavailability of this fruit [127].

Table 2. Main factors affecting the bioavailability and metabolism of phenolic compounds.

Environmental factors	Sun exposure, degree of ripeness, food availability.
Food processing	Thermal treatments, homogenization, lyophilization, cooking and culinary methods, and storage.
Food-related factors	Food matrix, presence of positive or negative effectors of absorption (*i.e.*, fat, fiber).
Interactions between food components	Bonds with proteins or with phenolic compounds with similar mechanism of absorption.
Chemical properties	Chemical structure, concentration in food, and amount introduced.
Host-related factors	Intestinal factors (*e.g.*, enzyme activity, intestinal transit time, and colonic microflora). Systemic factors (*e.g.*, gender and age, disorders and/or pathologies, genetics, and physiological condition, biological rhythms).

Adapted from D'Archivio and colleagues [83].

Phenolic compounds may be present in the matrix as single molecules, bound to cellular organelles or trapped in complex macromolecular matrices with other macronutrients such as carbohydrates or proteins, in effect, the food matrix also affects bioaccessibility and bioavailability [95]. Bioaccessibility, and consequently bioavailability of phenolic compounds significantly depend on the structure and form in which they are introduced into the organism, *e.g.*, through a complex food matrix or as purified isolates [95]. Direct interaction between phenolic compounds and some food components, including alcohol, carbohydrates, fat, fiber, proteins, can occur, affecting their absorption [128,129]. Processing of the food matrix (*i.e.,* drying or encapsulation), as well as the consistency of the food matrix

(liquid or solid) can also influence phenolic bioavailability [95]. Particularly, the effect of the food matrix on proanthocyanin bioavailability is evident due to the variability of proanthocyanin bioavailability in various foods [130]. Thus, the chemical structure of this compound, as well as the food matrix and interactions with other components of plant foods, play an important role in the release of proanthocyanins [131]. In this sense, most *in vitro* and *in vivo* studies in the literature suggest that proteins, dietary fiber, and minerals may have an unfavorable impact on phenolic bioavailability. In contrast, lipids, digestible carbohydrates, vitamins, alkaloids, carotenoids and other flavonoids may enhance phenolic bioavailability and metabolism [132]. Dietary fat effects the phenolic compounds bioavailability by increasing the intestinal passage time [95]. For instance, flavanone plasma behavior was different depending on whether it was administered as juice or co-administered with full-fat yogurt [133]. Different serum kinetic profile of catechin, resveratrol and quercetin was detected depending on their administration on juice, wine or vegetal homogenate [134]. As mentioned above, phenolic compounds bioavailability may be influenced by the covalent interaction between phenolic compounds and proteins [135], in fact, a decrease in the bioaccessibility and bioavailability of black tea catechin was observed due to the effect of protein-phenol interactions [136]. Moreover, transport competition between phenolic compounds with related transport mechanisms can exist and regulate the bioavailability and metabolism of phenolics [83]. Also, the phenolic compounds structure can have an essential impact on their bioavailability and metabolism [137]. For example, anthocyanins, due to their structure, can overcome acidic conditions in the stomach [138]. In fact, the chemical structure is the one that determines the rate and extent of phenolic compounds absorption, not the concentration [95]. Furthermore, host-related factors affecting bioavailability can be categorized into intestinal factors and systemic factors [83]. The intestinal factors represent probably the most important of the host-related factors [83]. As explained above, after intake of dietary phenolic compounds, absorption of some, but not all, components occurs in the small intestine [1,3,4]. In this regard, it has been shown that two mechanisms exist for glycoside hydrolyzation [83]. The first mechanism involves the action of floridizin hydrolase lactase and the second mechanism involves cytosolic β-glucosidase [83]. Furthermore, the hydrolyzing

activity of colonic microflora is of considerable relevance for the biological action of phenolic compounds, since the active metabolites are produced by the colonic microflora [83]. For instance, daidzein is converted to its active metabolite, *i.e.*, equol, in such a way [139]. Indeed, there is a substantial interindividual variability in the production of these active metabolites [83].

Finally, it has recently been demonstrated that the season of the year in which the food is consumed can modulate its phenolic bioavailability, such is the case of red grape, where it was observed that animals that consumed it during winter conditions presented a higher bioavailability of grape phenolics [140]. Indeed, circadian changes in the expression of phase II enzymes in mice have been reported, the contribution of photoperiod to the percentage of phase II flavonoid metabolites does not seem to be as relevant as its contribution to the modulation of small-weight phenolic acid metabolites such as cinnamic, phenylpropanoic, phenylacetic and benzoic acid derivatives as well as other metabolites [141].

2. Biological rhythms, chrononutrition and xenohormesis

The mammalian biological clock system modulates many physiological processes, such as daily rhythms of sleep–wake behavior, hormone secretion and metabolism. This system reacts to daily environmental changes, such as the light-dark cycle, food intake and drug administration [142]. Recorded recognition of the importance of biological rhythms in plants and animals dates back at least to 5000 B.C. [143]. It is therefore essential to understand the mechanisms of interaction between biological rhythms and their influence on the activity of bioactive compounds and, at the same time, the mechanisms of these compounds to modulate biological rhythms [143].

2.1. Biological rhythms

Life on earth goes through daily cyclical changes in specific circumstances, for instance, plants photosynthesize during the day, and nocturnal animals forage for food at night [144]. These cyclical changes, resulting from the Earth's rotation around its axis (photoperiod), its tilt (variations in day length) and its simultaneous rotation around the sun (seasonal changes), are crucial for

important evolutionary changes in life [145]. For any organism to function correctly, it is essential that it be able to control the timing of its biological functions. In this respect, light plays a fundamental role in key changes in the physiological and metabolic signaling pathways that trigger the response of organisms [146]. Actually, the major environmental signals that trigger biological clocks in most animals in nature are related to the light/dark alternation and photic signals [143]. The biological rhythms include circadian and circannual rhythms, which are a fundamental characteristic of all living organisms and their organelles as they are closely related to metabolism and nutrition [68]. Both circadian and circannual rhythms are driven by two main mechanisms: a) an intrinsic endogenous timing system that persists in the absence of environmental signals, with oscillating periods of approximately 24 hours or 365 days, respectively, driven by endogenous central and peripheral clocks, and b) environmental signals, called zeitgebers or time givers, that entrain these rhythms to external conditions, such as day length, *i*.e., photoperiods [147]. Daily changes in dietary patterns, physical activity and temperature are basic zeitgebers that synchronize various physiological and metabolic processes throughout the body [148]. Likewise, seasonal variations in barometric pressure, food availability, precipitation and weather can function as environmental cues to synchronize the circannual clocks [149].

2.1.1. Circadian rhythms

Almost all light-sensitive organisms, including humans, exhibit physiological changes in cycles of approximately 24 hours. These cycles are called circadian rhythms and involve the anticipation of food availability and the avoidance of predators [150]. Circadian clocks enable the anticipation of daily events, conferring a considerable advantage for saving time and the efficient use of energy [144]. Obviously, with respect to evolution, circadian rhythms appear to be related to the rotation of the Earth around its axis [143]. In mammals processes as diverse as temperature and blood pressure fluctuations, sleep-wake cycles, and glucose and lipid metabolism, are all under rhythmic control (**Figure 4**) [151].

In addition, several hormones implicated in metabolism, including insulin, glucagon, adiponectin, corticosterone, leptin and ghrelin, also show circadian oscillation [152]. Furthermore, the disruption of circadian rhythms is related to the

development of cancer, metabolic syndrome and obesity [152]. Actually, there is growing evidence that circadian misalignment is involved in weight gain and the development of obesity [153]. In addition, the cellular redox state, determined by food metabolism, and various nutrients, such as glucose, ethanol, adenosine, caffeine, thiamine, and retinoic acid, can disrupt circadian rhythms [152]. In this regard, animal experiments have shown that untimely feeding [154], a high-fat diet [155], jet lag [156] and shift work [157] alter circadian alignment and lead to metabolic disorders.

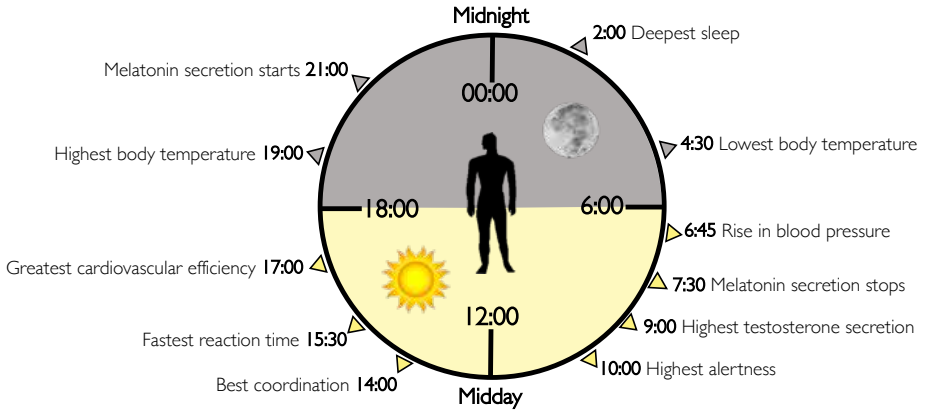


Figure 4. Schematic representation of key physiological circadian changes over the day. Adapted from Robinson and Reddy [151].

- Circadian clock

In all organisms, endogenous circadian rhythms are driven by a sequence of interconnected positive and negative transcription-translation feedback loops [158,159]. An internal biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN) in mammals, carefully monitors this temporal homeostasis by transmitting its time message to the whole organism [160], in other words, this master clock regulates circadian rhythms in mammals [152]. Effectively, the biological clock directs and maintains proper rhythms in endocrine and metabolic pathways required for organism homeostasis [161]. In fact, the SCN clock can run autonomously, without any external input, and can reset itself in response to environmental signals (zeitgebers) [161]. The SCN acts as a conductor for the peripheral clocks, which are believed to be present in all other tissues and cells of the body [162]. The clock is an intracellular transcriptional mechanism that shares

the same molecular components in SCN neurons and in peripheral cells, including liver, intestine and retina [152]. At the core of the molecular network that constitutes the biological clock are the central transcription factors CLOCK and BMAL1 [163], which drive the transcription of a wide range of clock-controlled genes by binding to E-box sites within their promoters [161]. Synchronization of peripheral clocks is crucial to ensure a coordinated physiology [162], in this sense, fasting-feeding cycles and rest-activity rhythms are the main synchronization signals for most peripheral clocks, indicating that temporal coordination of metabolism and proliferation is an important task of the mammalian timing system [150].

In addition, biological rhythms are responsible for daily food intake; the period of hunger and satiety is regulated by the central pacemaker, which resides in the SCN and connects with the tissues through bidirectional neuronal and humoral pathways [142]. Specially, the biological clock regulates food processing and energy homeostasis by regulating the expression and/or activity of enzymes involved in the metabolism of cholesterol, amino acids, lipids, glycogen and glucose [152]. At molecular level, biological clock modulates the expression of essential genes within several metabolic pathways [161]. A prominent example is the control by CLOCK:BMAL1 of Nampt (nicotinamide phosphoryl transferase) gene expression [164,165]. The enzyme NAMPT, which is a product of this gene, is linked to the control of cellular metabolism, inflammation and aging [166]. Furthermore, metabolism and food intake also feedback to modulate the biological clock [68,152]. It is important to note that the microbiota appears to play a key role in the clock-nutrition interaction [161].

2.1.2. Circannual rhythms

Circannual rhythms are long-term (tau ≈ 12 months) cycles of physiology and behavior that are crucial for life [167]. In fact, circannual rhythms appear to be related to the rotation of the Earth around the sun and represent an adaptative phenomenon from the perspective of the reproduction and survival of species [143]. This may explain why many biological activities, such as migration and reproduction, are restricted to one period of the year [144]. Indeed, circannual

rhythms are seasonally dependent oscillations and are a major selective force in the development of adaptive strategies and abilities to cope with environmental challenges [168]. The generation of circannual rhythms depends on tissue-autonomous, reiterated cycles of cell division, functional differentiation, and cell death [167]. As previously mentioned, cues such as day length, nutrition and social factors can synchronize circannual rhythms through hormonal influences, mainly through the thyroid and glucocorticoid axes [167].

- Seasonal molecular mechanisms

The molecular mechanisms underlying these circannual rhythms and their relationship to circadian clocks have not yet been completely clarified [169]. However, evidence is mounting that the transcriptional coactivator eyes absent 3 (EYA3), a clock-controlled gene product, plays a crucial role in the regulation of seasonal responses driven by photoperiod [169]. This protein functions in thyrotroph cells, located in the pars tuberalis (PT) region of the pituitary gland, which is recognized as the master regulator of seasonal biology in mammals [170]. In fact, EYA3 protein shows a circadian pattern in which its expression increases 12 h after the onset of darkness, as it is modulated by CLOCK and BMAL1 [169]. Hence, in long-day (LD) seasons, EYA3 reaches its peak in light conditions and coactivates the transcription of the gene encoding the thyroid-stimulating hormone beta-subunit (TSHβ) in the thyrotropic cells of the TP (Figure 5) [171]. PT-derived TSHβ acts locally, potentiating transcription of iodothyronine deiodinase type II (DIO2) in the tanycytes (T), a specialized ependymal cell of the third ventricle and whose processes leading to the hypothalamic parenchyma [171]. The increased enzymatic activity of DIO2 leads to the activation of thyroid hormones (TH), turning the inactive form thyroxine (T4) into the active form triiodothyronine (T3), which triggers other hypothalamic signals that regulate seasonal metabolic and reproductive adaptation to a LD state [168]. In contrast, in short-day (SD) seasons, EYA3 increases in dark conditions, decreasing markedly due to the repression of cyclic adenosine monophosphate release by melatonin. The resulting decrease in TSHβ and DIO2 activity, and the up-regulation of type III iodothyronine deiodinase (DIO3), deiodinating biologically active T3, results in

a substantial reduction in T3 availability in the hypothalamus, leading to a seasonal response different from the DS [168].

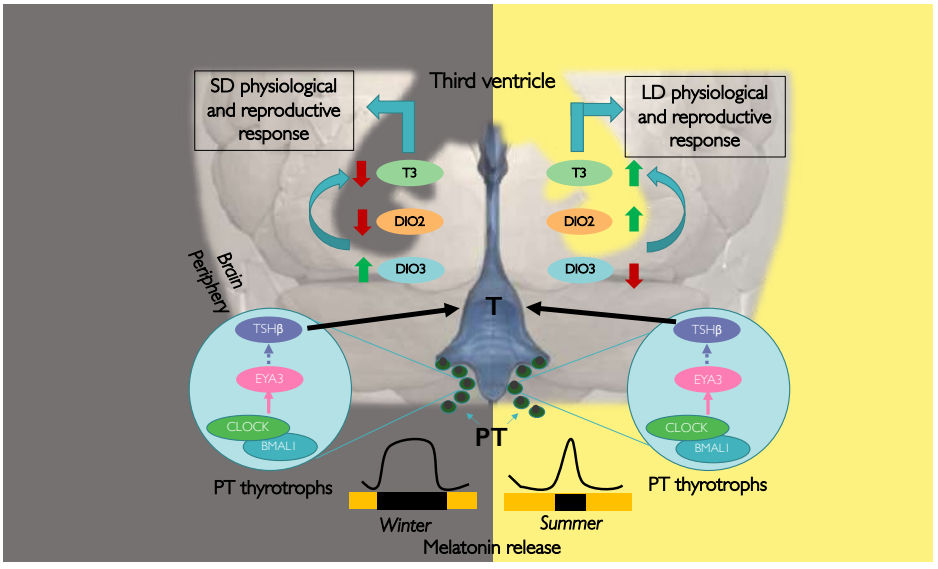


Figure 5. Molecular mechanisms involved in the physiological response to seasonal day length variations. Adapted from Wood and colleagues [168].

- **Seasonal physiological and metabolic variations**

Although humans living in modern, industrialized societies are insulated from seasonal fluctuations due to human-induced environmental alterations, such as the advent of artificial lighting, heating and air conditioning systems [172], a wide range of biological occurrences and processes with seasonal rhythms have been documented in numerous experimental and observational studies [173]. There is increasing evidence that circannual imbalance is implicated in weight gain and the development of obesity, in fact, in a recent study, a seasonal weight gain was observed in children during the summer as a result of social timing changes and the implications for seasonal patterns of weight gain [153]. In this regard, an association of seasonal variation in the prevalence of metabolic syndrome with insulin resistance has been found, resulting in significantly more subjects meeting criteria for metabolic syndrome in winter than in summer, with insulin resistance and increased blood pressure being the main components driving the findings [174].

It was reported that consuming fruits with a distinctive seasonal phenotype (in terms of bioactive compounds) produces a metabolic response that is dependent

on mammalian circannual rhythms [175]. In fact, local and seasonal fruits and vegetables are often described as tastier, fresher and of better nutritional quality than equivalent imported or out-of-season produce [176]. In this regard, the protective association between the seasonality of fresh fruit or vegetable salad consumption and the development of cancer and cardiovascular disease (CVD) was investigated in 1489 men and 1900 women, aged 35-75 years, with the result that frequent consumption of vegetable salad in winter was more protective than in summer for cancer in men, moreover, frequent consumption of salad vegetable in any seasons was considerably protective against CVD in women, but not against cancer. Frequent fresh fruit consumption in women was significantly protective of CVD but not quite significant, and only in winter, for cancer [177]. In addition, nutritional intervention trials conducted in Linxian, China, showed that nutrient intakes were low for selenium, zinc, vitamin B2 and calcium in both spring and autumn. A large seasonal variation was seen in the consumption of leafy vegetables and root vegetables, all of which might have contributed to the lower intake of vitamin A, vitamin C, protein and vitamin E in autumn [178]. Likewise, a recent study about seasonal consumption of polyphenol-rich fruits concluded that consuming rich-polyphenol fruits such as red grapes or sweet cherries may increase leptin sensitivity through the modulation of the hypothalamic leptin signal pathway in a photoperiod-dependent mode, mainly when consumed in short day photoperiod [179].

On the other hand, consuming fruit out-of-season could trigger erroneous signaling, leading to an alteration of the characteristic seasonal metabolism, which could lead to the development of obesity and related disorders [175]. In this line, new studies have reported that sweet cherry consumption exerts a remarkable season-dependent effect, inducing more changes when it was consumed out-of-season, especially in winter conditions, among these changes being an increased Had gene expression (soleus) and pAMPK levels (soleus and gastrocnemius) in rats fed a standard diet; another finding was an increase in whole-body fat oxidation and circulating levels of glucose and insulin in rats fed an obesogenic cafeteria diet [175]. Likewise, another study has also shown that consuming sweet cherries out-of-season influences the metabolism of adipose tissue and promotes

fat accumulation when accompanied by an obesogenic diet, producing changes in gene expression and morphology of white adipose tissue towards a phenotype prone to fat accumulation [180].

Another fruit that exhibits seasonal metabolic patterns is the orange (*Citrus x sinsensis*), which was the subject of a study demonstrating that its consumption out-of-season modulates fat accumulation, morphology and gene expression in the adipose tissue of Fischer 344 rats, in particular, Navelina orange from the southern hemisphere promotes a phenotype prone to fat accumulation when consumed in a short-day photoperiod, which could be explained by the xenohormesis theory [181]. Additionally, as for phenolic bioavailability, the results of a study where Fischer 344 rats were exposed to distinct photoperiods and administered with red grape produced organically or non-organically for 10-week, showed that conventional grape-administered rats had a more varied serum metabolite profile as a function of photoperiod exposure. Importantly, those rats exposed to winter light conditions, which the season of consumption of this fruit, exhibited a higher bioavailability of grape phenolics [140].

2.2. Chrononutrition

Recent evidence has associated the circadian timing system to metabolic physiology and nutrition, which is a relatively new area of interest [182]. This emerging concept, nominated as "Chrononutrition", was first mentioned in a Japanese book about nutrition and health published in 2005 [183]. Indeed, chrononutrition is a new research field in nutritional epidemiology that builds on the intimate relation between biological rhythms, nutrition and metabolism, along with the relationship between these factors and human health [184], which encompasses 3 dimensions of eating behavior: timing, frequency, and regularity; and also encompasses energy distribution, and the relative importance of these factors for metabolic health and chronic disease risk [184]. Particularly, this discipline proposes that nutrients or the timing of meals themselves could modulate the circadian clock system [144], and that desynchronization of biological rhythms could negatively influence timing and food choices [185]. Indeed, feeding behavior is the first element to be considered in the nutritional process of an organism [186].

Circadian rhythms in glucose and lipid metabolism, insulin responsiveness and sensitivity, energy expenditure and postprandial metabolism may favor dietary patterns characterized by an earlier temporal distribution of energy [184]. Research results in this field have also demonstrated that time-related eating patterns including mealtimes [187], nocturnal eating [188], energy distribution throughout the day [189] and the number of feeding episodes [190] may affect nutrient metabolism and be linked to metabolic and nutritional illness [185].

Regarding chrononutrition in the management of diabetes, research in this area has suggested that time of day is indicative of having an influence on the postprandial glucose response to a meal, therefore having a major effect on type 2 diabetes mellitus [191]. Such chrononutrition functions to optimize metabolism by timing nutrient intake to the acrophases of metabolic rhythms to improve whole-body insulin sensitivity and glycaemic control, and thereby positively impact metabolic health [191].

Most of the studies conducted in this field have focused primarily on the effects of meal timing [68]. In this sense, an ever-growing body of evidence in human studies reveals that timing of food intake across the day strongly affect metabolic health and general wellbeing [192]. Actually, recent evidence from chrononutrition studies verifies the importance of meal timing in energy balance and cardio-metabolic health and disease [193]. Humans are a diurnal species in which the light cycle stimulates wakefulness and feeding, and darkness initiates sleep and fasting. The mismatch between normal feeding/fasting, day/night and sleep/wake cycles can desynchronize central and peripheral regulation of metabolic processes and contribute to obesity and metabolic disorders [193]. Breakfast skipping and late night feeding have been associated with an increased risk of obesity and related health conditions [188]. In addition to significantly increasing the risk of obesity, a recent research found that acute breakfast skipping altered clock genes expression and led to an increase in postprandial glycaemic response [192].

Additionally, chrononutritional differences have been found between adolescents with and without delayed sleep-wake phase disorder [194]. Human dietary intervention studies seem to suggest that calories ingested at different times of the day have different effects on energy utilization, leading to differential weight

loss, even with isocaloric amounts [192]. Indeed, time of day of energy intake has been linked to obesity, since meals consumed in the evening have been associated with lower resting metabolic rate. Inconsistent timing and frequency of meals have also been linked to increased body weight and worse cardiometabolic health [195]. Furthermore, a new work with Brazilian adolescents showed that diet quality, nutrient intake and eating behaviors differed according to sleep duration among adolescents. In fact, the chrononutrition characteristics of sleep-deprived adolescents were marked by longer eating periods and time-interval between eating occasions than adolescents with adequate sleep duration [196]. In this line, another study on chrononutrition linked to children's body weight status found a higher proportion of energy and macronutrient intake at the main meals and a lower proportion during the afternoon and evening seems to be more beneficial for children's weight [197].

On the other hand, the chrononutrition during pregnancy has been studied, with emphasis on the nocturnal feeding of the mother. Here, it was found that unfavorable nutritional characteristics linked with nocturnal feeding have the potential to induce aberrant circadian rhythms in pregnant women, resulting in adverse metabolic and pregnancy outcomes [198]. Likewise, dietary interventions with antioxidant-enriched foods that take into account the principles of chrononutrition are of particular interest to the elderly, as they can help amplify the already powerful benefits of phytochemicals as natural tools with which to prevent or delay the onset of common age-related diseases [186]. Most importantly, the time of day influences both beneficial and undesirable effects, as different antioxidants tend to be absorbed in different proportions, depending on the time of day [186]. As the above studies demonstrate, chrononutrition may have therapeutic application for people with and at-risk of metabolic disease and generate health benefits within the general population [184].

Table 3 shows a compilation of health results of dietary interventions modulated by circadian and circannual rhythms.

Table 3. Health results of dietary interventions modulated by biological rhythms.

Dietary intervention	Experimental model	Biological rhythms	Health results	Reference
Catechin-rich green tea	Healthy young men	Evening	Decreased postprandial plasma glucose concentration	199
Polyphenol-rich grape-wine Extract	Mildly hypertensive males and females	Daytime	Lowered ambulatory systolic and diastolic blood pressures	200
Epigallocatechin-3-gallate	C57BL/6J mice	Night-time	Improved diet-induced metabolic derangement by regulating rhythmic expression of circadian clock genes in liver and adipose fat tissue	201
Grape seed proanthocyanidin extract	Male Wistar rats	Daytime	Modulated the plasma melatonin level	202
Oranges	Male F344	SD	Modulated fat accumulation, morphology, and gene expression in the adipose tissue	181
Resveratrol	Male Wistar rats	Night-time	Antioxidant	203
		Daytime	Pro-oxidant	
Red grapes	Standard- and cafeteria -fed F344	SD	Enhanced hypothalamic sensitivity to leptin	179
Sweet cherries	Standard-fed male F344	LD	Reduction of non-esterified free fatty acids in the blood.	175
		SD	Improved activation of fatty acid transport, β–oxidation related pathways, and circulating glucose and insulin levels	
	Cafeteria-fed male F344	SD	Increased detrimental impact of cafeteria diet in relation to glucose metabolism.	175
	Standard- and cafeteria -fed F344	SD	Enhanced hypothalamic leptin sensitivity	179

Abbreviations: F334, Fischer 344 rats; LD, long day photoperiod (18 h light/6 h dark); SD, short day photoperiod (6 h light/18 h dark). Adapted from Arola-Arnal [68].

The evolution of chrononutrition has been driven by the fact that individuals with circadian desynchronization, such as shift workers, have negative changes in their food consumption pattern and are more prone to develop metabolic and nutritional diseases such as obesity, dyslipidemia, metabolic syndrome, insulin resistance and type 2 diabetes mellitus [185]. Additionally, it seems that the modern environment, with exposures including artificial light, shift work, and ubiquitous food availability, predisposes individuals to circadian dysregulation and dysmetabolism [184]. Furthermore, there is considerable epidemiological evidence that shift work is associated with increased risk for obesity, diabetes and cardiovascular disease, perhaps as a result of physiological maladaptation to chronically sleeping and eating at abnormal circadian times [204].

2.3. Xenohormesis

Plants have been known for millennia to produce substances beneficial to human health such as phenolic compounds [205]. At the molecular level, it has become clear that many of these phytochemicals exert their effects by directly interacting and modulating specific enzymes or receptors [206]. In fact, most of the known beneficial health effects of edible plants are attributable to pharmacologically active substances in the stress response of plants [207]. Although the harmful properties of xenohormogenic phytochemicals are detrimental to plant-feeding microorganisms, insects and pests, at subtoxic doses ingested by humans as part of their diet, the same compounds are considered to induce mild cellular stress responses [208,209]. This, in turn, activates signaling pathways of the adaptive stress response, resulting in enhanced expression of genes encoding mostly cytoprotective proteins, including antioxidant enzymes, phase 2 detoxifying enzymes, protein chaperones, growth factors, mitochondrial proteins, etc. [207]. For example, oxidative stress caused by some flavonoids with prooxidant activity may contribute to their health-promoting activity by inducing important antioxidant enzymes, pointing to a beneficial effect of a putative toxic chemical reaction [210].

The phytochemicals produced by stressed plants activate sirtuin enzymes and prolong the life of fungi and animals, apparently mimicking the beneficial effects of caloric restriction [211]. For instance, resveratrol, a phenolic compound, activates the same pathways as calorie restriction, with early research showing the

compound was able to activate sirtuin enzymes in the yeast strain *Saccharomyces cerevisiae*, resulting in improved DNA stability and a dramatic 70% increase in lifespan [212]. A possible explanation is that sirtuin enzymes have evolved to respond to plant stress molecules as indicators of impending environmental deterioration, indeed, the sirtuin family seems to have first emerged in primordial eukaryotes, presumably to help them cope with adverse conditions [213]. This idea has become known as the Xenohormesis Hypothesis, the name stemming from a combination of the prefix xeno-(for stranger) with hormesis (a protective response induced by mild stress) [211]. The xenohormesis proposes that animals and fungi have evolved the ability to sense the signaling and stress molecules of other species, and that they are under selective pressure to do so (**Figure 6**) [205].

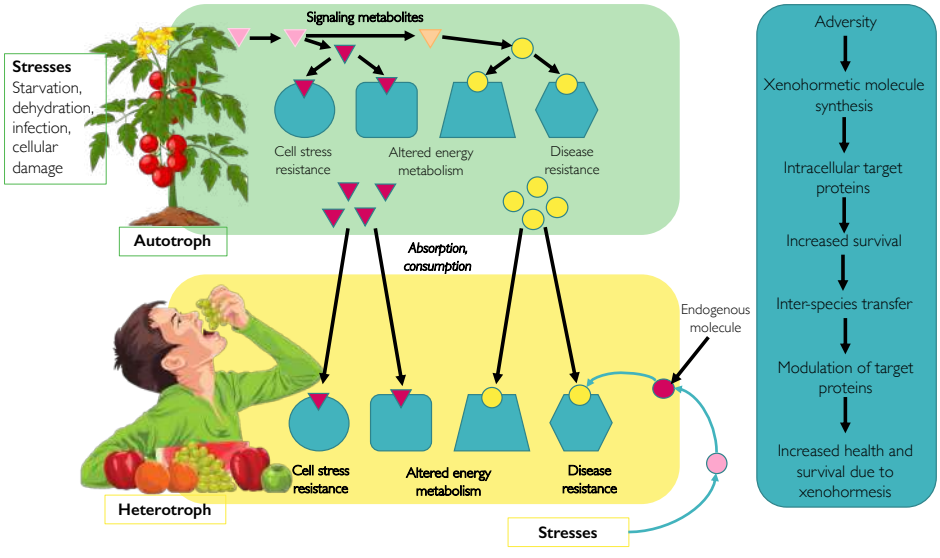


Figure 6. The xenohormesis hypothesis. Adapted from Howitz and Sinclair [205].

Basically, xenohormesis is a biological principle that refers to interspecific hormesis, whereby an animal or fungal species uses chemical signals from other species about the state of its environment or its food supply to mount a preemptive defense response that enhances its chances of survival [205], and explains how environmentally stressed plants produce bioactive compounds that can confer stress resistance and survival benefits to animals that consume them [214].

Indeed, it is called xenohormesis because the stress occurs in one organism and the beneficiaries are other organisms that have evolved to perceive these chemical signals [205]. This is why Howitz and Sinclar [205] propose that the common ancestor of plants and animals synthesized phenolic compounds, since the divergence of the phyla, there has been selection such that heterotrophs (animals and fungi) detect chemical signals about their environment from plants and other autotrophs (*i.e.*, organisms that obtain energy from light or inorganic chemical reactions) [205]. These chemical signals would give the heterotroph advance warning of the deteriorating environment, enabling it to prepare while conditions are still relatively advantageous [213]. Animals take advantage to exploit the information contained in the products of the sophisticated stress response of plants, that has developed as a result of their stationary lifestyle [207,214].

The xenohormesis predicts that many key mammalian enzymes and receptors will have evolved with binding pockets that permit modulation by molecules produced by other species. Moreover, health effects of many different molecules have been confirmed and putatively related to specific molecular targets [206]. Many non-nutritional dietary components activate stress responses and homeostasis mechanisms in animals [215]. The plant xenohormogenic compounds, when consumed, can promote longevity and fitness by activating the animal cellular stress response and can be applied in drug discovery, drug production and nutritional enhancement of the diet [214].

2.4. Plant-based foods intake patterns

Historically, fruits and vegetables have been produced and consumed in their immediate environment, during a specific and limited season based on climatic conditions of each crop and limited food transportation and storage technologies [68]. But these patterns are dynamic and constantly changing, in fact, year-round supply of fresh produce is now possible thanks to the intensification of agriculture, the use of new technologies, the expansion of natural production and growing seasons, and the increase in international trade [176]. Consequently, the expansion of world food markets, through globalization, has established a food culture with a wide variety of foods in many countries, where previously this was not possible [176]. Although this has led to a more varied diet in many countries,

this global demand has come at a high environmental cost, with increased energy use, loss of environmental biodiversity, loss of species and crop diversity, and different metabolic responses depending on when the food is consumed [68,175,176]. In fact, studies on human nutrition have shown that a nutritional transition is occurring worldwide, with people moving towards more affluent food consumption patterns [216].

Changing food preferences could be a significant demand shifter in the twenty-first century [217]. The relative change in consumption patterns across seasons and the populations vary, but, when environmental and health aspects of diets are considered, there is no apparent contradiction [176]. In this sense, the Mediterranean diet is a good model of this duality, consisting mostly of plant-based foods but does not exclude a small proportion of meat and other animal products, is closer to public health recommendations and has a lower environmental impact than the current average U.S. diet. [218]. Health issues have also become an increasingly important factor in consumer preferences for produce in recent years. Actually, eating more seasonal and local foods, especially fruits and vegetables, is one of the dietary challenges proposed to promote a more sustainable and healthier diet [176]. Seasonality can be considered as globally seasonal (*i.e.*, produced in the natural production season but consumed anywhere in the world) or locally seasonal (*i.e.*, produced in the natural production season and consumed within the same climatic zone) [176]. In this sense, seasonal foods are often associated with locally produced food, better quality or limited food choices, and for some are foods associated with annual cultural events [176]. In addition, seasonal plant-based foods can be less treated, tastier foods with a higher concentration of nutrients, they are also eco-sustainable foods, as they are often Km 0 [219]. As, the reduction in greenhouse gas emissions from eating seasonal is limited [220]. For centuries fresh produce has been stored to extend the period that food is available. While storage and transportation can be associated with the loss of some micronutrient and bioactive compounds of produce [221]. Some methods of preservation can actually increase the nutrient quality of a product; for example nutrients such as lycopene

and β-carotene in processed tomatoes are more bioavailable than in fresh tomatoes [127].

Furthermore, several epidemiological studies have reported differences in the amount of fruits and vegetables consumed according to the season of the year [222]. Moreover, Ziegler and colleagues [222] documented that vegetables are generally eaten year-round, whereas certain fruits are eaten primarily in a single season. A study demonstrated that for seasonal consumers in New Jersey frequency of consumption over a year was a time-weighted average of higher in-season consumption and the lower out-of-season consumption [222]. Moreover, changes in the diet toward more plant-based foods, toward meat from animals and toward foods processed in an energy-efficient manner offer an interesting and little explored area for mitigating climate change [218].

Among traditional local dietary patterns, the most famous is the Mediterranean [223]. The Mediterranean diet is characterized by high consumption of fruits, vegetables, seafood, nuts, legumes, whole grains, and olive oil, moderate intake of wine mainly within meals, and lower intake of red/processed meats, saturated fat, and sugary desserts and beverages [223,224]. Among the fruits, sweet cherry (*Prunus avium* L.) is one of the most widely consumed summer fruits across the temperate regions of Europe because of its health effects [46]. Moreover, among the vegetables, tomatoes (*Lycopersicon esculentum* Mill.) are a main component of this traditional diet, which have been related to health protection and longevity [127,224–226].

3. Phenolic-rich fruits

3.1. Sweet cherries

World production of cherries has been increasing steadily in many new and traditional regions [227]. Cherries belong to the genus Prunus, subgenus Cerasus, within the Rosaceae family, most of which are native to the Europe and Asia [43]. In turn, this subgenus is divided into eight sections, with section 1 (Eurocerasus) being the most relevant. The predominant species of Eurocerasus section are sweet cherries (*Prunus avium* L.), sour cherries (*Prunus cerasus* L.) and ground cherries (*Prunus fruticosa* Pall.) [43]. Furthermore, sweet cherries have been

categorized into subgroups depending on the color, shape and texture of the fruit [227]. Sweet cherries are harvested in the summer periods, where in general early-ripening cultivars predominate at lower latitudes, while late-maturing cultivars are grown in higher latitudes, such as western Canada and Norway [228]. In fact, sweet cherries are one of the few remaining seasonal fruit crops, and in many markets no other item creates as much seasonal in-store activity as fresh cherries [227]. The chemical composition of cherries varies according to cultivar, ripening stage, agricultural practices and environmental conditions [229]. The strong impact of environmental conditions is emphasized. In this regard, several studies have shown that the chemical composition of cherry cultivars is influenced by environmental conditions [229].

As far chemical composition, water is the major constituent of the fruit [43]; carbohydrates are the main chemical compounds in cherries, with contents of 12.2–17.0% [43]; sweet cherries are mildly acidic fruits with pH values between 3.7 and 4.2 [230]; protein content in cherries varied between 0.8 and 1.4% [43]; moreover, cherries are low-fat foods, their fat content varies between 0.2 and 0.7 g/100g of edible portion [43]; but interestingly, this fruit contains remarkable amounts of potassium and are considered a good source of this mineral [231], in addition, other minerals in cherries exist in low concentrations, such as phosphorous, calcium, magnesium and sodium and other minerals are also present at very low concentrations [43]. Furthermore, sweet cherries are a rich source of ascorbic acid (vitamin C), with mean values of 6–10 mg/100 g of FW [43]. Additionally, more than 100 volatile compounds have been identified, comprising the aroma of sweet cherries among free and glycosidically volatile compounds [231].

As a complement to this abundant chemical composition, sweet cherries have a large amount of bioactive compounds, so their regular consumption is related to a balanced diet and a wide spectrum of health benefits [45]. In this sense, sweet cherries are considered excellent sources of phenolic compounds [230–232]. These compounds are relevant to the quality of cherries because of their significant contribution to their appearance, flavor, aroma and health-promoting properties [45]. Specifically, sweet cherries consumption has been reported to reduce the risk

of cancer [233], as well as arthritis pain and inflammation [234], and provide protection against neurodegenerative diseases [235].

The phenolic compounds composition of sweet cherries has been reported by several studies, indicating that cherries contain substantial amounts of flavonoids, such as anthocyanins, flavan-3-ols and flavanols [43,232,236,237]. Anthocyanins are responsible for the characteristic red color of cherry skin and can be an indicator of the ripeness of this fruit [232]. Detailly, in sweet cherries, the major anthocyanins include cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside, followed by peonidin-3-*O*-rutinoside and peonidin-3-*O*-rutinoside [238]. In fact, the total anthocyanin content has been reported to range from 2 to 300 mg/100 g, as the concentration of these compounds depends on genotype, ripening stage, year of study, and agronomic and storage conditions [236,238]. Human intervention trials have provided evidence that cyanidins exhibit biologically relevant antioxidant properties [231]. Additionally, it is important to note that cherries are also rich in phenolic acids, in this line, derivatives of the hydroxycinnamic acids, mainly *p*-coumaroylquinic and neochlorogenic acid followed by chlorogenic acid, are the most abundant [45,46,232]. In fact, the former has been positively associated with the antioxidant potency of cherry extracts on human low-density lipoproteins [238]. Moreover, significant amounts of the flavonol, rutin (quercetin-3-*O*-ruthinoside), have been found in sweet cherries [43,232,237]. Flavan-3-ols detected in sweet cherry comprise epicatechin and catechin, in particular, there is a wide range of concentration of these compounds, reflecting the influence of agronomic and environmental conditions [43,44,237]. Likewise, a varied range of total phenolics concentrations has been reported [230–232,237], one of the possible causes could be the geographical origin of cherry cultivation [43]. For instance, it has been reported that the content of total phenolic ranged from 44.3 to 87.9 mg/100 g for cultivars grown in northern Europe [44], while for cultivars grown in a southern area of Europe it has been reported that the total phenolic content varied between 84.96 and 192 mg/100 g [45,230].

Table 4 summarizes the effects of cherries and products made from cherries on oxidative biomarkers of human health.

Table 4. The effects of cherries and products on oxidative biomarkers of human health.

Study Subjects	Treatment	Major Findings	Reference
10 well trained male athletes	30 mL tart cherry juice	Recovery of maximum voluntary contractions faster.	[239]
18 young, middle aged and elderly	Sweet cherry powder	Total sleeping time, immobility, and antioxidant capacity sweet cherry powder > basal level.	[240]
10 healthy women	280 g sweet cherry	↓ in plasma ORAC and FRAP, ↓ plasma oxidative stress and uric acid.	[234]
27 endurance trained runners or triathletes	Tart cherry	↓ markers of muscle catabolism, oxidative stress, and inflammation.	[241]
47 healthy adults	30 mL tart cherry	↑ FRAP	[242]
12 healthy older men and women	240 mL tart cherry juice	↓ oxidative stress in elderly	[243]
23 resistance trained men	480 mg tart cherry powder	↓ post-exercise muscle soreness. ↓ AST, ALT, and creatinine. No change in serum markers of oxidative stress and inflammation.	[244]
9 highly trained male Water polo players	30 mL tart cherry juice	No difference in measures of performance and recovery	[245]
16 trained cyclists	30 mL tart cherry juice	↓ lipid peroxidation.	[246]
30 volunteers	Cherry-based product	↑ urinary antioxidant capacity.	[247]

Adapted from Kelley and colleagues [232].

Among other aspects, the phenolic compounds in cherries stand out for their antioxidant power [232]. To date, several studies have concluded that cherries are a rich source of natural antioxidant substances [45,231,232]. In fact, a study performed by Tomás-Barberán and colleagues [45] indicated that sweet

cherries displayed antioxidant capacity in both hydrophilic and lipophilic fractions. Moreover, an *in vivo* assay demonstrated that cultivars with the highest levels of anthocyanins showed the greatest antiproliferative effects against human colon and gastric cancer cells [248]. In addition, a recent study investigated the effects of tart cherry juice on blood biomarkers of inflammation and oxidative stress, suggesting that the ability of tart cherry juice to reduce systolic blood pressure and low-density lipoprotein cholesterol in older adults, in part, may be due to its antioxidative and anti-inflammatory properties [249]. Similarly, other work demonstrated that Montmorency cherries may be efficacious in combating post-exercise oxidative and inflammatory cascades that can contribute to cellular disruption [246]. Finally, the review by Kelley and colleagues [232] about the health benefits of cherries summarized that cherry consumption decreased oxidative stress markers in 8/10 studies; inflammation in 11/16; exercise-induced muscle soreness and strength loss in 8/9; blood pressure in 5/7; arthritis in 5/5; and improved sleep in 4/4.

3.2. Tomatoes

Tomato fruit (*Lycopersicon esculentum* Mill.) is one of the most globally-consumed agricultural crops, being a key component of the Mediterranean diet [250]. Actually, tomato is the second most important vegetable crop next to potato [251]. Tomatoes belong to the genus Solanum, subgenus Lycopersicon, within the Solanacea family that includes many other plants of economic importance such as potatoes, eggplants, peppers and tobacco [252]. Today, this species is widespread throughout the world, representing the most economically important vegetable crop worldwide [252]. The three main producing countries are China, India and the United States of America, but consumption is highest in the Mediterranean and Arab countries [251].

The importance of this fruit does not lie in its nutritional value, but rather in the fact that it represents a significant source of phytochemicals endowed with important health-promoting properties, namely carotenoids (mainly lycopene and β-carotene), phenolic compounds (phenolic acids and flavonoids), vitamins

(ascorbic acid, tocopherols and vitamin A), glycoalkaloids (tomatine) and minerals (K, Mn, Ca, Cu and Zn) [41,253]. Moreover, so for phenolic compounds, the major compounds of this fruit include hydrocinnamic acids (mainly caffeic acid and its ester chlorogenic acid) and flavonoids such as naringenin and rutin [41,127]. In detail, in tomatoes, flavonoids are represented by flavanones, including naringenin glycosylated derivatives, and flavonols such as quercetin, rutin and kaempferol glycosylated derivatives [254].

However, as previously described, the content of bioactive compounds in tomatoes is influenced by environmental and genetic factors (cultivar or variety), geographical location, agricultural practices, processing conditions, among others [41,252,253,255,256]. For instance, a study comparing the nutritional quality of local tomatoes grown in two locations in Spain, reported that higher concentrations of caffeic acid, ferulic acid, *p*-coumaric acid, total phenolic content, and beta-carotene were observed in tomatoes grown in Barbastro. On the contrary, tomatoes grown in Montañana showed significantly higher concentrations of chlorogenic acid, ascorbic acid, and vitamin [41].

Importantly, caffeic and chlorogenic acid have *in vitro* antioxidant activity and might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds [257]. Curiously, the antioxidant mechanism of chlorogenic acid is analogous to that of lycopene [252]. Tomato phenolic compound are known for their capacity to act as free radical scavengers of reactive oxygen and nitrogen species [252], in fact, the accumulation of these species in the organism leads to oxidative stress, resulting from an imbalance between the generation and neutralization of reactive species in the cells [258]. In this sense, the antioxidant activity of phenolic acids stems from its ability to chelate transition metals and to scavenge free radicals, having a noteworthy effect on hydroxyl and peroxyl radicals, peroxynitrites and superoxide anions [259].

Furthermore, hydroxycinnamic acid derivatives show bioactivity based on the hydroxylation and methylation patterns of the aromatic ring [260]. Indeed, the free radical scavenging mechanism of hydroxycinnamic acids is analogous to that of flavonoids, attributed to its ability to give a hydroxyl hydrogen and resonance stabilization of the resulting radicals [261]. On the other hand, flavonoids bioactivity

is provided by the hydroxyl groups bound to the ring structures, in fact, flavonoids are able to protect DNA from hydroxyl radical damage, decrease tocopheroxyl radicals, activate or inhibit bioactive enzymes and reduce nitrosative stress [259]. Tomato rutin, also called vitamin P, exhibits antioxidant, anti-inflammatory and anticarcinogenic properties and therefore may reduce the fragility of blood vessels [262]. In addition, tomato quercetin is able to protect DNA from oxidative damage caused by the attack of hydroxyl and superoxide radicals and hydrogen peroxide [261].

