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Food Supplements and Functional Foods Assessment for Health and Nutrition

Volume I

Edited by
Laura Domínguez Díaz, Montaña Cámara and Virginia Fernández-Ruiz

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Editors

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About the Editors

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Preface

Dear colleagues,

The prevention of diet-related non-communicable diseases and their complications is currently one of the most important public health challenges worldwide with which the food industry has ever been faced. The societies of developed countries are engaged in a change in the concept of food, as consumers are increasingly demanding foods with added value beyond the provision of nutrients and the satisfaction of appetite—that is, food products which contribute to the achievement of optimal nutritional well-being, health status, and quality of life through reducing the risk of diseases and promoting the appropriate function of human organs and systems. In this context, food supplements and functional foods are increasingly considered in these new approaches as they contain functional ingredients with beneficial effects on human health.

In this reprint of the Topical Collection "*Food Supplements and Functional Foods Assessment for Health and Nutrition*", reviews address the potential benefits of phytonutrients in human healthcare and clinical nutrition as well as clinical trials assessing the potential role of functional foods and food supplements in therapeutic targets in different disorders and diseases.

Finally, we would like to express our gratitude and appreciation to the MDPI Reprint staff, the editorial team of *Nutrients* journal, especially Ms. Lilian Gao for her assistance and support, reviewers who took part in the peer review process for their professional work, and talented authors for their valuable contributions.

Laura Domínguez Díaz, Montaña Cámara, and Virginia Fernández-Ruiz
Editors

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Review

Clinical Evidence of the Benefits of Phytonutrients in Human Healthcare

Nicolas Monjotin [1], Marie Josèphe Amiot [2], Jacques Fleurentin [3], Jean Michel Morel [4] and Sylvie Raynal [5,*]

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Abstract: Phytonutrients comprise many different chemicals, including carotenoids, indoles, glucosinolates, organosulfur compounds, phytosterols, polyphenols, and saponins. This review focuses on the human healthcare benefits of seven phytochemical families and highlights the significant potential contribution of phytonutrients in the prevention and management of pathologies and symptoms in the field of family health. The structure and function of these phytochemical families and their dietary sources are presented, along with an overview of their potential activities across different health and therapeutic targets. This evaluation has enabled complementary effects of the different families of phytonutrients in the same area of health to be recognized.

Keywords: phytonutrients; healthcare; anthocyanins; organosulfur compounds; tannins; caffeine; flavonoids; carotenoids; phenolic acids

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1. Introduction

Phytochemicals are bioactive compounds generated from secondary plant metabolism in response to environmental changes [1,2]. Phytochemicals function as attractants for pollination or act as protectants against insect and pest attacks or exposure to various stresses, such as ultraviolet light [1,2]. In addition, phytochemicals contribute to the color, flavor, and aroma of plants and are recognized as having potential value in nutrition and human health. In fact, they are typically found in our diet through the intake of fruits, vegetables, whole grains, nuts, beans, herbs, tea, and coffee [3].

Numerous epidemiological studies have shown that high intakes of plant products are correlated with lower risks of chronic diseases and mortality, suggesting key protective roles of antioxidants [4,5]. In addition to the antioxidative vitamins C and E, plant-based diets provide numerous phytochemicals, also known as phytonutrients, that may contribute to the maintenance of good health, not only through their antioxidant activity, but also as anti-inflammatory and anticarcinogenic agents [6,7]. Phytonutrients comprise many different chemicals, including carotenoids, indoles, glucosinolates, organosulfur compounds, phytosterols, polyphenols, and saponins. The intake of phytonutrients among European populations appears to be highly variable [8], suggesting that theiy are benefits for consumers with a high adherence to World Health Organization dietary recommendations. The high variability of phytonutrient intake is related to the seasonal availability and affordability of healthy plant products. Our previous study estimated the levels of seven phytonutrients (phenolic acids, flavonoids, tannins, anthocyanins, carotenoids, organosulfur compounds, and caffeine) in a well-balanced French diet that met the requirements for

macro- and micronutrients to better identify any gaps in target phytonutrient intakes and recommend personalized nutritional strategies for maintaining good health [9].

Our present review focuses on healthcare targets for which benefits exist through an enrichment of the diet with at least one of the seven phytochemical families. Potential activities are presented on specific human healthcare targets. The first part of this review presents the structure and function of the seven phytochemical families and their dietary sources, while the second part describes their potential activities across different m.

2. Materials and Methods

An analysis of the scientific literature listed on PubMed up to December 2021 was undertaken using keywords related to the most common therapeutic indications in the following healthcare areas: digestive health, stress and sleep, immunity and ear, nose, and throat (ENT) diseases, vitality and cognition, and bones and joints (Table 1).

Table 1. Search terms/keywords used for PubMed literature search.

Therapeutic Area	Key Words
Bones and joints	Joint, rheumatism, osteoarthritis, arthritis, arthrosis, tendonitis, tendinitis, bone mineral density, bone mineral turnover
Digestive health	Abdominal bloating, gut health, constipation, diarrhea, chronic diarrhea, nausea, hepatic crisis, hepatic steatosis, irritable bowel syndrome, inflammatory bowel, syndrome, irritable colon, colitis
Immunity and ENT diseases	Chronic rhinitis, allergic rhinitis, rhinopharyngitis, sinusitis antritis, chronic sinusitis, sinus infection, non-productive cough, dry cough, chronic cough, sore throat, pharyngitis, angina, aphonia, hoarseness, seasonal allergy, allergic and pollen, influenza, flu, immunodeficiency, immune deficiency, immunodepression, immunity, immune defense
Stress and sleep	Stress, nervosity, anxiety, sleep, sleep disorder, drowsiness, insomnia, mood
Vitality and cognition	Attention deficit, hyperactivity, cognition disorder, cognition, memory, memory disorder, memory deficit, semantic memory, short-term memory, long-term memory, reactive agility, cognitive performance, physical activity, performance, training, exercise, muscle strength, strength recovery, muscle recovery, recovery, muscular recovery, muscular recuperation

ENT: ear, nose, and throat.

The details of a number of publications selected by phytochemicals and therapeutic area are presented in Table 2.

Table 2. Details of selected publications.

	Phenolic Acids	Flavonoids	Anthocyanins	Tannins	Organosulfur Compound	Carotenoids	Caffeine
Bones and joints	2	3	0	0	2	7	3
Digestive health	0	5	1	2	3	0	1
Immunity and ENT	0	3	0	2	1	2	1
Stress and sleep	0	2	1	1	0	4	3
Vitality and cognition	4	6	6	2	0	3	4

ENT: ear, nose, and throat.

We cross-referenced each keyword with each phytonutrient by filtering only the scientific literature on clinical studies. This search identified 23,830 articles. A second filter to eliminate duplicates, studies outside the scope of the search, or those not dealing specifically with phytonutrients resulted in the selection of 1012 publications. An in-depth analysis of these articles allowed us to identify 125 publications specifically addressing the therapeutic value of phytonutrients in the selected therapeutic areas and based on a robust experimental methodology. A total of 74 articles were included in this review, with

all others being discarded for lack of robustness or significance in the experimental results (Figure 1). This review presents the analysis of the identified articles.

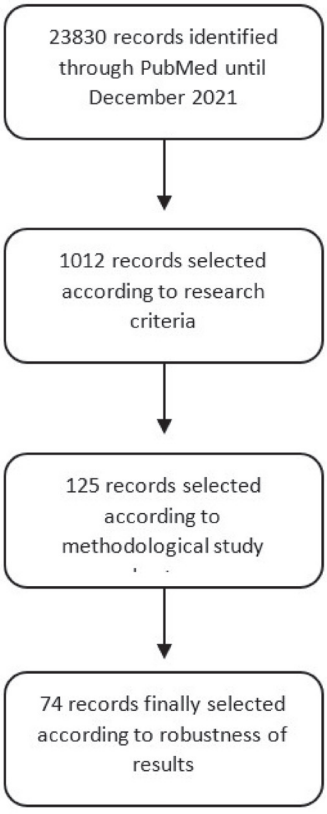

Figure 1. Literature search methodology.

3. Results

3.1. Phytonutrients

More than 10,000 phytonutrients have been identified in dietary plants [10–12]. Their concentrations differ greatly between species and cultivars, and also vary according to environmental conditions (light, soil, etc.), agricultural modes (fertilization and irrigation), storage, processing, and home uses [13]. A representation of the main families and chemical structures of phytonutrients found in dietary plants is shown in Figures 2 and 3. The properties of phytonutrients allow them to play a role in aspects of metabolic syndrome and associated mechanisms, notably inflammation and oxidation [14]. Experimental studies in cells or in animals have deciphered their mechanistic actions as being antioxidant, anti-inflammatory, antimicrobial, and anticancer in nature [15–19].

3.1.1. Phenolic Acids

Phenolic acids, or phenolcarboxylic acids, belong to the polyphenol family and are among the most widely distributed plant non-flavonoid phenolic compounds [20]. They have at least one carboxylic function and one phenolic hydroxyl [21].

This category includes hydroxybenzoic acid and its derivatives (gallic acid, vanillic acid, parahydroxybenzoic acid, syringic acid, and protocatechic acid), as well as cinnamic acid and its derivatives (ferulic, paracoumaric, caffeic, and sinapic acids) [22].

Phenolic acids are found in many foods such as artichoke, cereals, wheat flours, onions, coffee, kiwis, berries, apples, and citrus fruits [11,23]. In addition to dietary sources, phenolic acids can also be derived from the colonic microflora's secondary metabolism of other types of polyphenols [20].

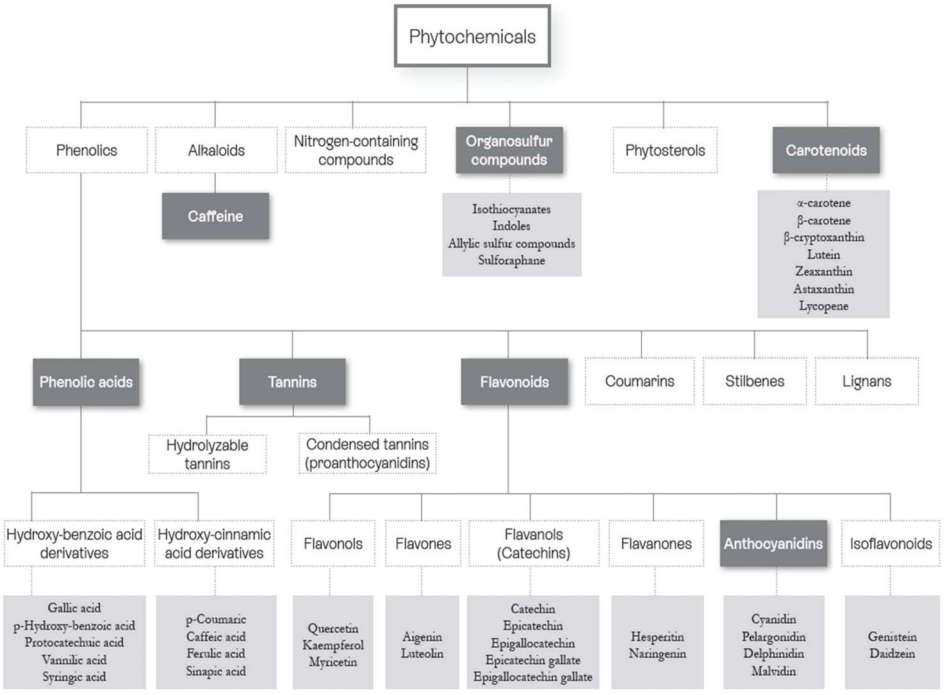

Figure 2. Classification of the main phytonutrient families (modified from [6]). Dark grey: Phytonutrients Families. Light grey: Phytonutrients examples.

3.1.2. Flavonoids

Flavonoids belong to the polyphenol family and include flavonols, flavones, flavanols, flavanones and isoflavonoids [21,24]. Anthocyanins are also part of the flavonoid family, but these are discussed in a separate paragraph in light of their specificities and therapeutic value. Flavonoids have a generic structure composed of two aromatic rings linked by three carbons: C6-C3-C6, a chain often closed in an oxygenated heterocycle called a C-ring [25]. The differences in the generic structure of the heterocyclic C-ring classify them as flavonols, flavones, flavanols, flavanones, anthocyanidins, or isoflavonoids [25]. Flavonoids are found in many plants and, as universal pigments of the yellow, red, and purple colors, are the molecules that give plants their 'colorful hues'. When they are not directly visible, flavonoids contribute to coloring through their role as co-pigments. This is the case of colorless flavones and flavonols that co-pigment and protect anthocyanosides. Flavonols are the flavonoids most widely found in foods, with quercetin and kaempferol being the main representatives of this group [11].

Flavonoids are present in a very wide variety of plants, albeit in relatively low concentrations [11,24]. The main sources of flavonoids are tea, onions, and apples, but they are found in many other colored plants [26]. Flavanones are found in tomatoes, citrus fruits, and herbs. Flavanols are found in olives, onions, cabbage, and lettuce. Flavones are found in celery and olives. Pears, red wine, and tea are good sources of flavanols. Finally, isoflavones are mainly found in soy products [12,26,27].