Several human interventions have suggested an association between the consumption of tomatoes and tomato-based foods and the prevention of chronic degenerative diseases induced by oxidative stress and inflammation [263]. The regular consumption of tomatoes and tomato-based foods has been related to numerous human health benefits [264]. In this sense, human intervention trials had provided evidence of several benefits of tomato bioactive compounds, either isolated or in combined extracts, including anticarcinogenic [263,265], cardioprotective [266], anticholesterolemic [267], antidiabetic [268], and hepatoprotective [269] effects among other health benefits, mainly due to its antioxidant [252,266] and anti-inflammatory [270] properties. Table 5 lists clinical trials investigating the effect of tomato and tomato product consumption against oxidative stress and carcinogenesis.

The bioavailability of tomato phenolic compounds is crucial to their physiologic effect [127]. Nevertheless, bioaccessibility and bioavailability of tomato compounds is influenced by the way tomatoes are consumed (*i.e.*, raw or processed), which affects their subsequent bioactivity [252]. In this line, the bioavailability of the tomato phenolic compounds can be affected by various food processing steps [252]. For example, a significant increase in plasma levels of chlorogenic acid and naringenin has been found after consuming cooked tomatoes compared to fresh tomatoes [271]. Additionally, a study demonstrated that plasma concentration and urinary excretion of naringenin glucuronide were both significantly higher after the consumption of tomato sauce than raw tomatoes, suggesting that mechanical and thermal treatments during tomato sauce manufacture may help to deliver

potentially bioactive phenolics from the food matrix more effectively than the addition of an oil component, thus increasing their bioavailability [272].

Table 5. Clinical trials investigating the effects of supplementation of tomato and its products on biomarkers of oxidative stress and carcinogenesis.

Study Subjects	Treatment	Major Findings	Reference
Fifty male athletes	75ml of tomato juice	Increased levels of glutathione peroxidase Decreased the levels of TBARS	[273]
Ten healthy women	60 g tomato purée per day for 21 days	Total plasma lycopene concentrations increased Lymphocyte DNA damage after *ex vivo* treatment with hydrogen peroxide decreased	[274]
Thirty-two patients diagnosed by biopsy with prostate carcinoma	Tomato sauce pasta	Increased apoptotic cells in benign prostate hyperplasia and in carcinomas Apoptotic cell death in carcinomas increased significantly with treatment Decreased *Bax* expression in carcinomas	[275]
19 healthy subjects	Spaghetti sauce, tomato juice, or tomato oleoresin per day for 1 week	25% decrease in LDL-TBARS 13% decrease in LDL-CD for all groups versus placebo	[276]
23 healthy volunteers	200 g tomato puree per day for 7 days	Significant decrease in LDL oxidizability in nonsmokers; no effects in smokers	[277]
22 healthy men	330 ml of tomato juice for 2 weeks	No effects on lipid peroxidation in plasma and feces	[278]
17 healthy subjects	Tomato juice per day for 4 weeks	Decreased lipid and protein oxidation	[279]

Continued on next page

Study Subjects	Treatment	Major Findings	Reference
26 healthy subjects	250 ml formulated tomato drink, or placebo drink, per day for 26 days	42% decrease in DNA damage in lymphocytes	280
22 healthy volunteers with different PON1-192 genotypes	330 ml of tomato juice per day for 2 weeks	Decreased lipid peroxidation in volunteers carrying the R-allele of the PON1-192 genotype; decreased plasma malondialdehyde in QR/RR subjects	281
37 healthy nonsmoking postmenopausal women	12 mg of synthetic lycopene, or 4 mg of synthetic lycopene as part of mixed carotenoids, or placebo, per day for 56 days	Decreased endogenous DNA damage in both carotenoid supplemented groups versus baseline and placebo	282

Adapted from Basu and Imrhan [265]. Abbreviations: TBARS, thiobarbituric acid reactive substances; LDL, low-density lipoprotein; CD, conjugated diene; PON1, Paraoxonase 1.

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II.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

Xenohormesis postulates that animals and fungi are able to adapt favorably to changing environmental conditions and increase their chances of survival due to the consumption of stress molecules from other species, such as phenolic compounds. These molecules are used as chemical signals that inform about environmental conditions to mount a preemptive defense response. It is well known that plant-based foods are an excellent source of phenolic compounds. However, phenolic signature of plant-based foods is conditioned by intrinsic and extrinsic factors, especially, plant genetics and cultivar, and environment conditions in which they were grown, including geographical origin of cultivation and farming management systems.

The 21st century is characterized by a significant change in food preferences, emphasizing the increased consumption of plant-based foods with higher nutritional value, health-promoting properties and that come from sustainable agricultural practices. Consequently, this has led to a preference for eating more seasonal and local foods, especially fruits and vegetables rich in phenolic compounds. In fact, phenolic compounds, when ingested, activate the mammalian stress response by generating physiological and metabolic changes that occur with a seasonal rhythmicity, mediated by daylight exposure. Hence, it is reasonable to assume that seasonal consumption of phenol-rich fruits and vegetables could induce different effects on the regulation of physiology and metabolism depending on the time of consumption.

Therefore, **we hypothesize that the seasonal consumption of phenolic-rich plant-based foods, especially sweet cherries, and tomatoes, from different geographical origins generates a differentiated pattern of bioactivity and phenolic bioavailability.**

Thus, the **main objective of this thesis was to evaluate whether geographical origin of cultivation, farming systems and seasonal consumption can condition the bioactivity and bioavailability of phenolic compounds from plant-based foods.**

To achieve this general objective, **specific objectives** were proposed:

1. **To evaluate whether organic farming system conditions the concentration of phenolic compounds and antioxidant capacity in local plant-based foods (Chapter 1).**

To develop this first objective, we conducted a comparative study on the composition of phenolic compounds and antioxidant capacity of local plant-based foods, *i.e.*, cultivated in a specific area of Tarragona, Spain, called Camp de Tarragona, and grown under organic or non-organic farming management systems **[Manuscript 1]**.

2. **To evaluate whether geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis affect their antioxidant capacity against oxidative stress in rats (Chapter 2).**

To meet this second objective, two tasks were proposed:

- **2.1.** To study whether geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks condition its antioxidant capacity against oxidative stress in rats **[Manuscript 2].**
- **2.2.** To evaluate the effects of geographical origin of cultivation and seasonal consumption of tomato cv. Ekstasis on its antioxidant capacity against oxidative stress in rats **[Manuscript 3].**

3. To evaluate whether geographical origin of cultivation and seasonal consumption of tomato cv. Ekstasis affect its phenolic bioavailability in rats (Chapter 3).

To achieve this third objective, three tasks were proposed:

3.1. To determine the phenolic profile of tomato cv. Ekstasis from two geographical origins of cultivation by uHPLC-MS^n [Manuscript 4].

3.2. To evaluate the pharmacokinetic profiles of phenolic compounds after acute consumption of tomato cv. Ekstasis from two geographical origins of cultivation in rats [Manuscript 4].

3.3. To evaluate the effect of seasonal consumption of tomato cv. Ekstasis from two geographical origins of cultivation on its phenolic bioavailability in rats [Manuscript 5].

III.

EXPERIMENTAL DESIGNS

EXPERIMENTAL DESIGNS

Three experimental designs were used to evaluate the main hypothesis and achieve the experimental goals previously stated in this dissertation.

1. **Evaluation of the effects of organic farming system on phenolic profile and on the antioxidant capacity of local plant-based foods.**

To evaluate the effect of farming systems, *i.e.*, ORG or NORG, on phenolic profile and antioxidant capacity, 13 local plant-based foods of the same cultivar grown ORG and NORG in Camp de Tarragona, Spain, were analyzed following the procedure outlined in **Figure 1**. In detail, the edible part of the plant-based foods was freeze-dried and ground, to subsequently determine the total content of phenols, anthocyanins, flavan-3-ols, flavonols as well as their antioxidant capacity by ORAC assay.

Figure 1. Procedure for the determination of the phenolic profile and antioxidant capacity of plant-based foods.

2. **Evaluation of the effects of geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis.**

To address whether the geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis affect their antioxidant capacity against oxidative stress, a 7-week chronic intake study was conducted. Here, sweet cherries from two geographical origins of cultivation, *i.e.*,

sweet cherries from Tarragona, Spain (local sweet cherry, LC) and sweet cherries from Cachapoal, Chile (non-local sweet cherry, NLC); together with tomatoes grown in two locations in Spain: in the northeast, Tarragona (local tomato, LT) and in the southeast, Almería (non-local tomato, NLT) were chosen. As illustrated in **Figure 2**, a total of 120 male Fischer 344 rats from were divided into 3 groups (n = 40) and exposed to simulation of three photoperiods to mimic the seasonal daylight hours (-DH): winter-DH (6 h light/day), spring/autumn-DH (12 h light/day) and summer-DH (18 h light/day), with ad libitum access to water and a standard chow diet for 4 weeks. Rats in each photoperiod were then divided into five groups (n = 8) and orally administered lyophilized LC, NLC, LT and NLT (100 mg /kg body weight (bw)/d) for 7 weeks. The control group received 42 mg of a sugar mixture solution (glucose:fructose, 1:1) per kg bw/d. After this period, animals were sacrificed by decapitation 1 h after the last administration, biological samples were obtained, in which the antioxidant status of the animals and biomarkers of oxidative stress were determined. In addition, the bioavailability and metabolism of tomato-derived phenolic compounds was studied after administration of both tomatoes, *i.e.*, LT and NLT.

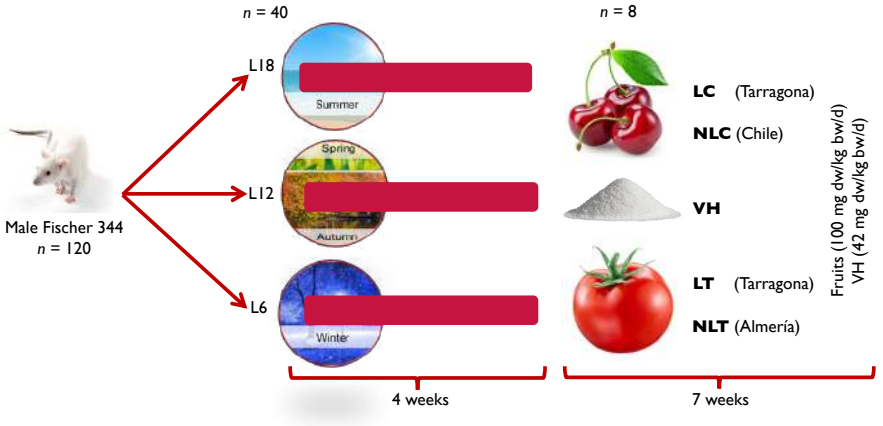

Figure 2. Experimental design to study bioactivity of sweet cherry and tomato, as well as phenolic bioavailability of tomato.

3. Acute serum profiling of the bioavailability of phenolic compounds of tomato cv. Ekstasis from two geographical origins of cultivation.

To study the phenolic pharmacokinetic profiles after consumption of tomatoes cv. Ekstasis from two geographical origins of cultivation, an acute administration study was performed in Wistar rats, as illustrated in **Figure 3**. The same tomato samples from experimental design 2 were used, *i.e.,* tomatoes grown in two locations in Spain: in the northeast, Tarragona (local tomato, LT) and in the southeast, Almería (non-local tomato, NLT). After the acute administration of 3 g of LT or NLT per kg bw, serum samples were collected at different times: 0, 2, 4, 7, 24 and 48 h. Extraction of tomato-derived phenolic metabolites and subsequent uHPLC-MS^n analyses of their phenolic metabolites in rat serum were performed.

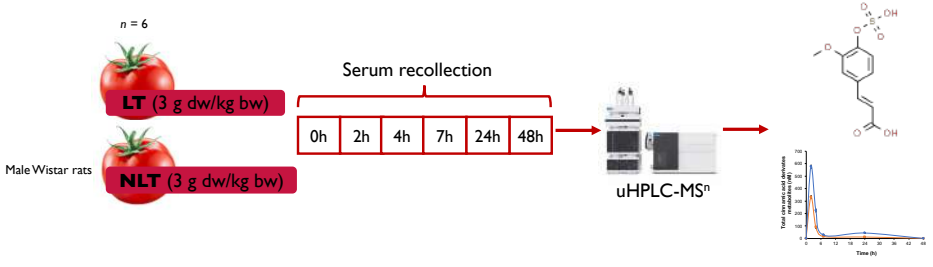

Figure 3. Experimental design to study the bioavailability and metabolism of local and non-local tomatoes.

4. Bioavailability serum profile of phenolic compounds after chronic consumption of tomato cv. Ekstasis from two geographical origins of cultivation.

To evaluate the effects of the geographical origin of cultivation of Ekstasis tomato and its chronic seasonal consumption on its phenolic bioavailability, extraction of tomato-derived phenolic metabolites from serum samples obtained from experimental design 2 and subsequent uHPLC-MS^n analyses were performed.

IV.

RESULTS

CHAPTER I

To evaluate whether organic farming system conditions the concentration of phenolic compounds and the antioxidant capacity in local plant-based foods.

ORGANIC VERSUS NON-ORGANIC PLANT-BASED FOODS - A COMPARATIVE STUDY ON PHENOLIC COMPOUNDS COMPOSITION AND ANTIOXIDANT CAPACITY

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Abstract

Phenolic compounds are phytochemicals produced by plants as stress response. Their synthesis can be modulated by organic (ORG) or non-organic (NORG) farming systems in which they are grown. To shed light on this issue, thirteen plant-based foods cultivated in ORG and NORG systems were compared in terms of antioxidant capacity, total content of phenolics, anthocyanins, flavan-3-ols and flavonols. The results showed that NORG fruits tended to have higher phenolic compounds content, whereas ORG fruits had more antioxidant capacity. NORG legume stood out for having higher values from all the parameters analyzed in comparison to its ORG equivalent. ORG nuts showed more flavan-3-ols and flavonols than their NORG counterparts, nonetheless, tended to be less antioxidant. ORG vegetables displayed higher phenolics and anthocyanins which reflected in higher antioxidant capacity than NORG ones. These findings suggest that farming systems differentially modulate phenolic compound composition and antioxidant capacity based on the plant species studied.

Keywords

Agricultural, farming, fruit, nuts, polyphenols, vegetables

Abbreviations

Cy3R, cyanidin-3-*O*-rutinoside; DMACA, *p*-dimethylaminocinnamaldehyde; fw, fresh weight; GA, Gallic acid; NORG, non-organic farming; ORAC, oxygen radical absorbance capacity; ORG, organic farming; PCC, phenolic compounds content; TAC, total anthocyanins content; TFaC, total flavan-3-ols content; TFoC, total flavonols content; TPC, total phenolic content.

1. Introduction

Studies show that consumers are now inclined to choose fruits and vegetables that are healthier and produced by a more sustainable and environmentally friendly agricultural system [1]. According to the Council of the European Union, organic farming respects rules based on fundamental principles, such as, prohibition of the use of genetically modified organisms (GMOs); prohibition on the use of ionizing radiation and limitation of the use of artificial fertilizers, herbicides and pesticides [2]. Recent production and market trends show how important organic products have become over the last decade. In the last ten years, organic farming has risen by more than 70% indicating a very dynamic and quickly rising sector [3]. This increase in the production of plant-based organic foods can be attributed to the consumer's preference for these products, possibly characterized by better taste and often produced in areas close to the place of consumption. In addition, it is supposed that they contain a higher content of beneficial and health-promoting substances [4]. However, when choosing between organic and non-organic products, consumers have doubts about the nutritional quality [4]. In this sense, a diet rich in fruits and vegetables has been associated with reducing the incidence of chronic diseases, due to the bioactive compounds they contain, notably phenolic compounds [5]. The phenolic compounds are the most predominant bioactive compounds in the diet, reaching values of up to 1 g/day [5]. Moreover, studies have provided evidence that consumption of phenol-rich foods generates protective effects against chronic diseases, including cardiovascular diseases, neurodegeneration and cancer [6]. Structurally, phenolic compounds can be classified as flavonoids and nonflavonoids; in turn, the main subclasses of flavonoids are anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols and isoflavones, while among the non-flavonoids of nutritional relevance outstands phenolic acids [6]. In nature, the phenolic compounds can be found in a wide range of dietary components and medicinal plants such as tea, fruits, and vegetables. In particular, the profile of phenolic compounds present in plants differs among species. In this regard, citrus fruits are rich in flavanones and flavones, high-colored fruits such as cherries, grapes, and berries are rich in anthocyanins and coffee and tea contain high

amounts of flavanols and flavan-3-ols, while yellow and red onions are especially rich source of flavonols; additionally, dietary sources of phenolic acids include fruits such as apricots, cherries and peaches, as well as other vegetal sources such as eggplant, cereals and spinaches [6–9]. The synthesis of phenolic compounds in plants can be influenced by environmental conditions such as water availability, soil fertilization and the mineral content of the soil. In particular, the phenolic profile of plant-based foods is known to differ according to systems of farm management, *i.e.*, organic vs. non organic [10]. Although it has not yet been proven that organic products contain the highest content of nutrients, they should certainly contain the lowest content of pesticides and substances harmful to health, such as nitrates [4]. Information supporting both agricultural practices can be obtained from the literature, however, the results of the research are inconclusive, and there is no enough evidence to affirm the differences in nutritional value between organically and non-organically grown plant-based foods. To shed light on this issue, the aim of this study was to evaluate the effects of organic (ORG) or non-organic (NORG) farming management systems on the antioxidant capacity and phenolic compounds content in plant-based foods grown in Tarragona - Spain.

2. Materials and methods

2.1. Chemicals and reagents

(+)-catechin, fluorescein, Folin-Ciocalteu reagent, gallic acid, *p*-dimethylaminocinnamaldehyde (DMACA), quercetin and trolox were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Cyanidin-3-*O*-rutinoside was acquired from PhytoLab (Vestenbergsgreuth, Germany). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Acros Organics (Geel, Belgium). Standard compounds were individually dissolved in acetone/Milli-Q water/acetic acid (70/29.5/0.5; *v/v/v*) and stored at −20 °C. All standard stock solutions were newly prepared every three months.

2.2. Plant-based foods samples

Samples of fruits: olive (*Olea europaea* L. cv. Arbequina), orange (*Citrus sinensis* L. cv. Navel), sweet cherry (*Prunus avium* L. cv. Burlat) and tomato (*Lycopersicon*

esculentum Mill. cv. Ekstasis and Tores); vegetables: onion (*Allium cepa* L. var. cepa cv. Figueres), sweet pepper (*Capsicum annuum* L. cv. Italia) and swiss chard (*Beta vulgaris* var. flavescens cv. Delta); nuts: almond (*Prunus dulcis* cv. Marcona), hazelnut (*Corylus* L. cv. Castanyera and Negreta) and walnut (*Juglans regia* L. cv. Serr); and legume: carob pods (*Ceratonia siliqua* cv. Banya de cabra) were harvested between Juny 2018 to February 2019 and donated by farmers and agricultural companies from Camp de Tarragona - Spain. Cultivars used in this study were selected by both organically (ORG) and non-organically (NORG) grown varieties available. According to the farmers, the plant-based foods were grown in strict compliance with the rules governing each of the cultivation systems. Approximately 2 kg of each plant-based food was randomly sampled. The samples were washed, and the edible part was separated, chopped, frozen in liquid nitrogen and ground. Then, the samples were freeze-dried for one week in a Telstar LyoQuest freeze-dryer (Thermo Fisher Scientific, Madrid, Spain). The powders were kept at room temperature and protected from light and humidity until use. The moisture content of the fresh samples was determined by the weight loss after heating (98 °C, 24 h) [11].

2.3. Extraction and quantification of phenolic compounds

2.3.1. Extraction of phenolic compounds

The extraction of phenolic compounds was carried out in line with the method described by Iglesias-Carres *et al.* [12]. Although this method was originally optimized specifically for grapes, unpublished results in which we compared several methods confirmed that this was the most suitable extraction methodology, since most of the families of phenolic compounds present in plant-based foods are extracted. Briefly, the extraction parameters were 80 mL/g, 65% methanol (1% formic acid), 72 °C and 100 min under agitation of 500 rpm.

2.3.2. Total phenolic content

Total phenolic content (TPC) of extracts was measured by the Folin-Ciocalteu method adapted from Nenadis *et al.* [13]. Briefly, 10 µL of the extract, 50 µL of Folin-Ciocalteu reagent and 500 µL of Milli-Q water were mixed and left in the dark for 3 minutes. Then, 100 µL of Na_2CO_3 (25%) was added and diluted to

1 mL with Milli-Q water. After 1 hour of incubation in dark, absorbance was measured at 725 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). Gallic acid (GA) was used as standard. The results are reported as mg GA Eq/100 g fresh weight (fw).

2.3.3. Total anthocyanins content

The total anthocyanins content (TAC) was assessed by the pH differential method [14]. Extracts were diluted with sodium acetate buffer (0.4 M, pH 4.5) and potassium chloride buffer (0.025 M, pH 1.0). Next, absorbance was read at 515 nm and 700 nm using an Eon BioTek spectrophotometer. TAC is expressed as milligrams of cyanidin-3-*O*-rutinoside equivalents per 100 grams of fresh weight (mg Cy3R Eq/100 g fw).

2.3.4. Total flavan-3-ols content

The total flavan-3-ols content (TFaC) of extracts was estimated by the DMACA method 15. Concisely, 100 µL of extract samples were mixed with 500 µL of DMACA solution (0.1% 1N HCl in methanol). After 10 min of incubation in dark, absorbance was assessed at 640 nm using an Eon BioTek spectrophotometer. Catechin concentrations were used to construct a calibration curve and TFaC values are shown as mg catechin Eq/100 g fw.

2.3.5. Total flavonols content

The total flavonols content (TFoC) was evaluated with the method described by Cacace *et al.* [16]. In brief, 250 µL of extract with 250 µL of 0.1% HCl in ethanol 95% and 4.55 mL of 2% HCl were mixed and could react for 15 minutes. Spectrophotometric measurements at 360 nm were performed. TFoC levels, which were calculated based on the standard curve of quercetin, are reported as mg quercetin Eq/100 g fw.

2.4. Antioxidant capacity

The antioxidant capacity of plant-based foods samples was determined by oxygen radical absorbance capacity (ORAC) assay described by Huang *et al.* [17]. Briefly, 25 µL of extract of samples were mixed with 25 µL of 73 mM 2,2′-Azobis(2-methylpropionamidine) dihydrochloride and 150 µL of 59.8 nM

fluorescein. The fluorescence intensity was assessed every 2 min for 120 min using an FLx800multi-detection microplate reader (Biotek, Winooski, VT, USA; λex = 485 nm and λem = 528 nm). Trolox concentrations were used to construct a calibration curve and the results are expressed as μmol Trolox Eq/ 100 g fw.

2.5. Statistical analysis

Student's *t*-test (SPSS, SPSS Inc., Chicago, IL, USA) was applied to assess any differences ($p < 0.05$) in the PCC results and ORAC values between ORG and NORG plant-based foods. Results are expressed as mean ± standard deviation (SD). In addition, to holistically evaluate the impact of the agricultural system, a chemometric analysis, specifically, principal component analysis (PCA) based on normalized concentrations was performed using the corresponding functions of MetaboAnalyst 5.0 software.

3. Results

Several authors have described phenolic compounds are naturally occurring and are synthesized by plants for their protection against aggressions. Thus the content depends on different intrinsic and extrinsic factors such as plant genetics and cultivar, soil composition and growing conditions, maturity state and post-harvest conditions, among others [5]. In addition, their abundance ranges at different levels in the same plant. Thus, every plant food contains a unique combination of several hundred of phenolic compounds [18]. In particular, the phenolic profile of fruits and vegetables is known to vary depending on farming practices, *i.e.*, organic or non-organic [10]. In fact, fruits, vegetables, nuts and legumes depending on the farming systems in which they are grown, *i.e.*, organic or non-organic, respond differently to stress factors, which could result in the differentiated synthesis of various phenolic compounds produced by the plant, as well as their particular distribution according to the way the plant uses them [4]. Although the use of organic agricultural practices would suggest that the plant is less protected to insects and other stressors, and therefore it is more stressed, leading to a higher induction of secondary metabolites, current studies do not make clear that an organic crop generates more phenolic compounds than a

non-organic one [5,10]. The literature reveals mixed outcomes in terms of phenolic composition and antioxidant capacity of organic and non-organic plant-based foods, which differ according to the bioactive compound studied and the type of food [5,10]. For this reason, in this study, we have selected thirteen plant-based foods from the same cultivar, which have been organically and non-organically cultivated in Tarragona - Spain. The total content of anthocyanins, flavan-3-ols, flavonols, phenolics and antioxidant capacity were determined in each of the plant-based foods to elucidate whether non-organic farming (NORG) generates the same influence on the synthesis of these compounds and antioxidant activity as organic farming (ORG).

3.1. Phenolic compounds content in organic and non-organic plant-based foods

The phenolic compounds content (PCC) in ORG and NORG plant-based foods can be observed in **Figure 1**, which shows that the thirteen plant-based foods from two farming systems evaluated in this study had a specific phenolic signature. Regarding the TPC (**Figure 1a**), there is a pattern of higher TPC in NORG plant-based foods, with higher values in 6 of the 9 samples. **Figure 1b** shows that the total anthocyanins content in plant-based foods was detected in 6 of the 13 samples, of which 67% varied statistically, although no trend in favor of either farming type could be stated. As expected, these compounds were not detected in any of the nuts or carob pod under analysis and in vegetables it was only detected in organic sweet pepper. Moreover, 54% of the plant-based foods showed differences on total flavan-3-ols content (TFaC) between ORG and NORG (**Figure 1c**), while the rest exhibited similar contents. Among those that differed statistically, 4 of 7 plant-based foods with the highest flavan-3-ols content were found to be NORG. Finally, the total flavonols content (TFoC) between ORG and NORG was statistically different in 9 of the 13 plant-based foods evaluated (**Figure 1d**). Here, 56% of plant-based foods with the highest TFoC values were found to be ORG.

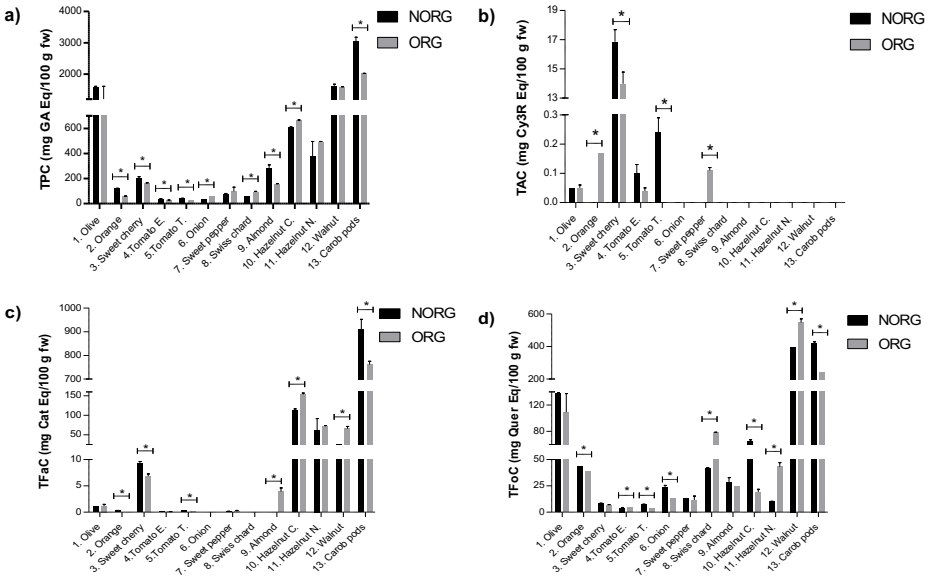

Figure 1. Phenolic compounds content in organic and non-organic plant-based foods. Samples analyzed per plant-based food (n = 3), analytical replicates per samples (qc = 3). Values are expressed as mean ± standard deviation (SD). * Statistical difference between farming practices by Student's t-test ($p < 0.05$). Abbreviations: Cy3R, cyanidin-3-*O*-rutinoside; Eq, equivalent; GA, gallic acid; Hazelnut C, hazelnut cv. Castanyera; Hazelnut N, hazelnut cv. Negreta; NORG, non-organic farming; ORG, organic farming; TAC, total anthocyanins content; TFaC, total flavan-3-ols content; TFoC, total flavonols content; Tomato E, tomato cv. Ekstasis; Tomato T, tomato cv. Tores; TPC, total phenolic content.

The data in **Figure 2** show the distribution of the variability of PCC (*i.e.*, TPC, TAC, TFaC and TFoC) from ORG plant-based foods compared to its NORG counterparts. It was found that 44% of the plant foods showed no variation between ORG and NORG. Among the PPCs that varied statistically between the ORG and NORG, 33% of the differences in TPC favored organically grown plant-based foods. The same trend was observed in 50% of the products in TAC, 48% in TFaC and 44% in TFoC. In brief, 41% of ORG plant-based foods were more abundant in the PCC. However, when considering all the differences as a whole, an average percentage difference in the abundance of organic samples was calculated for each PCC in question showing that TPC is -6%; TAC is +6%; TFaC is +7%; and TFoC is +20%. Interestingly, the organic farming was, on average, more abundant in all the phenolic fractions determined, except TPC.

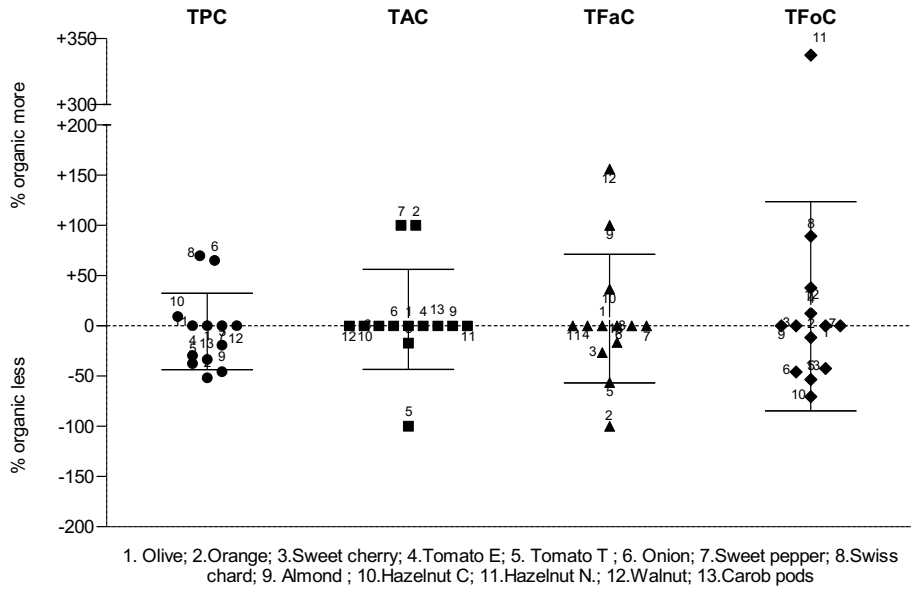

Figure 2. Phenolic compounds content in organic and non-organic plant-based foods. Each point representing the % variability in PPC of the same plant-based food and cultivar, which is labelled with a number. That is, plus and minus signs refer to more or less abundance of a PPC from an organic sample with respect to non-organic sample as the baseline for comparison. Abbreviations: Cy3R, cyanidin-3-*O*-rutinoside; Eq, equivalent; GA, gallic acid; Hazelnut C, hazelnut cv. Castanyera; Hazelnut N, hazelnut cv. Negreta; NORG, non-organic farming; ORG, organic farming; PPC, phenolic compounds content; TAC, total anthocyanins content; TFaC, total flavan-3-ols content; TFoC, total flavonols content; Tomato E, tomato cv. Ekstasis; Tomato T, tomato cv. Tores; TPC, total phenolic content.

3.1.1. Phenolic compounds content in organic and non-organic fruits

There is no statistical differences in PCC between ORG and NORG olives. Interestingly, ORG orange stood out for having 52% less abundance of TPC than NORG ones and this same trend continued for TFaC and TFoC, whereas the highest TAC was exhibited by the ORG oranges (0.17 mg Cy3R Eq/100 g fw) as NORG oranges lacked this family of compounds. The sweet cherries presented ~1.2-fold higher TPC, TAC and TFaC in the NORG samples, while showing equal proportions of TFoC between both types of farming. The tomatoes Ekstasis exhibited similar TAC and TFaC in both NORG and ORG, 1.4-fold higher TPC in NORG samples, but 1.1-fold higher TFoC was observed in ORG; otherwise, the tomatoes Tores stood out for presenting the highest PCCs in the

NORG samples than in the ORGs. Importantly, overall, there was a tendency to have higher TPC in NORG fruits (olive, orange, sweet cherry, and tomatoes), with values being higher in 4 of the 5 fruits analyzed. Moreover, TFaC displayed a tendency favoring NORG fruits, e.g., orange, sweet cherry, and tomato cv. Tores, where all statistical differences favored this type of farming.

3.1.2. Phenolic compounds content in organic and non-organic vegetables

ORG onions showed higher TPC (60.60 mg GA Eq/100 g fw) than their counterparts (36.68 mg GA Eq/100 g fw), but 1.8-fold lower TFoC. The sweet peppers showed equal amounts of total phenolics, flavan-3-ols and flavonols between ORG and NORG, and TAC was higher in ORG (0.11 mg Cy3R Eq/100 g fw) while its analogous NORG lacked the compound. The swiss chard samples did not show anthocyanins and flavan-3-ols, but 1.7-fold higher TPC and 1.1-fold higher TFoC were observed in ORGs than in the NORG equivalents. An opposite pattern compared to fruits was observed for vegetables (onion, sweet pepper and swiss chard) where the ORG samples were highlighted as having the highest contents.

3.1.3. Phenolic compounds content in organic and non-organic nuts and legumes

As far as nuts are concerned, the almonds showed higher TPC in NORGs (1.8-fold), higher TFaC in ORGs (3.92 mg catechin Eq/100 g fw), similar TFoC between NORGs and ORGs, however, no anthocyanins were found in either ORG or NORG. The hazelnuts Castanyera had higher TPC and TFaC in ORG (665.62 mg GA Eq/100 g fw and 153.50 mg catechin Eq/100 g fw, respectively), but higher TFoC in NORG (64.65 mg quercetin Eq/100 g fw). On the other hand, the hazelnuts Negreta had similar TPC and TFaC between both farming practices and higher TFoC in ORG (43.14 mg quercetin Eq/100 g fw). The walnuts had more flavan-3-ols and flavonols in ORG (65.54 mg catechin Eq/100 g fw y 548.24 mg quercetin Eq/100 g fw, respectively) and the same TPC between ORG and NORG. In general, the ORG nuts were characterized by the highest values of flavan-3-ols.

Finally, the legume, carob pods had 1.5-fold higher TPC, 1.2 -fold higher TFaC and 1.7-fold higher TFoC in NORG than in the ORG equivalent, and both carob pods lacked anthocyanins.

Among all plant-based foods studied, swiss chard and onion seem to be strongly affected by alteration in farming system, in fact, ORG swiss chard and onion exhibited 70% and 65%, more TPC than their NORG counterparts, respectively. In terms of anthocyanins content, ORG samples of orange and sweet pepper stood out for being 100% more abundant than their NORG ones, while ORG tomato cv. Tores was 100% less abundant. Moreover, the greatest variation in TFaC was displayed in walnut, here the ORG sample was 156% more abundant than its NORG counterpart; followed by almond and orange grown organically, which proved to be 100% more abundant and 100% less abundant, respectively. Moreover, hazelnut cv. Negreta farmed organically was characterized by the notable variability in the content of flavonols (TFoC), with 338% more abundance. Interestingly the lowest abundance (-70%) was observed in ORG hazelnut cv. Castanyera. Furthermore, it is important to note that, trend in the abundance of TAC and TFoC in organically grown tomato cultivars was opposite among the same species, resulting in more abundance in one cultivar and less abundance in another. The same pattern was observed for ORG hazelnut cultivars in TFoC abundance. It is important to highlight that the ORG legume (carob pod) exhibited lower abundance of all the PCCs, except for TAC, since this compound was not found in any of the sample types.

3.2. Antioxidant capacity of organic and non-organic plant-based foods

The antioxidant capacity of ORG and NORG plant-based foods determined by ORAC assay is displayed in Table 1. The ORAC values ranged from 336.0 to 6565.5 μmol Trolox Eq/100 g fw in ORG samples, while in NORG samples ranged from 338.0 to 5627.8 μmol Trolox Eq/100 g fw. Significant differences of the effects of farming systems on the antioxidant capacity were evident in 8 of the 13 samples studied, although a clear tendency favoring one of them was not observed, with half higher in the ORG samples and half in the NORG samples. Indeed, almond, carob pods, hazelnut H. and tomato T. grown non-organically

had 1.2-fold higher ORAC values than their ORG equivalents, while ORG hazelnut C. showed 1.1-fold higher ORAC values than its NORG analogous.

Table 1. Antioxidant capacity of organic and non-organic plant-based foods.

Plant-based foods		ORAC (μmol Trolox Eq/100 g fw)		*p* value
		ORG	NORG	
Fruits	1. Olive	2906.1 ± 525.7	3479.8 ± 9.3	0.09
	2. Orange	994.5 ± 69.7	860.3 ± 15.5	0.03
	3. Sweet cherry	1016.2 ± 3.5	843.8 ± 79.2	0.02
	4. Tomato E.	338.0 ± 7.2	336.0 ± 1.8	0.73
	5. Tomato T.	372.2 ± 9.7	446.5 ± 1.9	0.00
Vegetables	6. Onion	691.3 ± 39.6	686.5 ± 0.1	0.88
	7. Sweet pepper	430.4 ± 42.1	410.8 ± 32.6	0.47
	8. Swiss chard	584.0 ± 0.5	447.1 ± 0.6	0.00
Nuts	9. Almond	4093.6 ± 51.1	4827.6 ± 198.8	0.00
	10. Hazelnut C.	5349.2 ± 112.4	4728.1 ± 99.6	0.00
	11. Hazelnut N.	4194.7 ± 195.6	5095.4 ± 559.7	0.03
	12. Walnut	4757.9 ± 59.7	4833.3 ± 211.2	0.58
Legume	13. Carob pods	5627.8 ± 153.7	6565.5 ± 230.8	0.00

Samples analyzed per plant-based food (n = 3). Values are expressed as mean ± standard deviation (SD). Statistical difference between agricultural practices by Student's t-test ($p < 0.05$). Abbreviations: Hazelnut C, hazelnut cv. Castanyera; Hazelnut N, hazelnut cv. Negreta; NORG, non-organic farming; ORAC, oxygen radical absorbance capacity; ORG, organic farming; Tomato E, tomato cv. Ekstasis; Tomato T, tomato cv. Tores.

Similarly, swiss chard had 1.3-fold higher ORAC levels than its NORG counterpart, and organically grown oranges and sweet cherry had ORAC values 1.2-fold higher than their NORG equivalents. Carob pods were the plant-based food with the highest ORAC value among ORG (6565.5 μmol Trolox Eq/100 g fw) and NORG (5627.8 μmol Trolox Eq/100 g fw). On the other hand, Ekstasis tomato showed the lowest ORAC levels among samples of both types of farming (ORG, 336.0 and NORG, 338.0 μmol Trolox Eq/100 g fw). The data in **Figure 3** represent the distribution of variability of the significant ORAC values from ORG plant-based foods compared to NORG.

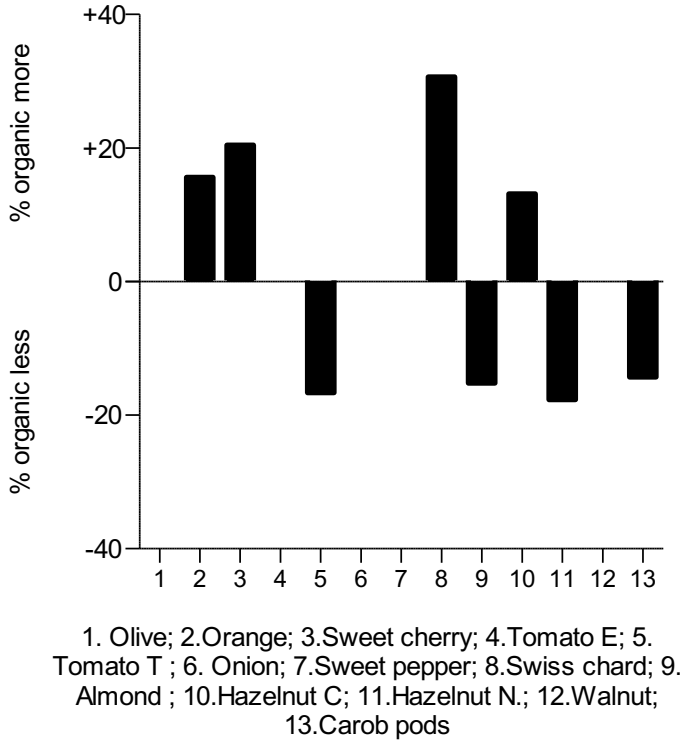

Figure 3. Distribution of variability of ORAC values of organic plant-based foods compared to the non-organic ones. Each point representing the % variability in ORAC values of the same plant-based food and cultivar, which is labelled with a number. That is, plus and minus signs refer to more or less abundance of an ORAC value from an organic sample with respect to non-organic sample as the baseline for comparison. Abbreviations: Hazelnut C, hazelnut cv. Castanyera; Hazelnut N, hazelnut cv. Negreta; ORAC, oxygen radical absorbance capacity; NORG, non-organic farming; ORG, organic farming; PPC, phenolic compounds content; Tomato E, tomato cv. Ekstasis; Tomato T, tomato cv. Tores.

3.3. Multivariate data analysis

Simultaneously all variables, including TPC, TAC, TFaC, TFoC and ORAC have been considered to perform the unsupervised method called principal component analysis (PCA), after plant-based foods data were preprocessed by autoscaling. PCA was used to find the directions that best explain the variance in plant-based foods without referring to class labels. **Figure 4** illustrates the PCA results between organically grown (ORG) and non-organically grown (NORG) plant-based food groups, including fruits (**Figure 4a**), vegetables (**Figure 4b**), and nuts and legume (**Figure 4c**), in 3D score plots of the first three principal

components (PCs), and PCA biplot between PC1 and PC2, showing the direction of the impact caused by the different parameters under study.

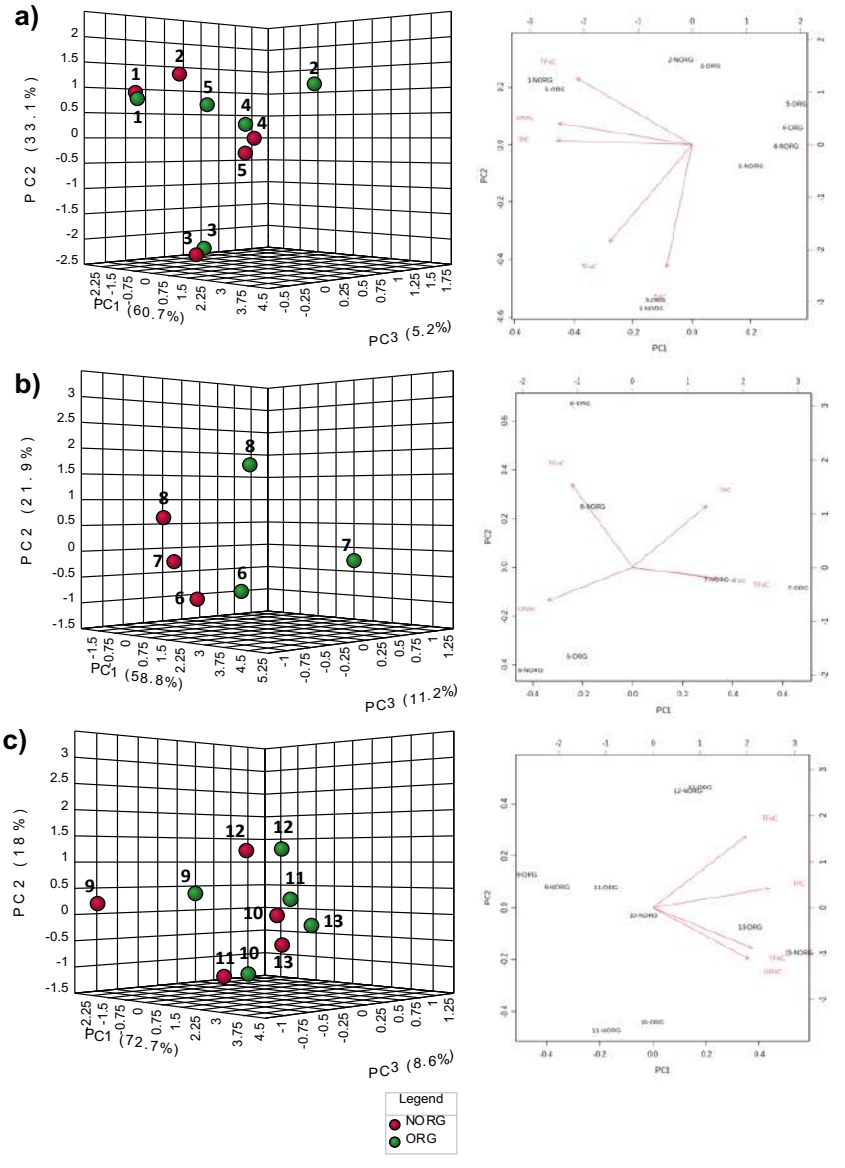

Figure 4. Patterns of separation between organically (ORG) and non-organically (NORG) grown plant-based foods across the three groups of samples**.** The left column corresponds to PCA score plot and the right column corresponds to PCA biplot between PC1 and PC2. a) PCA results of fruits samples. b) PCA results of vegetables samples. c) PCA results of nuts and legume samples. The number of data points corresponds to each sample: 1. Olive, 2. Orange, 3. Sweet cherry, 4. Tomato E., 5. Tomato T., 6. Onion, 7. Sweet pepper, 8. Swiss chard, 9. Almond, 10. Hazelnut C., 11. Hazelnut N., 12. Walnut, 13. Carob pods. Abbreviations: PCA, Principal Component Analysis; PC, Principal Component.