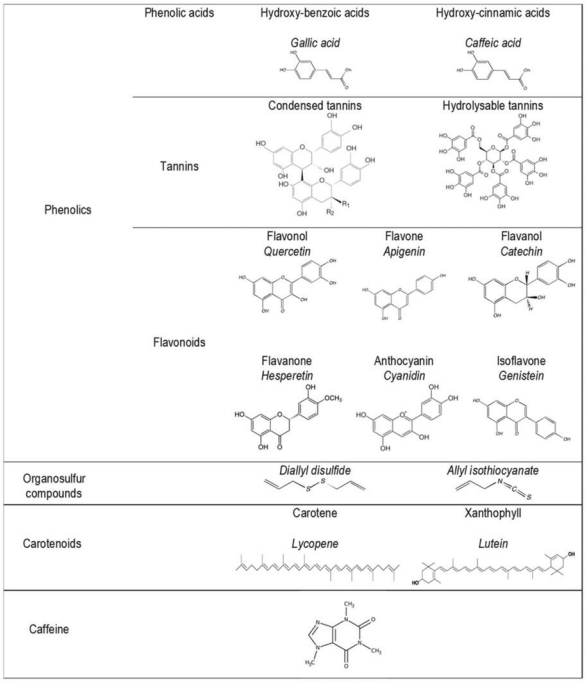

Figure 3. Main phytonutrient family chemical structures.

3.1.3. Anthocyanins

Anthocyanins are a subfamily of flavonoids and are derived from the general metabolism of flavonoids [21,24].

The most common anthocyanins are cyanidin, pelargonidin, delphinidin, and malvidin, and they are most commonly found in red-, pink-, blue-, or purple-colored fruits and vegetables [28]. The color of anthocyanins varies from orange to purple. By increasing the degree of hydroxylation, the absorbance wavelength is increased from orange-colored pelargonidin to purple delphinidin.

Anthocyanins are particularly concentrated in cherries, berries (such as blackcurrants, elderberries, and blueberries), and plums. They are also present in root vegetables such as beets and radishes, red onion bulbs, and in drinks such as fruit juices and red wine. Anthocyanins are also found in eggplant and red cabbage [12,29–31].

3.1.4. Tannins

Like flavonoids, tannins belong to the family of phenolic compounds. They differ in structure and biogenetic origin and are subdivided into two categories: condensed tannins and hydrolyzable tannins [32]. Condensed tannins, also known as catechins or proanthocyanidins, are oligomers or polymers of flavanols comprising units of flavan-3-ols linked together by carbon–carbon bonds of type 4 → 8 or 4 → 6 [24,30].

Tannins are non-hydrolyzable, but when treated with an acid under heat, they degrade into colored pigments formed of anthocyanidins [24,30,33]. Hydrolyzable tannins, unlike condensed tannins, have the capacity to cross the intestinal barrier after hydrolysis [12].

Plums, cocoa beans, carob beans, tea, and wine, as well as pomegranate bark, sorghum and barley seeds contain high levels of tannins [12,29,30,33].

3.1.5. Organosulfur Compounds

Organosulfur compounds include several classes of molecules with a similar basic chemical structure [12]. A carbon atom is surrounded by a glucose molecule via a sulfur bond, a sulfate group via the nitrogen atom of the oxime group and an aglycone, which varies according to the subclass and is derived from an amino acid [34].

The family of organosulfur compounds includes isothiocyanates, indoles, compounds derived from allyl sulfides and sulforaphanes [12,24]. Isothiocyanates are biologically active hydrolysis products of glucosinolates [34]. The two organosulfur compounds most commonly found in plant-based foods are glucosinolates and sulfur derivatives of garlic [12].

Glucosinolates are found in particular in *Brassicaceae* or cruciferous vegetables (cabbage, cauliflower, turnip, broccoli, black radish, mustard) and are present in varying quantities depending on the species, the part and the plant, as well as the cultivation and climatic conditions [29]. These compounds are responsible for strong odors and tastes.

Sulforaphane is found mainly in cruciferous vegetables (cabbage and broccoli) and isothiocyanate in mustard seeds [10]. Garlic is also a good source of sulfur compounds.

3.1.6. Carotenoids

Carotenoids are a large family of more than 800 different molecules, ranging in color from yellow-orange to red, and of which only approximately 20 are found in food [12,24]. The general structure of a carotenoid is a hydrocarbon chain of polyene composed of 9 to 11 double bonds, possibly terminating in rings [35]. Carotenoids are fat-soluble compounds divided into two classes: xanthophylls and carotenes. In the first class, we find molecules such as lutein, zeaxanthin, β-cryptoxanthin, and astaxanthin. As for carotenes, they are represented by α-carotene, β-carotene and lycopene.

The most extensively studied carotenoids are α-carotene, β-carotene, lycopene, lutein and zeaxanthin. The best known, β-carotene, is a precursor of vitamin A [26,35].

Carotenoids, which are highly sensitive to oxidation, are widely distributed in the natural environment: they accumulate in the chloroplasts of all photosynthetic tissues [36]. β-carotene, lutein, violaxanthin, and neoxanthin are present in the leaves of almost all plants. Carotenoids also accumulate in flower petals (common marigold, pansy, and French marigold), in fruits which may contain chloroplastic carotenoids or accumulate other derivative compounds (capsanthin and lycopene).

Carotenoids are found in carrots, spinach, tomatoes, herbs including parsley and basil, leafy greens such as lettuce and arugula, broccoli, kale, Brussels sprouts, squash and sweet potato, peppers, citrus fruits, seeds, some mushrooms, and in many other plants [29,37–39].

3.1.7. Caffeine

Caffeine is a molecule of the alkaloid family, also known as 1,3,7-trimethylxanthine [24,40]. Conversely, caffeine was included as a family in its own right because it accounts for a significant proportion of daily phytochemical intake, has well documented health benefits, and is routinely included in nutritional recommendations such as those issued by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) [41].

Caffeine is the most widely consumed psychoactive substance in the world and is found in coffee or kola nuts, tea or mate leaves, or guarana seeds [42].

3.2. Therapeutic Applications of the Value of Phytonutrients (Family Health)

3.2.1. Stress and Sleep

Most of the seven families of phytonutrients present a pharmacological effect either in sleep disorders or in the case of problems related to stress. Some have a beneficial effect such as anthocyanins, carotenoids, flavonoids, tannins, and caffeine. Others, on the contrary, are not indicated in the treatment of this health problem. A summary of the effects of phytochemicals in this therapeutic area is presented in Table 3.

Table 3. Summary of selected studies regarding the therapeutic area of stress and sleep disorders.

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Stringham (2018) [43]	Stress	Carotenoid (lutein, zeaxanthin)	59 young adults with oxidative stress and inflammation-implicated in stress (32 female) Mean age: 21.5 years USA	Double-blind, placebo-controlled trial 12 months treatment, control 6 and 12 months	T: Macular carotenoids (lutein, zeaxanthin and meso-zeaxanthin) 13 mg/day (n = 24), 27 mg/day (n = 25) C: Placebo (n = 10)	Stress, serum cortisol, physical and emotional health (questionnaire)	After 6 months and 12 months, stress cortisol and symptoms of suboptimal emotional and physical health were reduced ($p < 0.05$)	Carotenoid reduced oxidative stress and inflammation implicated in stress
Scholey (2012) [44]	Mood Neurocognitive effect	Flavonoid Epigallocatechine gallate (EGCG)	Volunteers without pathology (n = 31; 19 female) Mean age: 27.7 years Australia	Double-blind, placebo-controlled, crossover trial (1 week treatment, 1 week washout 1 week treatment)	One testing session, with second testing session one week after T: Green tea 300 mg free of caffeine (n = 31) C: Placebo (n = 31)	Brain activity and self-reported mood, cardiovascular function, and electroenceph alogram 120 min after intake	Increased self-rated calmness ($p < 0.04$) and reduced self-rated stress ($p < 0.017$) were reported	This pattern of results suggests that participants in the EGCC group may have been in a more relaxed and attentive state after consuming EGCC
Kell (2017) [45]	Mood	Carotenoid Crocins, safranal	121 patients with low mood but no depression (75 females) Age: 18–77 years Australia	Double-blind, parallel, randomized, placebo-controlled trial (4 weeks)	T: Affron® (saffron extract) 28 mg/day (n = 41) or 22 mg/day (n = 42) C: Placebo: (n = 38)	Mood, stress, anxiety, sleep; sleep quality index (SQI)	Decreased negative mood and symptoms relative to stress at 28 mg and no effect with 22 mg ($p < 0.001$) SQI: No effect	The use of Affron® (saffron extract) increased mood and managed stress without side effects
White (1980) [46]	Anxiety and muscle tension	Caffeine	36 student volunteers (Number of females and age not specified) USA	Double-blind, placebo-controlled trial	T: Caffeine citrate 300 mg 8 h intake and 11 h test (n = 19) C: Citric acid (Placebo) (n = 17)	Electromyography, reaction time and anxiety recorded 30 min after intake	Regular consumer of caffeine (376 mg) had higher muscle tension after 3 h then lower consumer (87 mg); brief abstinence of caffeine-induced anxiety in higher-consumption consumer	Brief abstinence from caffeine may produce anxiety

Table 3. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Bernstein (1994) [47]	Learning, performance, anxiety	Caffeine	21 healthy prepubertal children (9 females) Age: 8–12 years USA	Double-blind, placebo-controlled, crossover trial (1 week of treatment)	T: Low-dose caffeine 2.5 mg/kg or high-dose caffeine 5 mg/kg C: Placebo (n = 21)	Learning, performance, anxiety, test of attention, manual dexterity, short-term memory, processing speed, anxiety rating, level of caffeine in saliva	Reduced sluggishness reported with caffeine 5 mg ($p = 0.43$); increased target stimulus with T group vs. placebo ($p = 0.098$)	Caffeine can enhance performance on test of attention and motor task, but can cause increased anxiety
Grosso (2016) [48]	Depression	Caffeine	12 studies, 23 datasets, 346 913 individuals (8146 with depression; n umber of females and age not specified) Italy	Review and meta-analysis of observational study on depression	Dataset with coffee (n = 7) Dataset with tea (n = 6) Dataset with caffeine (n = 9)	Depression scale	J-shaped non-linear relation between coffee consumption and risk of depression; protective effect at 400 mL/day	Coffee has a protective effect against depression, which is only partially seen with tea and caffeine
Terauchi (2014) [49]	Menopausal symptoms, anxiety, sleep	Tannins Proan-thocyanidin	Females with menopausal symptoms (n = 91) Age: 40–60 years Japan	Double-blind, randomized, placebo-controlled study (8 weeks)	T: Caffeine 100 mg (n = 32) and 200 mg (n = 30) C: Placebo (n = 29)	After 4 weeks and 8 weeks: menopausal health questionnaire, anxiety, depression, sleep, blood pressure (BP), muscle mass	Significant result after 8 weeks of treatment: Decreased physical symptom score in the T group (high dose) ($p < 0.05$); hot flash score improved in the T group (high dose) ($p < 0.05$); lower score in Athens insomnia scale with T group (high dose) ($p < 0.01$); decreased anxiety score ($p < 0.01$), BP decreased in the T group (high and low dose) ($p < 0.01$); increased muscle mass in the T group (high and low dose) ($p < 0.05$)	Caffeine improved the physical and psychological impact of menopause

Table 3. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Umigai (2018) [50]	Sleep	Carotenoid	30 healthy men and postmenoposal women with mild sleep complaints (10 females) Age: 35–60 years Japan	Double-blind, randomized, placebo-controlled, crossover study (5 days baseline, 14 day intervention, 14 day washout period, 14 day intervention)	T: Crocetin 7.5 mg/day (n = 15) C: Placebo (n = 15)	Electroenceph alography, subjective sleep parameters, Ogury Shirakawa Azumi sleep inventory—MA score	Sleep maintenance ($p = 0.001$); feeling refreshed ($p = 0.007$); subjective sleep quality; no effect in sleep latency, sleep efficiency, total sleep time, or sleep after sleep onset	Crocetin contributes to sleep maintainance and sleep quality
Hachul (2011) [51]	Insomnia	Flavonoids	Postmenoposal women with insomnia (n = 38) Age: 50–65 years Brazil	Double-blind, placebo-controlled study (4 months)	T: Isoflavones 80 mg/day (n = 19) C: Placebo (n = 19)	Subjective and objective sleep parameters; polysomnography	Decreased intensity and number of hot flashes and frequency of insomnia fversus placebo; increased sleep efficiency ($p < 0.01$)	Flavonoids can reduce the symptoms of insomnia
Losso (2018) [52]	Insomnia	Anthocyanin	Male (5) or female (3) individuals (n = 8; 3 excluded due to apnea) (5 females) Age: >50 years USA	Placebo-controlled, crossover study Two weeks of twice daily dosing Two weeks washout	T: Cherry juice 240 mL titrated with cynanidins twice daily (n = 4) C: Placebo (n = 4)	Five validated questionnaires: Pittsburgh sleep quality index, (insomnia severity index, Epworth sleepiness scale, Beck depression inventory, state-trait anxiety inventory; blood test: kynurenine:tryptophan ratio, serum indoleamine 2, 3-dioxygenase, and prostaglandine E2	Increased sleep time for 84 min ($p < 0.01$) and sleep efficiency ($p < 0.03$) vs. placebo; increased tryptophan availability	Anthocyanins can increase sleep time and sleep efficiency
Kuratsune (2010) [53]	Insomnia	Carotenoid	Healthy adult men with a mild sleep complaint (n = 21) Age: 25–59 years Japan	Double-blind, placebo- controlled, crossover study (2 weeks of treatment, 2 week washout period, 2 weeks of treatment	T: Crocetin 7.5 mg (n = 10) C: Placebo (n = 10)	Actigraph; Mary's Hospital sleep questionnaire	Reduced wakening episode with crocetin vs. placebo ($p < 0.025$); trend for improved quality of sleep with crocetin	Crocetin can improve quality of sleep

Stress

Several studies included in this review assessed the benefit or risk of caffeine consumption in relation to stress [46–48]. Bernstein et al. evaluated the acute effects of caffeine consumption on learning mechanisms in children but also on stress [47]. According to the authors, the consumption of 2.5 or 5 mg/kg of caffeine lead to a slight increase in (self-rated) stress in children ($p = 0.098$). Nevertheless, this non-statistically significant result did not enable a clear claim to be made for a deleterious effect of caffeine on stress in children. In contrast, other studies have reported beneficial effects of caffeine in the management of stress. White et al. evaluated the effects of caffeine on muscular tension and anxiety and their work shows that when heavy coffee drinkers are deprived of caffeine for 3 h, their muscular tension and anxiety significantly increase, compared with low consumers, and that the consumption of caffeine brings these parameters back to the level of subjects treated with placebo [46]. According to the authors, it is therefore the lack of caffeine, and not the caffeine itself, that is responsible for the anxiety. A literature review of 12 observational clinical studies also investigated the effects of coffee, tea, or caffeine consumption on depression [48]. The results of this analysis suggested that a daily consumption of caffeinated coffee could play a protective role with respect to the symptoms of depression in a non-linear dose–effect relationship and with a maximum effect at a consumption of 400 mL of coffee per day. According to the authors, this effect may be due to a stimulation of the central nervous system by caffeine and an improvement of dopaminergic neurotransmission.

Other phytonutrients are of therapeutic interest in the management of stress. The beneficial effects of flavonoids were evaluated by Scholey et al., where the electroencephalogram (EEG) of subjects along with their perceived level of stress before and 120 min after administration of 300 mg of epigallocatechin gallate (EGCG) or placebo were investigated [44]. The results showed an increase in self-rated calmness and a reduction in self-rated stress, as well as EEG modifications, with EGCG use. According to the authors, the mechanism of action could be linked to an effect on nitric oxide (NO) synthesis associated with a modulation of cerebral vascular permeability.

Carotenoids have also shown beneficial effects in the management of stress [43,45]. Kell et al. evaluated the therapeutic benefits of saffron, particularly the crocin (carotenoid) it contains, in the management of mood, stress, and anxiety disorders [45]. After 4 weeks of treatment with 28 mg/day of saffron, subjects saw their stress and anxiety levels decrease and their mood improve significantly compared with placebo. Another study conducted by Stringham et al. sought to demonstrate the value of long-term carotenoid supplementation in the management of stress [43]. Carotenoids or placebo were administered for 12 months and the level of stress associated with cortisol levels was assessed. As early as 6 months, cortisol levels, stress, and anxiety were significantly reduced in subjects who were treated with carotenoids. The authors suggested a mechanism of action based on a direct antioxidant action of carotenoids in neural tissue leading to a decrease in the synthesis of stress-related hormones.

The mechanisms of action described in these studies are most often related to the antioxidant properties of phytonutrients. The antioxidant action of phytonutrients can be either directly linked to their chemical structure and exerted through a direct antioxidant action (hydrogen or electron transfer or chelation of transition metals) or via an indirect action (regulation of enzymatic activity, gene modulation) [54–59].

Sleep

The anthocyanin family, and particularly the cyanidin class, has been shown to have therapeutic value in sleep disorders. In a study by Losso et al., subjects who consumed 240 mL of cyanidin-titrated cherry juice for 2 weeks had an average increase in sleep duration of 84 min compared those receiving placebo ($p < 0.01$) [52]. According to the authors, the beneficial effects on sleep are related to the inhibitory activity of cyanidins on indoleamine 2,3-dioxygenase, an enzyme that degrades tryptophane.

Hachul et al. evaluated the effect of flavonoids, and in particular isoflavones, on the sleep quality of postmenopausal women suffering from insomnia [51]. Subjects received 80 mg of isoflavones or placebo every day for 4 months. A sleep analysis was carried out using polysomnography and questionnaires. The results obtained show a significant decrease in the number of episodes of insomnia at the end of treatment in patients treated with isoflavones versus placebo ($p = 0.006$) as well as an improvement in sleep efficiency ($p < 0.01$).

Tannins demonstrated therapeutic potential in the management of sleep disorders in postmenopausal women in a study conducted by Terauchi et al. [49]. In this study, the effect of 100 or 200 mg of proanthocyanidins (derived from grape seeds) on insomnia was compared with placebo when administered over an 8 week period. The results showed a significant decrease ($p < 0.01$) in the insomnia score after 8 weeks of treatment with 200 mg of proanthocyanidins. According to the authors, the mechanism of action of the active treatment may be linked to an antioxidant effect of the tannins which modulates gamma-aminobutyric acid (GABA)ergic activity, leading to significant hypnosedative and anxiolytic effects ($p < 0.01$).

The effect of carotenoids on the improvement of sleep quality was evaluated by Kuratsune et al. [53]. In this study, patients experiencing moderate sleep disorders received either extract of *Gardenia jasminoides* titrated to 7.5 mg per day of crocetin or placebo for two periods of 2 weeks separated by a washout period of 2 weeks. The patients' nocturnal activity measured by an actigraph showed a significant decrease in the number of waking episodes ($p < 0.025$), while the other parameters showed a trend towards improved sleep without reaching statistical significance. The same protocol was used in 2018 to evaluate the effect of crocetin on new parameters [50]. The results obtained showed no significant effect on electroencephalographic recordings, but an improvement in 'Sleepiness on rising' ($p = 0.011$) and 'Feeling refreshed' ($p = 0.007$) was reported. The mechanism of action supporting this effect is not fully understood but, according to the authors, it may be related to a modulation of the histaminergic system.

3.2.2. Immunity and ENT

Numerous studies have demonstrated the value of phytonutrients in the management of serious chronic diseases, one of the main causes of which is immune deficiency. However, in healthcare, the term immunity refers more to the notion of "maintaining natural defenses" in the context of benign conditions such as colds, allergic rhinitis, and other ENT pathologies. A summary of the effects of phytochemicals in this therapeutic area is presented in Table 4.

Table 4. Summary of selected studies regarding the therapeutic area of immunity and ENT diseases.

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Masuda (2014) [60]	Symptoms of Japanese Cedar Pollinosis (JCP)	Flavonoid (methylated catechin)	51 adults with JCP (36 female) Age: 20–65 years Japan	Randomized, double-blind, placebo-controlled trial (12 weeks)	T: 700 mL of 'Benifuuki' green tea containing O-methylated epigallocatechine gallate (EGCG (n = 26) C: 700 mL of 'Yabukita' green tea not containing O-methylated EGCG (n = 25)	Area under the curve (AUC) of symptom scores of nasal symptoms (sneezing, runny nose and nasal congestion), ocular symptoms (itchy eyes and tearing), and sore throat during the peak pollen season; quality of life (QoL)-related questionnaire; peripheral eosinophil (PE)	AUC: Significantly smaller with T group (runny nose [$p < 0.05$], itchy eyes [$p < 0.01$], tearing [$p < 0.01$]); QoL score: Significantly better in the T group ($p < 0.01$); PE: Suppressed in the T group	'Benifuuki' green tea containing O-methylated EGCG reduced symptoms of JCP and has potential as an alternative medicine for treating seasonal allergic rhinitis
Enomoto (2006) [61]	Allergic rhinitis	Tannins (procyanidins)	33 adults with moderate or severe persistent allergic rhinitis (24 female) Age: 15–65 years Japan	Randomized, double-blind, placebo-controlled trial (4 weeks)	T1: High polyphenols (200 mg per bottle) (n = 11) T2: Low polyphenols (50 mg per bottle) (n = 11) C: Placebo (n = 11)	Sneezing attacks (SA); nasal discharge (ND); swelling nasal turbinate (SNT); color, or inferior turbinate	SA and ND: Significant improvement ($p < 0.05$ and $p < 0.01$, respectively) for the T1 group; SA: Significant improvement for the T2 group ($p < 0.05$); SNT: Significant improvement for T1 and T2 groups (both $p < 0.05$)	Tannins (procyanidins) are effective in alleviating symptoms of persistent allergic rhinitis
Matsumoto (2011) [62]	Influenza infection	Flavonoid catechins and theanine	197 healthcare workers (152 female) Age: 21–69 years Japan	Randomized, double-blind, placebo-controlled trial (20 weeks)	T: Green tea catechins (378 mg/day) and theanine (210 mg/day (n = 98) C: Placebo (n = 99)	Incidence of clinically defined influenza infection (ICDII); incidence of laboratory-confirmed influenza infection (ILCII); time the patient was free from influenza infection (TFI)	ICDII: Significantly lower with T ($p = 0.022$); ILCII: Lower with T group but not significant ($p = 0.112$); TFI: Significantly different between the two groups ($p = 0.023$)	Taking green tea catechins and theanine may be effective prophylaxis for influenza infection

Table 4. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Müller (2016) [63]	Systemic effect in the context of live attenuated influenza virus (LAIV)-inoculation	Organosulfur compounds (sulforaphane)	29 adults (19 female) Mean age: m USA	Randomized, double-blind, placebo-controlled (4 weeks)	T: Broccoli sprout Homogenate (BSH) shake of 200 g (111 g of fresh broccoli sprouts + water) (n = 13) C: Alfalfa sprout homogenate (ASH) (n = 16)	Blood sample (BS); neutrophils (N); monocytes (Mo); macrophages (Ma); T cells; NKT cells and NK cells; cytoxic potential; granzyme B (GB)	NKT, T, NK cells were significantly reduced; NKT: Day 2 and Day 21, $p = 0.002$ and $p = 0.036$, respectively; T cell: Day 2, $p = 0.022$; NK cells: CD56 and CD158b expression ($p = 0.0084$ and $p = 0.0007$, respectively); NK cells: Significantly increasing CD16 expression ($p = 0.0095$) and CP (Day 2); BSH increased LAIV-induced GB production vs. ASH (Day 2, $p = 0.049$); BSH GB negatively associated with influenza RNA levels in nasal lavage fluid cells ($p = 0.088$)	Nasal influenza infection may induce complex changes in peripheral blood NK cell activation, and BSH increases virus-induced peripheral blood NK cell granzyme B production, an effect that may be important for enhanced antiviral defense responses
Nantz (2013) [64]	Immunity (γδ-T cell proliferation), cold and influenza	Tannins	45 adults (31 female) Age: 21–50 years USA	Randomized, double-blind, placebo-controlled, parallel intervention (10 weeks)	T: Powdered cranberry fraction (65–77% of proanthocyanins) (n = 22) C: Placebo (n = 23)	BS (γδ-T cells); peripheral blood mononuclear cell (PBMC) were cultured for 6 days with autologous serum and phyto-hemagglutinin stimulation; cold and influenza symptoms (CIS)	PBMC: γδ-T cells in culture were almost five times higher with T ($p < 0.001$); CIS: Significantly fewer symptoms of illness were reported ($p = 0.031$)	Consumption of the cranberry beverage modified the ex vivo proliferation of cells. As these cells are located in the epithelium and serve as a first line of defense, improving their function may be related to reducing the number of symptoms associated with a cold and flu

Table 4. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Crişan (1995) [65]	Acute and chronic rhinopharyngitis in children	Flavonoids	94 children Preschool (P, n = 47) Mean age: 6 years–3 months and 24 days Schoolchildren (S, n = 47) 9 years, 1 month and 4 d. (mean) Mean age: 9 years, 1 month and 4 days Romania	Randomized, double-blind, placebo-controlled (20 weeks)	T: NIVCRISOL (aqueous propolis extract rich in flavonoids) 1 mL/day (n = 61; 26 P, 35 S) C: Physiological salt solution (n = 33; 21P, 12 S):	Clinical observation (CO) (NC: Nasal catarrh, pharyngeal congestion, conjunctival mucosa congestion [CMC]); fever or any other state alteration (F); nasopharyngeal exudate monthly (5-months): virus isolation (VI), bacteriological and fungal examinations	CO: Significant lowering of the number of cases; decrease in the number of respiratory infections with general state alteration ($p < 0.01$); VI: Decrease and sometimes suppression of symptom (F and CMC) and altered general state ($p < 0.05$) for P and S; persistent cough ($p < 0.05$) only for S; reduction in the number of days of disease in cases with respiratory illness (2.5 times lower in treated P and 1.2 lower in treated S)	NIVCRISOL predominantly acts on viruses frequently encountered in nasopharyngeal exudate of subjects with acute or chronic rhinopharyngitis or apparent good health
Welsh (2010) [66]	Lung function	Caffeine	75 adults with mild to moserate asthma (16 female) Age: 16–73 years UK	Meta-analysis of randomized, controlled, crossover trials (seven studies: one study of exhaled nitric oxide concentration (FeNO) and six studies of pulmonary function test)	T: Oral caffeine or coffee (5–10 mg/kg of caffeine or 15 mg/kg of coffee) (n = 75) C: Placebo or decaffeinated coffee (n = 75)	Lung function (LF); forced expiratory volume in 1 s (FEV1); maximum mid-expiratory flow and specific airway conductance (FEF25–75); FeNO; maximal expiratory flow rates at 25 and 50% of viral capacity (Vmax50 and 25); symptoms, side effects and adverse effects SSEAE (heart rate [HR], blood pressure [BP])	Six trials (n = 55): caffeine improved LF for up to 2 h after consumption; FeNO: No significant difference ($p = 0.38$); FEV1: Small improvement (up to 2 h, 5%), two studies have a 12% and 18% mean difference ($p = 0.008$); FEF25–75: Small improvement with caffeine (up to 4 h); SSEAE: One study had a significant result on HR (decrease up to 9%) and BP (increase up to 12%)	Caffeine appears to improve airways function modestly, for up to four hours, in people with asthma. When testing lung function, people may avoid caffeine consumption