The PCA score plot of the fruits comparison presented an accumulative variance explained of 99% of the first three PCs, with no clear patterns of separation. PCA results showed that the principal component (PC1; 60.7%) separates the data mainly due to TAC followed by TFaC levels, while PC2 (33.1%) separates the data with respect to TPC and ORAC, and PC3 (5.2%) shares the same separating variables as PC2 (Figure 4a). Here, tomato T, *i.e.*, sample 5, was the fruit that stands out by presenting the greatest separation between those from organic farming (its distances to the origin are 1.9, 0.6, -0.6) and those from non-organic farming (its distances to the origin are 1.1, -0.3, 0.1). As stated previously, the ORG tomato T. varied by -100% in terms of TAC and -56% in terms of TFaC with respect to its NORG counterpart. Suggesting that anthocyanins content generates the greatest differentiation between organic and non-organic fruits. Similarly, the orange, *i.e.*, sample 2, was another fruit that stood out for presenting a large separation between those from organic and non-organic farming. In fact, as described above, this fruit varied significantly in all the parameters studied between the two types of farming.

On the other hand, the PCA score plot of the comparison between ORG and NORG vegetables showed the clear patterns of separation for all samples, as illustrated in Figure 4b. The first three PCs accounted for 91.9% of the cumulative variance explained, with principal component 1 (PC1; 58.8% of the variance) separating the data mainly by ORAC levels, followed by TFaC, while PC2 (21.9% of the variance) separates the data by ORAC levels, followed by flavan-3-ols content; and PC3 (11.2% of the variance) divides the vegetables mainly by ORAC and flavonols contents. This suggests that the most important factor in differentiating organically or non-organically grown vegetables is the ORAC value. It was observed that the data points corresponding to sample 8, *i.e.*, swiss chard, were the most distant from each other, namely ORG (-0.9, 1.7, 0.5 as distances to the origin) and NORG (-0.7, 0.6, -0.6 as distances to the origin), showing a strong impact of the farming system under which, it was grown, which in fact was the only vegetable that varied in ORAC levels between the two types of farming systems. In addition, the PC3 scores (11.2%) of the ORG and NORG vegetables were positive and negative, respectively. This result suggests that the PC3 score is

positively related to the ORG farming system. In this case, ORG vegetables that have large positive factor loadings in PC3 tend to increase TPC, TAC, TFoC and ORAC, and those with negative factor loadings tend to decrease TFaC in this type of cultivation.

The PCA score plot of the comparison between ORG and NORG nuts and legume showed that the principal component (PC1; 72.7%) separates the data mainly due to ORAC values and flavan-3-ols content, whereas PC2 (18.0%) separates the data mainly with respect to flavonols content and ORAC values, and PC3 (8.6% of the variance) divides nuts and legume mainly by flavan-3-ols and flavonols content (**Figure 4c**), with no clear pattern of separation. Here, hazelnut N., i.e., sample 11, which stands out by presenting the greatest separation between those from organic farming (its distances to the origin are -0.9, 0.2, 0.9) and those from non-organic farming (its distances to the origin are -0.9, -1.3, 0.0). In fact, as mentioned above, the ORG hazelnut N. varied by +338% in flavonols content and -18% in terms of ORAC with respect to its NORG counterpart. It appears that ORAC is the factor that generates the greatest differentiation between the ORG and NORG samples. It is important to note that in this group there was 1 feature with a constant or unique value in all the samples (anthocyanins were not detected) that was not taken into consideration for the PCA.

The PCs are determined by the contribution of all plant-based foods. The impact of a distinct plant-based food on the principal component is captured in its loading value. The plots of the PCA loadings (**Figures 4a, 4b, 4c**) display the contribution of single plant-based food to the global separation seen between samples. It becomes obvious from these plots that the differences are mainly driven by a small number of plant-based foods. Interestingly, the shared plant-based foods with the highest loading plot scores are almost identical between the three groups being TAC and TFaC for fruits group, ORAC and TFaC for vegetables group, and ORAC and TFaC for nuts and legume, are influencing PC1 in plant-based foods families.

4. Discussion

The results found indicate that most of the plant-based foods analyzed presented significant differences in total phenolic content, with a trend in favor of non-organic farming. The same trend was found in the study by Letaief *et al.* [19] in orange and orange juice. Similarly, the results obtained by Barrett *et al.* [20] demonstrated that tomato juice prepared from organically produced tomatoes on 4 farms was significantly lower in total phenolics content than conventional tomatoes, and these results were significantly different among specific growers. On the contrary, Tarozzi *et al.* [21] reported that organic red oranges cv. Tarocco have a higher phenolic content than integrated red oranges. In this vein, it has also been reported that tomatoes cv. Redondo were richer in total phenolics (+24%) than conventional ones [22]. Similarly, a study by Faller and Fialho [5] reported that organic fruits tend to have higher hydrolysable polyphenol contents than conventional ones. These different results may be because the fruits analyzed differ between their study and ours, only one coincides. In addition, it is known that farming practices gives rise to distinct patterns of phenolic profiles according to the plant species studied, being some species more susceptible to the induction of phenolic compounds synthesis than others [5]. This can be verified in the current research, where non-organic fruits exhibited higher total phenolic content, while in the case of vegetables it was exhibited by the organic ones.

There is scientific evidence of a correlation between a soil fertilization-dependent effect on flavonoid content [10]. In this way, more than half of the non-organically grown plant-based foods studied were notable for having higher total flavan-3-ols and flavonols contents than their organic counterparts. However, overall, the ORG samples exhibited 7% and 20% more abundance of flavan-3-ols and flavonols, respectively, than NORG ones. Thus, nitrogen provision can be based on inorganic and/or organic soil fertilization, and the reduction is theoretically possible due to the biochemical pathway that drives the synthesis of flavonoids [10]. Similarly, Lima *et al.* [18] informed that total content of flavonoids was higher in most of the analyzed conventional vegetables (zucchini squash, banana, potato, eggplant, orange, lime, mango, passionfruit and radish) possibly because of farming practice adopted. These findings are in line with the results obtained on

plums, where flavonols were considerably higher in conventionally farmed fruits [23]. However, differences in flavonols content because of varietal differences cannot be excluded, because flavonoid contents may show wide variations in different varieties of fruits and vegetables [4]. Indeed, in our study, non-organic tomatoes cv. Ekstasis and Tores stood out for their higher phenolic content than their organic counterparts, while Caris-Veyrat *et al.* [24] reported that organic tomatoes from three different varieties (Félicia, Izabella and Paola) had a higher polyphenol content than the conventional counterparts. About the total anthocyanins content, although no clear trend was observed in plant-based foods when comparing the two farming systems, on average, the organic samples proved to be 6% more abundant in anthocyanins than the non-organic samples. In this same way, other studies also identified no statistical differences in the content of phenolic compounds in relation to the type of farming system, such as the study comparing two eggplant cultivars [25] and another comparing strawberries [26].

Closely linked to phenolic content is the variation in antioxidant capacity [1]. It appears that, depending on the higher abundance of phenolic content in ORG fruits and vegetables, antioxidant compounds tend to increase in them. As it has been reported, synthesis of these compounds is enhanced in response to phytopathogenic infections, in accordance with their proposed role in plant defense mechanisms [4]. Infected plant tissue and resistant tissue have been identified as being characterized by a general change in metabolic pattern that includes the activation of phenol-oxidizing enzymes and peroxidases [27]. In this line, Wang and Millner [28] concluded that organic farming lead to an improvement in antioxidant activity in blueberries due to higher content of phenolic acids and anthocyanins compared to a non-organic farming. Likewise, Stracke *et al.* [29], found that organic apples exhibited on average 15% more antioxidant content than non-organic fruits. Most authors agrees that the type of farming system can influence the phytochemical composition of the plants and therefore, by implication, the level of antioxidant activity [4]. It has been described that organic farming has potential to influence the synthesis of antioxidants, increasing their levels and therefore increasing antioxidant capacity, due to the fact that this

farming system does not supply as much nitrogen as conventional fertilizers and also causes more stress to the plants than non-organic farming [4,29,30]. In addition, human and animal intervention trials have provided evidence of antioxidant effects of various phenol-rich organic foods. In fact, an Italian study aimed to compare the effects of an organic diet versus a conventional Mediterranean diet administered to 10 healthy men for 2 weeks. After consuming 14 days of the organic Mediterranean diet, a significant increase (21%) in the total antioxidant capacity of human plasma was observed over the conventional diet. In the same study, antioxidant activity was measured in several fruits and vegetables, as well as in wine and milk. In most of these products, the activity was higher in the organic product [31]. Another study was a fully controlled dietary intervention with organic or conventional diets given to 16 male and female volunteers, with the aim of comparing the intake and excretion of selected flavonoids, as well as plasma levels of known oxidative defense markers in both groups of volunteers. The organic diet resulted in higher urinary excretion of quercetin and kaempferol, while most markers of antioxidant defense did not differ between diets [32]. Moreover, several animal dietary intervention studies have been carried out investigating the health effects of organic vs conventional feed, such as the study of Rodrigues *et al.* [33] who observed different effects on the activity of antioxidant enzymes in the cerebellum of rats that consumed organic or non-organic juices in a chemical rat model of epilepsy. Lauridsen *et al.* [34] observed a higher reactivity of the immune system in the organically fed rats, indicated by the level of IgG in serum, as well as a lower level of fatty tissue and a more relaxed behavior. According to a study performed in the Netherlands, chickens fed an organic diet had lower body weights, higher immune reactivity and stronger catch-up growth after a challenge [35]. The results described above could suggest that organic plant-based food increases the resilience of living organisms. To confirm this, studies of effects on specific health markers are needed.

As mentioned above, the phenolic compounds content is known to be highly dependent on the cultivar and farming conditions [10]. In fact, among the plant-based foods studied, carob pods, both NORG and ORG, stood out for presenting the highest values of TPC, TFaC and ORAC. Additionally, NORG

carob pods also exhibited the highest TFoC, coinciding with what has previously been reported that carob pods contain considerable amounts of phenolic compounds with remarkable antioxidant properties [36]. Also, among the plant-based foods analyzed, orange seems to be more affected by alteration in farming system, since statistical differences were revealed between organic and non-organic oranges in all the determinations, without any clear trend in support of either farming system. But remarkably, the ORG orange showed higher anthocyanins content than the NORG orange, and the same pattern was evident in ORAC values. These results confirm that phenolic molecules may be important antioxidant components in explaining the observed activity. In addition, these findings are consistent with results reported by Tarozzi *et al.* [21], who reported that organic red oranges exhibited significantly higher total anthocyanins and total antioxidant activity than non-organic red oranges. Another case to emphasize is that of peppers, which showed the same pattern as oranges, statistically higher TAC, numerically higher TPC and TFaC, and consequently, numerically higher ORAC in ORG samples, showing conformity with what was proved by Muscolo *et al.* [37], that organic fertilizers enhanced the synthesis of total phenols, flavonoids, anthocyanins, as well as, antioxidant activities of red Topepo sweet peppers compared to those grown in unfertilized soil. Based on the results obtained for hazelnut and tomato cultivars, it appears that the type of cultivation system, organic or non-organic, has a differential influence between cultivars of the same species. Indeed, in a previous study comparing 23 broccoli cultivars, it was determined that phytochemicals studied individual compound concentrations responded differently and that the type of farming system, organic or non-organic, contributes to the variation in the concentration of theses phytochemicals [38].

5. Conclusions

The farming system, organic or non-organic, generated a different content of phenolic compounds and antioxidant capacity of plant-based foods, with no clear general pattern of differentiation. Thus, it is suggested that the effects of the type of cultivation tended to depend on the plant species studied and its cultivar. Specifically, the vegetable group showed a clear pattern of differentiation

between the two types of farming systems, where the highest abundance of antioxidant capacity and phenolic compounds, except TFaC and TFoC, was reflected in the organic samples. Indeed, consuming organic plant-based foods can promote health by providing more antioxidants with a lower risk of chemical contamination. In addition, fruits, vegetables, nuts, and legumes are good sources of beneficial phytochemicals, including phenolic compounds and antioxidants. And, as part of a healthy diet, these plant-based foods can help lower risk factors for non-communicable diseases.

Author Contributions

Álvaro Cruz-Carrión: conceptualization, formal analysis, investigation, writing-original draft preparation. Ma. Josefina Ruiz de Azua: investigation. Manuel Suárez: conceptualization, supervision, writing-original draft preparation, writing-review, and editing. Anna Arola-Arnal: conceptualization, supervision, writing-original draft preparation, writing-review, and editing.

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER II

To evaluate whether geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis affect their antioxidant capacity against oxidative stress in rats.

OBJECTIVE 2.1

To study whether geographical origin of cultivation and seasonal consumption of sweet cherries cv. Brooks condition their antioxidant capacity against oxidative stress in rats

OXIDATIVE STRESS IN RATS IS MODULATED BY SEASONAL CONSUMPTION OF SWEET CHERRIES FROM DIFFERENT GEOGRAPHICAL ORIGINS: LOCAL VS. NON-LOCAL

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Abstract

Sweet cherries (*Prunus avium* L.) are a source of bioactive compounds, including phenolic compounds, which are antioxidants that contribute to protection against oxidative stress. It is known that the composition of cherries is influenced by external conditions, such as the geographic origin of cultivation, and that biological rhythms have a significant effect on oxidative stress. Therefore, in this study, Fischer 344 rats were exposed to various photoperiods and were supplemented with Brooks sweet cherries from two different geographical origins, local (LC) and non-local (NLC), to evaluate the interaction of supplementation and biological rhythms with regard to the oxidative stress status. The results indicate that the two fruits generated specific effects and that these effects were modulated by the photoperiod. Consumption of sweet cherries in-season, independently of their origin, may promote health by preventing oxidative stress, tending to: enhance antioxidant status, decrease alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, reduce liver malondialdehyde (MDA) levels, and maintain constant serum MDA values and reactive oxygen species (ROS) generation.

Keywords

Antioxidant, cherries, phenolic signature, photoperiod, polyphenols, rhythms, seasons.

Abbreviations

ALT, alanine aminotransferase; AST aspartate aminotransferase; GSH reduced glutathione; L12, 12 h light/day, standard photoperiod, which simulated the spring/autumn light schedule; L18, 18 h light/day, long photoperiod, which simulated the summer light schedule; L6, 6 h light/day, short photoperiod, which simulated the winter light schedule; LC, local sweet cherry; MDA, malondialdehyde; NLC, non-local sweet cherry; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; TAC, total anthocyanins content; TBARS, thiobarbituric acid-reactive substances; TDF, total dietary fiber; TFaC, total flavan-3-ols content; TFoC, total flavonols content; TPC, total phenolic content.

1. Introduction

Many dietary phytochemicals, such as phenolic compounds, are synthesized as secondary metabolites that provide protection to plants against stresses [1]. Furthermore, these plant-derived chemicals consumed in relatively low doses activate adaptive cellular response signaling to produce stress resistance and other health advantages [2]. These molecules have been shown to be modulated by the environment, seasons, and various types of stress [3]. Hence, animals can use this signaling to anticipate seasonal changes and thus develop survival adaptations [4]. This phenomenon is known as xenohormesis and explains that bioactive compounds generated by plants under environmental stresses can provide resilience to stress and once consumed can generate survival benefits [2].

Humans routinely consume a wide range of phytochemicals from fruits, vegetables, and cereals. However, the daily consumption of fruits and vegetables differs widely around the world. Thus, a general pattern of increasing fruit and vegetable consumption in people with higher educational attainment is observed across all European Union Member States. In fact, in Spain, 77.4% of adults report eating fruits at least once a day [5]. Sweet cherry (*Prunus avium* L.) is one of the most widely consumed summer fruits across the temperate regions of Europe because of its good taste, health benefits, and comparatively low cost. Sweet cherries are seasonal and are available from May to August in Europe. However, in order to extend the natural harvest and marketing season for cherries, they are also imported from Turkey, Chile, Australia, or New Zealand [6].

Due to new eating styles and the relationship of fruit consumption with healthy effects and antioxidant capacity, cherries consumption has augmented [6]. Sweet cherries have been reported to be rich in anthocyanins, hydroxycinnamic acids, flavonols, and flavanols, which contribute to their health effects [6,7]. Polyphenols are secondary metabolites that have several activities. Thus, polyphenols from cherries manifest their health effects by various mechanisms, including antiproliferative and antioxidant effects [7]. However, phenolic composition is genotype-dependent and is influenced by climatic conditions, cultivars, harvesting season, and the environment (geographical origin) [8,9]. In fact, Serradilla *et al.* [9]

reported that the cultivars Napoleona Grappolo and Sonata grown in southern Europe display a higher total phenolic content than the cultivars Lapins and Ferprime grown in northern Europe.

The antioxidant function of cherries is highly associated with their anthocyanin content, which inhibits lipoperoxidation more efficiently than traditional antioxidants [6]. An optimal antioxidant status protects against oxidative stress, which is defined as an imbalance between oxidants and antioxidants, causing damage to lipids, proteins, and cellular macromolecules. Oxidative stress is known to have a significant photoperiod-dependent effect [10]. The daily organization of behavior and physiology in animals is modulated by the circadian system, which functions as an endogenous clock and entrains the rhythms to the seasonal photoperiod changes [11]. These changes imply daily endogenous variations in the generation of free radicals, including reactive oxygen species (ROS) and other oxidants, as a consequence of the circadian rhythms of metabolism and behavior. The changes also involve exogenous daily fluctuations in the external environment, such as lighting and temperature [12]. Interestingly, daily rhythms of glutathione and malondialdehyde concentrations have been reported to be present in the brain [10]. Furthermore, it has been shown that transition dairy cows have higher erythrocyte levels of thiobarbituric acid-reactive substances (TBARS) during the summer [13]. There is evidence of fluctuations in cortisol content in plasma from goats depending on seasonal and ambient temperature [14].

To investigate this topic, we carried out a proof-of-concept study in which Fischer 344 rats were exposed to short, standard, and long photoperiods to simulate winter, autumn/spring, and summer light schedules, respectively, and the animals were supplemented with sweet cherries from two different growing locations. The main objective was to investigate the antioxidant status and the levels of oxidative stress biomarkers in rats consuming sweet cherries from different geographical origins in-season and out-of-season.

2. Materials and methods

2.1. Chemicals and reagents

Folin–Ciocalteu and *p*-dimethylaminocinnamaldehyde (DMACA) reagents were acquired from Fluka/Sigma-Aldrich (Madrid, Spain). Gallic acid, quercetin, (+)-catechin, Trolox, fluorescein, 2-thiobarbituric acid, trichloroacetic acid, reduced glutathione (GSH), monochlorobimane, glutathione S-transferase, and 2′,7′-dichlorofluorescein diacetate were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Cyanidin-3-*O*-rutinoside was purchased from PhytoLab (Vestenbergsgreuth, Germany). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Acros Organics (Geel, Belgium).

The standard compounds to make up the calibration curve for the quantification of sweet cherry phenolics were prepared in acetone/water/acetic acid (70/29.5/0.5; *v*/*v*/*v*). Every three months, they were prepared again and stored protected from light at −20 °C.

2.2. Plant fruit material

Samples of sweet cherries (*Prunus avium* cv. Brooks) were used in this study. The Brooks sweet cherry was developed at the University of California-Davis and released to the public in 1987. This cultivar is a hybrid of Early Burlat and Rainier cherry, and it is characterized as a very high-quality, early-maturing sweet cherry that possesses the outstanding ability to develop large fruit [15]. These samples at commercial maturity were obtained from two different geographical origins: cherries from Tarragona, Spain (local sweet cherry, LC), were donated by the farmer in June 2018 (in-season consumption), and cherries from Cachapoal, Chile (non-local sweet cherry, NLC), were purchased in a local market in December 2018 (out-of-season consumption) (Supplementary Figure 1).

The edible part of the cherries was frozen in liquid nitrogen and ground. Then, the samples were freeze-dried for one week in a Telstar LyoQuest freeze-dryer (Thermo Fisher Scientific, Madrid, Spain) at −55 °C and ground (Moulinette 1, 2, 3, Moulinex); the powder was stored in amber flasks at room temperature until use.

2.3. Proximate composition

To determine the sugar content, the soluble solid content in fresh fruit was measured in °Brix with a hand-held refractometer. In addition, the dietary components of local and non-local sweet cherry were characterized according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC) [16]. The water content was measured as mass loss on heating (98 °C, 24 h). The ash content was calculated as the inorganic residue remaining after the water and organic matter were removed by heating (550 °C, 24 h). The protein was quantified by the Kjeldahl method (conversion factor 6.25), and the total lipid content was determined by continuous extraction with n-hexane in a Soxhlet extractor. The total dietary fiber (TDF) content was determined by treatment with heat-stable α-amylase, proteases from *Bacillus licheniformis*, and amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, Madrid, Spain) and by sequential weighing of the dry residue. The total carbohydrate content was calculated by subtracting the nutrient contents described above from the weight of LC and NLC. All assays were performed in triplicate using freeze-dried LC and NLC.

2.4. Extraction and quantification of the phenolic compounds

The phenolic compounds in LC and NLC were extracted according to Iglesias-Carres *et al.* [17], where the extraction conditions were 80 mL/g, 65% methanol (1% formic acid), 72 °C, and 100 min under agitation of 500 rpm. The total contents of phenolics (TPC), anthocyanin (TAC), and flavanol (TFaC) in the LC and NLC extracts were analyzed by the methods described by Iglesias-Carres *et al.* [17]. Total flavonol content (TFoC) was measured according to the method described by Iglesias-Carres *et al.* [18].

2.5. Experimental procedure in rats

A total of 72 male Fischer 344 rats from 7 to 8 weeks of age were purchased from Charles River Laboratories (Barcelona, Spain) and randomly subdivided into 3 groups (n = 24, each) depending on the light exposure regimen. The groups were subjected to simulation of various photoperiods, including 6 h light/day (short photoperiod L6, which simulated the winter light schedule), 12 h light/day

(standard photoperiod L12, which simulated the spring/autumn light schedule), or 18 h light/day (long photoperiod L18, which simulated the summer light schedule). Rats underwent photoperiod adaptation for 4 weeks. Thereafter, a daily supplementation period of 7 weeks with 100 mg LC dry weight (dw)/kg body weight (bw) [19], 100 mg NLC dw/kg bw, or vehicle (VH; glucose 21.2 mg/kg bw and fructose 21.2 mg/kg bw), in order to administer the same amount of sugars as those given to the cherry-supplemented rats, was implemented (n = 8, each). Supplementation was performed by voluntary oral administration between 8:00 a.m. and 9:00 a.m. (lights on at 7:00 a.m.) to avoid circadian interference. Throughout the experiment, the rats consumed a standard chow diet (AO4, Panlab, Barcelona, Spain) and tap water ad libitum. Rats were weighed every week, and their intake was monitored fortnightly. At the end of the experiment, the rats were deprived of food after supplementation with LC, NLC, or VH and were sacrificed by decapitation 1 h later. Blood was collected in non-heparinized tubes, incubated for 1 h at room temperature, and centrifuged (2000× g, 15 min, 4 °C) to obtain the serum. All collected organs and tissues were weighed and together with the serum were stored at −80 °C until use. The Ethical Committee of Animal Experimentation of the Rovira i Virgili University approved the experimental procedure (reference number 9495).

2.6. Biochemical analysis

2.6.1. Antioxidant status

- Oxygen radical absorbance capacity

The method described by Huang *et al.* [20] was applied to estimate the oxygen radical absorbance capacity (ORAC). In brief, 25 µL of 73 mM AAPH was injected into each well from a 96-well microplate containing 25 µL of serum and 150 µL of 59.8 nM fluorescein. The fluorescence intensity of the samples was evaluated using a FLx800 multiple detection microplate reader (Biotek, Winooski, VT, USA) with excitation at 485 nm and emission measured at 528 nm every 2 min for 120 min. ORAC levels, which were calculated based on the standard curve of Trolox, are reported as mmol Trolox eq/L.

- Reduced glutathione concentration

The hepatic levels of reduced glutathione (GSH) were assayed by the monochlorobimane fluorometric method [21]. Briefly, 10 µL of 100 µM monochlorobimane and glutathione *S*-transferase (1U/mL) solution was added to 90 µL of homogenized liver; the homogenate was then incubated at room temperature for 30 min. GSH levels, which were determined based on the standard curve of GSH (Sigma-Aldrich), were measured using an FLx800 multi-detection microplate reader (Biotek) with excitation at 360 nm and emission measured at 528 nm and are expressed as µmol GSH eq/g liver.

2.6.2. Oxidative stress biomarkers

- Alanine aminotransferase and Aspartate aminotransferase activity

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) based on the protocols of commercial kits (Química Clínica Aplicada S.A., Amposta, Spain). The ALT and AST values are expressed as IU/L.

- Malondialdehyde production

Malondialdehyde (MDA) levels were determined by the TBARS assay [22]. Here, 20% trichloroacetic acid in 0.6 M HCl (1:1, *v/v*) was added to the serum, and the tubes were kept in ice for 20 min. Samples were centrifuged at 1500× *g* for 25 min, and TBA (120 mM in 260 mM Tris, pH 7) was added to the supernatant in a ratio of 1:5 (*v/v*); then, the mixture was boiled at 97 °C for 30 min. Spectrophotometric measurements at 540 nm were performed at 20 °C. The MDA values are expressed as µmol MDA/L.

The liver MDA levels were determined according to the method previously described for the serum assay. The MDA levels are shown as nmol MDA/g liver.

- ROS generation

ROS generation was quantified using the method described by Gabbia *et al.* [23] with some modifications. Briefly, 50 µL of liver homogenate was mixed with 500 µL of solution of 2′,7′-dichlorofluorescein diacetate (10 µM) diluted 1:200 in Tris-

HCl buffer and the mixture was incubated for 40 min at 37 °C. The fluorescence intensity of the samples was assessed using an FLx800 multi-detection microplate reader (λex = 485 nm and λem = 528 nm). The ROS levels are expressed as FU/kg liver.

2.7. Statistical analysis

The results of the fruit characterization are expressed as the mean ± standard deviation (SD). Student's *t*-test was used to estimate significant differences ($p < 0.05$) between LC and NLC.

Biometric parameters and biomarker values (mean ± standard error of the mean, SEM) were subjected to a normality test and Levene's test, then data were submitted to two- and one-way analysis of variance (ANOVA) in order to determine whether differences among the means exist, and then to the least significant difference (LSD) post hoc test comparison to allow determining which means differ, using Statistical Product and Service Solutions (SPSS) software (SPSS Inc., Chicago, IL, USA). Values of ($p < 0.05$) were considered statistically significant.

3. Results

In the present study, we evaluated whether consumption of sweet cherries from different geographical regions influences metabolism, antioxidant status, and oxidative stress in Fischer 344 rats when the cherries are consumed in-season or out-of-season. The rats were exposed to various photoperiods: a short photoperiod (cherry consumed out-of-season), a standard photoperiod (cherry consumed out-of-season), and a long photoperiod (L18) (cherry consumed in-season). In addition, sweet cherries were characterized based on their proximate and phenolic composition. The samples were obtained at commercial maturity, soluble solids content was measured as an indicator of maturity, LC showed 8.43 ± 0.45 °Brix, and NLC had 9.13 ± 0.12 °Brix. In addition, color intensity, that is the most commonly used indicator of maturity, was evaluated (Supplementary Figure 1), depending on the cultivar: red sweet cherries (*e.g.*, Brooks) are commercially harvested when the skin color turns to solid bright red [6].

3.1. Proximate composition of local and non-local Brooks sweet cherry

The ash, protein, lipid, fiber, total carbohydrate, and sugar contents of LC and NLC were determined (Table 1). The ash values in both fruits were very similar; however, the remaining dietary components were statistically significantly different. In fact, LC had higher contents of protein, total lipids, and total dietary fiber that those in NLC. In contrast, NLC contained higher total carbohydrate and sugar contents. In this context, sweet cherries were dominated by carbohydrates (proportions higher than 79%), whereas the contents of ash, protein, and total lipids represented a small proportion of the LC and NLC proximate composition.

Table 1. Proximate composition of local sweet cherry (LC) and non-local sweet cherry (NLC).

Nutrients	LC	NLC	*p* value
Ash	2.05 ± 0.15	2.11 ± 0.35	0.81
Protein	6.41 ± 0.11	5.51 ± 0.07	0.01
Total lipid (fat)	1.01 ± 0.01	0.70 ± 0.07	0.03
Fiber, total dietary	10.95 ± 0.03	10.74 ± 0.06	0.04
Total carbohydrate, by difference	79.50 ± 0.18	80.78 ± 0.34	0.04
Sugar, total	43.30 ± 2.31	50.08 ± 0.68	0.02

The results are expressed as g/100 g dw ± SD (n = 3). SD: standard deviation.

3.2. Phenolic profiles of local and non-local Brooks sweet cherry

The data of Table 2 indicate that the two fruits from different geographical origins used in the study (LC and NLC) had a specific phenolic signature. Indeed, the LC had higher contents of total phenolic compounds, total anthocyanin, and total flavanol than the NLC. On the other hand, the NLC had a higher total flavonol content. It should be emphasized that both LC and NLC had high contents of total anthocyanin, representing approximately 16% of TPC. Nevertheless, the total flavanol and total flavonol contents of both fruits were low, at under 0.62 mg/g dw.

Table 2. Phenolic profiles of local sweet cherry (LC) and non-local sweet cherry (NLC).

Phenolic compounds	LC	NLC	*p* value
TPC (mg GA eq/g dw)	8.17 ± 0.20	7.64 ± 0.41	0.03
TAC (mg Cy3R eq/g dw)	1.31 ± 0.02	1.23 ± 0.03	0.02
TFaC (mg Cat eq/g dw)	0.44 ± 0.02	0.38 ± 0.03	0.02
TFoC (mg Quer eq/g dw)	0.55 ± 0.00	0.63 ± 0.05	0.04

The results are expressed as mg of phenolic components per gram of dry weight (mg/g dw) ± SD (n = 3). Abbreviations: TPC: total phenolic content, TAC: total anthocyanin content, TFaC: total flavanol content, TFoC: total flavonol content, GA: gallic acid, Cy3R: cyanidin-3-*O*-rutinoside, Cat: catechin, Quer: quercetin, SD: standard deviation.

3.3. Body composition and feeding test

Table 3 presents the biometric parameters and feeding tests of rats exposed to the three photoperiods and administered with LC, NLC, or VH for seven weeks. All the groups underwent a similar weight evolution. In addition, no significant changes were identified in most of the tissue weight and feeding tests after the administration of LC and NLC.

Interestingly, regardless of the treatment received, L6 photoperiod-exposed rats exhibited a lower skeletal muscle weight than both L12 and L18 photoperiod-exposed rats. Similarly, mesenteric white adipose tissue (MWAT) weight was modulated by photoperiod: the rats exposed to L12 had a higher MWAT weight than the L6 and L18 rats. Moreover, photoperiod had considerable effects on body fat (%). Indeed, L6 photoperiod-exposed rats had lower body fat than the rats exposed to the L12 and L18 photoperiods.

Table 3. Biometric parameters and feeding tests of Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with local sweet cherry (LC), non-local sweet cherry (NLC), or vehicle (VH).

	Short L6			**Standard L12**			**Long L18**			2wA
	LC	NLC	VH	LC	NLC	VH	LC	NLC	VH	
Biometric parameters										
Body weight gain (g)	57.1 ± 5.14	52.9 ± 3.15	44.6 ± 4.12	55.3 ± 1.24	52.9 ± 3.30	54.0 ± 3.50	50.0 ± 1.55	53.5 ± 4.00	51.5 ± 0.72	n.s.
Skeletal muscle (g)	3.64 ± 0.28	3.92 ± 0.16	4.13 ± 0.10	4.40 ± 0.07	4.18 ± 0.09	4.30 ± 0.10	4.24 ± 0.12	4.33 ± 0.15	4.43 ± 0.07	P
BAT (g)	0.45 ± 0.03	0.45 ± 0.05	0.40 ± 0.06	0.47 ± 0.03	0.39 ± 0.01	0.39 ± 0.03	0.34 ± 0.03	0.36 ± 0.05	0.44 ± 0.03	n.s.
EWAT (g)	8.61 ± 0.23	8.73 ± 0.45	9.01 ± 0.44	10.10 ± 0.93	8.94 ± 0.50	9.49 ± 0.47	9.20 ± 0.58	9.21 ± 0.57	9.88 ± 0.74	n.s.
IWAT (g)	2.87 ± 0.36	2.46 ± 0.21	2.83 ± 0.37	2.80 ± 0.21	2.50 ± 0.27	2.78 ± 0.19	2.52 ± 0.27	2.42 ± 0.22*	3.35 ± 0.37	n.s.
MWAT (g)	4.69 ± 0.26	4.64 ± 0.27	5.56 ± 0.50	6.05 ± 0.36	5.22 ± 0.38	5.52 ±0.30	5.39 ± 0.37	4.51 ± 0.24	5.12 ± 0.30	P
Body fat (%)	4.30 ± 0.19	4.43 ± 0.11	4.37 ± 0.16	4.68 ± 0.29	4.71 ± 0.20	4.78 ± 0.20	4.74 ± 0.23	5.11 ± 0.31	4.62 ± 0.16	P×T, P
Feeding tests										
Food intake (kcal/day)	56.9 ± 1.23	55.1 ± 1.62	54.1 ± 0.29	56.2 ± 1.13	53.8 ± 1.06	55.4 ± 1.15	54.0 ± 0.40	55.1 ± 1.00	54.9 ± 0.47	n.s.
FE (g/100 kcal)	2.76 ± 0.16*	2.64 ± 0.14*	2.16 ± 0.14	2.50 ± 0.04	2.54 ± 0.14	2.46 ± 0.18	2.64 ± 0.11	2.89 ± 0.16	2.75 ± 0.20	n.s.

Data are expressed as the mean ± SEM (n = 8). Body weight gain (g) was determined as the difference between final rat weight and weight at the beginning of the treatment. The skeletal muscle weight represents the total weight of the soleus and gastrocnemius muscles. Food efficiency (FE) was calculated as body weight gain per kcal consumed over the entire treatment and is expressed as g/100 kcal. *$p < 0.05$ vs VH in the photoperiod; two-way ANOVA was used to evaluate the differences between the groups. P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Abbreviations: L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, n.s.: not significant, BAT: brown adipose tissue, WAT: white adipose tissue, EWAT: epididymal WAT, IWAT: inguinal WAT, MWAT: mesenteric WAT.

3.4. Antioxidant status

The antioxidant status of Fischer 344 rats associated with LC and NLC consumption exposed to three different photoperiods is shown in **Figure 1**.

The antioxidant capacity was significantly affected by all the factors under evaluation (photoperiod, treatment, and photoperiod×treatment interaction) (**Figure 1a**). In this context, L18 photoperiod-exposed rats exhibited higher ORAC values than those exposed to L6 and L12 photoperiods. Moreover, rats that consumed cherries in-season (*i.e.*, L18 sweet cherry-supplemented rats) had higher ORAC values than the control animals; the values increased by 16.98% by the administration of LC and by 13.21% in the case of NLC. On the other hand, LC administration in the L6 photoperiod and NLC administration in the L12 photoperiod also increased (by 14.29% and 21.86%, respectively) the levels of ORAC compared to those in other groups within the same photoperiod.

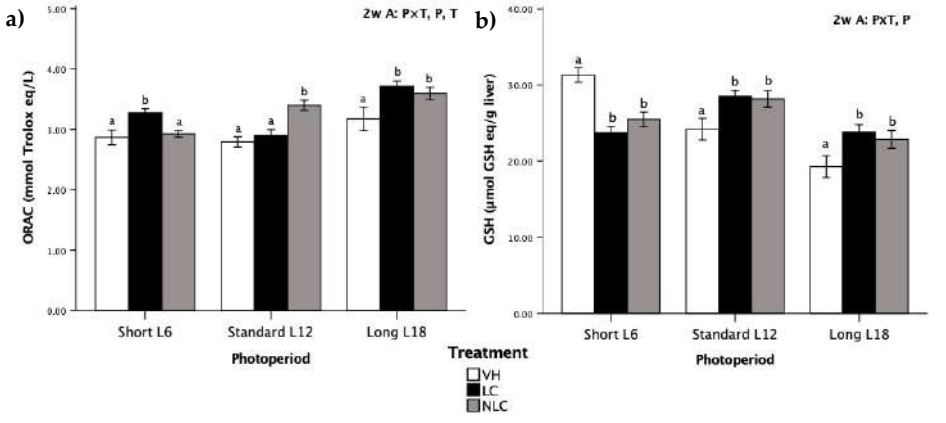

Figure 1. Levels of antioxidant status biomarkers in Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with local sweet cherry (LC), non-local sweet cherry (NLC) or vehicle (VH): (**a**) Oxygen radical absorbance capacity (ORAC); (**b**) Reduced glutathione (GSH) concentration. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA was performed to evaluate the differences between the groups. P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Different letters indicate significant differences between the treatments within each photoperiod estimated by one-way ANOVA.

The data in **Figure 1b** indicate that the photoperiod induced significant differences in GSH concentration. Rats exposed to the long photoperiod had a lower GSH concentration than those exposed to short and standard

photoperiods. Remarkably, both types of fruit had a similar impact on the pattern of GSH concentrations in all photoperiods; GSH levels were increased in rats exposed to the standard and long photoperiods (cherries consumed in-season) and decreased in rats exposed to the short photoperiod compared with the GSH levels in the VH rats.

3.5. Serum oxidative stress biomarkers

Each of those biomarkers was analyzed in the rat serum and was characterized by a specific and variable pattern (**Figure 2**). Regardless of the photoperiod exposition, consumption of sweet cherries diminished the ALT activity compared with that in VH supplementation (T effect, $p < 0.05$, two-way ANOVA). This reduction was significant in all groups except NLC L6 (**Figure 2a**). The enzymatic activity of AST was significantly different between the photoperiods; it was highest in L12 rats, followed by L6 rats, and was the lowest in L18 rats (P effect, $p < 0.05$, two-way ANOVA) (**Figure 2b**).

Furthermore, both LC and NLC administration produced a similar decrease in AST activity compared with that in the control group in L18 photoperiod-exposed rats (cherries consumed in-season). On the other hand, in the opposite photoperiod (L6), fruit administration had different effects depending on the origin of the fruit. The NLC group had an increase in the enzymatic activity of AST compared with that in the other two groups. No differences in the levels of serum MDA with regard to cherry administration were observed in any of the three photoperiods (**Figure 2c**). However, regardless of the treatment, L18 photoperiod-exposed rats had lower MDA levels than rats exposed to the short and standard photoperiods (P effect, $p < 0.05$, two-way ANOVA).

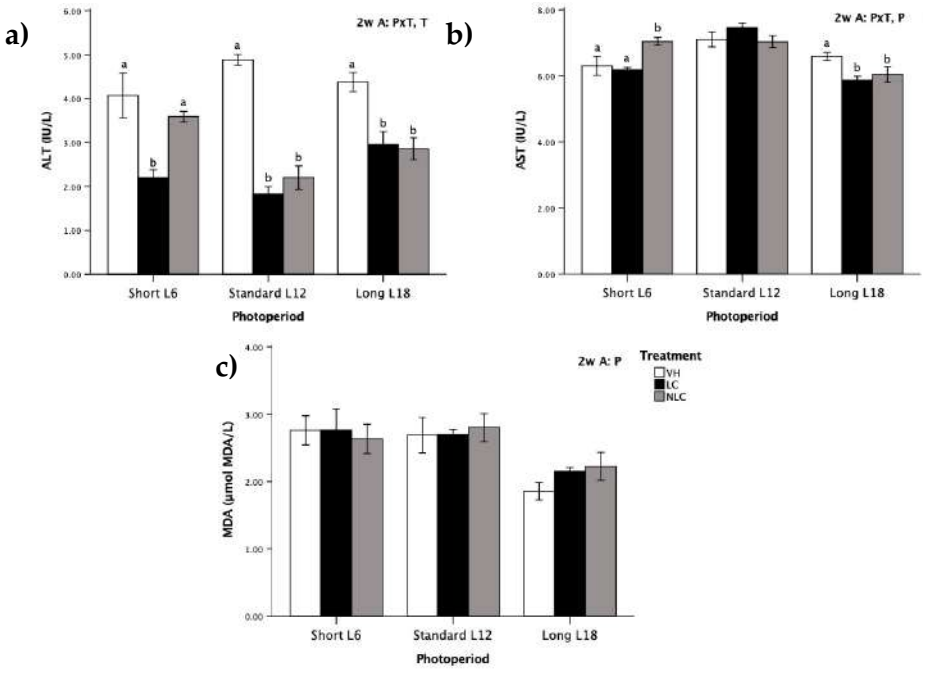

Figure 2. Serum oxidative stress biomarkers in Fischer 344 rats exposed to three photoperiods for 7 weeks and supplemented with local sweet cherry (LC), non-local sweet cherry (NLC), or vehicle (VH): (**a**) alanine aminotransferase (ALT) activity; (**b**) aspartate aminotransferase (AST) activity; (**c**) malondialdehyde (MDA) level. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA was used to evaluate the differences between the groups; thus, we could determine the effects of the photoperiod, P; the treatment, T; or the photoperiod × treatment interaction, P × T. Later, one-way ANOVA was used to evaluate significant differences among the treatments (LC, NLC, and VH) within each photoperiod exposure (Short L6, Standard L12, and Long 18); these differences are indicated with different letters. Abbreviations: L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, SEM: standard error of the mean, ANOVA: analysis of variance.

3.6. Liver oxidative stress biomarkers

MDA levels and ROS generation were quantified in the liver (**Figure 3**). MDA and ROS were influenced by photoperiod, treatment, and P × T ($p < 0.05$, two-way ANOVA), highlighting the impact of the performed treatments. Regardless of the photoperiod exposure, MDA levels were significantly different between the sweet cherry-supplemented rats (T effect, $p < 0.05$, two-way ANOVA) (**Figure 3a**). Independent of the photoperiod, NLC administration produced a decrease in the MDA concentration compared with that in the VH group. On

the other hand, LC supplementation reduced MDA levels compared with the levels of NLC and VH supplementation in L18 photoperiod-exposed rats (cherries consumed in-season). Interestingly, regardless of the treatment, L12 photoperiod-exposed rats had lower MDA and ROS values than rats exposed to both the L6 and L18 photoperiods.

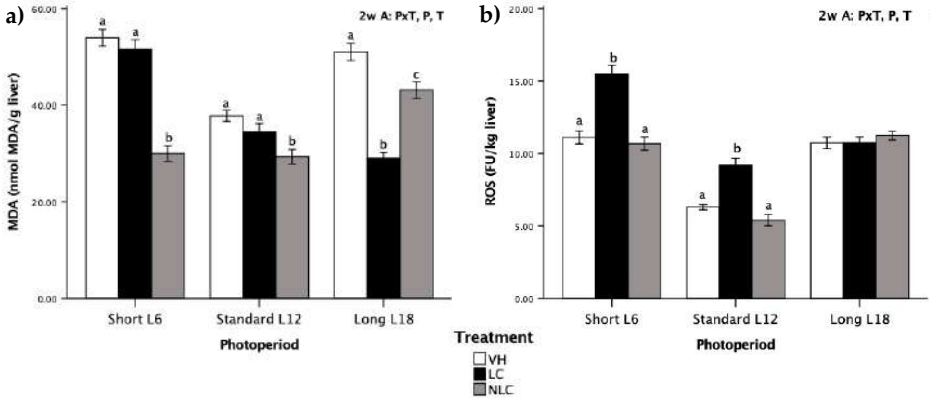

Figure 3. Liver oxidative stress biomarkers in Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with local sweet cherry (LC), non-local sweet cherry (NLC), or vehicle (VH): (**a**) malondialdehyde (MDA) level; (**b**) reactive oxygen species (ROS) generation. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA [3 × 3 factorial designs: treatment (LC, NLC, or VH) × photoperiod effect (L6, L12, or L18)] was used to evaluate the differences between the groups, thus we could determine the effects of the photoperiod, P; the treatment, T; or the photoperiod × treatment interaction, P × T. Later, one-way ANOVA was used to evaluate significant differences among the treatments (LC, NLC, and VH) within each photoperiod exposure (Short L6, Standard L12, and Long 18); these differences are indicated with different letters. Abbreviations: L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, SEM: standard error of the mean, ANOVA: analysis of variance.