Table 4. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Nourollahian (2020) [67]	Allergic rhinitis (AR)	Carotenoids	53 patients with AR (30 female) Mean age: 26.8 ± 9.3 years Iran	Randomized, double-blind, placebo-controlled trial (2 months)	T: Spirulina (2 g/day) (n = 26) C: Cetirizine (10 mg/day) (n = 27)	Cardinal rhinitis symptoms; sneezing; nasal congestion; rhinorrhoea (R); smell disturbance (SD); nasal itching (NI); sleep condition (SC); social activity (SA); daily work (DW); interleukin (IL)-10, -4, -1; interferon-gamma (IFN-γ)	No difference between the groups before the clinical presentations ($p > 0.05$); R, NI, and SD: Significant improvement for the T group ($p = 0.021$, $p = 0.039$ and $p = 0.030$, respectively); SC, DW, and SA: Significant improvement for the T group ($p < 0.05$); 1 month later: IL-1α, IL-1β, and IL-4 were significantly lower in the T group ($p < 0.001$, $p < 0.001$ and $p = 0.008$, respectively); IL-10: Significantly higher in the T group ($p = 0.049$)	Spirulina is more effective than cetirizine in improving cardinal symptoms of AR patients. Furthermore, spirulina can be considered as an alternative treatment in patients with AR
Cingi (2008) [68]	AR	Carotenoids	129 patients 75 (female) Age: 19–49 years Turkey	Randomized, double-blind, placebo-controlled trial (6 months)	T: Spirulina (2000 mg/day) (n = 85) C: Placebo (n = 44)	Symptoms and physical findings: ND; sneezing; nasal congestion (NC); NI	ND, sneezing, NC, and NI): Significantly improved ($p < 0.001$) with T vs. C	Spirulina is clinically effective on allergic rhinitis when compared with placebo. Further studies should be performed in order to clarify the mechanism of this effect

Phytonutrients, and in particular flavonoids, are of therapeutic interest in the field of immunity. In 1995, Crişan et al. evaluated the benefit of a propolis rich in flavonoids in the management and occurrence of colds in children [65]. In this study, children who received 1 mL of product/day by nasal instillation 7 days a month for 5 months had a significantly lower number of colds ($p < 0.01$) as well as a shorter duration of symptoms ($p < 0.05$) compared with children in the control group. In 2011, Matsumoto et al. evaluated the effect of taking capsules rich in catechin (378 mg/day) and theanine (210 mg/day) for 5 months on the prevention of influenza viral pathologies [62]. Their results showed that the 98 adults treated with the product developed significantly ($p = 0.022$) fewer influenza viral pathologies than those in the placebo group. The mechanism of action could be related to an inhibition of the adsorption of the virus to the host cell. The interest of catechins and more particularly of EGCG, the main flavonoid in green tea, has been confirmed by Masuda et al. [60]. In this double-blind clinical study of 51 adults, the effects of EGCG on allergic symptoms were evaluated. In the group that received 700 mL of an EGCG-rich drink daily for 12 weeks, the number of allergic symptoms, such as runny nose ($p < 0.05$), itchy eyes ($p < 0.01$), or tearing ($p < 0.01$) were significantly reduced compared with the control group. The authors suggested that EGCG may limit mast cell activation, thus reducing the synthesis of leukotrienes, histamine, and other inflammatory cytokines.

Organosulfur compounds are also of therapeutic interest in antiviral protection. In 2016, Muller et al. evaluated the effect of sulforaphane-rich broccoli administration on the immune system response to influenza vaccination. Their results show a significant decrease in the number of natural killer T (NKT), T, and N cells as well as a significant increase in the production of granzyme B (an antiviral protein) compared with the placebo group suggesting that sulforaphanes induce an improvement in defenses against viral infections [63].

Tannins also modulate the immune response in ENT pathologies, particularly allergic rhinitis [61]. A randomized, double-blind, placebo-controlled study of 33 patients with allergic rhinitis evaluated the benefit of apple tannins and, in particular, procyanidins on the symptoms of the pathology. The study reported a significant improvement in certain symptoms (sneezing attacks [$p < 0.05$] and nasal discharge [$p < 0.01$]) with treatment titrated to 200 mg/day of polyphenols compared with placebo.

In 2013, Nantz et al. evaluated the benefits of consuming cranberry juice rich in pro-anthocyanidins (65–77%) for 10 weeks in the management of ENT Winter pathologies [64]. They demonstrated that the use of active treatment significantly decreased flu symptoms compared with placebo ($p = 0.031$), along with a proliferation of γδ-T lymphocytes ($p < 0.001$), immune cells located in the respiratory epithelium, suggesting a strengthening of the first line of defense against viruses.

A Cochrane review has evaluated the effects of caffeine on respiratory parameters in asthmatic patients [66]. This analysis of seven randomized clinical studies and 75 subjects showed a significant improvement in respiratory parameters of up to 4 h, even with doses lower than 5 mg/kg.

Finally, the effect of carotenoids has been evaluated in several clinical studies [67,68]. In 2008, Cingi et al. evaluated the effect of the daily intake of 2 g of spirulina for 6 months versus placebo on symptoms associated with allergic rhinitis [68]. Data from 129 patients showed a significant improvement in nasal discharge, sneezing, nasal congestion, and itching compared with the control group (all $p < 0.001$). In 2020, Nourollahian et al. also evaluated the effects of a 2 g/day intake of spirulina for 2 months on allergic rhinitis symptoms and associated inflammatory parameters in comparison with cetirizine (control) [67]. The results showed a significant improvement of most of the monitored symptoms as well as an improvement of inflammation markers. The therapeutic benefits observed with carotenoids may be explained by an anti-inflammatory activity that regulates interleukin (IL)-4 and interferon (IFN)-γ expression and restores T helper (Th)1/Th2 balance [67,68].

The principal mechanisms of action involved in this area of health are therefore mainly related to the antimicrobial and anti-inflammatory properties of phytonutrients. Antimicro-

bial activity is based either on phytonutrients' direct destabilizing effects on the viral or bacterial membrane, which is well described for EGCG as an example [69–72], or by their inhibition of microbial enzymes or biofilms [73,74]. Anti-inflammatory activity, often associated with the antioxidant properties of phytonutrients, involves several main mechanisms of action. Families of phytonutrients such as anthocyanins (cyanidin and delphinidin), tannins (proanthocyanidin), flavonoids (quercetin), phenolic acids (ferulic acid), organosulfur compounds (sulforaphane), carotenoids (lycopene), and caffeine have been described as being able to inhibit activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, leading to a reduction in inflammation [28,55,75–79]. Flavonoids (luteolin and kaempferol), organosulfur compounds (sulforaphane), anthocyanins (cyanidin and delphinidin), phenolic acids (p-coumaric acid), carotenoids (lycopene), and tannins (proanthocyanidins) can also regulate the mitogen-activated protein kinase pathway, which is also widely implicated in inflammatory processes [28,80–84].

3.2.3. Digestive Health

Digestion is a therapeutic area that is very well represented in family health and includes many benign conditions and symptoms such as constipation, nausea, and diarrhea, but also chronic liver diseases [85–96]. Phytonutrients are again of great interest in this field. A summary of the effects of phytochemicals in this therapeutic area is presented in Table 5.

In 2016, Baek et al. demonstrated the beneficial effect of a flavonoid-rich extract on constipation parameters in a randomized clinical trial [85]. After 8 weeks of treatment, transit time in the colon was significantly reduced versus placebo, stool quality improved, and abdominal discomfort was reduced. The therapeutic effect was thought to be based on the ability of certain flavonoids to stimulate chloride channels and/or serotonin signaling leading to a secretion of water, electrolytes and mucin in the colon. A pilot study has also shown flavonoids present in grape juice to reduce nausea and vomiting during chemotherapy compared with placebo, without reaching the significance threshold [86]. Dryden et al. evaluated the therapeutic benefit of flavonoids in the management of ulcerative colitis and demonstrated that a 56 day administration of an extract rich in EGCG significantly increased ($p = 0.003$) the rate of remission of the pathology compared with placebo [87]. The observed therapeutic effect was believed to be based on the ability of EGCG to inhibit IkB kinase, thus blocking the activation and nuclear translocation of NF-kB, an important inflammation modulator.

Flavonoids are also of great interest in the management of liver pathologies. In 2013, a team investigated the value of isoflavone supplementation in obese postmenopausal patients as a complement to physical exercise [88]. This randomized, placebo-controlled, double-blind pilot study reported a benefit of isoflavone supplementation for 6 months compared with placebo, notably on the fatty liver index ($p < 0.01$) and γ-glutamyltransferase levels ($p < 0.01$). This health benefit was thought to be based on the ability of isoflavones to limit the oxidative stress found in liver pathologies. In 2019, another team evaluated the effects of hesperidin 1 g/day for 12 weeks on the components of non-alcoholic fatty liver disease [89]. Results showed a significant decrease in total cholesterol ($p = 0.016$), triglyceride ($p = 0.049$), hepatic steatosis ($p = 0.041$), and C-reactive protein levels ($p = 0.029$) versus placebo, probably due to an inhibition of the NF-kB pathway.

Tannins have also demonstrated therapeutic value in the management of digestive pathologies. In 2018, Venancio et al. evaluated the benefit of a daily intake of 300 g of gallotanin-rich mangoes on volunteers with chronic constipation [90]. An improvement in functional (stool frequency and consistency) and inflammatory parameters was demonstrated after 4 weeks of treatment. Tannins, particularly crofelemer, were also evaluated in the management of symptoms related to irritable bowel syndrome in a large randomized, controlled study [91]. After 3 months of treatment, functional parameters had not improved, but abdominal pain and discomfort had significantly improved ($p = 0.0076$) in those women treated with 500 mg of the product.

Table 5. Summary of selected studies regarding the therapeutic area of digestive health.

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Stomach								
Ingersoll (2010) [86]	Chemotherapy-induced nausea and vomiting	Flavonoids	77 adults with cancer (62 female) Mean age: 54.3 years USA	Double-blind, randomized clinical trial (1 week following each of four chemotherapy treatment cycles)	T: Grape juice (n = 40) C: No juice (n = 37)	Nausea and vomiting frequency, duration, and distress; quality of life; control over decision making; psychological state	No significant differences except for final anxiety and depression	Nausea and vomiting frequency, duration, and distress were lower for the treated group without any statistically significant difference over time
Intestine								
Biedermann (2013) [95]	Ulcerative colitis (UC)	Anthocyanins	13 patients with mild-moderate UC (3 female) Age: 19–61 years Switzerland	Prospective, non-blinded, non-controlled pilot trial (6 weeks)	T: Bilberry 160 g (4 trays per day equivalent to an average dose of anthocyanin of 840 mg/day)	Clinical activity index (CAI) with remission defined as CAI < 4; endoscopic Mayo score; short inflammatory bowel disease questionnaire (SIBDQ)	Remission was achieved in 63.4% of patients; significant reduction in the complete Mayo Score of at least two points in all patients; SIBDQ score was significantly higher at end of treatment for 81.8% of patients	Anthocyanins had a significant beneficial effect on inflammatory activity in UC

Table 5. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Dryden (2013) [87]	UC	Flavonoid (-)-epigallo-catechin-3-gallate (EGCG)	17 patients with mild-to-moderate UC (11 female) Mean age: 44.9 years USA	Randomized, double-blind, placebo-controlled trial (56 days)	T: Polyphenn E green tea low dose (n = 5) and high dose (n = 8) C: Placebo (n = 3)	UC disease activity index (UCDAI); inflammatory bowel disease questionnaire (IBDQ)	Significant improvement of UCDAI for 66.7% patients with T vs. 0% with C ($p = 0.03$); no effect for IBDQ	Administration of Polyphenon E resulted in a therapeutic benefit for patients with UC who were refractory to 5-aminosalicylic and/or azathioprine. Polyphenon E treatment resulted in only minor side effects
Mangel (2008) [91]	Diarrhea-irritable bowel syndrome (D-IBS)	Tannins (oligomeric procyanidins)	250 patients with D-IBS (185 female) Mean age: 50.2 years US	Double-blind, randomized trial: four groups comprising one placebo and three with different doses) (12 weeks)	T: Crofelemer (from Croton lechleri) 125 mg (n = 62), 250 mg (n = 59), 500 mg (n = 62) C: Placebo (n = 61)	Stool consistency (ST); stool frequency (SF); pain score (PS); pain and discomfort-free days (PFD and DFD)	ST: No difference; SF: Significant with 500 mg/day dose (−0.43 vs. −0.98 for C; p 0.03); PS: No difference; PFD and DFD: Significant improvement with 500 mg/day dose; benefits increased after 3 months	No improvement of ST, except females with D-IBS treated with crofelemer 500 mg/day. Crofelemer displayed improvement for PFD and DFD

Table 5. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Baek (2016) [85]	Transit and bowel function	Flavonoids Polyphenols	80 adults (71 female) Age: 19–39 years Korea	Randomized, double-blind, placebo-controlled trial (8 weeks)	T: *Ficus carica* paste (n = 40) Fiber: 1.7% Total phenolics compounds: 332 µg/g dry Total flavonoids: 44 µg/g dry C: Control paste (n = 40)	Colon transit time (CTT)	−38% with T vs. −24% with C, $p < 0.0001$	After 8 weeks of supplementation *Ficus carica* paste, there was a significant reduction in CTT and an improvement in bowel function. No adverse effects were reported
Venancio (2018) [90]	Constipation symptoms	Tannins Gallotannins	36 adults (28 female) Age: 18–65 years USA	Randomized trial (4 weeks)	Two T groups: Mango group (MG) (n = 19) Fibre group (FG) (n = 17) Intake of mango fruit (300 g)	Constipation symptoms; inflammatory biomarkers; hormones (gastrin); adipokines (interleukins [IL]); stool short-chain fatty acids (SCFA)	MG reported increased evacuation categorization; IL-6—23% in MG vs. FG ($p = 0.01$) IL-10—15.4% in MG vs. FG ($p = 0.03$); gastrin significantly increased in both MG and FG (+13% and +7%, respectively); valeric acid increased in both MG and FG ($p = 0.336$); endotoxin decreased in MG vs. FG ($p = 0.025$)	Mango consumption significantly improved constipation status, increased gastrin levels and fecal concentrations of SCFA (valeric acid), lowered plasma endotoxin and IL-6

Table 5. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Yanaka (2018) [93]	Bowel habits	Organosulfur compound: sulforaphane glucosinolates (SGS)	48 adults with constipation (44 female) Mean age: 35 years Japan	Randomized clinical trial (4 weeks)	Two T groups: Broccoli sprouts (BS) (n = 24) Alfalfa sprouts (AS) (n = 24) 20 g/day	Total constipation score: frequency of bowel movements, painful evacuation, incomplete evacuation, abdominal pain, duration of defecation attempt, assistance for evacuation, unsuccessful attempts of evacuation per 24 h; bacteria in stool samples	Significant reduction in constipation score for BS; significant effects on Bifidobacterium for BS and Lactobacillus for AS	A daily intake of 20 g/day of raw BS (4.4 mg/g SGS) for 4 weeks improves defaecation in healthy subjects. This effect was not demonstrated by an intake of the same amount of AS (no SGS). Beneficial effect of sulforaphane against chronic oxidative stress
Kaczmarek (2019) [94]	Gastrointestinal microbiota	Organosulfur compound: glucosinolates	18 healthy adults) (10 female) Age: 21–70 years USA	Controlled feeding, randomized, crossover study (18 day treatment periods separated by a 24 day washout)	T: Diet + 200 g of cooked broccoli and 20 g of raw radish per day C: Diet excluding Brassica vegetables	Fecal samples/ beta diversity Urine and plasma: metabolites	Increase in Bacteroidetes for T vs. C ($p = 0.03$); decrease in Firmicutes for T vs. C ($p = 0.05$)	Broccoli increased Bacteroidetes and decreased Firmicutes. Broccoli consumption increased the abundance of Bacteroides. *B. vulgatus* and *B. thetciiotciomicron* showed no change by treatment type

Table 5. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
				Liver				
Ruhl (2005) [96]	Chronic liver disease (CLD)	Caffeine	9849 adults (5995 female) Age: 25–74 years USA	Prospective (NHANES I) (mean 19 years)	Tea and coffee (>1, 1–2, >2 cups/day)	CLD	Multivariate-adjusted hazard ratio 0.36 (95% confidence interval: 0.17, 0.78) for >2 cups/day vs. <1 1 cup/day)	Coffee and tea drinking decreases the risk of CLD although the effect is limited to persons at increased risk of liver injury
Barsalani (2013) [88]	Hepatic steatosis	Flavonoïds Isoflavones	54 overweight to obese post-menopausal women (body mass index: 240 kg/m^2) Age: 50–70 years Canada	Randomized, double-blind, trial (6 months)	T: Exercise and soy isoflavones (70 mg/day) (n = 26) C: Exercise and placebo (n = 28)	Fatty liver index (FLI); plasma lipid profile; liver function enzymes: alanine aminotransferase(ALT), aspartate aminotransferase (AST), glutamyltransferase, alkaline phosphatase	All outcome parameters were improved in both T and C groups; significant improvements with isoflavones for glutamyltransferase and FLI after 6 months of treatment	In addition to exercise, isoflavones provided additional effects on FLI
Kikuchi (2015) [92]	Hepatic abnormalities	Organosulfur compound Glucoraphanin; sulforaphane precursor)	55 men with fatty liver Age: 30–69 years Japan	Randomized, placebo-controlled, double-blind trial (4 weeks)	T: Broccoli sprout extract 135 mg (approximately 310 μmol of glucoraphani per gram (n = 27) C: Placebo (n = 28)	Liver function markers: AST and ALT; γ-glutamyl transpeptidase (GTP); 8-hydroxydeoxyguanosine (8-OHdG)	Significant decrease in median (interquartile range) ALT before: 54.0 (34.5–79.0) vs. after T: 48.5 (33.3–65.3) international units (IU)/L; $p < 0.05$; significant decrease in GTP before: 51.5 (40.8–91.3) vs. after: 50.0 (37.8–85.3) IU/L; $p < 0.05$; 8-OHdG reduced in T not in C	Dietary supplementation with broccoli sprout extract containing the sulforaphane precursor is likely to be highly effective in improving liver function through reduction in oxidative stress

Table 5. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Cheraghpour (2019) [89]	Hepatic steatosis	Flavonoid Hesperidin (flavanone glycoside)	49 adults with non-alcoholic fatty liver disease (NAFLD) (grades 2 and 3) (22 female) Iran	Randomized, placebo-controlled, double-blind clinical trial (12 weeks)	T: Hesperidin 1 g (n = 25) C: Placebo (n = 24)	ALT, GTP); total cholesterol (TC); triglyceride (TG); hepatic steatosis (HS); high-sensitivity C-reactive protein (hsCRP); tumor necrosis factor-α (TNF-α); nuclear factor-κB (NF-κB)	Significant reduction in ALT ($p = 0.005$), GTP ($p = 0.004$), TC ($p = 0.016$), TG ($p = 0.049$), HS ($p = 0.041$), hsCRP ($p = 0.029$), TNF-α, ($p = 0.78$) NF-κB (no significant reduction)	Hesperidin supplementation accompanied with lifestyle modification was superior to lifestyle modification alone in the management of NAFLD at least partially through inhibiting NF-κB activation and improving lipid profile

The antioxidant activity of organosulfur compounds, particularly glucoraphanes, has been shown to improve liver function in patients with fatty liver after 4 months of treatment, possibly due to their antioxidant properties and ability to stimulate detoxifying enzymes by activating the NRF2 transcription factor [92]. In a study by Yanaka et al., the use of glucosinolates, particularly sulforaphanes contained in broccoli sprouts, provided a significant reduction in constipation versus placebo when consumed daily at 20 g/day (i.e., 4.4 mg/day of sulforaphane) for 21 days [93]. According to the authors, this effect was due to the antioxidant action of the sulforaphanes on the digestive tract. Other research teams have attributed the beneficial effects of organosulfur compounds in the digestive tract to a direct effect on the intestinal microbiota [94]. Daily consumption of 200 g of broccoli and 20 g of raw daikon radish for period of 18 days has been shown to result in a significant change ($p = 0.03$) in the composition of the intestinal microbiota versus a diet without organosulfur compounds; a significant decrease in firmicutes in favor of bacteroides ($p = 0.03$) compared with the control was noted.

Other phytonutrients may be of interest in the management of digestive pathologies. Biedermann et al. have shown that anthocyanins appear to be active in the management of ulcerative colitis by reducing certain symptoms as well as the Endoscopic Mayo Score [95]. This effect may be related to the anti-inflammatory activity of anthocyanins leading to a reduction in tumor necrosis factor-α (TNF-α) and IFNγ levels in mesenteric lymph nodes. A meta-analysis evaluated the protective potential of caffeine in this type of pathology and the authors suggested a protective effect of this phytonutrient in chronic hepatic pathologies, which may be due to its antioxidant action or modulating effect on insulin resistance [96].

3.2.4. Bones and Joints

Joints are a therapeutic area of interest in family health. Many products are marketed as a first-line treatment for benign tendon and joint disorders, but also as accompanying care for more serious chronic pathologies, such as osteoarthritis or rheumatoid arthritis. Some phytonutrients also demonstrate beneficial properties in this type of pathology while others can be deleterious. A summary of the effects of phytochemicals in this therapeutic area is presented in Table 6.

Table 6. Summary of selected studies regarding the therapeutic area of bones and joints.

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Ambrosini (2013) [97]	Fracture risk	Carotenoids	8320 individuals (664 female) Age: 40–62 years Australia	Observational studies (17 years)	T1: Synthetic all-trans β-carotene 30 mg/day T2: Synthetic all-trans β-carotene 0.75 mg/day T3: Retinol equivalents 7.5 mg/day as retinyl palmitate (25,000 international units)	Exploratory analysis of fracture (any fracture or osteoporotic fracture) risk as a secondary endpoint (the primary endpoint explored efficacy of retinol and β-carotene supplements for reducing the risk of mesothelioma and lung cancer in persons previously exposed to asbestos)	No increases in fracture risk after long-term supplementation with high doses of retinol and/or β-carotene (any fracture; $p = 0.17$; osteoporotic fracture, $p = 0.79$). NB: Previous cohort studies have reported positive associations between dietary retinol intake and fracture risk ($p = 0.002$)	This study observed no increases in fracture risk after long-term supplementation with high doses of retinol and/or β-carotene
Wu (2014) [98]	Fracture risk	Carotenoids	283,930 individuals (≈252,835 female) Age: 15–90 years China and others	Meta-analysis of prospective studies (n = 12)	Assess the effects of vitamin A (n = 8 studies) or retinol or β-carotene (n = 4 studies) on fracture risk (mainly of the hip)	Adjusted relative risk (RR); risk of hip fracture; risk of total fracture; relation between serum retinol level and hip fracture risk	A high intake of vitamin A and retinol increased the risk of hip fracture (RR [95% confidence interval (CI)]: 1.87 [1.31, 2.65] and 1.56 [1.09, 2.22], respectively). Low concentration of retinol increased RR (dose–response meta-analysis showed a U-shaped relationship between serum retinol level and hip fracture risk), but not a high intake of β-carotene (RR [95% CI] 0.82 [0.59, 1.14])	The meta-analysis suggested that blood retinol level is a double-edged sword for risk of hip fracture. To avoid the risk of hip fracture caused by too low or too high a level of retinol concentration, intake of β-carotene (provitamin A), which should be converted to retinol in blood, may be better than intake of retinol from meat, which is directly absorbed into blood after intake

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Lee (2014) [99]	Fracture risk	Caffeine	253,514 individuals (number of females not specified) Age: 25–103 years All countries, particularly Western countries (USA, Canada, Europe)	Systematic review and meta-analysis (n = 15)	Dose–response analysis to assess the risk of fractures according to the level of coffee consumption in the female population based on 12 939 fracture cases (cohort studies, n = 9; case–control studies, n = 6)	Urinary calcium excretion and expression of the protein receptor for vitamin D. RR of fracture	Daily coffee consumption is associated with an increased risk of fractures in women (RR of 1.02 per 2 cups to 1.54 per 8 cups per day) and a paradoxical decrease in risk in men	The meta-analysis suggested that daily consumption of coffee was associated with an increased risk of fractures in women and a contrasting decreased risk in men. However, future well-designed studies should be performed to confirm these findings
Connelly (2014) [100]	Knee osteoarthritis (OA)	Phenolic acid (rosmarinic acid)	46 women with OA Age: 48–72 years Canada	Randomized, parallel-arm, double-blind study (16 weeks)	Effects of consuming spearmint infusion rich in rosmarinic acid, twice daily, on knee OA T: High-rosA spearmint plant (280 mg/day. rosA) (n = 22) C: Placebo (26 mg/day. rosA) (n = 24)	Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC); short-form 36-item health survey (SF-36); 6-min walk test (6MWT); stair climb test (SCT)	Daily consumption of spearmint tea significantly improved stiffness and physical disability scores in adults with knee OA, but only the high-rosA tea significantly decreased pain; WOMAC: Significant decrease with T ($p = 0.002$) at 16 weeks and significant decrease for C at 8 weeks ($p = 0.04$), but not at 16 weeks ($p = 0.07$). SF-36: Significant only for QoL score ($p < 0.05$); SCT and 6MWT: No significant difference ($p = 0.43$ for T, $p = 0.44$ for C, and $p = 0.9$ between the group)	Consumption of high-rosA tea warrants further consideration as a potential complementary therapy to reduce pain in OA

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Law (2016) [101]	Bone mineral density (BMD), osteoporosis	Flavonoids	30 healthy subjects (18 female) Age: 40–80 years China, Taiwan	Randomized, double-blind, placebo-controlled trial (8 weeks)	T: 100 mL of onion juice (n = 16) C: Placebo (n = 14)	BMD; alkaline phosphatase (ALP); free radicals; total antioxidant capacity	Onion juice consumption showed a positive modulatory effect on the bone loss and BMD (inhibitory effects on the differentiation of osteoclasts) and can be recommended for treating osteoporosis	Onion juice consumption showed a positive modulatory effect on the bone loss and BMD by improving antioxidant activities and thus can be recommended for treating various bone-related disorders, particularly osteoporosis
Hu (2017) [102]	Rheumatoid arthritis (RA)	Carotenoids	227 incident RA patients and 671 matched controls (898 female) Age: 41–61 years USA	Prospective case–control study (10 years: Nurses' Health Study and Nurses' Health Study II)	To examine the associations between circulating carotenoids and future risk of RA	Measurement of plasma carotenoids (α-carotene, β-carotene, β- cryptoxanthin, lycopene and lutein/zeaxanthin) levels	No significant association was found between the level of circulating carotenoids and the risk of developing RA ($p = 0.93$)	Circulating carotenoids levels are not associated with reduced risk of RA. Further investigations using large prospective cohorts are warranted
Javadi (2017) [103]	RA	Flavonoids (Quercetin)	50 women with RA Age: 35–56 years Iran	Double-blind, placebo-controlled clinical trial (8 weeks)	T: Quercetin (500 mg/day) (n = 25), C: Placebo (n = 25)	Plasma levels of TNF-α; sedimentation rate; clinical symptomatology including early morning stiffness, morning and after-activity pain, tender and swollen joint counts; disease activity score 28 (DAS-28); physician global assessment (PGA); health assessment questionnaire (HAQ) at the beginning and end of the study	Clinical symptomatology: Significantly reduced early morning stiffness, morning pain, and after-activity pain ($p < 0.05$); TNF-α: Significantly reduced in the T group compared with C ($p < 0.05$); DAS-28 and HAQ: DAS-28 significantly decreased in the T group ($p = 0.04$), DAS-28 and HAQ scores decreased in the T group compared with C ($p = 0.001$ for both); PGA: No significant change	Quercetin 500 mg/day supplementation for 8 weeks resulted in significant improvements in clinical symptoms, diseases activity, hs-TNF-α, and HAQ in women with RA