ROS generation was influenced by photoperiod, treatment, and P × T ($p < 0.05$, two-way ANOVA). The pattern was similar between the L6 and L12 photoperiod-exposed rats, highlighting that LC-supplemented rats had higher ROS generation than rats supplemented with NLC or VH (Figure 3b). On the other hand, LC and NLC had no effect on ROS generation in rats exposed to the L18 photoperiod.

4. Discussion

The literature indicates that circadian and circannual rhythms of living beings play an essential role in preventing excessive oxidative stress [12]. Thus, in our study, male Fischer 344 rats were chronically exposed for seven weeks to three photoperiods to mimic the day length of different seasons: L6 (winter season), L12 (autumn and spring seasons), and L18 (summer season), and the animal diets were supplemented with freeze-dried sweet cherries from two different growing locations, local fruit (LC) and non-local fruit (NLC), to investigate whether the season in which the fruit is consumed modulates the antioxidant status and oxidative stress in the animals and whether the geographical origin of sweet cherry cultivation can result in different effects.

The data indicate that the intake of LC and NLC produced differentiated physiological and metabolic responses depending on the photoperiod. Circannual rhythms had an influence on the antioxidant status of rats. In fact, L18 photoperiod-exposed rats had the lowest reduced glutathione (GSH) concentration (as shows the two-way ANOVA significance regarding P factor); interestingly, this group of animals had the highest ORAC values. Thus, 6 h light/day conditions apparently increased the GSH levels in the rats, whereas the 18 h light/day conditions decreased the GSH levels in the rats. These results are in agreement with the data of Escribano *et al.* [10], who determined that light reduced the GSH levels in the healthy animals and dark periods had the opposite effect. A similar result was reported in juvenile gibel carp (*Carassius auratus*), in which antioxidant-related metabolites (GSH) were decreased after long-term exposure to light [24]. Several studies reported a day–night rhythm in the hepatic GSH concentrations in rats with daily oscillating patterns [25]. In the case of the ORAC assay results, our study determined that the total antioxidant capacity increases in the animals exposed to the summer light schedule in agreement with the results obtained by Morera-Fumero *et al.* [26], who showed that ninety-eight healthy subjects had significantly higher total antioxidant capacity levels in the summer than in winter at three time points studied (09:00, 12:00, and 00:00 h).

Oxidative stress biomarkers can be classified as molecules that are modified by interactions with ROS in the microenvironment and molecules of the antioxidant system that change in response to increased redox stress [27]. Specifically, the quantification of ALT and AST can provide information about liver damage and the onset of oxidative stress hepatotoxicity. In addition, MDA produced during ROS-mediated peroxidation of polyunsaturated fatty acids is a widely used marker of oxidative stress [10]. In this study, oxidative stress biomarkers had a notable photoperiod-dependent effect, except ALT activity. Certainly, the photoperiod had an influence on serum MDA levels, which were diminishing concomitant to an increase in the daily light hours, and in the liver MDA levels, only LC rats followed this behavior. These results are in agreement with a study by Baydas *et al.* [28], who showed that MDA values were progressively augmented in dark periods. A rhythm of lipid peroxidation was also described in *Drosophila* males, and the peak of lipid peroxidation coincided with the evening maximum of locomotor activity [29]. On the other hand, AST activity at the hepatic level was modulated by photoperiod exposure, reaching the lowest activity when rats were exposed to the long photoperiod (two-way ANOVA). This finding is in agreement with the data reported by Hardeland *et al.* [30], who demonstrated that mouse liver AST activity is at its maximum during the dark photoperiod; our data also concur with the results reported by Çevik and Aslan [31], who observed that prolonged dark periods enhance the oxidation of nutritional fatty acids and that this oxidation triggers an increase in the AST levels. In contrast, the activity of ALT was not modulated by exposure to the photoperiods. This finding agrees with the data obtained by Mariné-Casadó *et al.* [4], who previously detected a lack of differences in the ALT values between rats exposed to three photoperiods; similar results confirmed that increasing light photoperiod had no significant effects [32]. It is important to define the status of these biomarkers because ALT and AST are intracellular enzymes released from damaged hepatocytes into the blood following hepatocellular injury [30].

In this study, sweet cherries were administered daily to the experimental rats at a dose of 100 mg freeze-dried fruit/kg body weight. Recalculation of this dose using dose translation from animal to human [33] to estimate the intake for a 60 kg

human is equivalent to eating 5.12 g of fresh sweet cherries per day; this dose can contain a significant amount of nutrients and bioactive compounds that aid to achieve the daily intake recommended by the World Health Organization of at least 400 g of fruits and vegetables per day as part of a healthy eating pattern for the prevention of chronic diseases [34]. Eating fruits and vegetables is known to produce health benefits, and these effects are ascribed, at least in part, to their bioactive compounds; in particular, the sweet cherry fruit is a nutrient-dense food with a relatively low calorie content and significant amounts of important nutrients and bioactive food components such as fiber, polyphenols, carotenoids, vitamin C, and potassium, which have been reported to have beneficial health effects [7]. About the overall impact of fruit consumption on the animals, overall, the intake of sweet cherries favored the antioxidant status in Fischer 344 rats because it significantly enhanced the antioxidant capacity (ORAC) by 13.66% and numerically increased the concentration of GSH. The antioxidant capacity of cherries has been evaluated by several methods, most commonly by ORAC [6]. In this study, ORAC was selected to evaluate the antioxidant capacity because of the antioxidant mechanism assessed by hydrogen atom transfer [35].

Cherry consumption promotes health, due to the high concentrations of bioactive compounds (such as polyphenols, melatonin, carotenoids, and vitamins E and C), which contribute to their antioxidant and anti-inflammatory effects [7]. These results are related to the data of other studies, in which the intake of food stuffs rich in melatonin, such as cherries, grape juices, or beers, led to an increase in the plasma antioxidant capacity [36]; similarly, in another study, the short-term supplementation of Montmorency powdered tart cherries reduced immune and inflammatory stress and improved the maintained redox balance with a linear increase in the antioxidant activity at 24 and 48 h in aerobically trained individuals [37]. Similarly, cherry juice increased the total antioxidant status of the serum in recreational marathon runners by 10% over that detected in the placebo group, thus providing a broad spectrum of protection against inflammation and oxidative stress [38]. Sweet cherries are a significant source of various phenolic compounds that are associated with antioxidant activity due to their significant role in the stabilization of lipid peroxidation [7]. It has been attributed that one of the most

important benefits of cherry consumption is the increase in the bioavailability of antioxidants [39]. Bioavailability of antioxidants contributes to preserving the appropriate redox balance after over-generation of ROS [37]. ROS produced during aerobic metabolism is known to cause oxidative damage to the macromolecules [27]. Living organisms are protected from ROS by several defense mechanisms, including antioxidant enzymes and low-molecular weight antioxidants, such as GSH [35]. GSH forms a part of the antioxidant defense systems produced by the body to protect the cellular constituents from the damages caused by ROS [35]. Furthermore, it is very important that fruit supplementation to animals in this study had important effects on ALT activity and MDA concentration in the liver resulting in a remarkable reduction in ALT enzymatic activity of 34.62% and in MDA values of 20.08%. The tendency to reduce the concentration of MDA in the liver of rats supplemented with LC and NLC confirmed the antioxidant effects of sweet cherries. Other authors studied the addition of sweet cherry fruit and leaves to a high fat-cholesterol diet and reported that the hepatic enzymatic activity and MDA values decreased in Wistar rats and that oxidative stress was reduced [40]. Indeed, our results indicate that eating sweet cherries can improve liver function and lipid peroxidation. This effect can result from the presence of bioactive compounds, especially phenolic acids, in *Prunus avium* L. The literature data suggested that hydroxycinnamic acids reduce the activities of hepatic enzymes [40].

Genetic and environmental factors can modulate the phenolic composition of fruit, and various phenolic profiles can be encountered in fruits of the same variety originating from different geographical locations. In detail, especially relevant factors include geographical origin of cultivation, environmental conditions (preharvest day and night temperature, light intensity), ripeness, and post-harvest conditions. The nutritional composition of the harvested fruit can be modulated by growing conditions [8]. In fact, Viljevac *et al.* [41] concluded that geographical location had a strong impact on polyphenol and anthocyanin contents of various sour cherry cultivars. However, current globalization makes it possible to obtain fruits and vegetables from various regions all over the world and all year round. Therefore, in this study, we evaluated two Brooks sweet

cherry cultivars from different geographical regions administered for seven weeks. The proximate composition and phenolic profile of LC and NLC presented a different nutritional composition and a specific phenolic signature. The results indicate that the levels of TAC, which is the main group of phenolic compounds in sweet cherries [6,7,17], were significantly different between LC and NLC, demonstrating the environmental influence on anthocyanin synthesis. These differences in TAC are in agreement with the data on bilberry grown at various altitudes in the eastern Alps of Austria, where anthocyanin levels were lower at higher altitudes [42]. On the other hand, the notably higher content of TFoC in NLC can be caused by the temperature at the growing location, because the growing temperature in December 2018 of Chile was higher in comparison with Spain in Jun 2018 [43]. This finding is in agreement with the results found by Josuttis *et al.* [44], who in strawberries observed that higher temperatures increased the content of total flavonols. In the same way, TFaC was different between the two fruits, being higher in LC than NLC. We were unable to access the specific conditions under which the non-local cherries were harvested, and this could have an impact on the obtained results. Moreover, each fruit generated different effects on the antioxidant capacity and biomarkers of oxidative stress monitored in the rats supplemented with the fruits. Thus, regardless of photoperiod exposure, LC and NLC administration had statistically similar effects on the antioxidant status levels in rats; however, LC-supplemented rats had higher numerical values. This effect might be related to the fact that LC contained higher levels of fiber and phenolic compounds compared with that in NLC; similar differences were reported to improve the antioxidant status [45]. Similarly, ALT activity was remarkably decreased by LC supplementation. In contrast, NLC supplementation diminished the ROS values, and this effect may be a consequence of the antioxidant activity that scavenges ROS generated in the plasma. These biological activities of LC and NLC can be related to the individual and specific nutritional composition and phenolic signature of each fruit type.

According to xenohormesis theory, animals are able to adapt their physiology to the changes in the environmental conditions by consuming phytochemicals, chemical signals synthetized by plants. Consequently, the beneficial effects

provided by sweet cherries consumed in-season are different in the other two photoperiods with simulated out-of-season consumption; overall, the metabolic response generated by eating fruit with a seasonally distinctive phenotype depends on the circannual rhythms, and this response can be altered or distorted when the fruit is consumed out-of-season [11,46]. When LC and NLC are ingested in-season (L18, summer season), a notable increase in the antioxidant status of the animals housed under these conditions was observed. Another remarkable effect of the in-season consumption of sweet cherries was manifested as a reduction in the enzymatic activity of both ALT and AST. Moreover, L18 photoperiod-exposed rats experienced a substantial lowering of the MDA levels in the liver. It is important to emphasize that the in-season intake of cherry maintained constant levels of circulating serum MDA and ROS generation. Otherwise, out-of-season fruit eating causes an alteration in the characteristic seasonal metabolism [46]. Indeed, in this study, when the sweet cherries were consumed out-of-season, a markedly different pattern of antioxidant and oxidative stress biomarker values was observed. In fact, eating sweet cherries during the winter season lowered the GSH levels, which is opposite to the effect of in-season consumption. Moreover, the intake of NLC in L6 tends to increase the enzymatic activity of AST, compared with both LC and VH groups, while the consumption of the fruit out-of-season (winter, spring, or autumn) influenced the ROS levels: NLC consumption produced a tendency similar to that observed in the control group and LC intake increased the generation of ROS. Similarly, recent studies of our group have found that eating sweet cherries out-of-season may induce erroneous metabolic signaling in dietary-induced obese F344 rats, such as increases in whole-body fat oxidation and circulating levels of glucose and insulin [46]; changing the morphology of white adipose tissue, enhancing the cell area, and reducing the amount of adipocytes [47]; and, eventually, modulating the hypothalamic leptin system regulating *Agrp* and *Ptp1B* mRNA levels [48].

5. Conclusions

In summary, this study demonstrates that in-season consumption of sweet cherries can promote health by preventing an increase in oxidative stress. Sweet cherries enhance the antioxidant status, decrease the enzymatic activity of ALT

and AST, reduce the MDA levels in the liver, and maintain constant serum MDA levels and ROS generation. Otherwise, out-of-season consumption of the fruit can induce erroneous signaling. In terms of geographical origin, local and non-local Brooks sweet cherries had a different nutritional composition and a specific phenolic signature, thus generating differentiated and particular effects on the antioxidant status and oxidative stress in Fischer 344 rats when consumed in-season, with significant emphasis on reducing the liver MDA levels by supplementation with LC. Our results emphasize the significance of the consumption of local and seasonal fruits to achieve ideal health. However, further studies with different local and non-local cherries are needed to confirm these findings.

Author Contributions

Álvaro Cruz-Carrión: conceptualization, formal analysis, investigation, writing-original draft preparation. Ma. Josefina Ruiz de Azua: investigation. Miquel Mulero: conceptualization. Manuel Suárez: conceptualization, supervision, writing-original draft preparation, writing-review, and editing. Anna Arola-Arnal: conceptualization, supervision, writing-original draft preparation, writing-review, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary materials

(**a**) (**b**)

Supplementary Figure 1: Representative images of Brooks sweet cherries from two different geographical origins: (a) local sweet cherry (LC) from Tarragona, Spain; (b) non-local sweet cherry (NLC) from Cachapoal, Chile.

OBJECTIVE 2.2

To evaluate the effects of geographical origin of cultivation and seasonal consumption of tomatoes cv. Ekstasis on its antioxidant capacity against oxidative stress in rats

TOMATOES CONSUMED IN-SEASON PREVENTS OXIDATIVE STRESS IN FISCHER 344 RATS: IMPACT OF GEOGRAPHICAL ORIGIN

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Abstract

Tomato (*Lycopersicon esculentum* Mill.) constitutes an important source of health-promoting compounds including bioactive antioxidants, such as flavonoids, that can differ in terms of composition and quantity depending on the conditions that tomatoes are cultivated. Otherwise, biological rhythms modulate oxidative stress. Therefore, the aim of this study was to evaluate the antioxidant properties of seasonal consumption of tomatoes from two different geographical origins (local LT or non-local NLT) in Fischer 344 rats. The results show that LT and NLT have a specific phenolic signature and that each tomato give a particular response on biomarkers evaluated, which in turn showed a photoperiod-dependent effect. Remarkably, when tomatoes were administered in-season improved or sustained antioxidant biomarkers, thus reducing oxidative stress values. Noteworthy, the protective effect of tomatoes against oxidative stress depends on the geographical origin of the crop. Therefore, tomatoes consumed in-season may enhance health by preventing oxidative stress.

Keywords

Antioxidant status, biological rhythms, geographical origin, oxidative stress, photoperiod, polyphenols, season, tomato.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, reduced glutathione; L6, 6 h light and 18 h darkness, short day photoperiod; L12, 12 h light and 12 h darkness, middle-length day photoperiod; L18, 18 h light and 6 h darkness, long day photoperiod; LT, local tomato; MDA, malondialdehyde; NLT, non-local tomato; ORAC, oxygen radical absorbance capacity; P, photoperiod effect; P×T, photoperiod×treatment interaction effect; ROS, reactive oxygen species; T, treatment effect; TAC, total anthocyanins content; TFaC, total flavan-3-ols content; TFoC, total flavonols content; VH, vehicle.

1. Introduction

Polyphenols, such as flavonoids, are secondary metabolites produced by plants in response to environmental stress that are attributed with protective effects against chronic diseases [1]. In this sense, many studies suggest that dietary polyphenols may represent an important exogenous defence against oxidative stress, which is defined as the disproportion between generation and accumulation of reactive oxygen species (ROS) in the body [2]. This natural phenomenon can also be considered as an unequal balance between pro-oxidants and antioxidants [3]. Endogenous factors can influence the periodic generation of oxidants [2,3]. In this regard, animals have evolved to adapt to expected environmental changes, including changes in light. Most biological processes, including the activity of antioxidant enzymes, have endogenous oscillations of approximately 24 hours (circadian rhythms). However, the rhythms and internal synchronization of living beings can be disturbed by externally induced oxidative stress, which could affect healthy status [3]. In this sense, based on the xenohormesis theory, phytochemicals such as polyphenols, are believed to activate adaptive cellular response signalling, boosting stress tolerance and survival advantages to the animals that eat them [4]. This signalling can be used by animals to anticipate seasonal changes and create survival skills [5]. Considering that each plant has a distinguishing composition of polyphenols depending on the environment in which they were cultivated, it is plausible to believe that seasonal consumption of fruits rich in polyphenols could lead to significant variations in the regulation of physiology and metabolism depending on when they are consumed. In fact, it has been reported that both circannual and circadian rhythms affect the health outcomes of polyphenols [5]. In this regard, recent studies have shown that consumption out-of-season of sweet cherry produced an increase in whole-body fat oxidation and circulating glucose and insulin levels [6,7]. In addition, out-of-season intake of cherry altered the morphology of the white and the brown adipose tissue [7], and modified the hypothalamic leptin system [8], thus confirming a pronounced deleterious effect depending on the photoperiod. Likewise, out-of-season consumption of oranges also exhibited a photoperiod-dependent deterioration effect, such as dyslipidaemia and insulin resistance [9].

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop rich in phenolic compounds and carotenoids [10]. Indeed, foods such as tomatoes have been promoted as good sources of antioxidants, and new studies have assessed the effect of their intake on human health. In this sense, some studies show that eating tomato or its derivative products may exert a protective effect against oxidative stress in healthy elderly adults [11]. Moreover, supplementation of tomatoes has been shown to lower biomarkers of oxidative stress in healthy and type II diabetic patients [12]. However, tomato plants are grown around the world under different climatic conditions that determine the physicochemical properties of tomato fruit, which depends on cultivar, environmental and cultivation conditions among other factors [13]. Furthermore, the tomato is an annual plant that is harvested at various stages of ripeness and the storage conditions employed differ within each stage [13]. Today, globalization makes it possible to find in the market several varieties of tomatoes from different parts of the world all year round, as well as local varieties. These varieties have shown high levels of variation in agromorphological, genetic and organoleptic traits, but little is known about the variation in concentrations of functional compounds and their health effects [13]. Thus, we hypothesize that consumption of tomatoes exerts different antioxidant capacities and, consequently, different responses to oxidative stress depending on where they were grown (local LT or non-local NLT tomato), and also depending on the season of the year in which tomatoes are consumed . Considering all this, the objective of our study was to assess the antioxidant effects of seasonal tomato consumption from two different growing locations (LT and NLT) in Fischer 344 rats.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid, quercetin, (+)-catechin, trolox, fluorescein, 2-thiobarbituric acid, trichloroacetic acid, L-Glutathione reduced (GSH), monochlorobimane, glutathione *S*-transferase and 2′,7′-dichlorofluorescein diacetate and *p*-dimethylaminocinnamaldehyde (DMACA) reagent were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Cyanidin-3-*O*-rutinoside was purchased from

PhytoLab (Vestenbergsgreuth, Germany). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride was purchased from Acros Organics (Geel, Belgium). The standard solutions were prepared every 3 months and kept at −20 °C protected from light.

2.2. Fruit preparation and characterization

Tomatoes fruits (*Lycopersicon esculentum* Mill. cv. Ekstasis) were grown in conventional cultivation system and were purchased in the autumn, *i.e.*, November 2018, from a local market (Tarragona, Spain) at commercial maturity. Presenting the actual fruit commercialization process. The local tomatoes (LT) were cultivated in Tarragona (41°4'29.24'' N 1°3'8.78'' E; Spain) and the non-local tomatoes (NLT) were from Almería (36°50'17.3'' N 2°27.584' O; Spain). Tomatoes were frozen in liquid nitrogen, crushed and lyophilized by using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Barcelona, Spain) at −55 °C for one week. The powder was kept at room temperature and protected from light and moisture until use.

2.2.1. Proximate composition analysis

Proximate composition analysis, including ash, protein, total carbohydrate, total dietary fiber total lipid, and total sugar, was carried out by the methods previously detailed by Cruz-Carrión *et al.* [14]. The sugar content was determined by quantifying the total soluble solids (°Brix) present in fresh tomatoes using a handheld refractometer. All nutrients' values were expressed as gram of nutrient per 100 grams of dry weight (g/100 g dw).

2.2.2. Flavonoid quantification

Flavonoids were extracted using the following parameters: 80 mL/g, 65% methanol (1% formic acid), 72 °C and 100 min under agitation of 500 rpm [15]. Once extracted, the total anthocyanins content (TAC) was determined by pH differential method [16], total flavan-3-ols content (TFaC) was estimated by the DMACA method [17], and total flavonols content (TFoC) was quantified with the method described by Cacace *et al.* [18].

2.3. Experimental design

Seventy-two 7- or 8-week-old male F344 rats (Charles River Laboratories, Barcelona, Spain) were housed in pairs in cages at 22 °C and exposed to three photoperiods to mimic the day length of seasons of year: the winter (short day photoperiod, L6, 6 h light and 18 h darkness), the spring or autumn (middle-length day photoperiod, L12, 12 h light and 12 h darkness), emulating the tomato consumption season, and the summer (long day photoperiod, L18, 18 h light and 6 h darkness) (Figure 1).

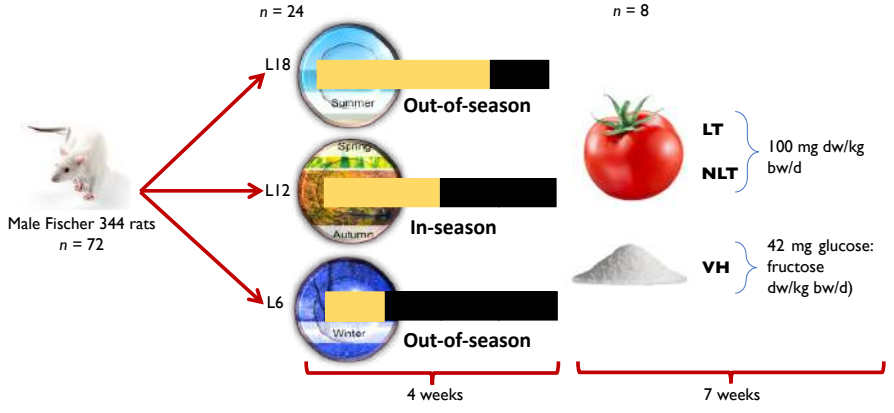

Figure 1. Graphical representation of the experimental design used in this study.

The groups (n = 24 per group) were subjected to a 4-week adaptation period during which they were fed ad libitum with a standard chow diet (AO4, Panlab, Barcelona, Spain). After this period, rats of each photoperiod were divided into three groups (n = 8 per group) according to the treatment received over seven weeks: either lyophilized LT or NLT at a dose of 100 mg per kg of body weight (bw), diluted in water. The vehicle group received 42 mg of a sugar mixture solution (glucose:fructose, 1:1) per kg of body weight, to administer the same amount of sugar as those given to the tomato-supplemented rats. Supplementation was performed by voluntary oral administration between 8:00 and 9:00 am (lights on at 7:00 am) to avoid circadian interferences. Body weight was recorded weekly and food intake biweekly. After seven weeks, 1 hour after the last treatment and being deprived of food, animals were sacrificed by decapitation. Blood was recollected in non-heparinized flasks and waited an hour at room temperature. Then, blood was centrifuged (2000×g, 15 min, 4 °C) to

obtain serum samples. Organs and tissues of interest were collected, weighed and frozen at –80 °C until analysis. The experiment followed the guidelines of the Animal Ethics Committee vide letter number 9495 of the University Rovira i Virgili (Tarragona, Spain).

2.4. Biometric parameters and feeding tests

Body weight gain (g) was calculated by the difference between final rat weight and weight at the beginning of the treatment, and body fat (%) was determined by the sum of the adipose tissues divided for the weight of the animal. The weight of the skeletal muscle corresponds to total weight of gastrocnemius and soleus muscles. Food efficiency was estimated as the increase in body weight per kcal consumed during treatment.

2.5. Biomarkers of antioxidant condition

2.5.1. Oxygen radical absorption capacity

The method described by Huang e*t al.* [19] was used to measure ORAC. Briefly, 25 µL of serum solution and 150 µL of 59.8 nM fluorescein were added to each well of a 96-well microplate. Then, 25 µL of 73 mM of the radical generator 2,2′-Azobis(2-methylpropionamidine) dihydrochloride were added. Trolox solution at different concentrations was used as a standard. The fluorescence was measured at λex = 485 nm and λem = 528 nm every 2 min for 120 min in FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, VT, USA). The ORAC values are expressed as mmol Trolox eq/L.

2.5.2. Reduced glutathione

The liver reduced glutathione (GSH) assay was performed following the method explained by Kamencic e*t al.* [20]. Briefly, 90 µL of homogenized liver were mixed with 10 µL of solution of 100 µM monochlorobimane and glutathione S-transferase (1 U/mL); the homogenate was incubated at room temperature for 30 min. The GSH values were measured in a FLx800 Multi-Detection Microplate Reader with excitation at 360 nm and emission measured at 528 nm, which were determined from the standard curve for GSH. The GSH levels are expressed as µmol of GSH eq/g liver.

2.6. Biomarkers of oxidative stress

2.6.1. Alanine aminotransferase and aspartate aminotransferase activities

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymatic activities in rat serum were estimated as recommended by the protocols of commercial kits (Química Clínica Aplicada S.A., Amposta, Spain), on an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) and the results are expressed as IU/L.

2.6.2. Malondialdehyde level

Malondialdehyde (MDA) concentration in liver samples were measured by the TBARS assay [21]. Liver homogenate was mixed with 20% trichloroacetic acid in 0.6 M HCl (1:1, *v/v*), and the tubes were kept in ice for 20 min. Samples were centrifuged at 1500×*g* for 25 min before adding TBA (120 mM in Tris 260 mM, pH 7) to the supernatant in a proportion of 1:5 (*v/v*); then, the mixture was boiled at 97 °C for 30 min. Spectrophotometric measurements at 540 nm were conducted at 20 °C. MDA concentrations were calculated as nmol MDA/g liver. This same method was used to estimate the MDA levels in serum and the values are expressed as µmol MDA/L.

2.6.3. ROS generation

The concentration of ROS in liver tissue was quantified using the method described by Gabbia *et al.* [22] with some modifications. Briefly, 50 µL of homogenized liver were mixed with 500 µL of a solution of 2′,7′-dichlorofluorescein diacetate (10 µM) diluted 1:200 in Tris-HCl buffer and the mixture was incubated for 40 min at 37 °C. FLx800 Multi-Detection Microplate Reader (λex = 485 nm and λem = 528 nm) was used to assess the fluorescence intensity of the samples. The ROS values are expressed as fluorescence units (FU)/kg liver.

2.7. Statistical analysis

Data are given as the mean ± standard deviation (SD). Student's *t*-test was used to estimate any statistical difference ($p < 0.05$) between LT and NLT.

Normality tests and Levene's test were performed at values of biometric parameters and biomarkers (mean ± standard error of the mean, SEM). Then, they were subjected to two-way analysis of variance (ANOVA) to determine the influence of the factors studied (P, T and P×T) and, within each photoperiod, were submitted to one-way ANOVA to establish any differences among the means, followed by the least significant difference (LSD) post hoc multiple comparison test to identify the means that differ, using SPSS (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Proximate composition of local and non-local Ekstasis tomatoes

Proximate composition analysis was carried out for the amounts of moisture, ash, total dietary fibre, protein, total lipids (fat) and total carbohydrate in the freeze-dried fruits, while sugar content was determined in fresh tomatoes (Figure 2).

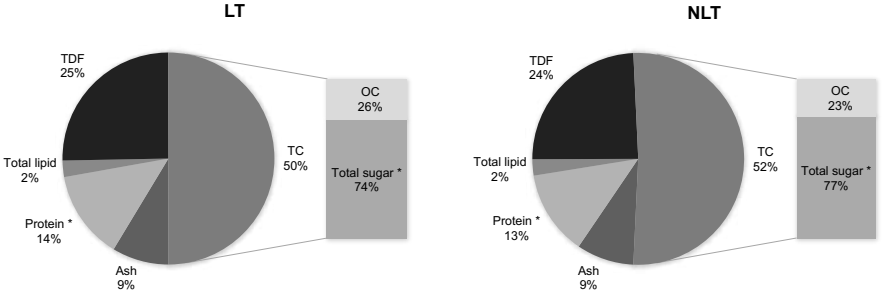

Figure 2. Proximate composition of local (LT) and non-local (NLT) Ekstasis tomatoes. Results are expressed as gram of nutrient per 100 grams of dry weight (g/100 g dw) ± SD ($n = 3$). *$p < 0.05$ LT vs. NLT by Student's *t*-test. Sugar content was determined by quantifying the total soluble solids (°Brix) present in fresh tomatoes using a handheld refractometer. The total carbohydrate (TC) content was calculated by difference, subtracting all the nutrient contents determined. Abbreviations: LT, local tomato; NLT, non-local tomato; OC, other carbohydrates; TDF, Total dietary fiber.

The water content in the LT was 94.95% and in the NLT was 94.86%. As seen in Figure 2, total carbohydrate was predominant in the composition, constituting approximately 50% of both fruits, followed by an important content of fibre (around 25%). On the other hand, the total lipid represented the smallest proportion (2.5%). LT differed significantly from NLT in the content of protein

and of sugar. Indeed, protein content was higher in LT while sugar content in NLT.

3.2. Phenolic profile of local and non-local Ekstasis tomatoes

LT and NLT showed a particular phenolic signature (Table 1). In fact, tomatoes varied significantly from each other in the content of flavan-3-ols, which was 1.1 times higher in LT. Regarding the distribution in flavonoids, among the families evaluated, flavonols were the most abundant in both tomatoes; on the contrary, the anthocyanins and flavan-3-ols of both fruits were low, under 0.03 mg/g dw.

Table 1. Phenolic profile of local (LT) and non-local (NLT) Ekstasis tomatoes.

Flavonoids	LT	NLT	*p* value
TAC (mg Cy3R eq/g dw)	0.026 ± 0.01	0.017 ± 0.01	0.245
TFaC (mg Cat eq/g dw)	0.021 ± 0.00	0.019 ± 0.00	0.000
TFoC (mg Quer eq/g dw)	0.772 ± 0.05	0.744 ± 0.00	0.465

The results are expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n = 3). Abbreviations: Cat, catechin; Cy3R, cyanidin-3-*O*-rutinoside; Quer, quercetin; TAC, total anthocyanins content; TFaC, total flavan-3-ols content; TFoC, total flavonols content.

3.3. Biometric parameters and feeding tests

The body composition and feeding tests of rats exposed to three photoperiods and supplemented with lyophilized LT and NLT or VH for 7 weeks are represented in Table 2. In the conditions of autumn/spring and summer is where significant differences due to the treatment were evidenced. For instance, inguinal white adipose tissue (IWAT) exhibited a smaller weight in autumn/spring when the rats were supplemented with LT compared to the control group, while in summer the NLT-supplemented rats stood out. Furthermore, animals fed tomatoes presented lower food intake than their controls, except for LT-rats in L6. In fact, in two photoperiods above mentioned, NLT supplementation was significantly highlighted. It is important to note that in all photoperiods, epididymal white adipose tissue (EWAT) weight was numerically lower in the tomato-fed animals than their respective control groups.

Table 2. Biometric parameters and feeding tests of Fischer 344 rats exposed to three different photoperiods and supplemented with vehicle (VH), local tomato (LT) or non-local tomato (NLT) for 7 weeks.

	L6 photoperiod			L12 photoperiod			L18 photoperiod			*2wA*
	VH	**LT**	**NLT**	**VH**	**LT**	**NLT**	**VH**	**LT**	**NLT**	
Biometric parameters										
Body weight gain (g)	44.63 ± 4.12	52.63 ± 3.14	52.88 ± 3.12	54.00 ± 3.50	50.25 ± 3.29	47.88 ± 2.64	51.50 ± 0.72	50.38 ± 2.19	44.50 ± 3.73	*n.s.*
Body fat (%)	4.37 ± 0.16	4.42 ± 0.19	4.58 ± 0.22	4.78 ± 0.20	4.47 ± 0.20	4.42 ± 0.16	4.62 ± 0.16	4.74 ± 0.12	4.64 ± 0.14	*n.s.*
EWAT (g)	9.01 ± 0.44	8.44 ± 0.56	8.43 ± 0.50	9.49 ± 0.47	8.52 ± 0.32	9.02 ± 0.59	9.88 ± 0.74	9.22 ± 0.37	9.25 ± 0.37	*n.s.*
IWAT (g)	2.83 ± 0.37	2.32 ± 0.30	2.09 ± 0.05	2.78 ± 0.19	2.20 ± 0.12*	2.29 ± 0.24	3.35 ± 0.37	2.68 ± 0.23	2.49 ± 0.23*	*T*
MWAT (g)	5.56 ± 0.50	5.38 ± 0.40	5.23 ± 0.54	5.53 ± 0.30	4.87 ± 0.40	4.85 ± 0.30	5.12 ± 0.30	5.64 ± 0.62	6.55 ± 0.71	*n.s.*
BAT (g)	0.40 ± 0.06	0.46 ± 0.04	0.41 ± 0.04	0.39 ± 0.03	0.38 ± 0.01	0.33 ± 0.01	0.44 ± 0.03	0.41 ± 0.03	0.36 ± 0.03	*n.s.*
Skeletal muscle (g)	4.13 ± 0.10	4.18 ± 0.04	4.06 ± 0.14	4.30 ± 0.10	4.19 ± 0.04	4.11 ± 0.12	4.43 ± 0.07	4.28 ± 0.10	4.23 ± 0.15	*n.s.*
Feeding tests										
Food efficiency (g/100 kcal)	2.16 ± 0.14	2.66 ± 0.09	2.69 ± 0.25	2.46 ± 0.18	2.38 ± 0.12	2.46 ± 0.17	2.75 ± 0.20	2.72 ± 0.07	2.65 ± 0.23	*n.s.*
Food intake (kcal/day)	54.14 ± 0.29	56.64 ± 2.11	54.08 ± 0.66	55.41 ± 1.15	53.01 ± 0.78	52.02 ± 1.36*	54.92 ± 0.47	52.18 ± 1.63	50.51 ± 1.62*	*T*

Data are expressed as the mean ± SEM (n = 8). Body weight gain (g) was calculated by the difference between final rat weight and weight at the beginning of the treatment, and body fat (%) was determined by the sum of the adipose tissues divided for the weight of the animal. The weight of the skeletal muscle corresponds to total weight of gastrocnemius and soleus muscles. Food efficiency was estimated as the increase in body weight per kcal consumed during treatment. Two-way ANOVA (2wA) was used to assess the effect of the factors examined (P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect). Then, within each photoperiod, a one-way ANOVA was carried out to identify any differences among the means: *$p < 0.05$ vs vehicle. Abbreviations: BAT: brown adipose tissue, EWAT: epididymal WAT, IWAT: inguinal WAT, L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, MWAT: mesenteric WAT, n.s.: no significant, WAT: white adipose tissue.

3.4. Antioxidant status biomarkers

The data in **Figure 3** expose the antioxidant status of F344 rats subjected to three photoperiods and chronically supplemented with local and non-local tomato. 2-way ANOVA showed that the antioxidant capacity was significantly affected by all the factors under evaluation, namely photoperiod, treatment and their interaction.

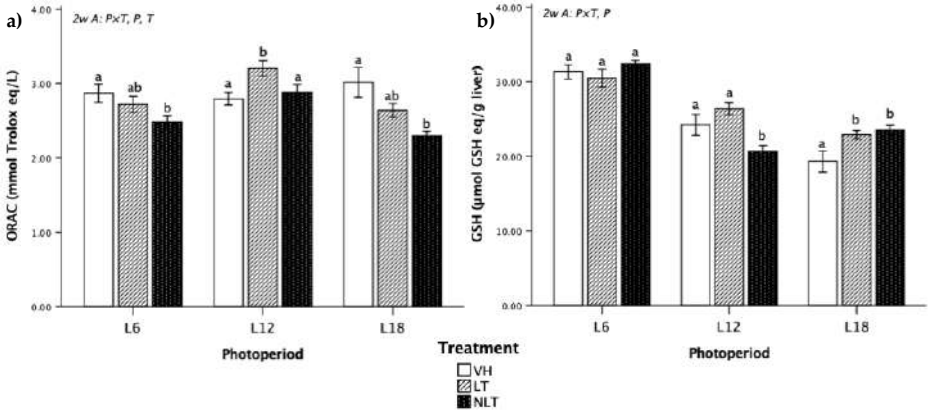

Figure 3. Antioxidant condition of Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with vehicle (VH), local tomato (LT) or non-local tomato (NLT): (**a**) Oxygen radical absorbance capacity (ORAC); (**b**) Reduced glutathione (GSH) concentration. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA analysis [3×3 factorial designs: treatment (VH, LT or NLT)×photoperiod effect (L6, L12 or L18)] was used to assess the effect of the factors examined: P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Different letters indicate significant differences among treatments within each photoperiod exposed by one-way ANOVA. Abbreviations: ANOVA: analysis of variance, L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, SEM: standard error of the mean.

In fact, in the tomatoes fruit consumption season (emulated by L12 photoperiod), LT supplementation significantly increased ORAC levels by 14.70% compared to VH rats, while out-of-season, both L6 and L18, supplementation with LT had a similar effect than with NLT. Remarkably, NLT consumption was never able to enhance serum ORAC levels. Thus, out-of-season NLT supplementation produced lower ORAC values than the VH group, while when NLT was consumed in-season its ORAC levels were equal to the VH group.

Interestingly, the control animals showed numerically the highest ORAC values when subjected to summer lighting conditions (L18).

Notably, the GSH concentration in liver increased as rats were exposed to less hours of light. In the consumption season (L12 photoperiod), NLT supplementation generated lower GSH values while the LT supplementation numerically increased the GSH values compared to the VH group. In summer conditions (L18 photoperiod), both LT and NLT supplementation, increased GSH values compared to the control group.

It was clearly observed that LT intake in-season, in our study represented by the L12 photoperiod, numerically increased the antioxidant status (ORAC and GSH values) of the animals studied.

3.5. Oxidative stress biomarkers

3.5.1. Liver oxidative stress biomarkers

MDA values and ROS generation were measured in liver tissues of F344 rats supplemented with local or non-local freeze-dried tomato or vehicle and exposed to three photoperiods (**Figure 4).** Indeed, both biomarkers were characterized by effects of P, T and P×T ($p < 0.05$, two-way ANOVA), emphasizing the strong influence of the treatments performed. Notably, it was observed that the administration of tomato significantly decreased the MDA values with respect to their controls, except in rats supplemented with NLT in the L12 photoperiod, where no significant differences were evidenced. In fact, the LT administration stood out for generating the lowest MDA values in rats in the L12 (in-season consumption) and L18 (out-of-season consumption) photoperiods; while in the L6 photoperiod, the lowest MDA levels were similarly attributed to the administration of both tomatoes. Regarding the generation of ROS, the highest levels were visualized in the animals exposed to L18 photoperiod, being similar in the three treatment groups. The treatment significantly affected this parameter, since, in L6 photoperiod, LT supplementation generated a decrease in the ROS generation with respect to its counterparts and in L12 photoperiod it produced similar values to the control group but lower than those generated by NLT supplementation. It is important to highlight that

when both tomatoes were consumed in-season (L12, autumn) they led to the greatest decrease in ROS generation compared to their out-of-season counterparts.

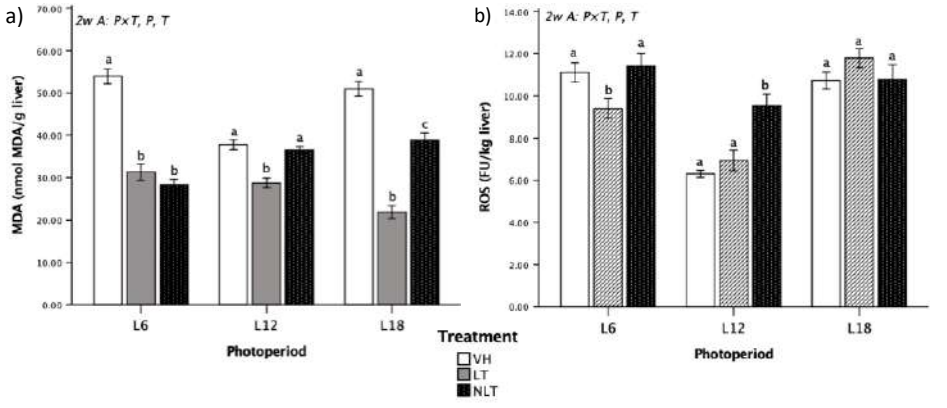

Figure 4. Liver oxidative stress biomarkers in Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with vehicle (VH), local tomato (LT) or non-local tomato (NLT): (**a**) malondialdehyde (MDA) level; (**b**) reactive oxygen species (ROS) generation. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA analysis [3×3 factorial designs: treatment (VH, LT or NLT)×photoperiod effect (L6, L12 or L18)] was used to assess the effect of the factors examined: P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Different letters indicate significant differences among treatments within each photoperiod exposed by one-way ANOVA. Abbreviations: ANOVA: analysis of variance, L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, SEM: standard error of the mean.

3.5.2. Serum oxidative stress biomarkers.

ALT and AST activities and MDA concentration were also measured in rat serum to determine the circulating level of these biomarkers. Results show that the levels of these biomarkers were affected by all the factors under assessment, including photoperiod, treatment (except in the case of AST activity) and the interaction of photoperiod×treatment, showing a distinct and particular pattern of behaviour for each of them (Figure 5). About the enzyme activities associated with hepatic injury, rats exposed to L12 photoperiod showed the highest activity of ALT and this activity was similar among the groups of this photoperiod while, in the other two photoperiods, ALT activity was significantly diminished when the animals consumed NLT. On the other hand, AST values were lower in animals fed LT when they were subjected to L12 photoperiod (in-season consumption),

but conversely, in L6 photoperiod (out-of-season consumption) this group had significantly highest AST value than the VH group. As far as MDA concentration is concerned, the lowest levels were found in rats exposed to L18 photoperiod, here, no differences between the groups were found.

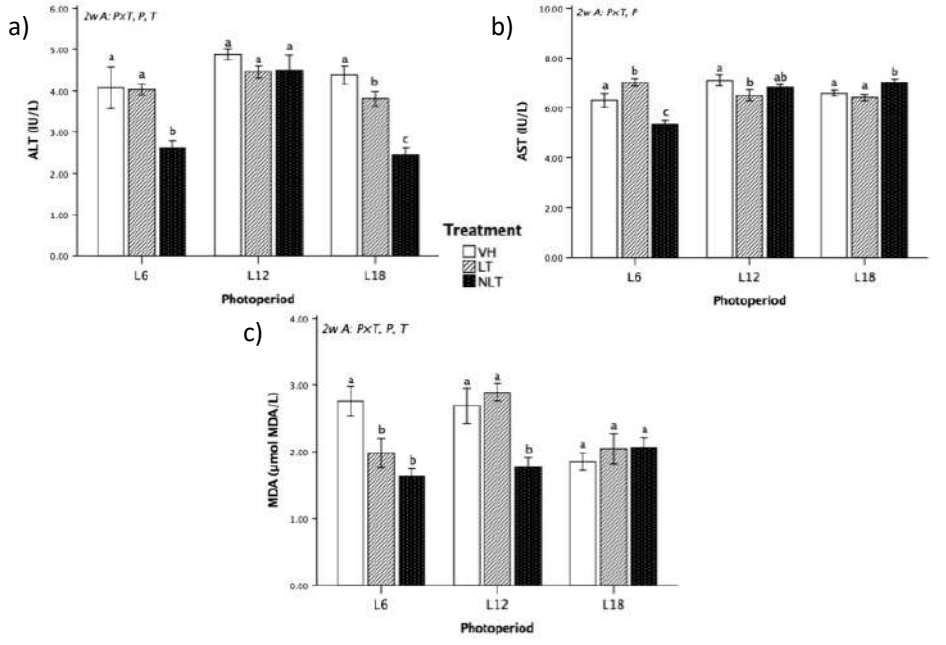

Figure 5. Serum oxidative stress biomarkers in Fischer 344 rats exposed to three different photoperiods for 7 weeks and supplemented with vehicle (VH), local tomato (LT) or non-local tomato (NLT): (a) alanine aminotransferase (ALT) activity; (b) aspartate aminotransferase (AST) activity; (c) malondialdehyde (MDA) level. Data are expressed as the mean ± SEM (n = 8). Two-way ANOVA analysis [3×3 factorial designs: treatment (VH, LT or NLT)×photoperiod effect (L6, L12 or L18)] was used to assess the effect of the factors examined: P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Different letters indicate significant differences among treatments within each photoperiod exposed by one-way ANOVA. Abbreviations: ANOVA: analysis of variance, L6: 6 h light/day, L12: 12 h light/day, L18: 18 h light/day, SEM: standard error of the mean.

In addition, in L6 photoperiod exposed rats, supplementation of both tomatoes decreased MDA levels compared to VH group whereas in L12 photoperiod this effect was carried out by the intake of NLT. Interestingly, animals that consumed NLT in-season had the highest ALT enzyme activity compared to their out-of-season counterparts, while animals that consumed LT in-season showed this behaviour with MDA values.