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Hosseinzadeh-Attar (2020) [104]	Knee OA	Organosulfur compound (garlic)	50 obese women (body mass index>30) with knee OA Age: 50–75 years Iran	Randomized, double-blind, placebo-controlled trial (12 weeks)	T: Daily odour-controlled garlic tablet 1000 mg (equivalent to 2500 mg of fresh garlic) containing 2.5 mg allicin (n = 23) C: Placebo (n = 25)	WOMAC questionnaire (including joint stiffness and physical function); visual analogue scale (VAS) for pain severity	WOMAC: Significant decrease in WOMAC total score ($p = 0.013$), joint stiffness ($p = 0.019$), and physical function ($p = 0.018$) in the T group compared with C; VAS: Marginal decrease ($p = 0.073$)	A 12 week garlic supplementation (1000 mg) exerted significant improvements in joint symptoms in obese women with knee OA. Future studies are required to address the potential better response of obese patients to interventions as well as relevant underlying mechanisms
Kim (2016) [105]	BMD, osteo-porosis	Carotenoids (β-carotene)	189 postmenopausal women Age: 50–75 years Korea	Cross-sectional study (6 months)	Relationship between nutritional intake (protein, carbohydrate, fat, micro, oligo elements and vitamins) and BMD	BMD T scores were measured at: lumbar spine, femoral neck, total hip; semiquantitative food-frequency questionnaire	Lumbar spine: Positively correlated with sodium, potassium, zinc, calcium, vitamin A, β-carotene and vitamin C ($p < 0.05$ for all); Femoral neck: Positive correlations with nutritional intake (protein, carbohydrate, fat, micro and oligo elements, and vitamins ($p < 0.05$ for all)); Total hip: Positive correlations with nutritional intake (protein, fat, vitamin, calcium, potassium, zinc, iron [$p < 0.05$ for all); β-carotene, Na and vitamin C had positive association with BDM-T scores ($p < 0.001$)	In postmenopausal Korean women, β-carotene, vitamin C, zinc, and sodium intakes were positively associated with bone mass Furthermore, frequency of vegetable consumption was positively associated with femoral neck and total hip T scores
Lambert (2017) [106]	BMD, osteo-porosis	Flavonoids (isoflavones aglycones)	2652 women analysis with postmenopausal or perimenopausal Age: 39–93 years Denmark	Systematic review of 26 randomized controlled trials	T: Isoflavone aglycones intakes C: Placebo	Weight mean difference (WMD) with the lumbar spine and femoral neck	WMD for lumbar spine: Isoflavone associated with a significant increase in BMD vs. placebo ($p < 0.00001$); WMD for femoral neck: Isoflavone associated with a significant increase in BMD vs. placebo ($p < 0.01$)	The effect appeared to be dependent on whether isoflavone treatments were in aglycone form. The beneficial effects against bone loss may be enhanced for isoflavone aglycones

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Li (2013) [107]	Hip fracture risk	Caffeine	4677 cases/159,307 controls Age: 50–70 years China, Europe, North America	Meta-analysis of prospective cohort studies and case–control studies (N = 6 cohort and N = 6 prospective studies)	Median coffee consumption	Establish the current evidence concerning the relationship between coffee consumption and hip fracture risk, according to study design and characteristics of study populations, and determine the potential dose–response pattern between coffee consumption and hip fracture risk	Pooled odds ratio: Increased hip fracture risk by 29.7% ($p = 0.09$) with high-dose caffeine result having no statistical significance. Subgroup analyses: Coffee consumption significantly increased hip fracture risk by 54.7% among women, by 40.1% for elderly participants aged >70 years, and by 68.3% for Northern Americans. Other subgroup analyses: Positive association between coffee and hip fracture risk. Follow-up duration also positively affected hip fracture risk (<13 years)	The meta-analysis provided insufficient evidence that coffee consumption significantly increases hip fracture risk. Coffee intake may increase hip fracture risk among women, elderly participants, and Northern Americans. No dose–response pattern was observed
Gambacciani (1997) [108]	BMD, osteoporosis	Flavonoids: isoflavones (ipriflavone)	80 postmenopausal women Age: 40–49 years Italy	Longitudinal, comparative (2 years)	T1: Ipriflavone (IP) (600 mg/day) (n = 20) T2: Conjugated equine oestrogens (CE) (0.3 mg/day) (n = 20) T3: Low-dose IP (400 mg/day) and CE 0.3 mg/day (IP+CE) (n = 20) C: Placebo calcium supplementation (500 mg/day) (n = 20)	Bone mass measurement; bone metabolism marker measurements (urinary excretion [UE] of hydroxyproline, plasma osteocalcin level [POL], and measure of vertebral bone density [VBD])	UE and POL in CE group: No modification of hydroxyproline; VBD: Significantly decreased with CE group and placebo ($p < 0.0001$); POL in IP and IP+CE: No modification;UE and VDB in IP and IP+CE: Significant decrease for UE ($p < 0.05$) and increase for VDB ($p < 0.05$)	Postmenopausal IP administration, at the standard dose of 600 mg/day, can prevent the increase in bone turnover and the decrease in BMD that follow ovarian failure. The same effect can be obtained with the combined administration of low-dose (400 mg/day) IP with low-dose (0.3 mg/day) CE

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Ambrosini (2014) [109]	Fracture risk	Carotenoids (β-carotene)	998 adults analyzed in cancer prevention program (335 female) Age: 15–80 years Australia	The Vitamin A Program, a cancer prevention program with supplementation of high-dose retinol and β-carotene (17 years)	From 1990 to 1996 randomly assigned to: T1: Retinol (7.5 mg /day) (n = 1006) T2: β-carotene (30 mg/day) (n = 1009) From 1996 to 2006, all assigned to: T1: Retinol (7.5 mg /day) (n = 1736)	Investigate plasma retinol and total carotene concentrations in relation to fracture risk	No convincing associations between plasma retinol concentration and fracture risk (hazard ratio [HR] 0.86 mmol/L; 95% CI: 0.65, 1.14) or osteoporotic fracture were observed (HR 0.97 mmol/L; 95% CI: 0.66, 1.43)	The possibility that higher plasma carotene concentrations may be associated with lower fracture risk is consistent with previous studies and warrants further study
Wetmore (2008) [110]	Bone mineral content (BMC) and BMD, osteo-porosis	Caffeine	625 women Age: 14–40 years USA	Prospective study	Associations between habitual caffeine intake and bone mass	BMC; BMD of total hip and lumbar spine	BMC and BMD: Intake >200 mg of caffeine per day had lower toal hip and lumbar spine ($p < 0.01$); caffeine intake not associated with either BMC or BMD ($p > 0.5$ for all models)	The data suggest that heavy habitual consumption of caffeinated beverages does not adversely impact bone mass among young women in general. Greater caffeine intake may be associated with lower BMC among depot medroxypro-gesterone acetate users
Pattison (2005) [111]	RA	Carotenoids	>25,000 subjects who completed a baseline 7 d diet diary (European Prospective Investigation of Cancer Incidence (13,975 female) Age: 45–74 years UK	Prospective study	Carotenes: β-carotene, β-cryptoxanthin, zeaxanthin	Longitudinal follow-up of inflammatory polyarthritis (IP), ascertained via the Norfolk Arthritis Register	88 cases of IP occurred: the mean of β-cryptoxanthin and zeaxanthin were 40% and 20% lower, respectively	These data are consistent with previous evidence showing that a modest increase in β-cryptoxanthin intake, equivalent to one glass of freshly squeezed orange juice per day, is associated with a reduced risk of developing inflammatory disorders, such as RA

Table 6. *Cont.*

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Rejnmark (2004) [112]	BMD, osteoporosis	Carotenoids (vitamin A)	2016 perimenopausal women Age: 48–52 years Denmark	Setting of the Danish Osteoporosis Prevention Study (5 year follow-up, case–control study)	Relationship between vitamin A and/or retinol at 0.53 mg/day. intake and BMD and fracture risk	BMD measurements: lumbar spine; femoral neck; cross-sectional analyses; longitudinal analyses	Cross-sectional and longitudinal analyses with femoral neck and lumbar spine: No associations between intakes ($p = 0.93$ with vitamin A and $p = 0.92$ with retinol) and BMD	During the 5 year study period, 163 subjects sustained a fracture (cases). Compared with 978 controls, logistic regression analyses revealed no difference in vitamin A intake. Thus, in a Danish population, average vitamin A intake was lower than in Sweden and the USA and not associated with detrimental effects on bone
Wattanathorn (2018) [113]	Risk factors of osteoporosis	Phenolic acids, gallic acid	45 healthy perimenopausal and postmenopausal women Age: 45–60 years Thailand	Double-blind, placebo-controlled, randomized trial (8 weeks)	T1: Combined extract of *M. Alba* and *P. odoratum* (50 mg/day) (n = 15) T2: Combined extract of *M. Alba* and *P. odoratum* (1500 mg/day) (n = 15) C: Placebo (n = 15)	Osteocalcin (OC); ALP; carboxy-terminal collagen cross-links (β-CTX); total phenolic compounds (TPC); clinical chemistry changes (CCC)	ALP, OC, TPC, and CCC: Significantly increased in T2 group ($p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.05$, respectively); CTX: Significant decrease ($p < 0.01$)	Clinical safety assessment failed to show toxicity and adverse effects. Therefore, herbal congee containing the combined extract of *M. alba* and *P. odoratum* leaves is a potential functional food that can decrease the risk of osteoporosis

More than 20 years ago, the beneficial effect of flavonoids, particularly ipriflavone, for the prevention of menopausal osteoporosis over the course of a long-term study was reported [108]. The authors concluded that there was an improvement in vertebral bone density with a supplement of ipriflavone 600 mg daily for 2 years, possibly caused by a limited bone resorption effect. The effect of flavonoids has been more recently verified in a study conducted by Law et al. [101]. After 2 months of daily treatment with 100 mL of onion juice rich in flavonoids and phenolic acids, patients with osteoporosis showed significant improvements in oxidation markers and positive modulation in bone loss. The mechanisms of action involved were related to the antioxidant properties of flavonoids as well as their ability to slow the differentiation of progenitors into osteoclasts. A systematic literature review of 26 randomized clinical studies and 2652 patients established a therapeutic benefit of isoflavones in the management of bone loss during menopause, with treatment significantly increasing bone density in the lumbar spine ($p < 0.0001$) and femoral neck ($p < 0.01$) [106]. An improvement in the clinical symptoms of rheumatoid arthritis with flavonoids has also been demonstrated in a randomized, controlled trial [103]. The use of quercetin administered at 500 mg/day for 8 weeks significantly improved the clinical (improvement of early morning stiffness, morning pain, and after-activity pain; $p < 0.05$ for all) and cytokinic profile of patients by inhibiting the NF-kB pathway and the release of associated inflammatory cytokines.

Carotenoids, particularly their anti-inflammatory activity, are of therapeutic interest in the prevention of chronic joint pathologies. A prospective study on more than 25,000 subjects evaluated the effect of carotenoid consumption on the risk of developing rheumatoid arthritis and concluded that an increase in the consumption of β-cryptoxanthin equivalent to a glass of orange juice reduces the risk of developing this pathology due to the antioxidant properties of this phytonutrient [111]. On the contrary, other studies indicate that there is no link between the amount of circulating carotenoids and the occurrence of inflammatory joint disease [102]. Similarly, there is no consensus on the effect of carotenoids on bone preservation. Kim et al. demonstrated that the intake of β-carotene was associated with an improvement in bone mass, particularly in the lumbar spine ($p < 0.05$), probably due to a stabilization of collagen synthesis and osteoblast differentiation [105]. In contrast, other studies have not indicated any interest in this family of phytonutrients for this indication [97,109,112] or suggested any effect that could be deleterious at high doses via the stimulation of osteoclasts and inhibition of osteoblasts [98].

Caffeine is also a phytonutrient for which there is no consensus on its therapeutic effects in the preservation of bone mass. The results of several meta-analyses, notably those conducted by Li et al. in 2013 and Lee et al. in 2014, indicate that caffeine consumption may induce a slight decrease in the risk of fracture in men and a slight increase in women, with a greater incidence in the elderly [99,107]. The mechanisms of action involved are not clearly defined and are sometimes conflicting. Caffeine has been described both as being able to inhibit osteoclastogenesis and limit osteoclast activity and, on the contrary, to promote it to the detriment of osteoblasts. Conversely, other studies indicate that there is no significant effect of caffeine on bone density or fracture risk [110,114].

A randomized, controlled clinical trial conducted by Wattanathorn et al. reported that the daily consumption of 1.5 g of a phenolic acid-rich extract for 8 weeks significantly increased the amount of markers involved in bone formation (osteocalcin, $p < 0.01$; alkaline phosphatase, $p < 0.05$) and decreased those of resorption (β-carboxy-terminal collagen cross-links, $p < 0.01$) versus baseline [113]. Phenolic acids may therefore be of interest in the management of osteoporosis, thus confirming the results reported by Law et al. (described previously) [101]. Another placebo-controlled study evaluated the therapeutic effects of twice-daily consumption of a spearmint infusion rich in rosmarinic acid (280 mg/day) for 16 weeks in 46 patients suffering from osteoarthritis [100]. Data from this study indicated a significant decrease in pain assessed via the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score in the rosmarinic acid supplemented group versus baseline, whereas there was no improvement in the placebo group. Of note,

an improvement in quality of life (QoL) was also reported in the supplemented group at 16 weeks.

The effect of organosulfur compounds was recently evaluated in a randomized, placebo-controlled clinical trial including 50 female patients with osteoarthritis [104]. The results showed that 1 g of garlic taken daily for a period of 12 weeks significantly reduced the WOMAC index ($p = 0.013$), joint stiffness ($p = 0.019$), and tended to reduce joint pain ($p = 0.073$) compared with placebo, due to the anti-inflammatory properties of organosulfur compounds.