4. Discussion

In recent studies, it has been shown that the activity of bioactive compounds or dietary components can be modulated by biological rhythms [5]. In this sense, there is a growing evidence that seasonal changes in health and disease, and biomarkers of oxidative stress are modulated by circannual pattern in accordance with the photoperiod [5,14,23]. Circannual rhythms in the redox status of organisms have been sparingly investigated [14,23]. Taking into account that tomato has been described to be an efficient and ubiquitously acting free radical scavenger and antioxidant [10,24] and considering the growing importance of biological rhythms, there is renewed interest in the possibility that the ability of organisms to defend against free radicals may vary according to the season in which the tomato is consumed. Our study evaluates the antioxidant properties of tomato from two different geographical origins concerning the ability of animals to resist oxidative damage over a seasonal period. For this, the effect of seasonal consumption of tomatoes from different growing locations (local and non-local) was evaluated in rats chronically exposed to three photoperiods, consisting of short photoperiod L6, middle-length photoperiod L12 and long photoperiod L18, to simulate winter, autumn/spring, and summer seasons, respectively.

The daily endogenous circadian rhythms of the activities of antioxidant enzymes and of low-molecular weight antioxidants have been described in have been described in various organisms [3]. Several studies suggest the relevance of biological rhythms to prevent excessive oxidative stress [3]. Effectively, antioxidant status of the animals studied was modulated by the photoperiod to which they were exposed. In fact, the GSH was higher in the rats exposed to the L6 photoperiod (emulating winter light conditions). This fact can be interpreted as an adaptive response to the oxidative stress generated by winter light schedule, resulting in a basic molecular mechanism of increased tolerance to environmental stress. This statement is supported by the findings of Escribano *et al.* [23] who claimed that GSH values increased when healthy animals were exposed to less light time and the opposite effect occurred when they were exposed to more light time. Thus, winter light schedule seemed to increase the rats GSH values whereas the summer light schedule decreased it, strengthening the evidence that

the concentration of GSH is modulated by light exposure. When there are environmental changes, as a response mechanism, the production of ROS in the cell is altered, but these fluctuations can also be the product of endogenous metabolic oscillations regulated by the biological clock [3]. Circannual variations are strongly evidenced in the control group, disregarding the treatment administered. Indeed, when the control animals were in the L12 photoperiod exhibited the lowest levels of ROS, showed significantly lower concentrations of liver MDA and displayed the highest ALT activity than their counterparts subjected to the other two photoperiods. Similar results were observed in our previous study, where F344 rats showed the lowest ROS values when subjected to the same conditions of the L12 photoperiod [14]. Similarly, results of the study by Guo *et al*. [25] showed that L12 photoperiod decreased MDA in broiler chickens. Moreover, the findings of ALT activity are similar to the obtained by Cerutti *et al*. [26] who concluded that ALT values were influenced by season in bulls. Specifically, authors showed that ALT values of clinically healthy Holstein cattle were higher in spring than in winter. Similarly, our results show that liver GSH presents a circannual rhythm of formation, as it seems that in the seasons with more light hours this biomarker decreased in the control animals. This finding is consistent with the results of Escribano *et al*. [23], who reported that healthy animals housed in light cycle showed lower GSH values than those in dark cycle. Finally, the photoperiod L18 was characterized by the lowest of MDA levels in the serum of the control animals compared with their counterparts in the other two photoperiods. This finding is in line with that reported by Baydas *et al*. [27] in pinealectomized rats, were the plasma MDA levels increased progressively in the dark photoperiod.

Consumption of fruit and vegetables have been linked with a protective action against different chronic diseases [1]. In fact, World Health Organization recommends a daily intake of at least 400 g of fruits and vegetables per day as part of a healthy eating pattern for the prevention of chronic diseases [28]. In our current study, tomatoes were administered daily to the experimental rats at a dose of 100 mg lyophilized fruit/kg body weight, using doses translation from animal to human [29] and estimating the intake for a 70 kg human, this dose is

equivalent to eating approximately 24 g of fresh tomatoes per day, which could constitute an important amount of the major dietary source of several micronutrients and phytochemicals. Abundant evidence exists showing that consumption of tomato (*Lycopersicon esculentum* Mill.) acts as an *in vivo* antioxidant, providing protection against lipid, protein and DNA damage [11]. In fact, tomatoes have been reported to be a rich source of antioxidant compounds [11]. These compounds may act synergistically to generate the antioxidant effect of the fruit. In this line, Hanson *et al.* [24] have reported that among tomato antioxidants, *i.e.,* lycopene, β-carotene, vitamin C and phenolic compounds, the latter were the most strongly related to antiradical power (r = 0.90) and inhibition of lipid peroxidation (r = 0.83), suggesting that phenolic compounds contribute greatly to the overall antioxidant activity of tomato fruit. Therefore, in our study we have focused on the phenolic compounds, in order to estimate the overall antioxidant potential of the tomatoes, specifically on the flavonoid subclasses although a limitation of the study is not to evaluate the other antioxidant compounds contained in tomato.

Interestingly, in our study, the supplementation of both tomatoes led to a numerical decrease in the weights of epididymal (EWAT) and inguinal (IWAT) white adipose tissues. This finding is in line with the study by Kim *et al.* [30], who concluded that rats fed high-fat diet (HFD) with tomato-wine and variable lycopene content had a reduction in adipose tissue. In fact, this effect of tomato seems to be mediated by inhibition of fatty acid synthesis and formation of lipid droplets. Therefore, a study proved that supplementation with tomato juice resulted in a decrease in fat tissue and peroxidative stress in young, healthy women in Taiwan [31]. Likewise, another study showed that a tomato product, vinegar, inhibited lipid accumulation in HFD rats [32]. Indeed, several human or animal intervention trials with tomatoes have revealed effects on lipid levels, antioxidants and inflammation. These findings support the evidence that another mechanism generated by this fruit to prevent oxidative stress is the reduction of WAT weight, since oxidative stress associates with intra-abdominal obesity [33]. The build-up of mitochondrial oxidative stress promotes obesity, and, together with the formation of ROS, influence the endocrine and metabolic activity of fat

cells in WAT [33]. In addition, another effect of tomatoes consumption was seen in the reduction of food intake of animals, the same result was reported by Friedman *et al*. [34] in mice fed tomato alkaloids.

The tomatoes used in this study were from the same cultivar (Ekstasis), cultured following conventional agricultural practices, but from different cultivation regions: LT was cultivated in the northeast of Spain (Tarragona) and NLT was cultivated in the southeast of Spain (Almería). The antioxidant concentration and chemical composition in a certain fruit or vegetable are strongly influenced by different factors such as the geographical and environmental differences [13]. Effectively, LT and NLT differed in sugar and protein content. In addition, genotype and environmental factors have significant effects on the content of the secondary metabolites in tomato fruits [35]. In fact, flavonoids profile of the two fruits was determined, resulting a specific and particular phenolic signature, significantly different in TFaC, demonstrating the environmental influence on flavonoids synthesis. These results are in line with the data in the literature, although the comparison is often difficult due to the strong influence of environmental factors typical and specific to each growing area. Indeed, a study by Asensio *et al*. [36] compared the "Rosa de Barbastro" tomato variety grown in two locations: Barbastro and Montañana, reporting that polyphenols was different between them. Similarly, Stewart *et al*. [37] showed that smaller cherry tomato fruits originating from warm sunny climates, such as Spain and Israel, contained higher concentration of flavonols than British fruits. Thus, also the differences in flavan-3-ols between the two tomatoes could be attributed to the fact that LT were harvested at the date of consumption, while NLT had to be harvested well before its commercial ripening stage in order to be transported to the place of consumption. Indeed, the time from harvest to consumption of the fruits can be up to several weeks if post-harvesting practices are used. During this period, the phytochemical responses of the harvested fruits to environmental factors can modify the rates of biological activity of bioactive compounds, such as flavonoids [38]. In fact, Buta and Spaulding [39] informed of high phenolic concentrations at the earliest stage of tomato growth, but these concentrations decreased quickly during post-harvest ripening. In addition, results from study by Verheul *et al*. [40]

show significant differences in tomato phenolics from harvest to sale, as related to postharvest conditions. Moreover, coincidentally with the study carried out by Vallverdú-Queralt *et al.* [41], the polyphenols of ketchups and tomato juices decreased during storage. Thus, several studies have proved that polyphenols of the foods change on storage due to oxidation of these compounds [42]. Indeed, literature confirms that bioactive compounds, *i.*e., flavonoids content in tomatoes is strongly degraded during post-harvest ripening conditions [38,40,41].

Current published data suggest that the timing of phenolic compounds consumption may also be a determining factor in the beneficial effects of these compounds [43]. For example, it has been shown that acute intake of catechin-rich green tea in the evening reduced postprandial plasma glucose concentrations compared to morning intake [43].

Each tomato (LT and NLT) was characterized by a specific phenolic signature and a distinctive nutritional composition, which generated different effects on the animals that consumed them. In fact, we showed that the antioxidant power of tomatoes was different depending on the crop origin of each sample and in accordance with the different content of main subclasses of flavonoids that each one contained. Indeed, in-season LT supplementation led to an increase in ORAC values relative to the control group, while out-of-season LT supplementation led to the same effect. On the other hand, LT supplementation was noted for increasing ORAC levels compared with NLT supplementation. Regarding to oxidative stress biomarkers, the supplementation of LT generated a reduction in liver MDA levels in those animals subjected to L12 and L18 photoperiod, and in L6, it generated the same effect of NLT supplementation. In addition, the administration of LT caused the generation of ROS to be reduced or held constant with respect to the control group. Similarly, the consumption of LT generated a decrease in the generation of ROS in F344 rats stabled in L6 and L12 in comparison with the consumption of NLT, while in L18 the two fruits presented the same effect. On the other hand, NLT supplementation tended to reduce ALT enzyme activity in L6 and L18. Interestingly, consumption of LT and NLT produced the same effect on GSH levels and serum MDA values, except in-season consumption. These marked effects could be related to the distinctive

phenolic distribution and proportion of each tomato, *i.e.*, to its phenolic signature. And, therefore it can be attributed to the antioxidant properties of its constituent polyphenols [24], this suggests that flavonoids make a key contribution to antioxidant power of tomatoes. It is well established that polyphenol-rich foods may increase plasma antioxidant capacity, and this effect depend on both their respective intakes and their bioavailability, which can vary greatly [42]. From the flavonoid subclasses studied, tomatoes differed significantly in total flavan-3-ols content, so the differences in antioxidant responses generated after consumption of tomatoes could be attributed to the difference found in this subclass. In addition, previous studies have shown a strong relationship between the phenolic acids contained in tomatoes, especially ferulic and caffeic acids, and their antioxidant capacity [44]. Therefore, these phenolic compounds may also be related with their antioxidant capacity. Moreover, when the tomatoes, LT or NLT, are consumed in-season (L12 photoperiod, autumn) they tend to improve or maintain constant ORAC levels; they tend to reduce enzymatic activity of AST and maintain constant enzymatic activity of ALT, and they tend to diminish or maintain constant liver and serum MDA values. Similar results were evidenced in our recent study where F344 rats were supplemented with sweet cherries and it was observed that their in-season consumption tends to enhance antioxidant status, decrease ALT and AST activities and reduce MDA concentration [14]. These tendencies can produce an optimal metabolic response, generating a balance between production and accumulation of ROS. When maintained at low or moderate concentrations, free radicals play several beneficial roles for the organism. For example, they are needed to synthesize some cellular structures and to be used by the host defence system to fight pathogens [2]. In contrast, when tomatoes are consumed out-of-season tends to lower numerically ORAC levels and it tends to increase enzymatic activity of AST (LT in L6 and NLT in L18); these responses can lead to a disruption in characteristic seasonal metabolism and regarding to oxidative stress can cause damage to many tissues, which can lead to a number of diseases over time. In addition, recent studies by our research group have shown that the consumption out-of-season of sweet cherry rich in polyphenols increased whole-body fat oxidation and circulating levels of glucose and insulin [6,7]; changed the morphology of white adipose tissue

[7]; and also modulated the hypothalamic leptin system [8]. Furthermore, when the sweet cherries were consumed out-of-season it produced GSH levels decreased and AST activity and ROS generation increased in F344 rats [14]. Likewise, the out-of-season consumption of Navelina oranges also exerted dyslipidaemia and insulin resistance [9].

5. Conclusions

We demonstrated that in-season consumption of tomatoes, regardless of their geographical origin, can produce an optimal metabolic response by preventing oxidative stress, showing a tendency to improve or maintain constant ORAC levels; reduce AST activity and maintain constant ALT activity and diminish or maintain constant liver and serum MDA values in F344 rats. Nevertheless, the consumption of tomatoes out-of-season could lead to erroneous signalling. In addition, the antioxidant power of tomatoes is strongly impacted by the geographical origin of the crop. These results highlight the importance of consumption of local and seasonal fruits to promote optimal health. Further studies focusing on the health impact of seasonal tomatoes consumption are needed to define more precise dietary recommendations for fruits and vegetables.

Author Contributions

Álvaro Cruz-Carrión: Conceptualization; Data curation; Formal analysis; Investigation; Methodology, Writing - original draft. Ma. Josefina Ruiz de Azua: Formal analysis; Investigation. Francisca Isabel Bravo: Conceptualization; Funding acquisition; Supervision; Writing - Reviewing and Editing. Gerard Aragonès: Conceptualization; Funding acquisition; Supervision. Begoña Muguerza: Conceptualization; Funding acquisition; Supervision; Manuel Suárez: Conceptualization; Funding acquisition; Investigation; Methodology; Supervision; Writing - Reviewing and Editing. Anna Arola-Arnal: Conceptualization; Funding acquisition; Investigation; Methodology; Supervision; Writing - Reviewing and Editing.

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Conflicts of interest

There are no conflicts to declare.

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CHAPTER III

To evaluate whether geographical origin of cultivation and seasonal consumption of tomato cv. Ekstasis affect its phenolic bioavailability in rats.

OBJECTIVES 3.1 and 3.2

To determine the phenolic profile of tomatoes cv. Ekstasis from two geographical origin of cultivation by uHPLC-MS^n

To evaluate the pharmacokinetic profiles of phenolic compounds after acute consumption of tomato cv. Ekstasis from two geographical origin of cultivation in rats

(POLY)PHENOLIC COMPOSITION OF TOMATOES FROM DIFFERENT GROWING LOCATIONS AND THEIR ABSORPTION IN RATS: A COMPARATIVE STUDY

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Abstract

Environmental factors, such as the growing location, have an important effect on tomato (poly)phenol content which in turn may reflect the putative health benefits related to the consumption of this foodstuff. The aim of this work was to address whether the growing location of tomato could generate a different (poly)phenol profile able to affect both the *in vivo* absorption and the (poly)phenol metabolite pattern upon tomato consumption. uHPLC-MS^n analyses allowed to obtain a detailed (poly)phenol profile of tomatoes from two locations in Spain, quantifying 57 (poly)phenolic compounds. Phenolic acids were by far the main (poly)phenol subclasses recovered in tomatoes, mainly caffeoyl derivatives representing more than 50% of the overall tomato (poly)phenol amount. However, local and non-local tomatoes showed a different concentration of their native (poly)phenols, which could be attributed to the diverse cultivation origin. Rat serum was analysed after an acute tomato feeding. Seven phenolic metabolites were quantified through uHPLC-MS^n, unravelling that all serum metabolites could be produced *via* caffeic acid, mainly by formation of sulfate and methyl-sulfate conjugates. No phase II metabolites of flavonoids were present in detectable amounts. Pharmacokinetic parameters were further evaluated, revealing different serum concentrations of phenolic metabolites between local and non-local tomato groups. The maximum peak serum concentrations, reached mainly after 2 hours of tomato ingestion, led to suppose that serum metabolites were mostly derived from absorption and metabolism in the upper gastrointestinal tract. The growing location of tomato fruits affected both the content of native (poly)phenols and their *in vivo* absorption, which in turn could affect their bioactivity and health benefit prospects of tomato intake.

Keywords

Geographical origin, intestinal absorption, kinetics, metabolites, polyphenols.

Abbreviations

AUC, area-under-the-curve; C_{max}, maximum serum concentration; LT, local tomato; MRT, mean residence time; n.d., not detected; n.q., not quantified; NLT, non-local tomato; T_{max}, time of peak serum concentration.

1. Introduction

There is considerable epidemiological evidence indicating that the consumption of diets rich in fruits and vegetables is associated with a reduction of chronic diseases, including cardiovascular diseases, neurodegeneration, and some types of cancer [1]. In this sense, it is well known that part of the health benefits of the Mediterranean diet could be attributed to the high content of fruits and vegetables rich in bioactive compounds [2]. Within this framework, tomatoes (*Lycopersicon esculentum*) represent an important part of the Mediterranean diet and their regular consumption has been consistently associated with a lower risk of several types of cancer and coronary heart disease [2,3]. Indeed, tomato is a rich source of nutrients and phytochemicals that are widely studied for their potential health properties, including fibre, minerals, vitamins C and E, carotenoids, chlorophylls, (poly)phenols, glycoalkaloids, and organic acids [4,5]. The tomato (poly)phenolic composition is genotype-dependent but it is also modulated by many agronomic, geographical and seasonal factors [6]. A recent study carried out with the variety of tomatoes "*Rosa de Barbastro*" showed that the location of cultivation influenced the concentration of (poly)phenols. Specifically, higher concentrations of caffeic acid, *p*-coumaric acid, ferulic acid and total phenolic content were found in tomatoes grown in an area while those grown in another area exhibited substantially higher concentrations of chlorogenic acid [4]. Furthermore, there is a vast literature on the role of (poly)phenols in the prevention of chronic diseases [1,7]. But it is essential that to fulfil this role, these bioactive compounds have to reach the target tissues in an effective concentration to exert their beneficial health effect [5].

Tomatoes contain quercetin, naringenin, rutin and chlorogenic acid as the main phenolic compounds It is important to keep in mind that the most polyphenols in the human diet are not necessarily the most active within the body, either because they have lower intrinsic activity or because they are poorly absorbed in the intestine, are highly metabolized, or are rapidly eliminated. In addition, metabolites found in the blood and target organs that result from digestive or hepatic activity may differ from native substances in terms of biological activity [7].

After ingestion, (poly)phenols are absorbed, distributed, and extensively metabolised. Absorption of some compounds into the circulatory system takes place in the small intestine [1]. In the course of absorption and before passing to the bloodstream, (poly)phenols are conjugated in the small intestine and subsequently in the liver, i.e., the aglycones undergo some degree of phase II metabolism forming sulfate, glucuronide and/or methylated metabolites [1,7]. Recycling back to the small intestine via biliary excretion may occur due to enterohepatic recirculation [1]. The unabsorbed phenolic fraction reaches the colon undergoing an extensive catabolism by the resident bacteria [1]. In fact, (poly)phenol metabolites rather than their native forms are those who have been attributed the health benefits [8]. Therefore, a thorough knowledge of the bioavailability of (poly)phenolic compounds in tomatoes is essential to understand their health effects. In this context, characterization of (poly)phenolic compounds present in tomatoes is of great interest. Thus, the present study aimed at investigating the (poly)phenol content of tomatoes cv. Ekstasis from two geographical origins and evaluating both (poly)phenol absorption and the circulating metabolites through an acute rat feeding study.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and reagents purchased from VWR International (Milan, Italy) were LC grade or LC-MS grade.

Salicylic, *p*-coumaric, caffeic, dihydrocaffeic, 3-caffeoylquinic. 4-caffeoylquinic, 5-caffeoylquinic acids, rutin and quercetin-3-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vitexin was purchased from Extrasynthese (Genay Cedex, France). Caftaric acid was from PhytoLab GmbH & Co. (Vestenbergsgreuth, Germany). Vanillic acid-4-β-D-glucoside was from Cayman Chemical (Ann Arbor, MI, USA). Quercetin-3′-sulfate was kindly provided by Professor Alan Crozier (University of Glasgow, United Kingdom).

The following standard compounds were from Toronto Research Chemicals (Toronto, ON, Canada); they are named according to the nomenclature proposed by Kay *et al.* [9] while the commercial names and catalogue number are

provided brackets. 3′-hydroxycinnamic acid-4′-glucuronide (caffeic acid 4-β-D-glucuronide, Catalogue N° C080020); 3′-methoxycinnamic acid-4′-sulfate (ferulic acid 4-*O*-sulfate, F308920); 3′-methoxycinnamic acid-4′-glucuronide (ferulic acid 4-*O*-β-D-glucuronide, 308910); 3-(4′-hydroxyphenyl)propanoic acid-3′-glucuronide (dihydrocaffeic acid 3-*O*-β-D-glucuronide, D448705); 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate (dihydro caffeic acid 3-*O*-sulfate, D448710); 3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide (dihydroferulic acid 4-*O*-β-D-glucuronide, D448315); 3-(4′-methoxyphenyl)propanoic acid-3′-glucuronide (dihydroisoferulic acid 3-*O*-β-D-glucuronide, D448940); 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate (dihydroferulic acid 4-*O*-sulfate, D448915).

2.2. Tomato sampling and proximate composition analysis

Tomatoes fruits (*L. esculentum* cv. Ekstasis) conventionally grown in two locations in Spain: in the northeast, Tarragona (41°4′29.24″ N 1°3′8.78″ E; local tomatoes, LT) and in the southeast, Almería (36°50′17.3″ N 2°27.584′ O; non-local tomatoes, NLT), were obtained from a local market in Tarragona at maturity. Whole fruits were frozen in liquid nitrogen and grounded. The homogenates were then freeze-dried for one week at −55 °C using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain). The tomatoes powder was kept dry and protected from humidity and light exposure until use. The dietary components of LT and NLT used in this study are detailed in Supplementary Table 1.

2.3. Extraction of (poly)phenolic compounds in freeze-dried tomatoes

The (poly)phenolic compounds from LT and NLT were extracted according to Mena *et al.* [10] with modifications. Briefly, 50 mg of each lyophilized tomato were added to 1 mL of 80% aqueous methanol acidified with 0.1% formic acid. Then, mixtures were strongly shaken for 1 min and sonicated for 10 min in an ultrasonic bath and vortexed again. After, samples were centrifuged at 16,600× *g* for 10 min at 5 °C and the supernatants were collected. The residues were re-extracted twice more with 1 mL of the same solvent, following the protocol indicated above. Finally, supernatants were diluted (1:4 *v/v*) in water acidified with

0.1% (*v*/*v*) formic acid, vortexed and centrifuged at 16,600× *g* for 10 min at 5 °C before uHPLC-MS^n analyses.

2.4. Animal experimental study

Male Wistar rats were housed at 22 °C with light/cycle of 12 h and were fed *ad libitum* with a standard chow diet (AO4, Panlab, Barcelona, Spain). The animals were randomly separated into two groups: the LT-administered (n = 6) and the NLT-administered (n = 5). After an 8-hour fasting period, rats were acutely administered, by intragastric intubation, a dose of 3 g of LT or NLT per kg body weight (bw). Blood samples were obtained from the saphenous vein using nonheparinized vials (Sarstedt, Barcelona, Spain) before (0 h) and 2, 4, 7, 24 and 48 h after tomatoes administration. After, blood samples were centrifuged (2000 × *g*, 15 min, 4 °C) to collect the serum and then stored at – 80 °C until use. Samples were pooled (LT, n = 6 and NLT, n = 5) to acquire sufficient volumes for chromatographic analysis. Furthermore, according to Margalef *et al.* [11], pooling of biological samples increases homogeneity and sensitivity and, consequently, this allows the detection of all potential metabolites. This experiment was conducted in strict compliance with the institutional guidelines for the care and use of laboratory animals and was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 9495).

2.5. Extraction of tomato-derived (poly)phenolic metabolites in rat serum

Tomato-derived (poly)phenolic metabolites in serum were extracted as previously described by Ardid-Ruiz *et al.* [12].

2.6. uHPLC-MS^n analyses of tomato-derived (poly)phenols and their (poly)phenolic metabolites in rat serum

The samples were directly analysed by ultra-high performance liquid chromatography (uHPLC) coupled with mass spectrometry (MS), using an Accela uHPLC 1250 apparatus equipped with a linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA), fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc.). Analyte separation was performed using an Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8

µm particle size) column coupled with a precolumn Acquity UPLC HSS T3 VanGuard (2.1 × 5 mm, 1.8 µm particle size) (Waters, Milford, MA, USA). All analyses were carried out in negative ionization mode.

Tomato-derived (poly)phenols present in the tomato samples were analyzed using full-scan negative ionization mode with a data-dependent MS^3 method scanning from *m/z* 100 to 2000. The mobile phase consisted of (A) 0.1% (*v/v*) formic acid in acetonitrile and (B) 0.1% (*v/v*) formic acid in water. The chromatographic and mass spectrometer conditions were the same reported by Ricci *et al.* [13], except for sweep gas that was set to 5 (arb. units). The LC-MS characteristics are reported in the **Supplementary Table 2**. The quantification of (poly)phenolic compounds in freeze-dried tomatoes was performed using calibration curves of each standard compound or by using the most structurally related compound as reported in **Supplementary Table 2**. Stock solutions of standard compounds used for quantification of tomato-derived (poly)phenols were prepared in methanol or dimethyl sulfoxide and adequately diluted with water acidified with 0.1% (*v/v*) formic acid to build the calibration curves.

On the other hand, serum (poly)phenolic metabolites were analysed by reducing the formic acid concentration in mobile phase to increase the sensitivity for phenolic acid metabolites. In detail, the mobile phase consisted of (A) 0.025% (*v/v*) formic acid in acetonitrile and (B) 0.025% (*v/v*) formic acid in water by applying the same LC gradient used for analysis of (poly)phenolic compounds in tomato powders. The MS parameters were the same reported by Calani *et al.* [14], except the Collision Induced Dissociation (CID) used that was set to 35 for all metabolites. Serum metabolite profiling after tomato (poly)phenol intake was evaluated through target full MS/MS analyses by monitoring the specific deprotonate molecule (**Supplementary Table 3**), and then quantified by setting the MS in the Selected Reaction Monitoring (SRM) mode. Where possible, (poly)phenolic metabolites were quantified using a calibration curve prepared with a reference compound. When such standards were not available, metabolites were quantified using a structurally related compound (**Supplementary Table 3**). Stock solutions of standard compounds used for quantification of tomato-derived (poly)phenolic metabolites were prepared in

methanol or dimethyl sulfoxide and adequately diluted with 50% (*v*/*v*) aqueous methanol acidified with 0.1% (*v*/*v*) formic acid to build the calibration curves. The limit of detection (LOD) and quantification (LOQ) for all used standards were evaluated. LODs and LOQs were calculated based on the minimal accepted values of the signal-to-noise (S/N) ratio of 3 and 10, respectively.

Helium gas was used for MS/MS experiments. For the quantification of (poly)phenolic metabolites, any compound present at the 0 h time-point was subtracted from the serum concentration at all other time-points. All instrumental data were acquired using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.).

2.7. Pharmacokinetic parameters of tomato-derived (poly)phenolic metabolites

Maximum serum concentration of tomato-derived (poly)phenolic metabolites from 0 to 48 h post dose was defined as C_{max}, with T_{max} being the time at which C_{max} was reached. The area-under-the-curve (AUC_{0-48}) serum concentration-time at a 48-hour interval, representing the exposure of the organism to tomato (poly)phenols; and the mean residence time from the time of dosing to the time of the final quantifiable concentration (MRT_{0-48}) were also determined. The calculations of all kinetics parameters were performed by non-compartmental analysis using PKSolver, an add-in program in Microsoft Excel [15].

2.8. Statistical analysis

Student's *t*-test (SPSS, SPSS Inc., Chicago, IL, USA) was used to estimate any differences in the (poly)phenolic composition of LT and NLT, and any differences in tomato-derived (poly)phenolic metabolites and kinetic parameters. Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Tomatoes-derived (poly)phenolic compounds

The native (poly)phenolic compounds present in lyophilized tomatoes is shown in Table 1. Fifty-seven (poly)phenolic compounds were quantified in LT or NLT. The predominance of phenolic acids was noted, reaching 73% of the total.

Specifically, phenolics belonging to the hydroxycinnamic acids were the most abundant phenolic acids, while hydroxybenzoic acids were the least numerous.

In detail, although the total (poly)phenolic compounds did not vary significantly between the two types of tomatoes, 57% of the compounds quantified in tomatoes showed statistical differences. In particular, LT displayed higher quantities of flavonoids than NLT (1.4-fold), while NLT was noted for containing 1.2-fold higher concentrations of phenolic acids. This difference was mainly due to the higher levels of free phenolic acids and hydroxycinnamoylquinic acids, the latter representing 39 to 45% of total phenolic acids. On the contrary, total caffeic and dihydrocaffeic acid derivatives were similar between the two types of tomatoes, while most caffeic acid derivatives compounds varied significantly between LT and NLT. Moreover, total free phenolic acids were more abundant in NLT. Indeed, all free phenolic acids, except *p*-coumaric acid, were significantly higher in NLT; interestingly, one of them, dihydrocaffeic acid, was only detected in NLT although at very low level. Regarding hydroxybenzoic acid derivatives, 2 out of 3 were statistically more abundant in LT, but the total of these phenolic acids did not show significant differences. In addition, although the sum of all the hydroxycinnamic derivatives did not show significant differences, individually, 67% of the forms varied significantly between LT and NLT. In addition, caffeic acid-*O*-hexoside III was the most abundant in LT, while caffeic acid-*O*-hexoside I was the most abundant in NLT. Hydroxycinnamoylquinic acids, which were the predominant tomato-derived phenolics in the current study, were recovered at higher total concentration in NLT than LT, with tricaffeoylquinic acid and dicaffeoylquinic acid III standing out as being significantly 2.2 and 2.1 times higher in NLT, respectively. Finally, the total concentration of phenylpropanoic acid glycosides was similar between both types of tomatoes, in fact, only dihydroferulic acid-*O*-hexoside and hydroxyphenylpropionic acid-*O*-hexoside varied, the former being higher in LT and the latter in NLT. Overall, 53% of the total (poly)phenol content in LT are represented by phenolics containing caffeoyl groups while in NLT these species reached 62% of the total (poly)phenol fraction.

Regarding flavonoids, they represented only 12% and 8% of the total (poly)phenols in LT and NLT, respectively. Nine of the 15 detected were significantly different between LT and NLT, rutin, the most flavonoid in both tomatoes, being significantly 1.3-times higher in LT. However, other flavonoids, such as kaempferol derivatives, were significantly more abundant in NLT.

Table 1. Concentration of (poly)phenolic compounds in local (LT) and non-local (NLT) Ekstasis tomatoes. The results are expressed as μg/g dw ± SD (*n* = 3).

Compound	LT	NLT
Flavonoids		
Kaempferol-*O*-rutinoside [a]	3.51 ± 0.48	9.27 ± 1.25*
Kaempferol-*O*-rutinoside-*O*-pentoside [a]	4.81 ± 0.58	11.22 ± 2.02*
Luteolin-*O*-hexoside-*C*-hexoside [a]	34.76 ± 3.6	21.2 ± 1.02*
Naringenin	1.64 ± 0.27	2.18 ± 0.47
Naringenin chalcone [b]	1.12 ± 0.20	1.43 ± 0.11
Phloretin 3′,5′- di-C-β-glucopyranoside [c]	107.97 ± 11.5	51.19 ± 4.19*
Quercetin-*O*-dihexoside [a]	5.87 ± 0.76	5.20 ± 0.34
QHRP-*O*-hexoside [a]	1.79 ± 0.43	1.24 ± 0.56
QHRP-coumaric acid [a]	21.16 ± 1.13	10.13 ± 1.57*
QHRP-ferulic acid [a]	10.98 ± 0.4	6.86 ± 0.12*
QHRP-sinapic acid [a]	4.24 ± 0.16	3.66 ± 0.37
QHRP-syringic acid [a]	6.55 ± 0.81	8.28 ± 1.54
Quercetin-*O*-rutinoside-*O*-hexoside [a]	2.52 ± 0.27	0.87 ± 0.13*
Quercetin-*O*-rutinoside-*O*-pentoside [a]	81.03 ± 2.80	64.25 ± 5.82*
Rutin	111.33 ± 1.11	86.38 ± 11.77*
Total, flavonoids	399.32 ± 16.15	283.37 ± 30.38*
Caffeic and dihydrocaffeic acid derivatives		
Caffeic acid derivative I [d]	33.24 ± 1.12	65.55 ± 17.50*
Caffeic acid derivative II [d]	28.10 ± 2.15	14.81 ± 3.34*
Caffeic acid derivative III [d]	13.95 ± 0.68	24.97 ± 6.96
Caffeic acid derivative IV [d]	4.19 ± 0.10	5.37 ± 0.47*
Caffeic acid derivative V [d]	4.79 ± 0.41	4.91 ± 0.98

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Compound	LT	NLT
Caffeoylmalic acid [d]	62.93 ± 3.55	66.80 ± 9.10
Dihydrocaffeic acid derivative [f]	38.14 ± 2.12	32.09 ± 5.61
Total, caffeic and dihydrocaffeic acid derivatives	185.34 ± 10.14	214.49 ± 43.97
Free phenolic acids		
Caffeic acid	27.30 ± 3.03	40.92 ± 1.25*
Dihydrocaffeic acid	n.d.	8.72 ± 2.73*
p-Coumaric acid	15.57 ± 1.14	16.19 ± 2.02
Salicylic acid	29.04 ± 3.22	54.39 ± 12.34*
Total, free phenolic acids	71.90 ± 7.39	120.22 ± 18.34*
Hydroxybenzoic acid derivatives		
Dihydroxybenzoic acid-*O*-pentoside [g]	24.44 ± 1.46	18.30 ± 1.64*
Hydroxybenzoic acid-*O*-hexoside [g]	40.36 ± 1.28	33.59 ± 3.08*
Syringic acid-*O*-hexoside [g]	37.53 ± 0.93	37.53 ± 5.59
Total, hydroxybenzoic acid derivatives	102.34 ± 3.66	89.42 ± 10.30
Hydroxycinnamic acid derivatives		
Caffeic acid-*O*-hexoside I [g]	169.42 ± 5.07	235.81 ± 8.06*
Caffeic acid-*O*-hexoside II [g]	67.52 ± 4.03	60.86 ± 5.82
Caffeic acid-*O*-hexoside III [g]	194.60 ± 4.23	159.57 ± 19.72*
Coumaric acid derivative	14.08 ± 1.85	15.00 ± 1.15
Coumaric acid-*O*-hexoside I [g]	87.86 ± 2.09	132.38 ± 1.72*
Coumaric acid-*O*-hexoside II and III [g]	161.79 ± 7.83	113.07 ± 0.57*
Dicaffeoyl-*O*-hexoside [g]	72.09 ± 1.97	109.38 ± 14.05*
Ferulic acid-*O*-hexoside [g]	72.85 ± 2.58	24.30 ± 2.35*
Sinapic acid-*O*-hexoside [g]	27.05 ± 2.16	31.30 ± 3.01
Total, hydroxycinnamic acid derivatives	867.26 ± 31.81	881.67 ± 56.44
Hydroxycinnamoylquinic acids		
3-*O*-Caffeoylquinic acid	30.09 ± 4.38	44.24 ± 4.39*
4-*O*-Caffeoylquinic acid	223.02 ± 7.43	247.29 ± 7.59*
5-*O*-Caffeoylquinic acid	200.14 ± 33.63	280.73 ± 9.04

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Compound	LT	NLT
Caffeoylquinic acid-*O*-hexoside I [j]	39.64 ± 2.68	37.24 ± 5.62
Caffeoylquinic acid-*O*-hexoside II [j]	52.89 ± 5.94	49.50 ± 5.53
Coumaroylquinic acid [j]	93.38 ± 9.33	96.80 ± 4.70
Dicaffeoylquinic acid I [i]	88.27 ± 3.34	103.29 ± 8.83
Dicaffeoylquinic acid II [h]	39.97 ± 1.91	68.08 ± 1.60*
Dicaffeoylquinic acid III [i]	75.77 ± 2.30	155.79 ± 0.01*
Dicaffeoylquinic acid-*O*-hexoside [h]	44.75 ± 2.45	37.09 ± 1.56*
Feruloylquinic acid [j]	31.76 ± 1.34	31.81 ± 2.03
Tricaffeoylquinic acid [h]	177.11 ± 3.55	390.57 ± 61.65*
Tricaffeoylquinic acid-*O*-hexoside [i]	53.05 ± 4.62	17.93 ± 0.69*
Total, hydroxycinnamoylquinic acids	1149.83 ± 82.90	1560.35 ± 113.23
Phenylpropanoic acid-glycosides		
Dihydrocaffeic acid-*O*-hexoside I [g]	63.44 ± 3.84	74.47 ± 4.54
Dihydrocaffeic acid-*O*-hexoside II [g]	144.74 ± 4.37	160.71 ± 14.94
Dihydrocaffeoyl-caffeoyl-*O*-hexoside [g]	79.63 ± 4.81	95.00 ± 18.17
Dihydroferulic acid-*O*-hexoside [g]	152.76 ± 9.93	94.48 ± 3.04*
Hydroxyphenylpropionic acid-*O*-hexoside [g]	145.74 ± 6.55	171.36 ± 0.72*
Total, phenylpropanoic acid-glycosides	586.30 ± 29.49	596.02 ± 41.42
Total, (Poly)phenolic compounds	**3362.30 ± 189.92**	**3745.54 ± 315.02**

* indicates a significant difference ($p < 0.05$) between LT and NLT by the Student's *t*-test. Abbreviations: n.d., not detected; QHRP, Quercetin-*O*-hexoside-*O*-rhamnoside-*O*-pentoside.

[a] Tentatively quantified using the calibration curve of rutin.
[b] Tentatively quantified using the calibration curve of naringenin.
[c] Tentatively quantified using the calibration curve of vitexin.
[d] Tentatively quantified using the calibration curve of caffeic acid.
[e] Tentatively quantified using the calibration curve of caftaric acid.
[f] Tentatively quantified using the calibration curve of dihydrocaffeic acid.
[g] Tentatively quantified using the calibration curve of vanillic acid-glucoside.
[h] Tentatively quantified using the calibration curve of 3-*O*-caffeoylquinic acid.
[i] Tentatively quantified using the calibration curve of 4-*O*-caffeoylquinic acid.
[j] Tentatively quantified using the calibration curve of 5-*O*-caffeoylquinic acid.

3.2. (Poly)phenolic metabolites in rat serum after tomatoes administration

Serum concentration of tomato (poly)phenol metabolites analysed in rat after acute oral administration of 3 g/kg bw of either LT or NLT are listed in **Table 2**.

In total, 17 compounds corresponding to the most likely detectable metabolites in the samples were monitored (Supplementary Table 3) [5]. As a result, a total of 7 (poly)phenolic metabolites were identified and quantified in rat serum, which occurred mainly as sulfate and methyl-sulfate conjugates, except for 4′-hydroxycinnamic acid-3′-glucuronide. In terms of (poly)phenol intake, LT and NLT provided 10.09 mg and 11.24 mg of (poly)phenols per kg of bw, respectively. The pharmacokinetic profiles of the sum of all detected tomato (poly)phenol metabolites in rat serum presented similar behaviors for LT and NLT treatments (Figure 1a), and this included a pronounced serum peak at 2 h, decreasing sharply until 7 h and then, in the case of LT, it decreased progressively until 48 h where no metabolite was detected, while in the case of NLT a slight serum peak reappeared at 24 h, detecting only one metabolite at 48 h. However, substantial differences were noted between treatments. The overall serum metabolite concentration varied statistically after ingestion of LT and NLT at each of the time points studied (Table 3). In fact, NLT presented an overall metabolite concentration equal to 619.5 nM 2 h after administration, whereas LT presented a lower overall serum concentration (367.1 nM).

Moreover, the overall metabolite serum concentration was observed at higher levels in NLT in all time points. It is important to note that the overall metabolite concentration at 24 h in NLT was 52.70 nM, which represents a 3.8-fold increase in serum with respect to LT group (14.10 nM). This trend is consistent with the higher intake of phenolics containing caffeoyl groups in NLT rodents than LT ones, especially for caffeoylquinic acids. Indeed, all circulating phenolic metabolites could be derived from caffeic acid metabolism, although native ferulic, dihydrocaffeic and dihydroferulic acids in tomatoes could be subjected to direct phase II metabolism concurring thus to increase the overall phenolic metabolite concentration in bloodstream. Instead, no phase II metabolites of flavonoids have been detected in serum samples, in keeping with the very low amounts of flavonoids provided by both LT and NLT.

Table 2. Concentration of tomato-derived phenolic metabolites in a pool of rat serum at 2, 4, 7, 24, and 48 h after the ingestion of 3 g/kg bw local (LT) or non-local (NLT) Ekstasis tomatoes. Data expressed as mean values ± SD (*tr* = 3).

Metabolite	I	Serum concentration (nM)				
		2 h	4 h	7 h	24 h	48 h
Cinnamic acid derivatives						
3´-methoxycinnamic acid-4´-sulfate	LT	143.42 ± 0.76*	39.05 ± 0.38*	7.64 ± 0.24*	9.67 ± 0.60*	n.d.
	NLT	211.17 ± 3.96	66.83 ± 2.14	11.86 ± 0.39	41.19 ± 0.35	n.d.
4´-hydroxycinnamic acid-3´-glucuronide [a]	LT	29.96 ± 0.80*	4.31 ± 0.22*	0.76 ± 0.00*	n.d.	n.d.
	NLT	54.14 ± 3.30	14.27 ± 1.41	0.60 ± 0.03	n.d.	n.d.
4´-methoxycinnamic acid-3´-sulfate [b]	LT	10.18 ± 0.05*	4.80 ± 0.09*	1.46 ± 0.03*	0.43 ± 0.03*	n.d.
	NLT	19.72 ± 0.54	11.63 ± 0.69	2.49 ± 0.09	0.90 ± 0.07	n.d.
Hydroxycinnamic acid sulfate I [c]	LT	16.64 ± 0.53*	2.98 ± 0.40*	0.73 ± 0.10	0.08 ± 0.08	n.d.
	NLT	31.92 ± 0.17	10.88 ± 0.79	1.16 ± 0.14	0.18 ± 0.01	n.d.
Hydroxycinnamic acid sulfate II [c]	LT	132.31 ± 7.63*	40.02 ± 0.25*	4.76 ± 0.85*	0.43 ± 0.11*	n.d.*
	NLT	259.05 ± 6.23	119.36 ± 6.90	11.98 ± 0.14	1.42 ± 0.07	0.34 ± 0.30
Phenylpropanoic acid derivatives						
3-(3´-methoxyphenyl)propanoic acid-4´-sulfate	LT	12.79 ± 0.22*	8.71 ± 0.57	2.71 ± 0.00	1.84 ± 0.25*	n.d.
	NLT	21.32 ± 0.45	7.62 ± 0.92	1.92 ± 0.37	4.96 ± 0.12	n.d.
3-(4´-hydroxyphenyl)propanoic acid-3´-sulfate	LT	21.81 ± 0.59*	25.71 ± 0.52*	6.44 ± 0.27*	1.66 ± 0.11*	n.d.
	NLT	22.23 ± 0.25	25.96 ± 0.13	4.68 ± 0.09	4.04 ± 0.09	n.d.
Total metabolites	LT	367.11 ± 10.59*	125.58 ± 2.43*	24.49 ± 1.49*	14.10 ± 1.17*	0.00 ± 0.00*
	NLT	619.54 ± 14.90	256.56 ± 12.98	34.69 ± 1.25	52.70 ± 0.71	0.34 ± 0.30

Values in a column with * are significantly different ($p < 0.05$) between LT and NLT ingestion by the Student's *t*-test. Abbreviations: I, intervention; n.d., not detected; tr, technical replicates.

[a] Tentatively quantified using the calibration curve of 3′-hydroxycinnamic acid-4′-glucuronide.

[b] Tentatively quantified using the calibration curve of 3′-methoxycinnamic acid-4′-sulfate.

[c] Tentatively quantified using the calibration curve of 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate.

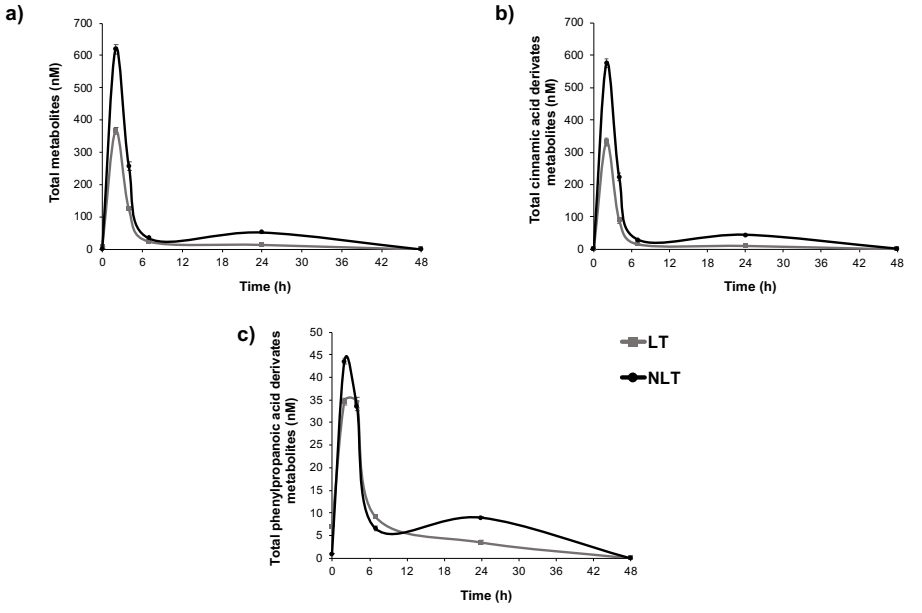

Figure 1. Serum pharmacokinetic profiles of phenolic metabolites after LT and NLT administration: (**a**) total metabolites; (**b**) total cinnamic acid metabolites; (**c**) total phenylpropanoic acid metabolites. Concentrations (nM ± SD) were quantified using a uHPLC-MS^n method in a pool of rat serum at 2, 4, 7, 24 and 48 h after the consumption of 3 g/kg bw of tomatoes.

3.2.1. Cinnamic acid derivatives

Cinnamic acid derivatives represented the main group of metabolites detected and quantified. Among them, 3′-methoxycinnamic acid-4′-sulfate and hydroxycinnamic acid-sulfate II were the major (poly)phenolic metabolites in both LT and NLT, their levels being much lower after LT administration. These metabolites determined the kinetic profile of this metabolite group in serum. The serum kinetic profile was similar in the first 7 h following administration of LT or NLT to rats, both showed a very noticeable peak at 2 h, after that, LT intake continued to gradually decrease while NLT intake showed another weakly pronounced serum peak at 24 h (**Figure 1b**). Both LT and NLT had a lack of detection of these metabolites at 48 h, except for hydroxycinnamic acid-sulfate II, recovered at trace level after NLT intake. Glucuronide conjugates of this group were not detected at any time points after LT and NLT administration, except for 4′-hydroxycinnamic acid-3′-glucuronide, which was quantified during the first 7 hours after tomato ingestion.

3.2.2. Phenylpropanoic acid derivatives

The serum kinetic profile of total phenylpropanoic acid derivatives, which corresponds to two metabolites that only occurred as sulfate conjugates, was slightly different from the profile of cinnamic acid derivatives (**Figure 1c**). Indeed, concerning LT administration, similar serum metabolite concentrations were observed at 2 and 4 h after administration and then decreased progressively until undetectable at 48 h, while as regards NLT administration, it showed a pronounced peak at 2 h, decreasing until 7 h, then a slight peak at 24 h and a void of detection at 48 h. It is important to note that the circulating metabolites at 4 and 7 h post-consumption of NLT was lower than LT. This differed from the pattern observed in the rest of the time points studied and in cinnamic acid derivatives that were higher for NLT at all time points evaluated.

3.3. Pharmacokinetic parameters after tomatoes administration

Table 3 shows the pharmacokinetic parameters of the 7 metabolites detected in serum after tomatoes administration. The C_{max} values of LT-derived metabolites ranged from 10.2 to 143.4 nM, while NLT-derived metabolites ranged from 19.7 to 259.1 nM; being higher in those of NLT than LT, except for 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate which did not vary significantly between treatments. These C_{max} values were reached for most of the metabolites at 2 h after tomatoes administration (T_{max}), except for 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate that reached its maximum concentration at 4 h. These T_{max} values led to suppose an absorption and metabolism mainly in the small intestine rather than colon. Furthermore, like the C_{max} values, the area-under-the-curve (AUC_{0-48}) was greater for all metabolites of NLT than LT, except for 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate which did not differ. Indeed, the highest AUC_{0-48} was observed for 3′-methoxycinnamic acid-4′-sulfate, reaching in NLT group a value 1.9 times higher than LT one. Furthermore, 3 of the 7 metabolites differed statistically in terms of mean residence time (MRT_{0-48}) after consuming LT or NLT, with higher MRT values after consuming NLT.

Table 3. Pharmacokinetic parameters of phenolic metabolites in a pool of rat serum after the ingestion of 3 g/kg bw of local (LT) or non-local (NLT) Ekstasis tomatoes. Data expressed as mean values ± SD (*tr* = 3).

Metabolite	I	C_{max} (nM)	T_{max} (h)	AUC_{0-48} (nM×h)	MRT_{0-48} (h)
3-(3´-methoxyphenyl)propanoic acid-4´-sulfate	LT	12.79 ± 0.22*	2	90.06 ± 3.97*	13.96 ± 1.19*
	NLT	21.32 ± 0.45	2	122.96 ± 4.06	33.46 ± 3.91
3-(4´-hydroxyphenyl)propanoic acid-3´-sulfate	LT	25.71 ± 0.52	4	186.36 ± 3.75	7.93 ± 0.22*
	NLT	25.96 ± 0.13	4	190.57 ± 1.82	15.75 ± 0.25
3´-methoxycinnamic acid-4´-sulfate	LT	143.42 ± 0.76*	2	543.08 ± 5.20*	11.34 ± 0.54*
	NLT	211.17 ± 3.96	2	1058.20 ± 12.42	30.32 ± 0.26
4´-hydroxycinnamic acid-3´-glucuronide	LT	29.96 ± 0.80*	2	71.84 ± 1.05*	2.47 ± 0.02
	NLT	54.14 ± 3.30	2	144.86 ± 3.03	2.55 ± 0.06
4´-methoxycinnamic acid-3´-sulfate	LT	10.18 ± 0.05*	2	50.53 ± 0.64*	7.13 ± 0.22
	NLT	19.72 ± 0.54	2	100.99 ± 3.15	7.23 ± 0.42
Hydroxycinnamic acid-sulfate I	LT	16.64 ± 0.53*	2	48.71 ± 0.40*	3.72 ± 0.58
	NLT	31.92 ± 0.17	2	104.21 ± 0.36	3.63 ± 0.01
Hydroxycinnamic acid-sulfate II	LT	132.31 ± 7.63*	2	415.88 ± 22.19*	3.36 ± 0.06
	NLT	259.05 ± 6.23	2	969.55 ± 33.37	4.37 ± 0.43

Values in a column with * are significantly different ($p < 0.05$) between LT and NLT ingestion by the Student's *t*-test. Abbreviations: AUC, area-under-the-curve serum concentration-time; C_{max}, maximum serum concentration; I, intervention; MRT, mean residence time; T_{max}, time of peak serum concentration; tr, technical replicates.

Furthermore, like the C_{max} values, the area-under-the-curve (AUC_{0-48}) was greater for all metabolites of NLT than LT, except for 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate which did not differ. Indeed, the highest AUC_{0-48} was observed for 3′-methoxycinnamic acid-4′-sulfate, where NLT intake was 1.9 times higher than LT intake. Furthermore, 3 of the 7 metabolites differed statistically in terms of mean residence time (MRT_{0-48}) after consuming LT or NLT, among them, higher MRT values were evidenced after consuming NLT.

4. Discussion

This work aimed to characterize the (poly)phenol composition of two tomatoes cultivated at different locations and to elucidate whether the geographical origin of tomato cultivation can modulate (poly)phenols bioavailability and metabolism in rats. To ensure that the only independent variable was the cultivation location, Ekstasis tomatoes, conventionally grown in two locations in Spain: in the northeast (local tomato LT) and in the southeast (non-local tomato NLT), were used. To our knowledge, the findings of the present study give the most detailed information on tomato (poly)phenol characterization as well as their absorption and metabolism in rats.

The (poly)phenolic profiles of both tomatoes were consistent with the major (poly)phenolic subclasses in several tomato varieties [3,4,6,16]. In our work, 4-*O*-caffeoylquinic acid was the most abundant compound in LT, while tricaffeoylquinic acid was the most abundant in NLT, also coinciding with the most abundant compounds found in four tomato varieties in northeastern Portugal homegardens [16]. Among quantified flavonoids, phloretin 3′,5′-di-*C*-β-glucopyranoside and rutin were the predominant compounds in both tomatoes comprising 49 to 55% of the total flavonoid content, in agreement with these results found by Slimestad *et al.* [17] in different tomato types. Total flavonoid content varied significantly between LT and NLT, being higher in the former, which is consistent with the fact that flavonoid content varies according to the origin of the crop [4,17,18]. On the other hand, NLT was characterized by a higher total phenolic acid content compared to its local counterpart, this is in agreement with Asensio *et al.* [4] who revealed significant effects of location in the

concentration of some phenolic acids, *i.e.*, caffeic, chlorogenic, ferulic and *p*-coumaric acid, from Spanish traditional tomato. Similarly, our results are in line with studies conducted by San José e*t al.* [19] where they found that the environment plays a key role in determining the phenolic composition of eggplant fruits.

The *in vivo* absorption of (poly)phenolics compounds after LT and NLT acute administration was evaluated in Wistar rat serum at different times. It is well documented that dose administration and food matrix are key factors modulating the bioavailability and metabolism of (poly)phenolic compounds [20,21]. In order to assess whether the consumption of tomato fruit from two distinct geographical origins, *i.e.,* LT or NLT, has a differential impact on their (poly)phenolic bioavailability, we administered the same amount of lyophilized LT or NLT (3g/kg bw) to the animals. Blood collection points were defined on the basis that early collection times (*i.*e., 2 to 4 h) provide information on the absorption from the small intestine, while later time points (*i.e.,* 7 to 48 h) provide information on their colonic metabolism [22]. It should be noted that different studies have evaluated the bioavailability and metabolism of tomato (poly)phenols [2,5,23–25], but the effect of crop origin on tomato (poly)phenol bioavailability, to our knowledge, has not been previously evaluated. In the present study, a total of seven metabolites were quantified in rat serum following consumption of LT or NLT. Cinnamic acid and phenylpropanoic acid metabolites detected in this study were mainly recovered as sulfate and methyl-sulfate conjugates. Actually, when comparing glucuronide and sulfate or methyl-sulfate metabolites, independently of the intervention, both sulfate and methyl-sulfate conjugate forms were clearly more prevalent than the glucuronide ones. Indeed, sulfation is generally a higher-affinity, lower-capacity pathway than glucuronidation, so that when the ingested dose increases, a shift from sulfation toward glucuronidation occurs [7]. Our results contrasted with the trend observed for the "*liso rojo rama*" tomato variety, where four metabolites of phenolic compounds were identified in plasma and the glucuronide form was clearly more prevalent [2]. This fact may be attributed to differences in dosing, metabolites quantified, tomato varieties and model used [1]. Moreover, nM C_{max} were attained in ≤ 4 h for all metabolites, indicating

absorption from the small intestine, most of which had a relative short MRT_{0-48} as they were rapidly removed from the bloodstream.

In this work, relevant differences in the (poly)phenolic absorption and metabolism in rat serum after LT and NLT acute administration were identified. When comparing kinetic parameters, the highest C_{max} values of all metabolites were evidenced after NLT administration. Similarly, the exposure to tomato (poly)phenols (AUC_{0-48}) was markedly higher when animals were administered with NLT. The reason for these varying metabolite profiles can be attributed to the fact that the metabolites identified could be derived from phenolic acids and NLT was characterized as having the highest amount of this phenolic subclass, mainly caffeic acid. It was observed that after ingestion of LT or NLT, total (poly)phenolic metabolites are metabolized and absorbed in a similar, but not identical, manner. In fact, the highest concentrations were reached at 2 h, with a second less pronounced peak at 24 h, always showing a higher concentration after NLT administration, while at 7 h the concentrations were closest (LT 25 nM and NLT 34 nM). The 24-h peak serum could be explained by the action of colon microbiota towards dicaffeoyl- and tricaffeoylquinic acids, that reached higher content in NLT than LT, concurring thus to a slight increase of serum phenolic metabolites in NLT rats than LT ones. The factors influencing variability in the appearance of metabolites in serum, which may be related to other dietary components of tomatoes such as non-digestible carbohydrates, should be explored along with the actions of the absorbed metabolites [26]. In this sense, LT contained a higher protein content, and as is known, (poly)phenols are known to form complexes with proteins, resulting in changes in the structural, functional and nutritional properties, and digestibility of both compounds [27]. Trombley e*t al*. [28] have suggested that bioavailability of plant (poly)phenols may be influenced by the covalent interaction between (poly)phenols and proteins. Indeed, it has been speculated that high amounts of protein may limit the availability and fermentation of (poly)phenols and the formation of metabolites from the microbiota through complexation [21]. This could partially explain the lower metabolite levels found after 24 h of LT tomato consumption. This statement is consistent with other studies in which a decrease in the bioavailability of black tea

(poly)phenols was observed due to the effect of protein-phenol interactions [30]. However, the mechanisms of interactions between tomato (poly)phenols and proteins should be investigated.

At the level of metabolite groups, specifically, with regard to caffeic acid derivatives, *i.e.,* hydroxycinnamic acid sulfate I and II, and 4′-hydroxycinnamic acid-3′-glucuronide, higher concentrations of metabolites were identified after NLT ingestion. The reason could be attributed to the fact that NLT showed higher concentrations of chlorogenic acids, mainly 3-*O*-, 4-*O*- and 5-*O*-caffeoylquinic acids. Literature data regarding the absorption of chlorogenic acid in its intact form are still fragmentary and not exhaustive [30]. Moreover, the highest serum concentration of cinnamic acid derivatives was found at 2 h after tomatoes administration, and this was similar for both LT and NLT. In both cases, 3′-methoxycinnamic acid-4′-sulfate accounted for greater than 91% of the cinnamic acids found in serum at 2 h, the serum appearance of 3′-methoxycinnamic acid-4′-sulfate was largely due of caffeic acid conjugations by rat catechol-*O*-methyltransferases and sulfotransferases. Lastly, the content of phenylpropanoic acid derivatives peaked a second time in serum 24 h after tomatoes administration in both treatment groups, especially in NLT. The higher total phenylpropanoic acid-glycosides content in NLT could contribute to this higher concentration. In particular, regarding the other main phenylpropanoic acid-glycoside catabolites, dihydrocaffeic acid derivatives (3-(3′-hydroxyphenyl)propanoic acid-4′-sulfate and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate), it has been suggested that these compounds are able to scavenge intracellular reactive oxygen species [1], which may contribute to the known antioxidant capacity of tomatoes, among other biological activities. It is important to note that the bioavailability and metabolism of (poly)phenolic compounds are the principal limiting factors of their bioactivity [23]. Therefore, the differences described in this work could be related to relevant variations in the biological effects generated by consuming tomatoes produced in different areas.

5. Conclusions

This study demonstrated that the differences in the (poly)phenolic and nutritional composition of Ekstasis tomatoes from two geographical origins of cultivation led to different (poly)phenolic kinetic profiles in rat serum. As a result of these differences on absorption and metabolism of tomato (poly)phenols, it is suggested that the health-promoting effects of consuming tomatoes could differ depending on their growing location. Lastly, further human intervention trials are required to corroborate these results and to correlate individual tomato (poly)phenols with its putative health effects.

Author Contributions

Álvaro Cruz-Carrión: performed the sample analyses and wrote original draft. Ma. Josefina Ruiz de Azua: contributed with investigation. Luca Calani: performed the sample analyses. Pedro Mena: funded acquisition, supervised. Daniele Del Rio: funded acquisition, supervised. Manuel Suárez: designed the research, funded acquisition, supervised, wrote – reviewed. Anna Arola-Arnal: designed the research, funded acquisition, supervised, wrote – reviewed. All authors critically revised the paper and read an approved the final manuscript.

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Conflicts of interest

There are no conflicts to declare.

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Supplementary materials

Supplementary Table 1. Nutritional composition of local (LT) and non-local (NLT) Ekstasis tomatoes.

Nutrients	LT	NLT
Ash	8.61 ± 0.28	8.76 ± 0.12
Carbohydrate, total; by difference	75.18 ± 0.14	75.74 ± 0.14
Fibre, total dietary	25.26 ± 0.00	24.31 ± 1.18
Sugars, total	36.98 ± 0.93	39.57 ± 0.92*
Protein	13.54 ± 0.04	12.96 ± 0.00*
Total lipid (fat)	2.51 ± 0.03	2.49 ± 0.01

The results are expressed as g/100 g dw ± SD (n = 3). * Indicates a significant difference (p < 0.05) between LT and NLT by the Student's t-test. Table adapted from Cruz-Carrión *et al*.

Supplementary Table 2. uHPLC-MS^n characteristics of (poly)phenolic compounds and phytochemicals detected in tomatoes.

Compound	RT	[M-H]- (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
Salicylic acid	7.41	137	93	
Glutamic acid	0.83	146	128, 102	128: 128, 84, 110, 82
Protocatechuic acid	3.59	153	109	
p-Coumaric acid	6.00	163	119	
Phenylalanine	2.77	164	147, 72	147: 103
Isopropylmalic acid	4.52	175	115, 113, 157, 85, 129	115: 71
Caffeic acid	5.08	179	135	
Dihydrocaffeic acid*	4.85	181	137, 119, 109, 58	
Azelaic acid	7.11	187	125, 169, 97	125: 97, 105, 83
Naringenin chalcone	8.90	271	151, 177, 125, 107, 165	
Naringenin	9.00	271	151, 177, 125, 107, 165	
Eriodictyol	8.00	287	151, 125	
Dihydroxybenzoic acid-*O*-pentoside	4.10	285	153, 109, 207	153: 109
Caffeoylmalic acid	5.38	295	179, 133, 231, 115	179: 135
Hydroxybenzoic acid-*O*-hexoside	2.48	299	137	93
Quercetin	8.20	301	179, 151, 273, 257, 193, 107	
Homoeriodictyol	9.20	301	151, 177, 165, 107, 233, 255, 286	
Feruloyl tyramine	7.78	312	297, 178, 135, 148, 270	297: 253, 178, 148, 176
Coumaric acid-*O*-hexoside I	4.05	325	163, 119	163: 119
Coumaric acid-*O*-hexoside II	4.67	325	163, 119	163: 119
Coumaric acid-*O*-hexoside III	4.69	325	163, 129	163: 119

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Compound	RT	[M-H]- (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
Hydroxyphenylpropionic acid-*O*-hexoside	4.37	327	165, 207	165: 121, 93, 59, 119
Trihydroxy-octadecenoic acid	9.44	329	229, 211, 311, 293, 171	229: 211, 125
Coumaroylquinic acid	5.26	337	191, 163, 173	191: 127, 93, 109, 85, 155, 173
Caffeic acid-*O*-hexoside I	3.98	341	179, 135	179: 135
Caffeic acid-*O*-hexoside II	4.30	341	281, 251, 221, 179	281: 221, 179, 135
Caffeic acid-*O*-hexoside III	4.48	341	179, 251, 281, 233, 135	179: 135
Dihydrocaffeic acid-*O*-hexoside I	4.27	343	181, 137	181: 137, 119
Dihydrocaffeic acid-*O*-hexoside II	4.53	343	181, 137	181: 137, 119
3-Caffeoylquinic acid	3.95	353	191, 179, 135	191: 93, 85, 173, 127, 111
5-Caffeoylquinic acid	4.63	353	191, 179	191: 127, 173, 85, 93, 111
4-Caffeoylquinic acid	4.71	353	173, 179, 191	
Ferulic acid-*O*-hexoside	4.38	355	193	149, 178, 134
Dihydroferulic acid-*O*-hexoside	4.65	357	195	151, 136, 123, 177, 119, 180, 108, 59
Syringic acid-*O*-hexoside	6.00	359	197, 153	197: 153
Feruloylquinic acid	5.54	367	191	
Sinapic acid-*O*-hexoside	4.55	385	223, 325, 208	223: 208, 179, 164
Benzyl alcohol-*O*-hexoside-*O*-pentoside	4.90	401	269, 239, 161, 131	269: 161, 159, 101, 125, 143, 85
(Iso)pentyl-dihexose	4.78	411	249, 365, 161, 321	249: 161, 101, 99, 125, 113
Coumaric acid derivative	7.63	409	163, 119	163: 119
Dihydrocaffeic acid derivative	7.84	427	181, 325, 343, 137	181: 137, 119, 109
Benzyl alcohol-*O*-dihexoside	4.50	431	269, 161, 179, 323, 221, 143	269: 161, 101, 99, 125, 113

Continued on next page

Compound	RT	[M-H]- (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
Caffeic acid derivative I	6.44	487	323, 443, 221, 179	323: 221, 179, 177, 203, 263, 135, 161
Caffeic acid derivative II	6.88	487	323, 443, 179, 263, 307, 409, 161	323: 179, 221, 263, 203, 161, 280, 158, 135
Caffeic acid derivative III	6.80	497	179, 317, 173, 215, 323	179: 135
Dicaffeoyl-*O*-hexoside	6.34	503	323, 179, 341, 221	323: 179, 221, 177, 161, 135
Dihydrocaffeoyl-caffeoyl-*O*-hexoside	6.61	505	343, 323, 181, 161	343: 181, 137
Ferulic acid derivative**	6.96	511	193, 197, 173, 149, 317, 431, 257	193: 149, 134
Caffeoylquinic acid-*O*-hexoside I	3.68	515	353, 191, 323, 341	353: 191, 179; 191: 155, 85, 173, 93
Caffeoylquinic acid-*O*-hexoside II	4.18	515	323, 353, 191	323: 161, 179
Dicaffeoylquinic acid I	6.35	515	353, 335, 469, 179, 173	353: 173, 179, 191, 135
Dicaffeoylquinic acid II	6.58	515	353, 179	
Dicaffeoylquinic acid III	6.75	515	353, 395, 203, 299, 173	353: 173, 179, 191, 135
Caffeic acid derivative IV	7.56	523	179, 199, 343, 479, 361	179: 135
Caffeic acid derivative V	6.50	563	179, 383, 323, 245, 287, 503	179: 135
Kaempferol-*O*-rutinoside	6.30	593	285	257, 241, 267, 197, 229, 163, 151
Phloretin-C-dihexoside	5.90	597	357, 477, 387, 417, 459, 489	
Luteolin-*O*-hexoside-C-hexoside	5.70	609	429, 285, 447, 489, 309, 257	429: 339, 309, 284, 257
Quercetin-3-*O*-rutinoside	6.00	609	301, 343	301: 179, 151, 273, 257

Continued on next page

Compound	RT	[M-H]- (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
Quercetin-*O*-dihexoside	5.40	625	300, 301, 445, 505, 463, 271, 343, 179	300: 271, 255, 179, 151
Caffeoylquinic acid-*O*-dihexoside	3.05	677	515, 353, 631, 377, 191	515: 353, 191
Dicaffeoylquinic acid-*O*-hexoside	5.82	677	515, 497, 353	515: 353, 203, 299, 179
Tricaffeoylquinic acid	7.90	677	515, 497, 353, 299, 203	515: 353, 335, 173, 179, 191, 299
N-Tris-(dihydrocaffeoyl) spermine	5.65	693	529, 571, 365, 407, 449	
Kaempferol-*O*-rutinoside-*O*-pentoside	5.81	725	593, 575, 284, 285, 327, 459	593: 327, 357, 285
QHRP	5.49	741	300, 609, 301, 591, 343, 475	300: 271, 255, 179, 151
Quercetin-*O*-rutinoside-*O*-hexoside	4.52	771	609	301, 300, 271
Tricaffeoylquinic acid-*O*-hexoside	5.32	839	677, 515	677: 515, 353
QHRP-coumaric acid	6.73	887	741, 723, 665, 591, 300	741: 609, 300, 343, 301, 591, 475, 447, 271
QHRP-*O*-hexoside	4.30	903	741	300, 609, 301, 591, 475, 271, 343
QHRP-ferulic acid	6.72	917	741, 723, 593	741: 300, 609, 591, 475, 343, 271
QHRP-syringic acid	7.21	921	741, 877, 723, 283	741: 609, 300
QHRP-synaptic acid	6.55	947	741, 723, 755, 609	741: 609, 591, 300, 301, 343, 475
Tricaffeoylquinic acid-*O*-dihexoside***	4.88	1001	839, 677, 515	839: 515, 677, 341, 335, 365
Tomatoside A	7.45	1081.6	919.7, 757.6	919.7: 757.7, 594

Abbreviations: CQA, caffeoylquinic acid; QHRP, Quercetin-*O*-hexoside-*O*-rhamnoside-*O*-pentoside. *: not detected in LT; **: not detected in NLT; ***: not detected in NLT.

Supplementary Table 3. Rat serum metabolites of tomato (poly)phenolic compounds monitored through uHPLC-MS^n.

Metabolite	RT	[M-H]- (*m/z*)	LOD (nM)	LOQ (nM)	MS^2 ions (*m/z*)
3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide	4.49	371	10	50	**175**, 113, 195
3-(3′-methoxyphenyl)propanoic acid-4′-sulfate	4.6	275	1	5	**195**, 149
3-(4′-hydroxyphenyl)propanoic acid-3′-glucuronide	4.33	357	10	50	**181**, 175, 113, 137
3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate	4.4	261	5	10	**181**, 135, 137
3-(4′-methoxyphenyl)propanoic acid-3′-glucuronide	4.96	371	10	50	**175**, 195, 113
3′-hydroxycinnamic acid-4′-glucuronide	3.68	355	10	50	**179**, 175, 135, 113
3′-methoxycinnamic acid-4′-glucuronide	4.12	369	10	50	**175**, 193, 113, 178
3′-methoxycinnamic acid-4′-sulfate	4.7	273	50	250	**193**, 229, 149
4′-hydroxycinnamic acid-3′-glucuronide	4.37	355	10	50	**179**, 175, 135
4′-methoxycinnamic acid-3′-glucuronide	4.89	371	10	50	**175**, 113, 193
4′-methoxycinnamic acid-3′-sulfate	5.06	273	50	250	**193**, 149
Hydroxycinnamic acid-sulfate I	4.36	259	5	10	**179**, 215, 135
Hydroxycinnamic acid-sulfate II	4.65	259	5	10	**179**, 215, 135
Naringenin-4′-glucuronide	6.74	447	1	5	**271**, 175
Naringenin-7-glucuronide	6.64	447	1	5	**271**, 175
Quercetin-3-glucuronide	5.96	477	1	5	**301**, 179
Quercetin-3′-sulfate	7.46	381	1	5	**301**, 179

The fragment ions are reported in their order of relative abundance. Quantifier ions are reported in bold. Standard of both naringenin-glucuronides have been only used for identification because of their lower purity than standard of quercetin-3-glucuronide. Abbreviations: LOD, limit of detection; LOQ, limit of quantification; RT, retention time.

OBJECTIVE 3.3.

To evaluate the effect of seasonal consumption of tomatoes cv. Ekstasis from two geographical origin of cultivation on their phenolic bioavailability in rats

IMPACT OF SEASONAL CONSUMPTION OF LOCAL TOMATOES ON THE BIOAVAILABILITY OF (POLY)PHENOLS IN FISCHER RATS

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In preparation

Abstract

Consuming phenol-rich fruits and vegetables, including tomato, is associated with beneficial health outcomes. The health effects of phenolics compounds have been attributed to the products of their metabolism. The bioavailability of these compounds can be modulated by several factors. This study aims to evaluate the effect of a seasonal consumption of local tomatoes on their (poly)phenols bioavailability. For this, (poly)phenolic bioavailability was analyzed by uHPLC-MS^n after tomatoes chronic consumption in Fischer rats exposed to three photoperiods to mimic the seasonal daylight schedule. Moreover, tomatoes from two locations in Spain (LT, local tomatoes from Tarragona and NLT, non-local tomatoes from Almería) were evaluated. The bioavailability of tomato (poly)phenols depended on the photoperiod to which the rats were exposed, significantly varying the metabolite concentrations between seasons. Some metabolites were even not detected in winter. In-season consumption of tomato allowed to obtain the highest amounts of total circulating metabolites. In addition, the origin of the tomato administered generated marked differences in metabolic profiles, being after NLT ingestion, where higher amounts were detected. We conclude that in-season consumption of tomato increases their (poly)phenols bioavailability in Fischer rats, whereas local tomatoes consumption showed lower circulating metabolites than non-local tomatoes. Thus, the tomato cultivar and the seasonal daylight schedule affects the bootability of tomato (poly)phenols that could also affect to their bioactivity.

Keywords

Metabolites, phenolic compounds, photoperiod, seasonal consumption, tomato.

Abbreviations

CAD: cinnamic acid derivatives; DH: daylight hours; F344: Fischer 344 rats; LT: local tomato; n.d.: not detected; NLT: non-local tomato; PAD: phenylpropanoic acid derivatives.

1. Introduction

It has long been appreciated that diets rich in fruits and vegetables promote health, reduce the risk of both cancer and cardiovascular disease, and are correlated with increased longevity [1]. Among these diets, the Mediterranean diet could be highlighted, as it is well known that part of its health benefits could be attributed to the high content of fruits and vegetables rich in bioactive compounds [2]. In this regard, tomato (*Lycopersicon esculentum* Mill.) represents an essential part of the Mediterranean diet and are worldwide among the most consumed vegetables [3]. Tomatoes play an important role in the human diet as their regular consumption has been associated with a reduced risk of several types of cancer and coronary heart disease [2,3]. Tomatoes have high antioxidant capacity which can be attributed to the high levels of carotenoids, (poly)phenolic compounds, vitamins C and E [4]. Specifically, tomatoes contain a number of (poly)phenolic compounds, *i.e.*, flavonoids and phenolic acids, which are mainly represented by flavanones (naringenin derivatives), flavonols (quercetin, rutin, and kaempferol derivatives), and cinnamic acid derivatives (chlorogenic, caffeic and ferulic acids) [5,6]. (Poly)phenolic composition data in tomatoes vary due to genetic, agronomical and environmental factors, being the growing location and storage conditions after post harvesting among the most relevant [3,4,7]. In this line, it has been observed that the total phenolic content of tomatoes grown at 30°~60°N latitude was significantly higher than those at 0°~30°N latitude. It is considered that low latitude or high temperature of the geographical origins may lead to the low (poly)phenolic compounds accumulation [4]. Likewise, data on phenolic characterization of Spanish tomato grown in two locations revealed significant effect of location in most compounds. Tomatoes grown in traditional areas showed a significantly higher concentration of total (poly)phenolic content and some phenolic acids, such as caffeic acid, ferulic acid, and *p*-coumaric acid [3]. Also, changes in the phenolic content of tomato products during storage have been reported, in fact, the total polyphenol content decreased during storage of tomato ketchups and juices, the most significant decrease was observed for quercetin followed by caffeic and ferulic acids, while glycosylated polyphenols showed higher stability during storage [8].

After tomato ingestion, dietary (poly)phenols appear in small concentrations in the circulatory system as phase II metabolites. Some parent compounds or their metabolites pass into the colon, where they are degraded by the local gut microbiota [9]. There is extensive literature showing that these bioactive compounds have to reach the target tissues in an effective amount to exert health-promoting effects [10]. In fact, these health benefits are attributed to their metabolites rather than to their natural forms [11]. Hence, a thorough knowledge of the bioavailability of tomato (poly)phenolic compounds is crucial to understand their health effects. Several dietary factors may impact (poly)phenol bioavailability, in addition to endogenous factors such as microbiota and digestive enzymes, the food matrix can also significantly affect the bioavailability of polyphenols [12]. In this sense, a growing number of studies indicate that gastrointestinal host physiology exhibits circadian variation [13,14]. It has previously been described that the activities of certain enzymes throughout the small intestine of rats exhibited circadian fluctuations [15]. Likewise, it has been reported that intestinal microbiota exhibit diurnal oscillations in composition and function [13]. Therefore, the alteration of the host physiology condition the bioavailability and phenolic metabolism [16]. One study with grapes reported a relationship between metabolites derived from organic and conventional grapes and the photoperiods to which the animals were exposed, concluding that the bioavailability of red grape polyphenols in Fischer 344 rats is influenced by grape cultivar and the exposure of the animals to different photoperiods emulating spring or fall, summer and winter light exposure [11]. In this context, the study of tomato (poly)phenolic compounds, as representative of the Mediterranean diet, is of great interest. Accordingly, this study aimed to determine whether in-season consumption of local Ekstasis tomatoes influences the (poly)phenolic bioavailability and metabolism. As our knowledge this is the first time that the impact of circannual rhythms in the bioavailability of tomato phenolic compounds is evaluated.

2. Materials and methods

2.1. Chemicals and reagents

All extraction and LC-MS grade solvents were acquired from VWR International (Milan, Italy). 3′-methoxycinnamic acid-4′-sulfate, 3′-methoxycinnamic acid-4′-glucuronide, 3′-hydroxycinnamic acid-4′-glucuronide, 3-(4′-methoxyphenyl)propanoic acid-3′-glucuronide, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, 3-(4′-hydroxyphenyl)propanoic acid-3′-glucuronide, 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide were bought from Toronto Research Chemicals (Toronto, ON, Canada). Quercetin-3-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin-3′-sulfate was provided by Prof. Alan Crozier (School of Medicine, Dentistry and Nursing, University of Glasgow, Glasgow, United Kingdom; and Department of Nutrition, University of California, Davis, Davis, CA, USA).

2.2. Tomato samples

Mature, conventional tomatoes (*Lycopersicon esculentum* Mill. cv. Ekstasis) from two regions of Spain: southeast (Almería, 36°50'17.3" N 2°27.584' O; non-local tomatoes NLT) and northeast (Tarragona, 41°4'29.24" N 1°3'8.78" E; local tomatoes LT) were obtained from a local produce store. The whole tomatoes were frozen in liquid nitrogen and grounded, then freeze-dried for one week at −55 °C using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain). The lyophilized samples were stored in a dry and dark place until use. The phenolic composition and concentrations of the tomatoes showed in Table 1, and the nutritional characterization, that can be consulted in Supplementary Table 1, were first determined (Cruz-Carrión *et al.*).

Table 1. Concentration of phenolic compounds in local (LT) and non-local (NLT) Ekstasis tomatoes. The results are expressed as μg/g dw ± SD (n = 3).

Compound	LT	NLT
Flavonoids		
Kaempferol-*O*-rutinoside [a]	3.51 ± 0.48	9.27 ± 1.25*
Kaempferol-*O*-rutinoside-*O*-pentoside [a]	4.81 ± 0.58	11.22 ± 2.02*
Luteolin-*O*-hexoside-*C*-hexoside [a]	34.76 ± 3.6	21.2 ± 1.02*
Naringenin	1.64 ± 0.27	2.18 ± 0.47
Naringenin chalcone [b]	1.12 ± 0.20	1.43 ± 0.11
Phloretin 3′,5′- di-C-β-glucopyranoside [c]	107.97 ± 11.5	51.19 ± 4.19*
Quercetin-*O*-dihexoside [a]	5.87 ± 0.76	5.20 ± 0.34
QHRP-*O*-hexoside [a]	1.79 ± 0.43	1.24 ± 0.56
QHRP-coumaric acid [a]	21.16 ± 1.13	10.13 ± 1.57*
QHRP-ferulic acid [a]	10.98 ± 0.4	6.86 ± 0.12*
QHRP-sinapic acid [a]	4.24 ± 0.16	3.66 ± 0.37
QHRP-syringic acid [a]	6.55 ± 0.81	8.28 ± 1.54
Quercetin-*O*-rutinoside-*O*-hexoside [a]	2.52 ± 0.27	0.87 ± 0.13*
Quercetin-*O*-rutinoside-*O*-pentoside [a]	81.03 ± 2.80	64.25 ± 5.82*
Rutin	111.33 ± 1.11	86.38 ± 11.77*
Total, flavonoids	399.32 ± 16.15	283.37 ± 30.38*
Caffeic and dihydrocaffeic acid derivatives		
Caffeic acid derivative I [d]	33.24 ± 1.12	65.55 ± 17.50*
Caffeic acid derivative II [d]	28.10 ± 2.15	14.81 ± 3.34*
Caffeic acid derivative III [d]	13.95 ± 0.68	24.97 ± 6.96
Caffeic acid derivative IV [d]	4.19 ± 0.10	5.37 ± 0.47*
Caffeic acid derivative V [d]	4.79 ± 0.41	4.91 ± 0.98
Caffeoylmalic acid [d]	62.93 ± 3.55	66.80 ± 9.10
Dihydrocaffeic acid derivative [f]	38.14 ± 2.12	32.09 ± 5.61
Total, caffeic and dihydrocaffeic acid derivatives	185.34 ± 10.14	214.49 ± 43.97
Free phenolic acids		
Caffeic acid	27.30 ± 3.03	40.92 ± 1.25*
Dihydrocaffeic acid	n.d.	8.72 ± 2.73*

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Compound	LT	NLT
p-Coumaric acid	15.57 ± 1.14	16.19 ± 2.02
Salicylic acid	29.04 ± 3.22	54.39 ± 12.34*
Total, free phenolic acids	71.90 ± 7.39	120.22 ± 18.34*
Hydroxybenzoic acid derivatives		
Dihydroxybenzoic acid-*O*-pentoside [g]	24.44 ± 1.46	18.30 ± 1.64*
Hydroxybenzoic acid-*O*-hexoside [g]	40.36 ± 1.28	33.59 ± 3.08*
Syringic acid-*O*-hexoside [g]	37.53 ± 0.93	37.53 ± 5.59
Total, hydroxybenzoic acid derivatives	102.34 ± 3.66	89.42 ± 10.30
Hydroxycinnamic acid derivatives		
Caffeic acid-*O*-hexoside I [g]	169.42 ± 5.07	235.81 ± 8.06*
Caffeic acid-*O*-hexoside II [g]	67.52 ± 4.03	60.86 ± 5.82
Caffeic acid-*O*-hexoside III [g]	194.60 ± 4.23	159.57 ± 19.72*
Coumaric acid derivative	14.08 ± 1.85	15.00 ± 1.15
Coumaric acid-*O*-hexoside I [g]	87.86 ± 2.09	132.38 ± 1.72*
Coumaric acid-*O*-hexoside II and III [g]	161.79 ± 7.83	113.07 ± 0.57*
Dicaffeoyl-*O*-hexoside [g]	72.09 ± 1.97	109.38 ± 14.05*
Ferulic acid-*O*-hexoside [g]	72.85 ± 2.58	24.30 ± 2.35*
Sinapic acid-*O*-hexoside [g]	27.05 ± 2.16	31.30 ± 3.01
Total, hydroxycinnamic acid derivatives	867.26 ± 31.81	881.67 ± 56.44
Hydroxycinnamoylquinic acids		
3-*O*-Caffeoylquinic acid	30.09 ± 4.38	44.24 ± 4.39*
4-*O*-Caffeoylquinic acid	223.02 ± 7.43	247.29 ± 7.59*
5-*O*-Caffeoylquinic acid	200.14 ± 33.63	280.73 ± 9.04
Caffeoylquinic acid-*O*-hexoside I [j]	39.64 ± 2.68	37.24 ± 5.62
Caffeoylquinic acid-*O*-hexoside II [j]	52.89 ± 5.94	49.50 ± 5.53
Coumaroylquinic acid [j]	93.38 ± 9.33	96.80 ± 4.70
Dicaffeoylquinic acid I [i]	88.27 ± 3.34	103.29 ± 8.83
Dicaffeoylquinic acid II [h]	39.97 ± 1.91	68.08 ± 1.60*
Dicaffeoylquinic acid III [i]	75.77 ± 2.30	155.79 ± 0.01*
Dicaffeoylquinic acid-*O*-hexoside [h]	44.75 ± 2.45	37.09 ± 1.56*

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Compound	LT	NLT
Feruloylquinic acid [j]	31.76 ± 1.34	31.81 ± 2.03
Tricaffeoylquinic acid [h]	177.11 ± 3.55	390.57 ± 61.65*
Tricaffeoylquinic acid-*O*-hexoside [i]	53.05 ± 4.62	17.93 ± 0.69*
Total, hydroxycinnamoylquinic acids	1149.83 ± 82.90	1560.35 ± 113.23
Phenylpropanoic acid-glycosides		
Dihydrocaffeic acid-*O*-hexoside I [g]	63.44 ± 3.84	74.47 ± 4.54
Dihydrocaffeic acid-*O*-hexoside II [g]	144.74 ± 4.37	160.71 ± 14.94
Dihydrocaffeoyl-caffeoyl-*O*-hexoside [g]	79.63 ± 4.81	95.00 ± 18.17
Dihydroferulic acid-*O*-hexoside [g]	152.76 ± 9.93	94.48 ± 3.04*
Hydroxyphenylpropionic acid-*O*-hexoside [g]	145.74 ± 6.55	171.36 ± 0.72*
Total, phenylpropanoic acid-glycosides	586.30 ± 29.49	596.02 ± 41.42
Total, (Poly)phenolic compounds	**3362.30 ± 189.92**	**3745.54 ± 315.02**

* indicates a significant difference ($p < 0.05$) between LT and NLT by the Student's *t*-test. Abbreviations: n.d., not detected; QHRP, Quercetin-*O*-hexoside-*O*-rhamnoside-*O*-pentoside.

[a] Tentatively quantified using the calibration curve of rutin.
[b] Tentatively quantified using the calibration curve of naringenin.
[c] Tentatively quantified using the calibration curve of vitexin.
[d] Tentatively quantified using the calibration curve of caffeic acid.
[e] Tentatively quantified using the calibration curve of caftaric acid.
[f] Tentatively quantified using the calibration curve of dihydrocaffeic acid.
[g] Tentatively quantified using the calibration curve of vanillic acid-glucoside.
[h] Tentatively quantified using the calibration curve of 3-*O*-caffeoylquinic acid.
[i] Tentatively quantified using the calibration curve of 4-*O*-caffeoylquinic acid.
[j] Tentatively quantified using the calibration curve of 5-*O*-caffeoylquinic acid.

2.3. Study design

The study and protocol were approved by Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 9495). A total of 72 male Fischer 344 (F344) rats from 7 to 8 weeks of age were divided into 3 groups ($n = 24$) and exposed to simulation of three photoperiods to mimic the seasonal daylight hours (-DH): winter-DH (6 h light/day), spring/autumn-DH (12 h light/day) and summer-DH (18 h light/day), with ad libitum access to water and a standard chow diet (AO4, Panlab, Barcelona, Spain) for 4 weeks. Then, rats of each photoperiod were divided into three groups ($n = 8$) and treated by voluntary oral administration for 7 weeks with

lyophilized LT and NLT (100 mg /kg body weight (bw)/d, equivalent to 0.34 and 0.37 mg total polyphenols/kg bw/d of LT and NLT, respectively). The control group received 42 mg of a sugar mixture solution (glucose:fructose, 1:1) per kg bw, to provide the equivalent amount of sugar as those given to the tomato-supplemented rats. After this period, animals were sacrificed by decapitation 1 h after the last administration, blood samples were obtained and centrifuged (2000×g, 15 min, 4 °C) to collect serum and stored at - 80 °C until use (**Figure 1**).

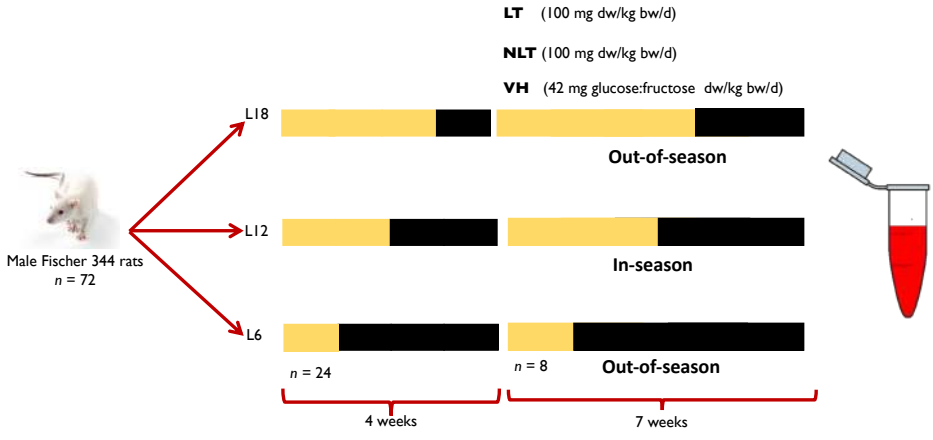

Figure 1. Experimental design of this study.

2.4. uHPLC MSⁿ analyses of tomato-derived (poly)phenolic metabolites in rat serum

The (poly)phenolic metabolites of LT and NLT in rat serum were extracted as reported previously by Ardid-Ruiz *et al.* [17]. The samples were directly analysed by ultra-high performance liquid chromatography (uHPLC) coupled with mass spectrometry (MS), using an Accela uHPLC 1250 apparatus equipped with a linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA), fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Serum metabolite profiling after tomato (poly)phenol intake was evaluated through target full MS/MS analyses by monitoring the specific deprotonate molecule (**Supplementary Table 2**). The analysis and quantification parameters of serum metabolites after tomato polyphenol intake were the same as we previously detailed (Cruz-Carrión *et al.*).

2.5. Statistical analysis

Results are presented as mean values ± standard error (n = 8). For all metabolites found in serum, a two-way analysis of variance (ANOVA) was performed to determine the influence of the factors studied (P, T and P×T), and then, individually, each intervention was subjected to a one-way ANOVA to establish any differences between means across the three photoperiods, followed by the least significant difference (LSD) post hoc multiple comparison test to identify means that differed. In addition, Student's t-test was used to estimate any differences between LT and NLT metabolites within the same photoperiod. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL, USA) software package.

3. Results

In this research we evaluate the effect of the in-season consumption of local Ekstasis tomatoes (specifically, in autumn emulated by exposing rats at 12 h of light a day) on the tomatoes (poly)phenolic bioavailability. For this purpose, rats were exposed to three photoperiods (winter-DH (6 h light/day), spring/autumn-DH (12 h light/day) or summer-DH (18 h light/day)) to simulate seasonal light schedules and administered 100 mg/kg bw of LT or NLT for 7 weeks. Serum samples were collected one hour after the last administration to evaluate the bioavailability and metabolism of tomatoes (poly)phenols.