3.2.5. Energy and Vitality

The energy and vitality field is a therapeutic area of great interest in family health and includes several indications. It not only covers problems related to fatigue or recovery, but also symptoms associated with cognition. Numerous studies reveal the significant benefit of phytonutrients in this vast area of health. A summary of the effects of phytochemicals in this therapeutic area is presented in Table 7.

Table 7. Summary of selected studies regarding the therapeutic area of energy and vitality.

First Author (Year)	Target	Phytochemicals	Participants (Total Number, Female Number, Age Years) Country	Study Type (Duration)	Exposure, T (Number Treated) C (Number Control) Phytochemicals	Outcome Parameter	Results	Comments
Borota (2014) [115]	Recognition performance and memory consolidation	Caffeine	160 individuals (80 female) Age: 18–30 years USA	Randomized, double-blind, placebo-controlled trial (48 h duration)	T (n = 122): 100 mg, n = 77) 200 mg, n = 35 300 mg, n = 10 C (Placebo), n = 38	Salivary samples: caffeine metabolites; hippocampal memory (HM)–dependent task, particularly taxing pattern separation	Caffeine metabolites: Significant increase at the 1 h and 3 h time points, which then returned to baseline amounts over a 24 h washout period HM: More likely to call lure items 'similar' rather than 'old' vs. placebo in rates of target hits or foil rejection ($p = 0.04$)	Caffeine enhanced performance 24 h after administration according to an inverted U-shaped dose–response curve. Caffeine enhanced consolidation of the initial study session such that discrimination during retrieval was improved
Carvalho-Peixoto (2015) [116]	Physical performance enhancement	Anthocyanins	14 athletes (all male) Mean age: 26 ± 6 years Brazil	Simple-blinded, randomized intervention study (four visits on four separate days)	T: Anthocyanins beverage of 300 mL containing 4% acaï (anthocyanins 27.6 mg) C: Placebo	Control of muscle (CM); cardiorespiratory responses (CR); time to exhaustion (TE); oxidative stress biomarkers (OSB)	CM: Reduction in perceived exertion ($p < 0.05$) CR: enhancement ($p < 0.05$) TE: Increased mean difference: 69 s (95% confidence interval [CI]: −296, 159; t = 2.2; $p = 0.045$) OSB: Attenuation of the metabolic stress induced by exercise ($p < 0.05$)	Anthocyanins beverages may be a useful and practical ergogenic aid to enhance performance during high-intensity training
Alharbi (2016) [117]	Cognitive function alertness and mood	Flavonoids	24 men Age: 30–65 years UK	Randomized, double-blind, placebo-controlled, crossover (2 day exposure, 2 week washout)	T: Enriched orange juice (240 mL) containing flavonoids 272 mg C: Placebo	Immediate word recall (IWR); simple and complex finger tapping (SCFT); digit symbol substitution test (DSST); continuous performance test (CPT); serial sevens (SS); positives and negative affect scale (PANAS); contrast sensitivity (CS); delayed word recall (DWR)	No significant differences between drinks for any dependent variables. From a 2x2 ANOVA: no significant main effects or interactions for IWR, DSST, SS, PANAS, CS, DWR; SCFT: Significant mean change from baseline across time points, higher following flavonoid-rich drink (mean 1.4, SE 0.8) vs. placebo (mean −0.6, SE 0.5); CPT: Significant difference at 6 h ($p < 0.05$) but no significant difference at 2 h	Executive function and psychomotor speed significantly improved after the flavonoid-rich drink compared with control

Table 7. *Cont.*

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Bazzucchi (2019) [118]	Neuromuscular function impairment caused by acute eccentric exercise-induced muscle damage	Flavonoids	12 young men Mean age: 26.1 ± 3.1 years Italy	Randomized, double-blind, crossover (3 weeks of exposure, 14 days of washout)	T: Quercetin 1000 mg/day C: Placebo	Maximal voluntary isometric contraction (MVIC); force-velocity (FV); electromyography (EMG); isometric strength (IS); resting arm angle (RAA); arm circumference (AC); plasma creatine kinase (PCK); lactate dehydrogenase (LDH)	MVIC: Significant increase in IS recorded compared with baseline (+4.7%, $p < 0.05$); EMG, RAA, AC: Torque and muscle fibre conduction velocity (MFCV) decay significantly lower with T compared with C ($p < 0.001$); IS, FV, and MFCV significantly lower with C than T ($p < 0.001$); PCK, LDH: No significant findings	Quercetin supplementation appears to attenuate the severity of muscle weakness caused by eccentric-induced myofibrillar disruption and sarcolemma action potential propagation impairment
Saitou (2018) [119]	Cognitive function	Phenolic acids (chlorogenic acid [CGA])	38 healthy volunteers with subjective memory complaints (17 female) Age: 50–69 years Japan	Randomized, double-blind, placebo-controlled-parallel group (16 weeks)	T: CGA drink with dry green coffee extract without caffeine and rich in chlorogenic acids (caffeoylquinic acids [CQA] 67.5%, feruloylquinic acid [FQA] 13.8%, and dicaffeoylquinic acids 18.6%) CQA + FQA = 300 mg (n = 20) C: Placebo without CQA (n = 18)	Cognition vital signs (Cognitrax); verbal memory test (VBM); visual memory test (VIM); finger tapping test (FTT); symbol digit coding (SDC); stroop test (ST); shifting attention test (SAT); CPT; blood sample: levels of apolipoprotein (A1); transthyretin (TTR)	VBM: CGA scores lower vs. placebo group ($p = 0.093$); Cognition: CGA group significant increase ($p < 0.05$); SDC: scores for errors tended to be higher ($p = 0.080$) at 16 weeks; SDC, FTT/ ST, SAT, and CPT: significantly higher changes with CGA vs. placebo ($p = 0.080$, $p = 0.071$, $p = 0.063$, and $p < 0.05$, respectively); A1: higher score with CGA vs. placebo ($p = 0.07$); TTR: significantly higher in CGA group vs. placebo group ($p < 0.05$)	CGAs may improve some cognitive functions, including attention as well as motor speed, which would help in the efficient performance of complex tasks. Blood concentration of TTR and ApoA1 increased after the CGA treatment, which might reflect the improved cognitive functions observed in the neuropsychological tests

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Bowtell (2017) [120]	Brain task-related activation, cognitive function, and resting perfusion	Anthocyanins	26 healthy older adults (13 female) Mean age: 68.3 ± 1.7 years UK	Double-blind, randomized, controlled trial (12 weeks)	T: Blueberry concentrate (anthocyanidins 387 mg) (n = 12); C: Placebo (n = 14)	Magnetic resonance imaging (MRI); serum measurements; cognitive tests	MRI: Significant increase in brain activity for T vs. C group ($p = 0.001$); Serum measurements: Significant improvement in grey matter perfusion for T vs. C group in the parietal ($p = 0.013$) and occipital ($p = 0.031$) lobes; Cognitive tests: Improvement in working memory for T vs. C group (2-back test) ($p = 0.05$)	Supplementation with an anthocyanin-rich blueberry concentrate improved brain perfusion and activation in brain areas associated with cognitive function in healthy older adults
Kesse-Guyot (2014) [121]	Brain aging, particularly cognitive disorder	Carotenoids	2983 middle-aged adults (1381 female) Age: 45–60 years France	Randomized, double-blind, placebo-controlled, primary prevention trial (5–7 years, continuation of an 8 year study)	T: Carotenoid-rich dietary pattern (CDP) C: No supplementation	Cognitive test performance (6 neuropsychological tests); recall tasks (RT); backward digit span task (BDST); trail-making test (TMT); semantic fluency task (SFT)	CDP associated with a higher composite cognitive test performance ($p = 0.02$); RT, BSDT, TMT, and SFT: Significant results (all p for trend <0.05)	Upon confirmation in other settings, these findings may argue that sufficient quantity and variety of colored fruits and vegetables in one's diet may help to maintain brain health during ageing

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Cook (2017) [122]	Physiological responses	Anthocyanins	13 healthy men Mean age: 25 ± 4 years UK	Randomized, double-blind, crossover trial (7 day intake separated by a 14 day washout)	T: New Zealand blackcurrant extract 600 mg/day (CurraNZ) C: Placebo (anthocyanins 210 mg/day)	Isometric maximal voluntary contractions (iMVC) (from 0 and 120 s and from 30% to 100% iMVC) measured by: EMG; near-infrared spectroscopy (NIRS); hemodynamics; ultrasound	iMCV at 100%: No effect ($p = 0.732$); Hemodynamics: Significant result with 30% ($p < 0.001$); iMCV: Total peripheral resistance, systolic, diastolic, and mean arterial pressure were lower with increased cardiac output and stroke volume; EMG: Lower muscle oxygen saturation ($p < 0.001$) and root mean square (at 45 s, $p = 0.05$, at 60 s, $p = 0.034$, and at 75 s, $p = 0.015$); NIRS: No significant results; Ultrasound: Increase in femoral artery diameter at 30 s—30% iMVC (6.9%, $p = 0.009$), 60 s—30% iMVC (8.2%, $p = 0.8$), 90 s—30% iMVC (7.7%, $p = 0.021$), and 120 s—30% iMVC (6.0%, $p = 0.022$)	Seven-day intake of 600 mg of New Zealand blackcurrant extract containing 210 mg anthocyanins, with the final intake 2 to 3 h before testing, increased vasodilation during sustained submaximal isometric exercise in young adult healthy men
Falcone (2018) [123]	Nootropic effects	Phenolic acids	142 healthy, recreationally active adults (44 female) Mean age: 27.5 ± 7.9 years USA	Randomized, double-blind, placebo-controlled, parallel trial (90 days)	T: Proprietary spearmint extract (PSE) (n = 73) 900 mg; C: Placebo (n = 69)	Number of hits and average reaction time (stationary and multi-directional test); complete blood count (CBC)	Average reaction time: Significant with PSE at Day 7 ($p = 0.049$) and Day 30 ($p = 0.049$); Stationary: Between group differences at Day 30 ($p = 0.040$) and Day 90 ($p = 0.002$); Multi-directional: Between group differences at Day 30 ($p = 0.007$) and Day 90 ($p = 0.026$); CBC: No significant difference	The findings of the current study demonstrate that consumption of PSE 900 mg improved specific measures of reactive agility in a young, active population

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Gratton (2020) [124]	Cerebral cortical oxygenation and cognition improvement	Flavonoids	18 healthy men Mean age: 23.9 ± 7.3 years USA	Randomized, double-blind, placebo-controlled, crossover trial (two visits with a 2 week washout)	T: High flavanol (HF) intake cocoa drink comprising epicatechin 150 mg and catechin 35.5 mg; C: Low flavanol (LF) intake placebo drink delivering <4 mg of both monomers	Cortical hemoglobin concentration; flow-mediated dilatation (FMD); functional near-infrared spectroscopy (fNIRS); cerebral CO_2 reactivity (CCR); double-strop task (DST)	fNIRS: Blood oxygenation most evident in lateral frontal region; HF intake lead to earlier and larger response: significant interaction between intervention and latency of response reach 90% maximal oxygenation ($p = 0.002$); FMD: significant increase of 1% after HF intake ($p < 0.001$); DST: Significant difference between LF and HF conditions ($p = 0.029$); CCR: No significant improvement	Using dietary strategies containing plant-derived flavanols is useful for enhancement of blood oxygenation and cognitive performance in healthy populations, as well as for populations at higher risk of cognitive impairment or to help recovery from and treatment of brain injuries and disease
Grgic (2018) [125]	Muscle strength and power	Caffeine	294 individuals (51 female) Age: 16−34 years Global	Systematic review and meta-analysis of randomized, double-blind studies (N = 20)	T: Caffeine 4.3–6.5 mg/kg (caps, liquid, or gel). C: Placebo	Upper and/ or lower body exercise (muscle strength); monitor unit recruitment; vertical jump magnitude	Upper and lower body exercise: Caffeine improved both strength and power (standardized mean difference [SMD] = 0.20, 95% CI: 0.03, 0.36; $p = 0.023$; and SMD = 0.17; 95% CI: 0.00, 0.34; $p = 0.047$, respectively). A subgroup indicated caffeine significantly improved upper body exercise (SMD = 0.21; 95% CI: 0.02, 0.39; $p = 0.026$)	Meta-analyses showed significant ergogenic effects of caffeine ingestion on maximal muscle strength of upper body and muscle power. Future studies should more rigorously control the effectiveness of blinding (female group and different type of caffeine)