3.1. (Poly)phenolic metabolites in serum after tomatoes administration

Contents of serum tomato (poly)phenolic metabolites are detailed in Table 2 and Figure 2. The serum tomato (poly)phenolic metabolites profile found in these animals reflects the metabolites of the two last tomatoes doses administered to rats (*i.e.,* 1 h and 25 h after tomatoes administration). As a result, a total of 7 (poly)phenolic metabolites were identified and quantified or tentatively quantified in rat serum. Indeed, cinnamic acid derivatives (CAD) and phenylpropanoic acid derivatives (PAD) detected in this study occurred mainly as sulfate. None of the parent (poly)phenols present in the tomato were detected in the rat serum. CAD, the greatest family of phenolic metabolites found (71%), included the only phase II glucuronidated metabolite, *i.e.*, 4′-hydroxycinnamic

acid-3′-glucuronide, which was detected in all the photoperiods after consumption of both tomatoes.

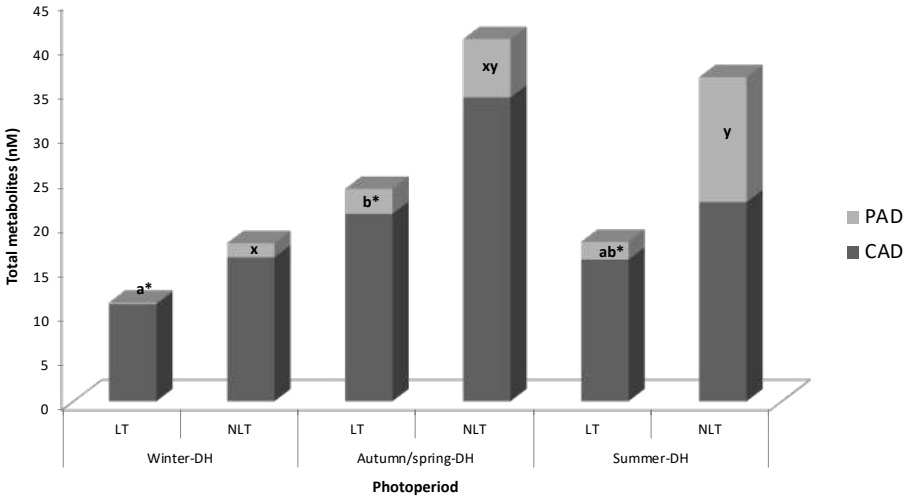

Figure 2. Distribution of (poly)phenolic metabolites in rat serum after 100 mg/kg bw/d administration of local (LT) or non-local (NLT) Ekstasis tomatoes and exposed to seasonal daylight schedules. * indicates statistical difference of PAD concentrations ($p < 0.05$) between LT and NLT ingestion within each photoperiod estimated by Student's *t*-test. Values with different letters (a, b, c; for LT consumption) and (x, y, z; for NLT consumption) indicate different PAD concentrations ($p < 0.05$) between the photoperiods, estimated by one-way ANOVA. CAD concentration was no statistic different by one-way ANOVA or Student's *t*-test. Abbreviations: CAD, cinnamic acid derivatives; DH, daylight hours; PAD, phenylpropanoic acid derivatives.

Table 2. Concentration of tomato-derived (poly)phenolic metabolites in serum of rats exposed to three seasonal daylight schedules after the ingestion of 100 mg/kg bw/d local (LT) and non-local (NLT) Ekstasis tomatoes. Results are expressed as the mean values in nM ± SEM (n = 8).

Metabolite		Serum concentration (nM)						
		Winter-DH		Spring/autumn-DH		Summer-DH		2wA
		LT	NLT	LT	NLT	LT	NLT	
CAD	3´-methoxycinnamic acid-4´-sulfate	n.d. ^{a*}	3.84 ± 3.84 ^{x}	11.33 ± 5.61 ^{b}	18.53 ± 9.08 ^{x}	8.80 ± 4.93 ^{b}	13.06 ± 6.07 ^{x}	P
	4´-hydroxycinnamic acid-3´-glucuronide	3.45 ± 1.70 ^{a}	2.03 ± 1.21 ^{x}	3.04 ± 1.71 ^{a*}	7.23 ± 3.40 ^{x}	2.37 ± 1.40 ^{a}	2.62 ± 1.19 ^{x}	n.s.
	4´-methoxycinnamic acid-3´-sulfate	n.d. ^{a*}	0.21 ± 0.13 ^{x}	0.60 ± 0.25 ^{b}	0.47 ± 0.24 ^{x}	0.33 ± 0.16 ^{b}	0.54 ± 0.15 ^{x}	P
	Hydroxycinnamic acid sulfate I	0.20 ± 0.10 ^{a}	0.85 ± 0.22 ^{x}	0.88 ± 0.24 ^{b}	0.73 ± 0.22 ^{x}	0.41 ± 0.14 ^{ab}	0.51 ± 0.15 ^{x}	n.s.
	Hydroxycinnamic acid sulfate II	7.24 ± 3.71 ^{a}	9.23 ± 2.05 ^{x}	5.20 ± 1.32 ^{a}	7.17 ± 1.34 ^{x}	3.99 ± 1.33 ^{a}	5.63 ± 2.11 ^{x}	n.s.
PAD	3-(3´-methoxyphenyl)propanoic acid-4´-sulfate	n.d. ^{a*}	1.42 ± 0.95 ^{x}	0.78 ± 0.35 ^{b*}	2.60 ± 1.20 ^{xy}	0.54 ± 0.52 ^{b*}	9.36 ± 3.69 ^{y}	P, T
	3-(4´-hydroxyphenyl)propanoic acid-3´-sulfate	0.19 ± 0.19 ^{a}	0.17 ± 0.17 ^{x}	2.03 ± 0.83 ^{a}	3.95 ± 1.31 ^{xy}	1.47 ± 0.76 ^{a}	4.68 ± 2.02 ^{y}	P
	Total metabolites	**11.26 ± 5.26 ^{a}**	**17.76 ± 6.97 ^{x}**	**23.86 ± 7.19 ^{a*}**	**40.69 ± 14.22 ^{x}**	**17.90 ± 7.50 ^{a*}**	**36.40 ± 12.56 ^{x}**	**n.s.**

* denotes significant difference (p < 0.05) between LT and NLT ingestion within each photoperiod estimated by Student' s t-test. Values with different letters (a, b, c; for LT consumption) and (x, y, z; for NLT consumption) indicate statistical differences (p < 0.05) between the photoperiods, estimated by one-way ANOVA. Two-way ANOVA was used to evaluate the differences between the groups. P, photoperiod effect; T, treatment effect; P×T, photoperiod×treatment interaction effect. Abbreviations: CAD: cinnamic acid derivatives; Winter-DH: winter daylight hours, 6 h light/day; spring/autumn-DH: spring/autumn daylight hours, 12 h light/day; summer-DH: summer daylight hours, 18 h light/day; n.d.: not detected; PAD: phenylpropanoic acid derivatives.

3.2. Effects of photoperiod in the (poly)phenolic metabolites in serum after tomatoes administration

Remarkably, 3′-methoxycinnamic acid-4′-sulfate was significantly affected by photoperiod effect (P), resulting the animals in winter-DH, regardless of the treatment received, contained less concentration than those in the other photoperiods. It was not detected in the group of animals exposed to the winter DH and LT administration. Importantly, the same pattern was observed for 4′-methoxycinnamic acid-3′-sulfate after LT consumption. As for PAD, the total PAD concentration was strongly impacted by the photoperiod effect. In this sense, the total PAD values found in the serum of rats exposed to winter-DH were significantly lower than those of rats exposed to summer-DH. This metabolites group, which includes 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, was found in most of the groups studied, except the former, which was not detected in those animals fed with LT and exposed to winter-DH. Regardless of tomato type, both metabolites were affected by the photoperiod effect, being the concentration of 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate of animals stabled in summer-DH was significantly higher than that of animals stabled in winter-DH. While the 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate concentration of animals stabled in winter-DH was significantly lower than that of animals stabled in the other two photoperiods.

In fact, the concentration of tomato-derived polyphenolic metabolites varied according to in-season (autumn-DH) and out-of-season (summer-DH or winter-DH) consumption of local and non-local tomatoes. In fact, different patterns in metabolite concentrations were observed after consumption of each type of tomato. The mean of total metabolites showed a tendency (p = 0.079, two-way ANOVA) to be less concentrated in the serum of animals exposed to the winter-DH, than those exposed to the autumn/spring-DH.

No statistical variation in CAD concentration was observed between the animal groups exposed to the three seasonal daylight hours after consumption of both LT and NLT. About LT consumption, total PAD concentration was statistically

lower after out-of-season consumption of LT than when it was consumed in-season (Figure 2). In addition, 3′-methoxycinnamic acid-4′-sulfate, 4′-methoxycinnamic acid-3′-sulfate and 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate were not found in the serum of rats after out-of-season consumption of LT (specifically when LT was consumed in winter-DH), thus differing statistically from their counterparts in the other photoperiods. Similarly, the concentration of hydroxycinnamic acid sulfate I found in the serum of rats that consumed LT out-of-season (winter-DH) was lower than those that consumed the fruit in-season. On the other hand, regarding NLT consumption, total PAD values were statistically lower when NLT was consumed out-of-season (specifically winter-DH) than when it was consumed in-season (spring/autumn-DH). Additionally, the PAD, *i.e.*, 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, individually also varied after the time of NLT consumption, in fact, both showed the same pattern, with higher concentrations being detected when NLT was consumed in summer-DH than in the opposite season-DH, but NLT consumed in-season generated the same concentrations as out-of-season. Additionally, 3′-methoxycinnamic acid-4′-sulfate amounts were lower when NLT was consumed out-of-season (winter-DH) than in-season.

It is important to note that the contribution of each group of metabolites (*i.e.*, CAD and PAD) in the total tomato-derived polyphenolic metabolites in rat serum was also dependent on exposure to seasonal daylight hours, as illustrated in Figure 2, resulting in a more noticeable effect after NLT consumption. Indeed, it appears that as animals are exposed to more hours of light, the total PAD amount increases after NLT consumption, rising from 9% to 16% to 39% in winter-DH, autumn/spring-DH and summer-DH respectively. This pattern is opposite to that observed for total CAD concentration, which goes from 91% to 84% to 61% as daylight hours increase. On the other hand, after LT consumption, total PAD concentration (2% of total metabolites) was found to be 57.3 times lower than total CAD concentration in rats exposed to winter-DH, interestingly PAD values increased up to 12% in rats exposed to autumn/spring-DH but relapsed in animals exposed to summer-DH.

3.3. Effects of the geographical origin of tomatoes in their (poly)phenolic bioavailability

The same whole Ekstasis tomatoes cultivated grown in two locations of Spain, *i.e.*, LT and NLT, were studied due to their particular (poly)phenolic profile (Table 1). Total (poly)phenolic metabolites in serum after NLT administration were higher than those of their LT-administered counterparts, irrespective of photoperiod exposure. Particularly, this effect was significant in autumn/spring-DH-stabled and summer-DH-stabled rats, in which the total metabolite concentration was 1.7-fold and 2.0-fold higher after NLT ingestion, respectively. The greatest number of statistical differences between the concentration of (poly)phenolic metabolites post-consumption of LT and NLT was evidenced in animals housed in winter-DH, in fact all three differences favoured NLT intake, as the absence of detectable amounts of 3′-methoxycinnamic acid-4′-sulfate, 4′-methoxycinnamic acid-3′-sulfate and 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate in serum was observed after LT intake. Followed by those animals exposed to autumn/spring-DH, where the concentration of 4′-hydroxycinnamic acid-3′-glucuronide and 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate in rat serum was significantly higher after NLT administration. And last but not least, animals housed in summer-DH differed statistically 17.3-fold in 3-(3′-methoxyphenyl)propanoic-4′-sulfate acid concentration after consuming NLT.

4. Discussion

Regular consumption of fruits and vegetables rich in (poly)phenols, including tomatoes, is related to beneficial health effects. In fact, their bioactivities have been mainly attributed to their metabolites [9,12]. A growing body of scientific evidence has reported that in-season fruit consumption produces optimal metabolic responses, while out-of-season consumption may induce erroneous signaling [18–20]. Emphasizing that local fruit consumption may lead to an enhanced antioxidant metabolic response against oxidative stress [19]. Therefore, bioavailability studies of (poly)phenols, as well as the factors that may modulate them, is important to understand the mechanism(s) of the health effect [12]. In this framework, a recent study by our group revealed that important physiological

changes in bioavailability and metabolism of red grape (poly)phenolic compound may occur under exposure to different photoperiods [11]. To the best of our knowledge, there are no studies that evaluate the effect of different light exposure regimens on the bioavailability of tomatoes (poly)phenolic compounds. Therefore, in this study the bioavailability of tomatoes (poly)phenolic compounds, was investigated by using uHPLC-MS^n to analyse serum collected after 1h of a chronic ingestion of 100 mg/bw/d of conventionally grown freeze-dried Ekstasis tomatoes from two locations of Spain. This dose is equivalent to consuming approximately 24 g of fresh tomatoes per day for a 70 kg human, which could constitute an important amount of the major dietary source of several micronutrients and phytochemicals [21]. In terms of (poly)phenol intake, this dose of LT and NLT resulted in 0.34 and 0.37 mg total (poly)phenols/kg bw/d, respectively. The chronic ingestion was performed to healthy Fischer 344 rats subjected to simulation of three seasonal daylight hours for 7 weeks. Fischer 344 rats were selected due to their physiological sensitivity to photoperiod [22]. From the total of 7 compounds identified in rat serum, most were phenolic acid derivatives, probably because tomatoes are mostly composed of phenolic acids, 88% in LT and 92% in NLT. No parent (poly)phenols were detected in the rat serum, which is indicative of no cleavage of the sugar moieties, which generates a low probability of absorption of even minute amounts and in turn indicates an effective phase II metabolism of the aglycones forming sulfate and glucuronide metabolites [9]. In this framework, sulfate conjugates predominated among the metabolites found. This suggests that a greater action of sulfotransferases than uridine-5′-diphosphate glucuronosyltransferases and catechol-*O*-methyltransferases in forming conjugated [9]. Furthermore, CADs, the predominant group of phenolic compounds in both tomatoes, were the highest number of tomato-derived metabolites found in rat serum. This corroborates with the vast literature that cinnamic acid and its derivatives constitute one of the largest and most ubiquitous groups of plant metabolites [23].

The circannual rhythms are an intrinsic timekeeping system that regulates numerous physiological, biochemical, and behavioural processes at intervals of approximately 12 months [24]. By regulating such processes, the circannual rhythm

allows organisms to anticipate and adapt to continuously changing environmental conditions [24]. In this regard, seasonal variations in the effects of bioactive compounds have been reported as a function of its time of administration, including its effects on a set of physiological circadian rhythms, *i.e.*, the temporal structure of the organism [25]. Thus, as evidenced with these results, the bioavailability and metabolism of tomatoes (poly)phenols depends on the photoperiod to which the rats are exposed. In this regard, one of the metabolites strongly impacted by photoperiod was 3′-methoxycinnamic acid-4′-sulfate, commonly referred to as ferulic acid-4′-sulfate or 4-hydroxy-3-methoxycinnamic acid, which was less concentrated in animals exposed to winter-DH. Moreover, a similar pattern was observed for one of the isomers of ferulic acid (4′-methoxycinnamic acid-3′-sulfate) which was less concentrated in animals consuming LT and exposed to winter-DH. This is in keeping with the report of Iglesias-Carres *et al.* [11] that ferulic acid bioavailability shows seasonal variations. In fact, it was only detected in one photoperiod after the administration of conventional grapes. The ferulic acid absorption mechanism, due to its low molecular weight and high hydrophilicity, was probably the paracellular pathway reported for other active ingredients with similar characteristics [2]. In this line, the bioavailability of ferulic acid from tomatoes in humans has been investigated by monitoring the pharmacokinetics of its excretion in relation to intake, with the result that peak time for maximal urinary excretion was approximately 7 h and the recovery of ferulic acid in urine was 11-25% of that ingested [26]. In fact, it has been reported that ferulic acid significantly reduced cadmium-induced hepatic and renal oxidative stress markers and restored antioxidant defense levels in the liver and kidney of male Wistar albino rats [27]. In addition, total PAD was also strongly impacted by the seasonal light schedule to which the animals were subjected, thus demonstrating a higher absorption of PAD in summer-DH animals than in winter-DH animals. Similarly, the findings of Iglesias-Carres *et al.* [11] also indicated that total PAD detected in serum post-ingestion of organic and conventional grapes was higher in rats exposed to the L18 photoperiod (summer-DH) than in those exposed to the opposite photoperiod (winter-DH).

As for fruit consumption, traditional fruit consumption was marked by the natural harvest season. Today, however, fruits such as tomatoes are marketed throughout the year, giving the option of consuming them out-of-season [10]. But each fruit has a distinctive composition of (poly)phenols that depends on intrinsic and environmental factors, including growing location and harvest season [3]. It has been suggested that seasonal consumption of polyphenol-rich fruits could lead to significant variations in the regulation of mammalian circadian rhythm-dependent physiology and metabolism and that ingestion of fruits out-of-season could trigger an alteration of the characteristic seasonal metabolism [18,28]. Along these lines, the values of total metabolites detected in serum after ingestion of both types of tomatoes was numerically higher when tomatoes were administered in-season (autumn-DH) than out-of-season (winter-DH). This same pattern was observed in the total amount of PAD. These results agree with those of Iglesias-Carres *et al.* [11], who found that the total amount of metabolites present in rats exposed to the L6 photoperiod (traditional grape consumption season), regardless of grape variety administration, was higher than when exposed to the L12 or L18 photoperiods. When both LT and NLT tomatoes were consumed in-season, higher concentrations of PAD, including 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, were detected. These metabolites could have been formed because of the action of colonic microbiota. In this regard, the anti-inflammatory properties of microbiota catabolites derived mainly from benzoic acid, phenylacetic acid and phenylpropanoic acid after consumption of blueberries have been studied by Russell *et al.* [29], although contradictory inter-individual results were observed. These results suggest that the anti-inflammatory effect of blueberry (poly)phenolics in the colon may depend on microbial catabolism that may vary somewhat because of the diversity of colonic microbiota composition from person-to-person. In addition, colonic metabolites of chlorogenic acids such as dihydroferulic acid [3-(3′-methoxyphenyl)propanoic acid-4′-sulfate] showed high antioxidant activity in a study carried out by Gómez-Ruiz *et al.* [30]. Likewise, dihydroferulic acid and 3-(3′-methoxyphenyl)propanoic acid, chlorogenic acid, and colonic catabolites of anthocyanins partially counteracted oxidative stress-induced platelet hyper-

reactivity, and were also able to reverse the negative effects of hormonal stress-induced platelet hyper-reactivity [31]. The results regarding the other main catabolite of chlorogenic acid, dihydrocaffeic acid derivatives, *i.e.*, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, indicate that this compound is able to eliminate intracellular reactive oxygen species (ROS) [32]. Here, dihydrocaffeic acid also increased nitric oxide (NO) synthase activity in a dose-dependent way in cultured cells, with a matching rise in endothelial NO synthase protein. These results suggest that dihydrocaffeic acid may act as an intracellular antioxidant. The antioxidant properties of dihydrocaffeic acid were corroborated in another study in which it reduced cytotoxicity and the production of proinflammatory cytokines in human cells after UV irradiation [33]. Furthermore, Miene *et al.* [34] demonstrated that dihydrocaffeic acid was able to up-regulate glutathione *S*-transferase T2 and decrease cyclooxygenase-2 expression in human adenoma cells. DNA damage induced by cumene hydroperoxide was markedly decreased by the catabolite. These findings may contribute to the chemopreventive power of (poly)phenols after degradation in the distal gastrointestinal tract. In addition, chlorogenic acid-derived catabolites, including dihydrocaffeic acid and dihydroferulic acid, tested in combination at physiological concentrations were found to protect human neuronal cells against oxidative stress in an *in vitro* experimental model [35].

In this study, significant differences were identified in the bioavailability and metabolism of (poly)phenols in serum of Fischer 344 rats after chronic administration of tomatoes of different geographical origin of cultivation: LT, local tomato and NLT, non-local tomato. When comparing the metabolites amount after LT and NLT ingestion, the concentration of total metabolites was higher following NLT ingestion in rats exposed to autumn/spring-DH and those exposed to summer-DH. Likewise, all metabolites that varied statistically within the same seasonal daylight schedule were higher after chronic NLT ingestion than their LT equivalent. These significant variations may be associated with the fact that the metabolites found are mostly phenolic acid derivatives and NLT stood out as having the highest amount of this family of phenolic compounds. However, LT was noted for having a higher flavonoid content, possibly because local fruits are harvested close to their consumption date, which reduces their

transport and/or storage time. But the factors influencing the variability in the appearance of their metabolites in serum, which may be related to other dietary components of tomatoes such as non-digestible carbohydrates, should be explored along with the actions of the absorbed metabolites [36]. Indeed, LT contained a higher numerical content of dietary fiber, and as is known, it binds to (poly)phenolic compounds under gastrointestinal conditions, preventing their absorption in the small intestine and promoting the increased passage of these compounds to the colon [12]. Similarly, LT was distinguished by a higher protein content, in this regard, (poly)phenols are known to form complexes with proteins, resulting in changes in the structural, functional and nutritional properties, and digestibility of both compounds [37]. In this sense, Trombley *et al.* [38] have suggested that bioavailability of plant (poly)phenols may be influenced by the covalent interaction between (poly)phenols and proteins. Indeed, it has been speculated that high amounts of protein may limit the availability and fermentation of (poly)phenols and the formation of metabolites from the microbiota through complexation [12]. This statement is consistent with other studies in which a decrease in the bioavailability of black tea (poly)phenols was observed due to the effect of protein-phenol interactions [39]. However, the mechanisms of interactions between tomato (poly)phenols and proteins should be investigated.

5. Conclusions

These findings provide useful information on the fate of (poly)phenols in the body following ingestion of tomatoes grown at two different locations. In fact, the bioavailability of tomato-derived (poly)phenols showed a seasonal daylight schedule-dependent effect, with significant variations between groups. In addition, the highest amounts of total metabolites were found in rat serum after in-season consumption of both tomatoes. The amounts of metabolites found differed considerably post-consumption of LT and NLT, presumably because of variations in the phenolic and nutritional composition of each fruit. Further studies focusing on human and microbial metabolites are needed to establish the mechanisms through which tomato-derived (poly)phenols may exert their health-promoting effects.

Author Contributions

Álvaro Cruz-Carrión: performed the sample analyses and wrote original draft. Ma. Josefina Ruiz de Azua: contributed with investigation. Luca Calani: performed the sample analyses. Pedro Mena: funded acquisition, supervised. Daniele Del Rio: funded acquisition, supervised. Manuel Suárez: designed the research, funded acquisition, supervised, wrote - reviewed. Anna Arola-Arnal: designed the research, funded acquisition, supervised, wrote - reviewed. All authors critically revised the paper and read an approved the final manuscript.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary Table 1. Proximate composition of local (LT) and non-local (NLT) tomatoes.

Components	LT	NLT
Ash	8.61 ± 0.28	8.76 ± 0.12
Protein	13.54 ± 0.04	12.96 ± 0.00*
Total carbohydrate, by difference	75.18 ± 0.14	75.74 ± 0.14
Total dietary fiber	25.26 ± 0.00	24.31 ± 1.18
Total sugars	36.98 ± 0.93	39.57 ± 0.92*
Total lipid (fat)	2.51 ± 0.03	2.49 ± 0.01

The results are expressed as g/100 g dw ± SD (n = 3). * denotes a significant difference ($p < 0.05$) by the Student's t-test. Table adapted from Cruz-Carrión *et al.*

Supplementary Table 2. Rat serum metabolites of tomato (poly)phenolic compounds monitored through uHPLC-MS^n.

Metabolite	RT	[M-H]- (*m/z*)	LOD (nM)	LOQ (nM)	MS^2 ions (*m/z*)
3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide	4.49	371	10	50	**175**, 113, 195
3-(3′-methoxyphenyl)propanoic acid-4′-sulfate	4.6	275	1	5	**195**, 149
3-(4′-hydroxyphenyl)propanoic acid-3′-glucuronide	4.33	357	10	50	**181**, 175, 113, 137
3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate	4.4	261	5	10	**181**, 135, 137
3-(4′-methoxyphenyl)propanoic acid-3′-glucuronide	4.96	371	10	50	**175**, 195, 113
3′-hydroxycinnamic acid-4′-glucuronide	3.68	355	10	50	**179**, 175, 135, 113
3′-methoxycinnamic acid-4′-glucuronide	4.12	369	10	50	**175**, 193, 113, 178
3′-methoxycinnamic acid-4′-sulfate	4.7	273	50	250	**193**, 229, 149
4′-hydroxycinnamic acid-3′-glucuronide	4.37	355	10	50	**179**, 175, 135
4′-methoxycinnamic acid-3′-glucuronide	4.89	371	10	50	**175**, 113, 193
4′-methoxycinnamic acid-3′-sulfate	5.06	273	50	250	**193**, 149
Hydroxycinnamic acid-sulfate I	4.36	259	5	10	**179**, 215, 135
Hydroxycinnamic acid-sulfate II	4.65	259	5	10	**179**, 215, 135
Naringenin-4′-glucuronide	6.74	447	1	5	**271**, 175
Naringenin-7-glucuronide	6.64	447	1	5	**271**, 175
Quercetin-3-glucuronide	5.96	477	1	5	**301**, 179
Quercetin-3′-sulfate	7.46	381	1	5	**301**, 179

The fragment ions are reported in their order of relative abundance. Quantifier ions are reported in bold. Standard of both naringenin-glucuronides have been only used for identification because of their lower purity than standard of quercetin-3-glucuronide. Abbreviations: LOD, limit of detection; LOQ, limit of quantification; RT, retention time.

V.

GENERAL DISCUSSION

GENERAL DISCUSSION

Phenolic compounds are a group of phytochemicals closely related to plant stress and secondary resistance in animals [1]. The key enzymes for the synthesis of these bioactive compounds react to external stressors as part of the plant defense system. In fact, it is well documented that the infection and environmental challenges can condition accumulation and composition of phenolic compounds in plants [2,3]. When ingested, these compounds, may provide antioxidant and anti-inflammatory effects, as well as therapeutic effects against aging, cancer, type 2 diabetes, neurodegenerative and renal diseases [4]. However, it has been reported that these effects can be modulated by seasonal rhythmicity [5]. Therefore, the hypothesis of this thesis was that the seasonal consumption of phenolic-rich plant-based foods, specifically sweet cherries, and tomatoes, from different geographical origins generates a differentiated pattern of phenolic bioactivity and bioavailability. In order to prove it, our main objective was to evaluate whether the geographical origin of cultivation, farming systems and seasonal consumption can condition the bioactivity and bioavailability of phenolic compounds in plant-based foods.

In this framework, the ingestion of organic foods has been associated with health implications, partially due to their content of health-promoting phytochemicals, such as phenolic compounds [6–11]. In this regard, decreased applications of pesticides and fungicides in organic farming may induce plants to increase the production of their own defense and stress systems, which in turn could elicit beneficial responses when consumed by animals and humans [12]. In contrast, modern agriculture, which is characterized by the use of pesticides and ample irrigation, produces fruits and vegetables with different phenolic profiles [12]. To shed light on this issue, **our first objective was to elucidate whether the ORG conditions the concentration of phenolic compounds and the antioxidant capacity in local plant-based foods [Manuscript 1]**. Here, we demonstrated that ORG and NORG practices generate a differentiated concentration of phenolic

compounds and antioxidant capacity depending on the plant species studied (fruits, vegetables, nuts, or legume) and its cultivar, with no prevalence from either farming systems type. Interestingly, the vegetables showed a clear pattern of differentiation between the two types of farming systems; indeed, ORG vegetables had a higher content of phenols and anthocyanins, which was reflected in a higher antioxidant capacity. This is consistent with the review by Heimler and colleagues [13], that pointed out a significant effect of farming systems on the phenolic composition of vegetables, whereas farming systems minimally affect the polyphenol content of fruits. In nuts and fruits, no correlations were detected between higher contents of phenolic compounds and antioxidant capacity, which could be attributed to the fact that plant-based foods contain many different antioxidant components that have not been evaluated, such as vitamin C, vitamin E, carotenoids and others [14].

Although the effect of the farming system generated an unclear trend and had only minor impact on dynamics in the accumulation of phenolic compounds in local plant-based foods, it is important to take into account that there are other possible factors, including environmental conditions, intrinsic to the growing location, that intervene and affect the phenolic compounds biosynthetic pathway [13,15–17]. In this sense, when fruits are consumed, regardless of whether they were produced by organically or non-organically systems, provide a chemical signature of the state of the environment in which the plant was cultivated [18]. Based on the xenohormesis theory, these signals provide beneficial responses, giving advance warning of deteriorating environmental conditions, allowing heterotrophs to prepare for adversity while conditions are still favorable [18,19]. In this context, out-of-season consumption of fruits generate erroneous xenohormetic responses [5,20–22], and it is plausible to assume that this erroneous signals are also produced at eating fruits from another geographical origin of cultivation. Therefore, **our second objective was to evaluate whether the geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis affect their antioxidant capacity against oxidative stress in rats [Manuscripts 2 and 3]**. These fruits were selected as representative of local products from the area of Camp de Tarragona and are part of the Mediterranean diet [23]. In this regard,

some authors argue that the xenohormesis theory may explain, at least in part, why the Mediterranean diet, rich in local/seasonal products, is apparently so healthy [24,25]. Actually, the Mediterranean diet is characterized by high consumption of fruits, vegetables, nuts, legumes, etc. [23,26]. Among them, tomato (*Lycopersicon esculentum* Mill.), a climacteric fruit, is associated with health protection and longevity [26–29]. On the other hand, sweet cherry (*Prunus avium* L.), is a non-climacteric fruit, characterized to be one of the most widely consumed summer fruits across the temperate regions of Europe and with described health benefits [30,31].

It was determined that both sweet cherries and tomatoes showed differences in the phenolic and nutritional composition between local and non-local fruits. The sweet cherries (LC) and tomatoes (LT) grown in Camp de Tarragona, Spain, were considered local fruits, while the sweet cherries grown in Cachapoal, Chile (NLC), and the tomatoes grown in Almería, Spain (NLT), were considered non-local fruits [**Manuscripts 2 and 3**]. This corroborates that environmental conditions, intrinsic to the location where plants grow, affect the biosynthetic pathway and accumulation of phenolic compounds [13,15,32,33]. It is also important to bear in mind that non-local fruits have to be subjected to post-harvest treatments, as well as transport conditions until they reach the point where they are marketed; in turn, in our case non-local fruits had a higher sugar content, which suggests a higher state of maturity, as in general there is an increase in sugars as the fruit ripens [34]. In this line, as it is well documented that post-harvest conditions can modulate the nutritional and phenolic composition of the fruits and vegetables [35–39]. A recent study evaluated the effects of temperature and storage time on the quality and compositional traits of three cherry tomato cultivars, reporting that the total polyphenol content of the fruits varied during storage time, peaked at 7 days and then decreased by 16% at 14 days of storage, with the highest values recorded at 10 °C [40]. Likewise, during cold storage of cherries, a 41-52% decrease in total anthocyanin content was evidenced after 15 days at 1°C and 95% RH in two sweet cherry cultivars, which may indicate no net anthocyanin biosynthesis [39]. However, the exact transport and storage conditions to which our non-local fruits were subjected are unknown, so it is

important to consider that different accumulation patterns of phenolic compounds within storage conditions could be produced [41–43].

Marked and distinct antioxidant xenohormetic responses were evidenced after consumption of local and non-local fruits, which could be attributed to the characteristic content of phenolics, and nutritional compounds present in each type of fruit [Manuscripts 2 and 3]. In this respect, the in-season consumption of LC, simulated by summer daylight hours, generated a significant decrease in liver MDA levels with respect to their NLC counterparts. The reason for this different response could be the fact that LC contained a higher anthocyanin content, since, according to the literature, around 92% of the antioxidant activity of fruits is provided mainly by two classes phenolic compounds, anthocyanins and hydrolyzed tannins [44]. Furthermore, the in-season consumption of LT, simulated by autumn/spring daylight hours, increased the levels of biomarkers of antioxidant status and caused a significant decrease in liver MDA and reactive oxygen species (ROS) values while a remarkable decrease in serum MDA was evidenced after NLT consumption. On the other hand, when fruits were consumed out-of-season, different patterns of bioactivity were evidenced.

When comparing in-season or out-of-season fruits consumption, regardless of the origin, different patterns of antioxidant responses were evident in Fischer 344 rats [Manuscripts 2 and 3]. Specifically, some biomarkers of antioxidant status were significantly modulated by the photoperiods. This is in concordance with the report by Wei and colleagues [45], which showed that light intensity can affect the antioxidant system. Moreover, daily rhythms of some enzymes related to antioxidant defense system and oxidative stress have been demonstrated [46–49]. Regarding fruit consumption, it was observed that, in most cases, the intake of fruits (sweet cherries and tomatoes) contributed to improve the antioxidant status of the animals against oxidative stress. This is consistent with the results found in the literature, that fruit and vegetable intake can decrease oxidative stress and inflammation in patients [50]. Moreover, it seems that these effects are enhanced when the fruit is eaten in its natural season of consumption emulated by the photoperiod. In this respect, it is worth mentioning that season of Brooks sweet cherry consumption is summer [30] and that of the Ekstasis tomato is

autumn [51]. Thus, when sweet cherries were consumed in-season, the levels of serum antioxidant biomarkers increased; another beneficial xenohormetic response was a reduction in liver enzyme activity; in addition, a reduction in liver malondialdehyde (MDA) levels was also observed [Manuscript 2]. This result is consistent with a study that showed that hypercholesterolemic rabbits fed with cornelian cherry had a significant decrease in MDA, compared to that of the high cholesterol-fed group [52]. Regarding in-season consumption of tomatoes, a decrease and/or maintenance of oxidative stress biomarker levels in serum was evidenced [Manuscript 3]. To our knowledge, there are no studies related to the antioxidant xenohormetic responses against oxidative stress generated by the seasonal intake of cherries and tomatoes. However, there have been recent studies by our group evaluating other xenohormetic responses to seasonal consumption of fruits. They have reported that out-of-season fruits consumption could induce erroneous signaling leading to a disruption in the characteristic seasonal metabolism. Whereas in-season consumption could lead to optimal health effects [5,20–22].

Because the greatest number of different antioxidant responses were observed with LT and NLT administration, both when consumed in- and out-of-season, it was essential to perform **phenolic characterization by uHPLC-MS^n and to study the phenolic pharmacokinetic profiles after acute consumption of LT and NLT in rat serum** [Manuscript 4] to identify the tomatoes bioavailable phenolic compounds in both tomatoes. As stipulated by Bohn [53], a better understanding of the polyphenols bioavailability is essential to understand their bioactivity. Qualitatively no differences were observed among LT and NLT, except for the appearance of dihydrocaffeic acid in NLT, which was not detected in LT fruits. Quantitatively, LT showed higher amounts of flavonoids, but NLT was notable for containing higher concentrations of total phenolic acids, this difference was mainly due to higher levels of free phenolic acids and hydroxycinnamoylquinic acids [Manuscript 4]. Considering that NLT contained a higher amount of sugars, pointing to its higher maturity stage, it has been also reported that this links to an increase of certain phenolic acids during fruit ripening [54–56]. Assessing accumulation changes of flavonoids, Caputo and colleagues [54] reported that the

highest rutin levels were found at the earliest stage of tomato maturity and observed a slight decrease during ripening. In our study, the highest rutin concentration was found in LT, *i.e.*, the fruit at the earliest stage of ripening. In addition to the influence of ripening stage on the accumulation of phenolic compounds, and as emphasized above, the agronomic factors have a strong influence on the biosynthesis of these compounds. In this line, Asensio and colleagues [15] reported significant variations in the concentrations of chlorogenic, ferulic and *p*-coumaric acid when comparing Rosa de Barbastro tomatoes grown in Barbastro and Montañana locations.

The unique concentration pattern of phenolic compounds, from LT and NLT, could partially explain the different antioxidant responses observed following their chronic consumption [Manuscript 3]. Phenolic compounds and carotenoids are the main biologically active compounds present in ripened tomatoes [57]. Our results suggest that LT containing a higher amount of flavonoids could be related to a higher antioxidant bioactivity, which are able to scavenge peroxyl radicals [58]. In addition, individual tomato compounds have been reported to be significantly related to antioxidant capacity, such as ferulic and caffeic acids, which in the study differed statistically among tomato types [59]. There are also lines of evidence suggesting that certain combinations of natural antioxidants have synergistic antioxidant activity, *i.e.,* the actual inhibitory activity of the mixture is greater than the activities of the individual antioxidants [60]. In this line, Shixian and colleagues [60] mentioned that polyphenols present in tomato contribute to the synergistic antioxidative effects observed with lycopene, in fact, phenolic compounds may play a role in preventing the degradation of lycopene. For instance, when four normolipidemic subjects aged 30-45 years, were treated with the polyphenols glabridin, rosmarinic acid and carnosic acid in combination with lycopene, inhibition of low-density lipoprotein oxidation was observed to a greater extent than the calculated additive effects by 32%, 32% and 15%, respectively [61].

As a product of this differentiated accumulation of xenohormetic compounds in either LT or NLT, particular pharmacokinetic profiles of tomato-derived metabolites were observed after acute consumption of these fruits [Manuscript 4]. Seven phenolic metabolites (*i.e.,* 3-(3′-methoxyphenyl)propanoic acid-4′-

sulfate, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, 3′-methoxycinnamic acid-4′-sulfate, 4′-hydroxycinnamic acid-3′-glucuronide, 4′-methoxycinnamic acid-3′-sulfate, hydroxycinnamic acid-sulfate I and hydroxycinnamic acid-sulfate II) were detected in rat serum, which reached their maxima concentration at first 4 h, indicating efficient absorption from the small intestine. There were substantial quantitative differences in the metabolites profile obtained, highlighting the higher concentration of metabolites after NLT ingestion. In this sense, according to Bohn [53], the food matrix and dosing, among other factors, can also significantly affect the bioaccessibility, absorption and subsequent metabolism of polyphenols. As previously stated, the food matrix of LT and NLT presented differences in terms of protein and sugars, the former being higher in LT and the latter in NLT. Indeed, it has been suggested that bioavailability of phenolic compounds may be conditioned by the covalent interaction between phenols and proteins [53,62], moreover, high amounts of protein may limit the bioavailability and the formation of metabolites from the microbiota through complexation [63].

Once the pharmacokinetics of local and non-local tomato-derived phenolic metabolites in rat serum during the first 48 h after consumption of both fruits are known, it is essential to study **their bioavailability and metabolism after in- and out-of-season consumption of both local and non-local tomatoes [Manuscript 5] to link these molecules with the observed effects [Manuscript 3],** since as previously stated, the metabolites of phenolic compounds are believed to be the bioactive forms to which the health effects are attributed [64]. In this regard, after chronic administration of LT and NLT for 7 weeks to rats exposed to different photoperiods, the same metabolites were identified as after acute consumption. In another study, similar results were found in three cultivars of raw tomatoes grown in Almería, in south-east Spain, where, among the metabolites identified, the most abundant were phenolic acids [65]. When comparing qualitatively the metabolic phenolic profile, no differences were observed between LT and NLT, except for the appearance of 3′-methoxycinnamic acid-4′-sulfate, 4′-methoxycinnamic acid-3′-sulfate and 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate after out-of-season NLT administration, specifically in winter daylight hours, which was not detected after LT consumption. As it is well known,

metabolites are the end products of cellular regulatory processes and their levels can be considered as the ultimate response of biological systems to genetic or environmental changes [65]. There is considerable epidemiological evidence indicating that significant physiological changes in the bioavailability and metabolism of phenolic compounds occur under exposure to various photoperiods [65–68]. In terms of quantification, total metabolites were more highly concentrated after in-season NLT intake. The greatest number of differences between metabolite concentrations after LT and NLT administration occurred in winter daylight hours, with the highest concentrations in all cases after NLT administration. Thus, as evidenced with our results, the bioavailability and metabolism of tomatoes phenolic compounds depends on the photoperiod to which the rats are exposed [Manuscript 5].

The metabolites and interactions amongst these may possibly be responsible for the health benefits deriving from the tomatoe consumption [65]. Specifically, the antioxidant power of phenolic metabolites has been reported, *e.g.*, chlorogenic acid metabolites such as 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate showed a higher antioxidant activity than other coffee compounds [69–71]. Furthermore, it has also been shown that 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate metabolites when tested in combination at physiological concentrations has proven to be effective in protecting human neuronal cells against oxidative stress in an *in vitro* experimental model [70]. The detection and characterization of intrinsic antioxidant bioactive compounds in tomatoes, specifically phenolic compounds, and their metabolites, suggested that these compounds in the form of metabolites could be associated with the observed antioxidant activity against oxidative stress described in Manuscript 3.

Considering that natural antioxidants exist in nature in combination, and a combination of different antioxidants can act synergistically [61,72], the findings obtained in our studies suggest that the unique and marked bioactivity shown by local and non-local fruits, in addition to being attributed to their phenolic profile and nutritional composition, could also be attributed to the antioxidant effect of other bioactive compounds present in these fruits, and synergistic effects among them. Therefore, further studies are needed to evaluate the synergistic

antioxidant effects of phenolic compounds derived from Brooks sweet cherries and Ekstasis tomatoes with other antioxidant compounds present in these fruits.

Finally, based on the obtained results, our research points to a promotion of the regular consumption of seasonal and local phenolic-rich fruits, on the basis that it could enhance the beneficial impact of the diet and contribute to optimal health.

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VI.

CONCLUSIONS

CONCLUSIONS

This research evaluated how farming systems, together with the geographical origin of the cultivation, and seasonal consumption can condition the content, bioactivity, and bioavailability of phenolic compounds in plant-based foods. Based on quantitative and qualitative phenolic and antioxidant analyses, as well as animal experiments, it can be concluded that:

1. Organic farming systems differently affected the profile of phenolic compounds and the antioxidant capacity in local plant-based foods, depending on the species and plant cultivar analyzed, with no clear differentiation patterns. Remarkably, organic vegetables were found to be more susceptible to the induction of phenolic compound biosynthesis, presenting a clear pattern of differentiation between organic and non-organic farming systems.

2. The geographical origin of cultivation of sweet cherry cv. Brooks and tomato cv. Ekstasis influences their phenolic profile.

    2.1. Local and non-local fruits exhibited different phenolic composition, specifically, local fruits showed a higher content of flavan-3-ols and anthocyanins compared to non-local ones.

    2.2. A quantitatively different phenolic profile between tomatoes from diverse geographical origins of cultivation was determined by uHPLC-MS^n. Local tomato had a higher flavonoid content, but non-local tomato contained a higher phenolic acid content.

3. The evaluation of the effects of geographical origin of cultivation and seasonal consumption of sweet cherry cv. Brooks and tomato cv. Ekstasis on antioxidant capacity, demonstrated that these factors condition the *in vivo* antioxidant capacity of these fruits against oxidative stress.

    3.1. In-season consumption of both sweet cherries (simulated by summer daylight hours) and tomatoes (simulated by autumn/spring daylight hours), regardless of their cultivation origin, enhanced the antioxidant status of rats against oxidative stress.

    3.2. Out-of-season consumption of both sweet cherries and tomatoes resulted in different patterns of antioxidant bioactivity depending on the origin of the fruit.

    3.3. Consumption of local sweet cherry and local tomato showed favorable differences in antioxidant bioactivity, enhancing health benefits compared to their non-local counterparts.

4. The geographical origin of the tomato cv. Ekstasis cultivation and its seasonal consumption conditioned its phenolic bioavailability.

    4.1. The different concentration of phenolic compounds and matrix factors in tomatoes from two geographical origins of cultivation condition the phenolic bioavailability and pharmacokinetic profile. Specifically, a higher concentration of metabolites was detected after acute ingestion of non-local tomato linked to its lower protein content, probably due to a less covalent interaction limiting its bioavailability.

    4.2. The bioavailability of tomato-derived phenolic compounds showed seasonal variations. In fact, the highest concentrations of total metabolites were detected after chronic in-season consumption of both tomatoes. This correlates with the fact that a trend of higher antioxidant bioactivity was detected after in-season consumption of tomatoes, irrespective of the geographical origin of cultivation.

Based on the results obtained in this thesis, it is suggested that consuming more in-season and local phenolic-rich foods is a possible way to move towards sustainable and healthy consumption patterns, considering that local fruits have a higher phenolic bioavailability and antioxidant capacity, which could lead to health benefits.

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- **Cruz-Carrión, A.**, Ruiz de Azua, MJ., Mulero, M., Arola-Arnal, A., Suárez. M. (2020). Oxidative stress in rats is modulated by seasonal consumption of sweet cherries from different geographical origins: local vs. non-local. Nutrients, 12, 2854;doi:10.3390/nu12092854.
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- **Cruz-Carrión, A.**, Calani, L., Ruiz de Azua, MJ., Mena, P., Del Rio, D., Suárez, M., Arola-Arnal, A. (Poly)phenolic composition of tomatoes from different growing locations and their absorption in rats: a comparative study. Submitted to Molecular Nutrition & Food Research.

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POSTER COMMUNICATIONS

- Colom-Pellicer, M., **Cruz-Carrión, A.**, Arola-Arnal, A., Suárez, M., Aragonès, G. (2019). Time-of-day dependent effect of grapeseed procyanidins on white adipose tissue function in diet-induced obese rats. Conference poster, *ICPH2019 KOBE The 9th International Conference on Polyphenols and Health* on November 28 – December 1, 2019, in Kobe, Japan.
- Escobar-Martínez, I., Rojas-Criollo, M., **Cruz-Carrión, A.**, Bravo, F., Arola-Arnal. A., Suárez, M. (2019). Administration time significantly affects bioavailability of grape seed proanthocyanidins extract in Fischer 344 rats. Conference poster, *ICPH2019 KOBE The 9th International Conference on Polyphenols and Health* on November 28 – December 1, 2019, in Kobe, Japan.
- Colom-Pellicer, M., Aguado, M., **Cruz-Carrión, A.**, Arola-Arnal, A., Suárez, M., Aragonès, G. (2019). Time-of-day dependent effect of bioactive compounds on white adipose tissue metabolism and

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- Colom-Pellicer, M., Aguado, M., **Cruz-Carrión, A.**, Arola-Arnal, A., Suárez, M., Aragonès, G. (2019) Time-of-day dependent effect of bioactive compounds on white adipose tissue metabolism and expandability in obese rats. Conference poster, *Alimentòmica, XI Seminar on Food and Healthy Lifestyles* at July 23-24, 2019, in Barcelona, Spain.
- **Cruz-Carrión. A.**, Rojas-Criollo. M, Iglesias-Carres. L, Arola-Arnal. A, Suárez, M. (2018) Circadian rhythms affect the absorption and metabolization of phenolic compounds from grape seed proanthocyanidins extract. Conference poster, *INVINOTEC II National Congress of Wine Biotechnology* at October 18-20, 2018, in Tarragona, Spain.

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- **Cruz-Carrión. A,** Rojas-Criollo. M, Iglesias-Carres. L, Arola-Arnal. A, Suárez, M. (2018) Circadian rhythms affect the absorption and metabolization of phenolic compounds from grape seed proanthocyanidins extract. Oral conference presentation, *INVINOTEC II National Congress of Wine Biotechnology* at October 18-20, 2018, in Tarragona, Spain.

Review

CHRONONUTRITION AND POLYPHENOLS: ROLES AND DISEASES

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Abstract

Biological rhythms can influence the activity of bioactive compounds and, at the same time, the intake of these compounds can modulate biological rhythms. In this context, chrononutrition appears as a research field centered on the study of the interactions between biological rhythms, nutrition and metabolism. This review summarizes the role of phenolic compounds on the modulation of biological rhythms, focusing on its effects in the treatment or prevention of chronic diseases. Heterotrophs are able to sense chemical cues mediated by phytochemicals such as phenolic compounds, promoting their adaptation to environmental conditions. This is what is called xenohormesis. Hence, the consumption of fruits and vegetables rich in phenolic compounds exert several health benefits mainly attributed to the product of their metabolism. However, the profile of phenolic compounds present in plants differs among species and is highly variable depending on agricultural and technological factors. In this sense, the seasonal consumption of polyphenol-rich fruits could induce important changes in the regulation of physiology and metabolism, due to the particular phenolic profile that contain. This fact highlights the need of studies that evaluate the impact of these specific phenolic profiles on health in order to stablish more accurate dietary recommendations.

Keywords

Biological rhythms, chrononutrition, diseases, health benefits, metabolic syndrome, nutrition, polyphenols.

1. Introduction

1.1. Biological rhythms (circadian and seasonal)

Biological rhythms, present in all the organisms and including circadian and circannual rhythms, are closely related to metabolism and nutrition. In this framework, light plays a pivotal role performing relevant changes on the physiological and metabolic signaling pathways that drive the behavior of the organisms [1,2]. Moreover, light modulates seasonal specific behaviors such as reproduction, migration, hibernation, germination or blossom according to the period and organism [3–5]. The presence of endogenous rhythms is clearly an advantage in the evolution since cyanobacteria, which represents one of the earliest and primitive organisms that present this type of regulatory processes [6]. Nevertheless, although it is known that these reactions are highly regulated by the light-induced gene expression, the mechanisms involved are still not clearly identified. In this sense, there is growing evidence that factors affecting the biological clock, such as gene polymorphisms of the core clock machinery and seasonal changes of the light-dark cycle, exert a marked influence on the physiological activity.

In the last years, it has been observed that the biological rhythm can influence the biological activity of bioactive compounds or nutrients from the diet. Moreover, the intake of those products can also modulate the biological rhythms. In this line, some authors have reported that the first meal after a long fasting, like breakfast, is important for synchronization of the clock [7]. Furthermore, it is worth to highlight that bioactive compounds such as polyphenols from diet can also interact with the clock, establishing an accurate time-point for their consumption according to the active signaling pathways [8]. Recently, it has been reported a link between circadian rhythms, microbioma and obesity in which the gut microbiome is understood as an endocrine system that, linked with circadian rhythms, may have an influence in obesity [9]. In addition, the consumption of functional foods has been shown to have beneficial effects to prevent or ameliorate many chronic physiological diseases such as obesity and associated metabolic syndrome at least in part through the modulation of the rhythms [10]. All

these appreciations show the importance of rhythms in the physiological and metabolic response of our body and how we can use this knowledge to prevent or ameliorate pathologies as metabolic syndrome.

1.2. Molecular mechanism

Circadian rhythms determine that all processes present maximum and minimum values of functionality throughout the day. Currently, it is accepted that the molecular mechanism that drives biological rhythms is composed of a set of two interlocking transcriptional-translational *feed-back loops*, CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1). CLOCK/BMAL1 heterodimer is the common central clock in all cells and stimulates transcriptional activity of three Period (*Per*) and two Cryptochrome (*Cry*) genes [9]. The PER/CRY heterodimer acts as a negative *loop feed-back* of Clock/Bmal1 transcriptional expression [11]. Moreover, the CLOCK/BMAL1 heterodimer activates *Bmal1* gene expression. Additionally, there are two *loops feed-back Rorα* and *Rev-erbα*, whose expression is regulated by CLOCK/BMAL1. RORα and REV-ERBα act as activator and inhibitor of the *Bmal1*, respectively (**Figure 1**).

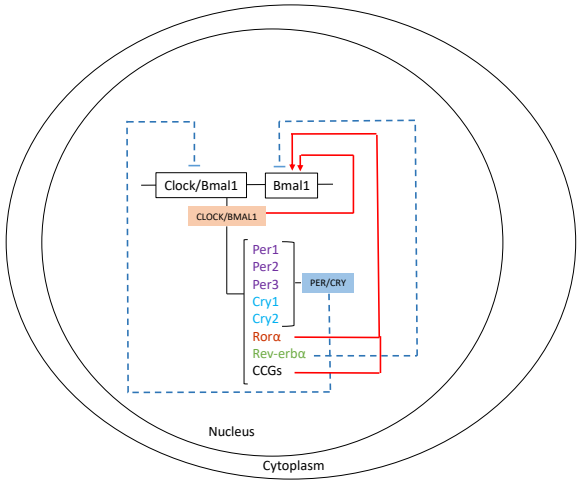

Figure 1. Molecular mechanisms of biological rhythms. CLOCK/BMAL1 heterodimer is the central clock in all cells and stimulates transcriptional activity Period (*Per*) and Cryptochrome (*Cry*) genes, whose heterodimer act as a negative loop feed-back of Clock/*Bmal1* transcriptional expression. The two loops feed-back *Rorα* and *Rev-erbα* expression is regulated by CLOCK/BMAL1.

In addition to NAD, other major intracellular biochemical components with a clock regulator function are monophosphate-activated protein kinase (AMPK) and cyclic adenosine monophosphate (cAMP). In this line, there are specific CCGs according to the cell type [12]. Thus, the molecular clock controls the cellular functionality and metabolism due to the CCGs through metabolic pathways or transcriptional factors and nuclear receptors, which induce the expression of enzymes and other metabolic factors [13,14]. Furthermore, light regulates all this process triggering the activation of different signaling pathways in the body (**Figure 2**). On this regard, melatonin plays a pivotal role during dark-cycle turning off the light-cycle metabolism.

It is well established that mutants in CLOCK and BMAL1 present abnormal metabolic phenotype, showing obesity, metabolic syndrome and type 2 diabetes. These disorders are due to hypoglycaemia and elevated glucose clearance after acute glucose administration in the pancreas [15], hyperglycaemia and lowered glucose tolerance in the pancreas [16], decreased insulin-dependent glucose metabolism in the skeletal muscle [17] and abnormal polyunsaturated fatty acid secretion from the adipocytes [18] regulating hypothalamic appetite centres in white adipose tissue-specific clock disruption. In recent years, our group have studied the circannual rhythms in metabolic homeostasis analysing the seasonal fluctuations on glucose and lipid metabolisms [19] as well as several comorbidities related to obesity, including hypertension and insulin-resistance. In this line, our studies were focused on how biological rhythms can modulate these pathological parameters by regulating the circadian rhythm-related genes *Cry1*, *Bmal1*, *Per2* and *Nr1d1* signaling pathways [20]. The present review summarizes the role of polyphenols on modulation of biological rhythms, with potential interest in the treatment or prevention of chronic diseases as metabolic syndrome.

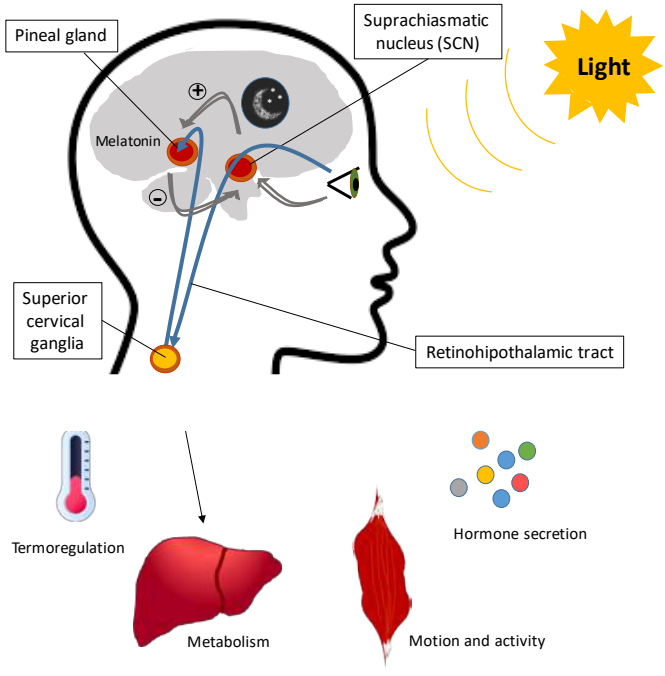

Figure 2. Effect of the light on biological rhythms. Light regulates the biological rhythms through activation the suprachiasmatic nucleus, which actives superior cervical ganglia, which triggers different signaling pathways in the body such as modulating termoregulation, metabolism, motion and activity and hormone secretion. In dark period, pineal gland synthesizes melatonin, which inhibits the suprachiasmatic nucleus action.

2. Chrononutrition

2.1. Definition

Over the last years, there has been increasing recognition of the biological clock impact on nutrition, having different effects on energy balance and metabolism, and influencing health and diseases. This concept has led to the development of a new discipline known as chrononutrition [21], which was first mentioned in a Japanese book about nutrition and health published in 2005 [22]. Since then, chrononutrition has emerged as a research field focused on the study of the interactions between biological rhythms, nutrition and metabolism [21]. Specifically, it involves not only how the timing of food intake and biological rhythms may affect health, metabolism and nutrition but also how nutrition (composition and size of meal) may affect our internal clock system [23].

The majority of the clinical studies carried out in this field are mainly focused on the effects of meal timing. In this regard, it has been observed that meal timing patterns such as skipping breakfast, higher energy intake during the evening and higher eating and snack frequency, are linked with a higher risk of being overweight or obese and with adverse metabolic effects in humans [24]. In fact, changes in circadian eating patterns result in increased energy intake in both human and rodents [25–30]. Moreover, differential effects on weight loss have been shown after meal timing variations in overweight and obese women [31]. On the other hand, short sleep duration has been associated with the development of different chronic diseases such as hypertension, type 2 diabetes or obesity [32–34]. The mechanisms underlying these effects are not fully understood yet. However, in the last years there have been different studies that have provided new insights. Thus, circadian rhythm alterations lead to increased glucose and insulin levels as well as increased arterial pressure, decreased leptin level, sleep deficiency, and altered cortisol secretion rhythm [35]. Leptin signaling, which modulates satiety, exhibits circadian variation and may link clock genes fluctuation with metabolic diseases such as diabetes and obesity [35]. Indeed, unusual food intake timing has been linked to altered satiety signals and reduced serum leptin level [25,36,37]. This reduction in leptin level leads to increased appetite while reducing energy expenditure and, therefore, it promotes the development of obesity and others metabolic disorders [35]. Another factor that may explain the influence of circadian clocks on metabolism modulation are the effects on thermogenesis and energy expenditure [25]. Thus, thermogenesis is induced by meal intake and it shows a circadian rhythm, being highest in the morning, followed by the afternoon and night [25]. Hence, this may explain why skipping breakfast is linked to increased body weight. Recently it has been shown that this increased diet-induced thermogenesis in the morning may be due to the circadian rhythmicity of circulating norepinephrine and epinephrine with increased values in the morning and which are known to influence food intake modulation [38,39]. Moreover, in a study with healthy individuals, the same meal consumed in the evening determined a lower resting metabolic rate (RMR) and increased glycemic/insulinemic responses, suggesting circadian variations in the energy expenditure and metabolic pattern [40]. The hunger hormone ghrelin, has

also shown a circadian oscillation, being minimum in the morning, and modulating energy expenditure and thermogenesis by suppression of brown fat thermogenesis [38].

2.2. Macronutrients and bioactives

On the other hand, as mentioned above, dietary patterns may affect our biological rhythms [23,41]. Hence, the expression levels of clock genes, such as Per1/Per2, showed variations with daytime feeding in liver [42,43]. Moreover, absence of feeding rhythmicity together with absence of adrenal hormones depleted hepatic clock genes rhythms [44]. Nutrients and food factors can also modulate cellular circadian clocks or clock systems throughout the body. Thus, high-fat diet (HFD) consumption led to increased food ingestion during the light period in mice, which may contribute to alterations in body weight regulation, and to altered circadian patterns of circulating metabolic markers such as leptin and insulin, clock-regulating nuclear factors RORα and PPARα, hypothalamic neuropeptides AgRP and NPY and factors involved in lipid metabolism [45]. In addition, HFD has been reported to alter AMPK kinase signaling, which is activated in situations of energy shortage, leading to aberrant clock gene expression in comparison with normal diet [46]. This HFD-mediated AMPK signaling alteration was improved in mice with time-restricted feeding, besides equivalent calories consumption as those with ad libitum access, leading to protection against obesity, hyperinsulinemia, hepatic steatosis, and inflammation [47,48]. In addition, HFD in mice under constant darkness alters also the circadian locomotive rhythms, increasing their locomotive activity [45]. In humans, changes in dietary carbohydrate and fat percentage, from 55% carbohydrate-30% fat to 40% carbohydrate-45% fat, led to alterations in cortisol circadian oscillation, inflammatory and metabolic gene expression profiles and *PER* gene expression rhythms in monocytes [49]. This study highlights the importance not only of the type of diet but also of its constituents' proportion. High salt (HS) diets have also been shown to affect biological rhythms. Thus, HS feeding showed alterations in intrarenal circadian rhythmicity with significant delay in the peak expression of Bmal1 and Cry1 and Per2 expression suppression in the renal inner medulla [50].

Recently, gut microbiota has emerged as a key factor in metabolism modulation and its potential influence on circadian rhythms is critical as it regulates the energy derived from food and modulates the levels of host- and diet-derived products [51]. Thus, changes in gut microbiota induced by diet can affect the gut clock influencing the organism's homeostasis. Indeed, gut microbiota composition undergoes circadian oscillations in both mice and humans [51,52]. Microbiota seems also to influence several neuronal functions and, therefore, it may be possible that the central clock or different brain areas may be receiving "nutritional" information in a cyclic manner through the gut-microbiota interplay [51,53,54].

Regarding individual nutrients contained in the foods, the potential effect of some amino acids residues on sleep/wake cycle alterations have been evaluated. Thus, the consumption of 3 g L-Serine 30 min before bedtime combined with a bright-light exposure during the morning has been shown to be useful to enhance light-induced phase resetting in humans [55]. Moreover, it is known that tryptophan is involved in sleep regulation since it is precursor of serotonin and melatonin, hormones involved in sleep latency and quality, respectively [56]. Thus, consumption of tryptophan-enriched cereals (60 mg tryptophan twice a day in the morning and at night) has shown to increase sleep efficiency, actual sleep time and immobile time in elderly people aged 55–75 years old [56]. Moreover, consumption of cereals enriched with tryptophan, adenosine-5′-phosphate, and uridine-5′-phosphate at night increased sleep efficiency in infants 8-16 months old with sleep problems [57]. In addition, a diet rich in cherries or cherry juices, which contain a high concentration of tryptophan, serotonin and melatonin, can also produce beneficial effects on sleep/wake rhythms in both middle-aged and elderly people, for example reducing insomnia or increasing the sleep time and efficiency [58–61]. However, their beneficial effects are dependent on the type of cherry cultivar [58]. In addition to tryptophan, other compounds contained in cherries could be involved in their effects such as phytomelatonin, original melatonin of the plant, or polyphenols [59,60]. Moreover, fruits rich in serotonin may also help with sleep problems. Thus, the consumption of 2 kiwis 1 hour before bed for 4 weeks has been shown to improve the sleep quality in adults with sleep disturbances [62].

2.3. Xenohormesis

Besides nutrients, heterotrophs are able to sense chemical cues mediated by non-nutrients synthesized by plants under adverse conditions, also called phytochemicals, promoting the adaptation capacity of these organisms. This process is known as xenohormesis [63,64]. In fact, some of these phytochemicals such as polyphenols have been associated with prevention and/or treatment of chronic diseases such as hypertension, cancer, diabetes, obesity and other medical conditions [65].

3. Phenolic compounds: eating patterns and diseases

Regarding to xenohormesis, one of the most important groups of phytochemicals is the composed by phenolic compounds. These are secondary metabolites produced by the plants as response to several types of stress: drought, high or low temperatures, microbial infections or herbivores, amongst others [66–70].

There are thousands of phenolic compounds described in plants that have in common the presence of at least one phenolic ring (more than 8,000 described structures) [71]. These compounds can be divided in two main groups regarding their chemical structure: flavonoids and non-flavanoids. Among these two groups, flavonoids are the most widely distributed compounds [72].

Supplementary Figure 1 shows the main classes of phenolic compounds within these two categories. In general, these compounds can be found in plants as polymeric forms or/ and linked to sugars [73]. For example, the high molecular weight condensed tannins, also known as proanthocyanidins, are polymers composed of repetitions of flavanol units [74].

There are several health benefits derived from the consumption of fruits and vegetables rich in phenolic compounds [72,75]. Furthermore, these compounds confer bitterness, astringency, flavor, color, and oxidative stability to fruits and vegetables. In this sense, polyphenols are the main responsible for red colors and bitterness of wine.

3.1. Phenolic contents in vegetable products

Fruits, cocoa products and beverages, like tea and wine, are the main sources of phenolic compounds in the human diet [72,76]. These compounds are located throughout all the plant, including roots, leaves and fruits. Thus, leaves and stems contain higher amounts of these compounds, primarily in their monomeric form, while polymeric polyphenols are present in vacuoles, leaf, epidermis, flowers and fruits.

Supplementary Table 1 shows the values of total polyphenol content determined by Folin-Ciocalteau assay and obtained from Phenol-Explorer database [77–79]. Amongst the fruits and vegetables included in Table 1, cocoa, beans and walnuts are those with the highest polyphenol content (5624.23, 1234.38 and 1574.82 mg/100 g FW, respectively). Other sources rich in phenolic compounds are pomegranates, plums, strawberries and oranges, which polyphenol content range from 400 to 280 mg/100 g FW, approximately. It has to be taken into account that these values are the average of few reported independent studies, so these values can differ due to several factors.

The profile of phenolic compounds present in plants differs among species. In this regard, citric fruits are rich in flavanones and flavones like naringenine and hesperidin [80], while high-colored fruits such as cherries, grapes and berries are rich in anthocyanins like cyanidin or malvidin [81] and beverages such as wine, coffee and tea contain high amounts of flavanols like epigallocatechin gallate, catechin and epicatechin [82]. Actually, these last compounds are usually found in the vacuolar juices and in the epidermis [72,83]. Among the non-flavanoids, lignans (e.g. pinoresinol and matairesinol) are widespread in seeds and nuts [83].

3.2. Factors affecting polyphenol composition

As previously explained, several factors can affect the content of phenolic compounds within the same species. In fact, both agricultural and technological factors have shown to influence the phenolic profile in plants. Within these two groups, variety, environment, soil fertilization, irrigation system and ripening stage are the main agricultural factors. On the other hand, postharvest treatments arise within the technological factors.

Among all the factors, variety is one of the most important because it strongly conditions the profile of phenolic compounds. For example, in the bibliography more than 7500 different apple varieties are described [84] and as Kalinowska *et al.* describe [85], the content of their phenolic compounds deeply varies among them. For instance, the total phenolic content can range between 56 mg GAE/100g FW in Gala apple to 221 mg GAE/100g FW in Panaia apple [86]. Interestingly, these two are red varieties. Francini and Sebastiani (2013) suggest that the genetic variability between different apples has an important impact on the phenolic profile [87]. Some of these changes can be explained by the expression of genes involved in the biosynthetic pathway of phenolic compounds, which differ within varieties. For example, in grapes, a lack of the expression of anthocyanidin 3-*O*-glucosyltransferase 2 (UFGT) gene, an enzyme crucial for the synthesis anthocyanins [88], is observed in the white varieties compare to the red ones.

Environmental factors also modulate the phenolic composition of fruits and vegetables. For instance, water availability, temperature, light exposure, and soil salt are described as important modulators of phenolic synthesis [66–70]. In fact, fruits from the same variety cultivated in different areas present different content of phenolic compounds [89]. For example, Häkkinen and Törrönen observed significant differences in the total phenolic content in berries cultivated in Finland (49.3 mg/100g FW) compared to those cultivated in Poland (36.1 mg/100g FW) [89]. These results highlight the importance of environmental factors in the content of phenolic compounds in plants.

Finally, regarding agronomical factors, in organic cultivation controversial results had been observed concerning the phenolic content. Hence, some studies have shown that this agricultural practice increases the amount of phenols in the cultivar. For example, Stracke *et al.* showed that "Golden Delicious" apple from organic cultivars had 14-19% increased phenolic content compared to apples from non-organic cultivars [90]. However, in the study of Winter *et al.* [91], authors concluded that not consistent differences exists between organic and conventional cultivars. In another study, Mulero *et al.* [92] described that although significant differences were observed between unripe organic and non-organic

grape cultivars, these differences disappeared when grapes reached ripening stage. Therefore, more research is needed in this field.

Concerning technological factors, these include all the post harvesting treatments such as cleaning, storage, antimicrobial treatments and minimal processing. In this sense, the mixture of gasses used during the storage can modulate the synthesis of phenolic compounds. Moreover, wounding of plants during the harvesting induces the production of phenolic compounds as mechanism of defense [93].

Taking in consideration all these facts, it is evident that the profile of phenolic compounds of fruits and vegetables is highly variable. Therefore, it is plausible to consider that local fruits, cultivated in specific area and following determined agricultural practices, will have a specific profile of phenolic compounds. In relation with the xenhormesis theory, the external signals conferred by these plant polyphenols may prepare the body for the local upcoming situation. All this may reinforce the consumption of local products as it may provide some benefits to the organism compared to other fruits.

3.3. Polyphenols and diseases

As it can be seen in **Supplementary Table 1**, the beneficial effects of fruits and vegetables rich in phenolic compounds are wide and these phytochemicals exert anti-inflammatory, antioxidant and anti-carcinogenic activities, cardioprotective and anti-hypertensive, among others. The mechanism underling these effects are based on the capacity of these compounds to modulate basic biochemical pathways related to inflammation, lipid and energy metabolism [94]. Moreover, these compounds can also exert its action through epigenetic mechanism. For instance, these have been seen to regulate miRNA expression [95].

The health effects of fruits and vegetable consumption are partially attributed to specific polyphenols that can act as the bioactive compounds in each plant. For example, the flavanols (+)-catechin and (-)-epicatechin are some of the main bioactives present in very common fruits such as apples, cocoa and grapes [72,82,94,96–108]. Otherwise, in citric fruits like in oranges or lemons, these activities are related with the flavanones hesperidin and naringenin [72,97,109–111]. It is important to highlight that the health effects of polyphenols are mainly attributed to the

product of their metabolism. In this sense, polyphenols are extensively conjugated in the small intestine and the liver by specific enzymes to generate glucuronide, sulphate and methylated derivatives. Most of the polyphenols are not absorbed in the small intestine and reach the colon, where they are subjected to the activity of the gut microbiota [112,113]. Therefore, in addition to the agricultural and technological factors that influence the content of polyphenols in plants, host internal factors such as the microbiota or enzymes activities may also have an impact on the effects of these compounds.

3.4. Eating patterns

Traditionally, fruits and vegetables were produced and consumed during a limited season, due to the climatic conditions of each cultivar. For example, ripening of cherries and strawberries were limited to spring, whereas oranges ripening limited production to autumn and winter. However, nowadays these eating patterns are changing because of the globalization that allows the consumption of vegetables and fruits products throughout all the year. For example, Spain is the major producer of oranges in the European Union (EU), with an estimated production of 55%. However, this production is limited to autumn and winter season. Thus, during the rest of the year, oranges in the EU are imported from countries from the south hemisphere such as South Africa, Argentina and Uruguay [114]. This allows maintaining in the markets this product all the year. In addition, the production of fruits and vegetables in greenhouses also contributes to avoid seasonality of some products. For example, tomatoes and cucumbers can be found in the marked during all the year due to this agriculture practice [115]. However, it has to be taken into account that some of these fruits and vegetables that are imported usually are collected before reaching maturity (climacteric fruits). Moreover, those that are produced in greenhouse suffer different postharvesting treatment than fruits traditionally cultivated. Thus, considering that these are important factors affecting phenolic synthesis, the content of phenolic compounds can change and consequently this may also modify the health effects derived of its consumption.

4. Mechanisms implicated in the modulation of metabolism by seasonal consumption of polyphenol-rich fruits

As explained above, taking into account that each plant contains a distinctive composition of (poly)phenols based on the environment in which they were harvested, it is plausible to believe that seasonal consumption of polyphenol-rich fruits could induce marked changes in the regulation of physiology and metabolism depending on when they are consumed. Actually, it has been confirmed that both circannual and circadian rhythms influence the health outcomes of polyphenols. Figure 3 schematizes the interaction between gene regulation of biological rhythms, seasonal variation of plant polyphenols composition, and health seasonal effects. However, there is still a lack of information about this fact, and scarce studies can be found in the literature. Table 1 shows a compilation of dietary interventions and their health effects modulated by circadian and circannual rhythms.

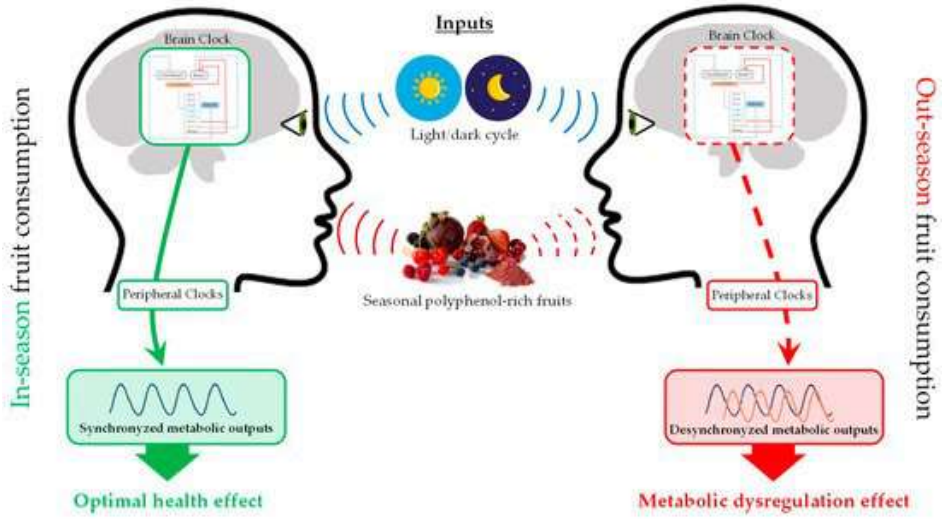

Figure 3. Interaction between gene regulation of biological rhythms, seasonal variation of plant polyphenols composition, and health seasonal effects.

4.1. Circannual rhythms

In this sense, recent studies performed in dietary-induced obese F344 rats, demonstrated that the consumption for ten weeks of sweet cherry *Prunus avium L.* exerted a marked deleterious photoperiod-dependent effect increasing whole-body fat oxidation and circulating levels of glucose and insulin when it was

consumed out of season [116,117]. These effects were partially explained by a downregulation of the phosphorylated levels of the downstream post-receptor target of insulin Akt2 and an enhancement of fatty acid transport and β-oxidation-related pathways in skeletal muscle [116]. In addition, the consumption of *Prunus avium L.* out of season changed the morphology of white adipose tissue, increasing cell area and decreasing the number of adipocytes, which was mainly attributed to the downregulation of the expression of key genes involved in adipose tissue fat metabolism [117]. Interestingly, in brown adipose tissue, the fatty acid transporter *Cd36* was also downregulated, suggesting a lower capacity of brown adipocytes to absorb and oxidize fat (less thermogenic activity) [117]. Finally, in central nervous system, the consumption of *Prunus avium L.* also modulated the hypothalamic leptin system regulating *Agrp* and Ptp1B mRNA levels only when it was consumed out of season [118].

In a similar way, the consumption out of season of navelina orange (*Citrus x sinsensis*) for ten weeks in the same animal model also exacerbated the deleterious effects induced by an obesogenic diet, such as dyslipidemia and insulin resistance, increasing fatty acid synthesis in white adipose tissue and downregulating the lipid uptake and β-oxidation in brown adipose tissue [119].

Relevantly, several epidemiological studies show how biological rhythms misalignment can contribute to a wide variety of metabolic disorders, including obesity, dyslipidemia, insulin resistance and hypertension [120]. Therefore, the physiological and molecular changes elicited by seasonal fruit consumption could be partially explained by the modulation of the mammalian clock system. In fact, both *Prunus avium L* and *Citrus x sinsensis* consumption has been correlated to the modulation of mRNA levels of different peripheral clock-related genes such as *Nr1d1* in skeletal muscle, and *Per2* and *Cry1* in the liver and white adipose tissue of healthy rats when they are consumed out of season [116,117,119]. In accordance, previous studies performed by our group demonstrated that grape seed proanthocyanidins (PAs), a subclass of flavonoids with marked protective effect against diet-induced dyslipidemia and insulin resistance [94], could significantly modulate both central and peripheral biological rhythms in male Wistar rats. Specifically, an acute dose of 250 mg/kg body weight of PAs maintained elevated

melatonin levels (nocturnal) at the beginning of the light phase and altered the rhythmic oscillations of some important circulating metabolites in healthy rats [121]. This phenotypic alteration was concomitant with the regulation of the hypothalamic expression pattern of clock genes such as *Bmal1* and *Nampt* [121]. In addition, different physiological doses of PAs during four weeks in diet-induced obese rats also modulated peripheral clock components such as *Bmal1, Nampt, Sirt1* and NAD^+ levels in a positive dose-dependent manner in the liver, gut and white adipose tissue of these animals [8,122,123], suggesting that PAs could modulate physiology and metabolism by adjusting the peripheral and central molecular clock system in obese state.

Table 1. Dietary interventions and their health outcomes modulated by circadian and circannual rhythms.

Dietary intervention	Experimental model	Health outcomes	Time scale		Reference
			Circadian rhythms[a]	Circannual rhythms[b]	
Human models					
Catechin-rich green tea	Healthy young men	Reduced postprandial plasma glucose concentration	Evening (17:00 h)	n.a.	[124]
Polyphenol-Rich Grape-Wine Extract	Mildly hypertensive males and females	Lowered ambulatory systolic and diastolic blood pressure	Day-time	n.a.	[125]
Animal models					
Epigallocatechin-3-gallate	C57BL/6J mice	Ameliorated diet-induced metabolic misalignment by regulating the rhythmic expression of the circadian clock genes in the liver and fat adipose tissue	Night-time	n.a.	[126]
Grape seed proanthocyanidin extract	Male Wistar rats	Modulated the plasma melatonin level	Day-time	n.a.	[121]
Resveratrol	Male Wistar rats	Antioxidant	Night-time	n.a.	[127]
		Pro-oxidant	Day-time	n.a.	[127]

Continued on next page

Dietary intervention	Experimental model	Health outcomes	Time scale		Reference
			Circadian rhythms[a]	Circannual rhythms[b]	
Red grapes (Traditional consumption: L6)	Standard (STD)-fed and cafeteria (CAF)-fed male F344	Increased hypothalamic leptin sensitivity	n.a.	L6	[118]
Sweet cherries (Traditional consumption: L18)	STD-fed F344	Decreased blood nonesterified free fatty acids (NEFAs)	n.a.	L18	[116]
	STD-fed male F344	Increased activation of fatty acid transport, β-oxidation-related pathways, and circulating glucose and insulin levels	n.a.	L6	[116]
	CAF-fed male F344	Enhanced detrimental impact of CAF diet related to glucose metabolism.	n.a.	L6	[116]
	STD-fed and CAF-fed male F344	Increased hypothalamic leptin sensitivity	n.a.	L6	[118]

[a] Day-time (light cycle); night-time (dark cycle). [b] L6: Short-day photoperiod (6 h light/day); L18: long-day photoperiod (18 h light/day). n.a. information not available. F344, Fischer 344 rats.

4.2. Circadian rhythms

Several other plant (poly)phenols have been described to affect central and peripheral clock system. Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea (*Camellia sinensis*), has been demonstrated to possess beneficial effects on obesity-related parameters including decreased body weight, reduced cholesterol and triglyceride levels in the liver and plasma, and an improvement in glucose homeostasis [128]. Notably, in the search of mechanisms of action of EGCG, Mi *et al.* [126] recently demonstrated for the first time that EGCG may ameliorate these obesity-related metabolic alterations by regulating the rhythmic expression of the circadian clock genes in the C57BL/6J mice. Specifically, EGCG partially normalized the circadian expression levels of the

clock genes, such as *Clock*, *Bmal1* and *Cry1* by regulating the levels of *Sirt1* and *PGC1α* in both the liver and white adipose tissue. In addition, resveratrol, another body fat-lowering polyphenol found in grapes and red wine, restored the circadian desynchrony of lipid metabolism induced by a high-fat diet modifying the rhythmic expression of both clock genes (*Clock*, *Bmal1*, *Per2* and *Rev-Erbα*) and clock-controlled lipogenic genes (*Sirt1*, *Pparα*, *Srebp-1c*, *Acc1* and *Fas*) in white adipose tissue [129,130]. Cichoric acid, a polyphenol component from *Echinacea purpurea* that exhibits preventive effects on fatty liver disease, has also demonstrated a Bmal1 resistance to fatty liver accumulation by enhancing the Akt/GSK3β signaling pathways and modulating the downstream gene expressions involved in lipid metabolism [131]. Finally, cinnamic acid, another phenolic acid abundant in fruits that have attracted attention last years due to their antioxidant and antidiabetic properties [132], has been reported to shorten the circadian period of the molecular clock in differentiated neuronal cells as well as to reduce the free-running period of behavioral rhythms in mice [133].

All these studies might provide valuable data to design strategies of polyphenol-rich fruit consumption to counteract the metabolic alterations related to obesity by restoring the biological rhythms desynchrony induced by an obesogenic behavior, and ultimately normalizing the rhythmic expression of most important metabolic-related transcripts and metabolites. However, further comprehensive investigations are required to elucidate the underlying mechanisms through which (poly)phenols modulate the central and peripheral clock machinery.

5. Conclusions

The bidirectional interaction between phenolic compounds and biological rhythms strongly affects the beneficial effects derived from their consumption. In this sense, the different factors that modulate the synthesis of these compounds in plants are essential when evaluating the impact of this type of plant product on health. Different changes in metabolism may be observed in humans based on the consumption of seasonal products due to the particular phenolic profile that the product contains. This fact highlights the need for more studies focused on

the impact of these specific phenolic profiles on health that would allow us to define more precise dietary recommendations regarding fruits and vegetables.

Author Contributions

Conceptualization, M.S., and A.A-A.; Writing—Original Draft Preparation, M.S., A.A.-A., Á.C.-C., C.T.-F., J.Á.-R., G.A., M.M., F.I.B., B.M. and L.A. Writing—Review & Editing, M.S. and Á.C.-C. Project Administration, B.M.; Funding Acquisition, B.M. and A.A-A.

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Conflicts of Interest

The authors declare no conflict of interest.

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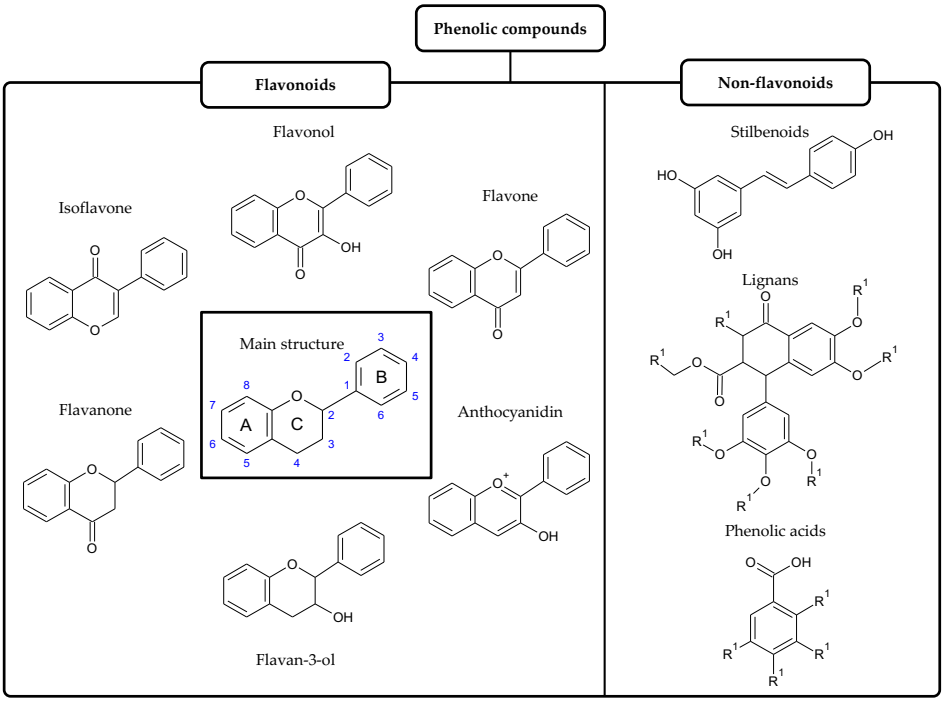
Supplementary materials



Supplementary Figure 1: Basic chemical structures of the main classes of phenolic compounds. According to their structure, phenolic compounds can be classified into two major families: flavonoids and nonflavonoids, and they can be further divided into several subclasses.

Supplementary Table 1. Total phenolic contents of relevant plant products, their most important bioactive phenols, and their health benefits.

Plant product	Scientific name	TPC (mg/100 g FW)*	Most important bioactive phenols	Plant health benefits	Reference
Apple (whole, raw)	*Malus domestica*	200.96	(+)-Catechin (-)-Epicatechin Quercetin Kaempferol	Anti-ulcer Anti-allergy Anti-oxidant Anti-hypertensive Anti-cancer Anti-diabetic Anti-inflammatory Cardioprotective	72,94,96,97,102,106–108
Apricot (raw)	*Prunus armeniaca* L.	133.00	Quercetin Kaempferol	Anti-oxidant Anti-cancer Anti-diabetic Anti-inflammatory	72,96,97,102,108
Broccoli (raw)	*Brassica oleracea* var. italica	198.55	Quercetin Myricetin	Anti-cancer	134
Cocoa, powder	*Theobroma cacao*	5624.23	(+)-Catechin (-)-Epicatechin	Anti-oxidant Anti-hypertensive Anti-diabetic Anti-inflammatory	72,94
Coffee (beverage, filter)	*Coffea arabica* L.	266.70 mg/100mL	Chlorogenic acid Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-inflammatory Cardioprotective	98–100,135,136
Common beans (whole)	*Phaseolus vulgaris* L.	1234.38	(+)-Catechin Quercetin Vanillin acid Ellagic acid Caffeic acid Ferulic acid Gallic acid Chlorogenic acid Sinapic acid	Anti-diabetic Anti-obesity Anti-oxidant Anti-inflammatory Chemoprotective	101

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Plant product	Scientific name	TPC (mg/100 g FW)*	Most important bioactive phenols	Plant health benefits	Reference
Grape (black)	*Vitis vinifera* L.	184.97	(+)-Catechin (-)-Epicatechin Quercetin Kaempferol Chlorogenic acid Caffeic acid *p*-Coumaric acid Resveratrol Proanthocyanidins	Anti-ulcer Anti-allergy Anti-proliferative Anti-oxidant Anti-hypertensive Anti-diabetic Anti-inflammatory Anti-cancer Anti-obesity Cardioprotective	72,94,108,135–139,96–100,102,106,107
Lemon	*Citrus limon*	59.80	Hesperidin Naringenin	Anti-oxidant Anti-inflammatory Anti-cancer Cardioprotective	72,97,110,111,140
Mango	*Mangifera indica* L.	144.77	Mangiferin	Anti-oxidant Anti-cancer Anti-diabetic Anti-allergic Anti-obesity	141,142
Nectarine (whole)	*Prunus persica* var. nucipersica	55.44	Chlorogenic acid Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-inflammatory Cardioprotective	98–100,135,136
Olive (green, raw)	*Olea europaea* L.	161.24	Tyrosol Hydroxytyrosol	Anti-cancer Anti-inflammatory Anti-oxidant Cardioprotective	143–147
Onion (red, raw)	*Allium cepa* L. var. cepa	102.83	Quercetin Kaempferol	Anti-ulcer Anti-allergy Anti-proliferative Anti-oxidant Anti-cancer Anti-diabetic Anti-inflammatory Cardioprotective	72,96,97,102,107,108,143

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Plant product	Scientific name	TPC (mg/100 g FW)*	Most important bioactive phenols	Plant health benefits	Reference
Orange (blond)	*Citrus sinensis* L.	278.59	Hesperidin Naringenin	Anti-oxidant Anti-inflammatory Anti-cancer Cardioprotective	72,97,110,111, 140
Parsley (fresh)	*Petroselinum crispum* Mill.	89.27	Apigenin	Anti-oxidant Anti-cancer Anti-inflammatory Cardioprotective	72,148,149
Peach (whole)	*Prunus persica* L.	279.08	Chlorogenic acid Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-inflammatory Cardioprotective	98–100,135,136
Pear (whole)	*Pyrus communis*	107.91	(+)-Catechin Chlorogenic acids	Anti-oxidant	107
Pomegranate (peel, juice, seed)	*Punica granatum* L.	410.03a	Anthocyanidins	Anti-oxidant Cardioprotective	150,151
Plum (fresh)	*Prunus domestica* L.	409.79	Chlorogenic acids Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-inflammatory Cardioprotective	98–100,135,136
Raspberry (red, raw)	*Rubus idaeus* L.	154.65	Cyanidin Malvidin Gallic acid	Anti-oxidant Anti-obesity Anti-inflammatory Anti-cancer Anti-diabetic Cardioprotective	72,106,152–160
Soybean (seed)	*Glycine Max* L. Merr.	354.00b	Genistein Daidzin	Anti-oxidant Anti-cancer Anti-hyperlipidemi	72,161–163
Spinach (raw)	*Spinacia oleracea* L.	248.14	Chlorogenic acid Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-inflammatory Cardioprotective	98–100,135,136

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Plant product	Scientific name	TPC (mg/100 g FW)*	Most important bioactive phenols	Plant health benefits	Reference
Strawberry (raw)	*Fragaria* L.	289.20	Cyanidin Malvidin Gallic acid	Pro-apoptotic effects Anti-proliferative Anti-oxidant Anti-obesity Anti-inflammatory Anti-cancer Anti-diabetic Cardioprotective	72,143,160,164,152–159
Sweet cherry (raw)	*Prunus avium* L.	174.90	(+)-Catechin Quercetin Cyanidin Malvidin Chlorogenic acid Caffeic acid *p*-Coumaric acid	Anti-oxidant Anti-obesity Anti-inflammatory Cardioprotective	72,98–100,107,135,136,153–155
Tangerine	*Citrus tangerina*	192.00	Hesperetin Naringenin	Anti-oxidant Anti-inflammatory Anti-cancer Cardioprotective	72,97,110,111,140
Tea (infusion)	*Camellia sinensis* L. Kuntze	104.48 (black) 61.86 (green) mg/100 mL	Gallic acid (+)-Catechin (-)-Epicatechin	Anti-oxidant Anti-cancer Anti-diabetic Anti-hypertensive Anti-inflammatory Cardioprotective	72,94,152,156–160
Walnut	*Juglans regia L.*	1574.82	Ellagic acid Gallic acid	Anti-diabetic Anti-oxidant	165,166

*Data from the Phenol-Explorer database [77–79]. [a] 167. [b] 168

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Plant-based foods farming systems, together with the antioxidant responses of seasonal consumption of local and non-local sweet cherries and tomatoes, as well as the phenolic bioavailability of tomatoes, are the topics of this thesis. The results obtained in this thesis shown that consuming in-season and local phenolic-rich foods leads to health benefits, supporting a movement towards more sustainable and healthy consumption patterns.