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Haskell-Ramsey (2018) [126]	Cognition and mood	Caffeine	59 individuals (29 female) split into two age groups: older (61–80 years; n = 30, 16 female) and younger (20–34 years; n = 29, 13 female) UK	Randomized, placebo-controlled, double-blind, counter-balanced crossover trial (three visits, 7 days of washout between each visit)	T1: Regular coffee (caffeine 100 mg) T2: Decaffeinated coffee (caffeine ≈5 mg) C: Placebo	Saliva sample; immediate word recallIWR; delayed word recall (DWR) and recognition; delayed picture recognition (episodic memory); numeric working memory (working memory); simple reaction time (SRT); digit vigilance (DV); rapid visual (RV; attention); subjective state (SStt)	Saliva sample: Confirmed adherence to caffeine abstention instructions, SRT decreased with regular coffee; RV: increased alertness compared with placebo ($p = 0.014$) and faster accuracy ($p = 0.009$); decaffeinated coffee increased alertness compared with placebo ($p = 0.0048$); DV: increased with regular coffee compared with decaffeinated coffee ($p = 0.01$); SStt: decreased tiredness ($p = 0.003$) and headache ratings were observed ($p = 0.0049$)	These findings suggest behavioural activity of coffee beyond its caffeine content, raising issues with the use of decaffeinated coffee as a placebo and highlighting the need for further research into its psychoactive effects
Imai (2018) [127]	Oxidative stress severe fatigue	Carotenoids	24 healthy volunteers (11 female) Age: 30–60 years Japan	Randomized, double-blind, placebo-controlled, two-way crossover trial (3-months with a 4 week washout between each period of supplementation)	T: 2 x capsules containing 3 mg of astaxanthin and 5 mg of sesamin (AS) (n = 12) C: Placebo (n = 12)	Visual analog scale (VAS); Calder fatigue questionnaire (daily subjective fatigue) [CFQ]; subjective feelings (SF); work efficiency (WE); autonomic nerve activity (ANA); OSB); safety; plasma AS concentration	CFQ: Significant improvement in time with AS vs. placebo from mental fatigue ($p < 0.01$); OSB: Significant decrease with AS vs. placebo ($p < 0.05$); VAS: Significant difference between AS vs. placebo group; Correlation coefficient between plasma AS and difference in VAS score: recovery 2 h-task 4 h was −0.451 ($p < 0.05$) and recovery 4 h-task 4 h was −0.502 ($p < 0.05$); SF and WE: No significant difference between AS and placebo; safety: no adverse effects	In conclusion, AS supplementation may be a candidate to promote recovery from mental fatigue which is experienced by many healthy people. Thus, antioxidative activity exhibited by AS could be a possible mechanism for its anti-fatigue effect

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Pilaczynska-Szczesniak (2005) [128]	Oxidative stress from an incremental rowing ergometer exercise	Anthocyanins	19 male athletes (rowing team members) Age: 20–24 years Poland	Randomized, double-blind, placebo-controlled trial (4 weeks)	T: Chokeberry juice (150 mL daily; 23 mg/100 mL of anthocyanins) (n = 9) C: Placebo (n = 10)	Redox; creatine kinase (CK); lactate levels (LA); thuibarbituric acid reactive substances (TBARS); super oxide dismutase (SOD); gluthanione peroxydase activity (GPx)	TBARS: Significantly lower with T group at 1 min ($p < 0.05$) and 24 h sample after exercise ($p < 0.05$); GPx: Significantly lower with T group at 1 min after exercise ($p < 0.05$); SOD: Significantly lower compared with placebo in the 24 h blood sample ($p < 0.05$); CK, LA, and redox: No significant result	These findings indicate that an increased intake of anthocyanins limits the exercise-induced oxidative damage to red blood cells, most probably by enhancing the endogenous antioxidant defense system
Lamport (2016) [129]	Cognitive function, driving performance, and blood pressure	Flavonoids and anthocyanins	25 healthy mothers Mean age: 43 ± 0.6 years UK	Double-blind, randomized, crossover, placebo-controlled trial (12 weeks with 4 weeks washout between each trial)	T: Concord grape juice 355 mL containing 777 mg total polyphenolics as a gallic acid equivalent (167 mg anthocyanins as malvidin equivalent and 334 mg proanthocyanidins as catechin equivalent) C: Placebo	Visual verbal learning test (immediate and delayed recall) (VVLT); visual spatial learning test (immediate and delayed recall) (VSLT); rapid visual information processing (RVIP); attention (psychomotor skill); blood pressure (systolic and diastolic) (BP); mood; driving performance (match speed and direction of a lead vehicle)	Immediate spatial memory (VVLT, VS.LT, RVIP; $p < 0.05$): Significant improvements main effect of condition recall was higher after Concord grape juice (mean: 12.72 items; standard error [SE]: 0.39) vs. placebo (mean: 12.57 items; SE: 0.36; $p < 0.05$); Driving performance: More accurate with Concord grape juice vs. placebo (mean correlation: 0.96; SE: 0.01; $p = 0.05$); Attention: Significantly higher with Concord grape juice ($p < 0.05$); mood and BP: No significant result	Cognitive benefits associated with the long-term consumption of flavonoid-rich grape juice are not exclusive to adults with mild cognitive impairment. Moreover, these cognitive benefits are apparent in complex everyday tasks such as driving. Effects may persist beyond the cessation of flavonoid consumption

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Mastroiacovo (2015) [130]	Cognitive performance	Flavonoids (flavanol)	90 elderly individuals (53 female) Age: 61–85 years Italy	Randomized, double-blind, controlled, parallel-arm study (8 weeks)	T1: High cocoa flavanols 993 mg (n = 30) T2: Intermediate cocoa flavanols 520 mg (n = 30) C: Low cocoa flavanols 48 mg (n = 30)	Mini-mental state examination (MMSE); trail making test (TMT) A and B; verbal fluency test (VFT); insulin resistance (IR); BP; lipid peroxidation (LP)	MMSE: No significant difference; TMT A and B: significant with high flavanols (-8.6 ± 0.4 and -16.5 ± 0.8 s, respectively) and intermediate flavanols (-6.7 ± 0.5 and -14.2 ± 0.5 s, respectively) differed from low flavanols (-0.8 ± 1.6 and -1.1 ± 0.7 s, respectively); VFT: Significant improvement in all group, but greater with high flavanols (7.7 ± 1.1 words/60 s) vs. intermediate flavanols (3.6 ± 1.2 words/60 s) and low flavanols (1.3 ± 0.5 words/60 s); IR ($p < 0.0001$), BP ($p < 0.0001$), and LP ($p = 0.001$) better with high flavanols and intermediate flavanols vs. low flavanols	Regular cocoa flavanols consumption can reduce some measures of age-related cognitive dysfunction, possibly through an improvement in insulin sensitivity. These data suggest that the habitual intake of flavanols can support healthy cognitive function with age
Duvnjak-Zaknich (2011) [131]	Agility performance and decision-making accuracy after simulated team-sport exercise	Caffeine	10 moderately trained male team-sport athletes Mean age: 21.6 ± 2 years Australia	Randomized, double-blind, counterbalanced trial (22 trials realized with a 1 week washout)	T: Caffeine (6 mg/kg) C: Placebo	Total time (TT); reactive agility time (RAT); decision time (DT); movement time (MT); decision-making accuracy	TT, RAT, MT, and DT: No interaction effect between trials (similar between time and conditions). Caffeine ingestion significantly increased TT (2.3%, $p = 0.001$), RAT (3.9%, $p = 0.001$), MT (2.7%, $p = 0.043$), and DT (9.3%, $p = 0.045$) vs. placebo	Caffeine ingestion may be beneficial to reactive agility performance when athletes are either fresh or fatigued

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Trombold (2010) [132]	Recovery of skeletal muscle strength after eccentric exercise	Tannins	16 recreationally active males Mean age: 24.2 ± 1.4 years USA	Double-blind, randomized, placebo-controlled crossover trial (two testing periods, each of 9 days, with a 14 day washout)	T: Pomegranate extract (POMx) 500 mL containing 650 mg of pomegranate (ellagitannins) C: Placebo (liquid)	IS (load cell); soreness (visual analog rank = subjective); BS: CK; myoglobin (Mb); interleukin 6 (IL-6); C-reactive protein (CRP)	IS: Significantly higher with POMx vs. placebo at 48 h ($p = 0.01$) and 72 h ($p = 0.009$) after exercise. BS (CK, Mb, IL-6, CRP): No significant changes	Supplementation with ellagitannins from POMx significantly improves recovery of IS 2–3 days after a damaging eccentric exercise
Whyte (2015) [133]	Cognitive performance	Anthocyanins	14 children (4 female) Mean age: 9.17 ± 0.6 years UK	Controlled and crossover trial (≈1 week with a washout of 7 days [minimum])	T: Flavonoid-rich blueberry (anthocyanins 143 mg) C: Placebo	Go-NoGo; ST; Rey's auditory verbal learning task (RAVLT); object location task (OLT); visual N-back (VNB)	Go-NoGo, ST, VNB, and OLT: No significant results (RT and accuracy responses). RAVLT: Significant results vs. placebo ($p < 0.001$)	Although findings were mixed, the improvements in delayed recall found in this pilot study suggested that, following acute flavonoid-rich blueberry interventions, school-aged children encoded memory items more effectively (vs. without)
Falcone (2019) [134]	Cognitive performance and nootropic effects	Phenolic acids	142 healthy, recreationally men and women (44 female) Age: 18–50 years USA	Randomized, double-blind, placebo-controlled, parallel design (90 days)	T: PSE 900 mg/day (n = 73) C: Placebo (n = 69)	FTT; SDC; complex attention (CA); ST; CPT; SAT; reasoning; sustained attention; four-part continuous performance test (FPCPT); Digi Span test; Leeds sleep evaluation questionnaire (LSEQ); quality of life (QoL); profile of mood states (POMS)	Sustained attention: Significant improvement with PSE at Day 30 ($p = 0.001$) and at Day 90 ($p = 0.007$); CA: Significant improvement with PSE at Day 7 ($p = 0.016$); SAT and FPCPT: Significant improvement in two individual tests vs. placebo ($p < 0.05$); QoL, POMS, and LSEQ: No significant difference with PSE vs. placebo	The current study demonstrates that chronic supplementation with PSE 900 mg improves cognitive performance in a young, active population, further supporting PSE as an efficacious nootropic

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Johnson (2008) [135]	Cognitive performance	Carotenoids	49 healthy, non-smoking women Age: 60–80 years USA	Randomized, double-blind intervention trial (4 months)	T1: Docosahexaenoic acid (DHA) 800 mg/d (n = 14) T2: Lutein 12 mg/day (n = 11) T3: Combination of DHA and lutein (n = 14) C: Placebo (n = 10)	Verbal fluency (VF); digit span forward and backward (DSFB); shopping list task (SLT); word list memory test (WLMT); memory in reality (MIR) apartment test; pattern comparison test (NES2 PC); ST; mood scale (NES2 MS)	VF: Significant improve of score for DHA, lutein, and combined treatment compared with placebo ($p < 0.03$); SLT: Learn significantly faster with the combined treatment ($p = 0.03$); trend for WLMT but not significant ($p = 0.07$); MIR (delayed recall): Significant improvement ($p = 0.02$); NES2 PC: Significant increase for the placebo group ($p = 0.04$); NES2 MS, ST, DSFB: No significant results	These exploratory findings suggest that DHA and lutein (carotenoid compounds) supplementation may provide cognitive benefits for older adults
Ataka (2007) [136]	Anti-fatigue effects	Tannins	18 healthy volunteers (9 female) Mean age: 39.1 ± 9.1 years Japan	Double-blind, randomized, placebo-controlled, three-way crossover trial (8 days)	T1: Applephenon® (1200 mg/day)—a rich procyanidins product T2: Ascorbic acid (1000 mg/day) C: Placebo	Physical performance test (PPT); subjective rate of fatigue level (visual analog from 0 to 100) (SRFL); BP; heart rate (HR); BS	PPT: No difference at baseline. Significant change in maximum velocity between 30- and 120-min trials: higher in the T1 group than C (rpm: +2, $p < 0.05$). T2 had no effect. SRFL, BP, HR, and BS: No significant differences	These results suggest that Applephenon® attenuates physical fatigue, whereas ascorbic acid does not

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Do Rosario (2021) [137]	Cognitive function	Anthocyanins	31 participants (9 female) with mild cognitive impairment (MCI) Mean age: 75.3 ± 6.9 years Australia	Randomized, double-blind, placebo-controlled trial (8 weeks)	250 mL fruit juice T1: High-dose anthocyanins (201 mg/day). T2: Low-dose anthocyanins (47 mg/day). C: Placebo	Microvascular function (MF), 24 h ambulatory blood pressure (ABP); serum inflammatory biomarkers (tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, c-reactive protein)	TNF-α: Significant reduction with T1 group ($p = 0.002$) vs. C and T2 ($p < 0.05$ for both). IL-6, IL-1β, c-reactive protein, MF, and 24 h ABP: Not altered by any treatment	A daily high dose of fruit-based anthocyanins for 8 weeks reduced concentrations of TNF-α in older adults with MCI. Anthocyanins did not alter other inflammatory biomarkers, microvascular function, or blood pressure parameters. Further studies with a larger sample size and longer period of follow-up are required to elucidate whether this change in the immune response will alter cardiovascular disease risk and progression of cognitive decline
Calapai (2017) [138]	Cognitive function	Anthocyanins	111 healthy older adults (58 female) Age: 60–72 years Italy	Randomized, double-blind, placebo-controlled trial (12 weeks)	T: Cognigrape® (250 mg/day: 30/40% of *V. vinifera* extract) (n = 57) C: Placebo (n = 54)	MMSE: temporal orientation, spatial orientation, immediate memory (IM), attention and calculation (AC), recall memory (RM), language, praxia visuo-constructive; Beck depression inventory (BDI); Hamilton anxiety rating scale (HARS); repeatable battery for the assessment of neuropsychological status (RBANS)	MMSE: Significantly improved with T ($p < 0.0001$) at baseline and vs. C group (AC ($p < 0.001$), language ($p < 0.05$), IM ($p < 0.0001$)). BDI, HARS: Significant reduction of 15.8% (BDI), and 24.9% (HARS) ($p < 0.0001$) and vs. C ($p < 0.0001$ for BDI and $p < 0.05$ for HARS). RBANS: Significantly improved with T at baseline and vs. placebo ($p < 0.0001$)	The results show that 12 weeks of Cognigrape® supplementation is safe, can improve physiological cognitive profiles, and can concurrently ameliorate negative neuropsychological status in healthy older adults

Flavonoids have been evaluated with respect to cognitive problems and show improvement in several studies [117,118,124,129,130]. In 2015, Mastroiacovo et al. evaluated the effect of daily flavanol consumption on cognition in a randomized controlled trial of 90 elderly patients [130]. Their results showed that daily consumption of a flavonoid-rich beverage (993 mg/day) for 8 weeks significantly improved results obtained during exercises assessing cognitive function, particularly the trail making test and the verbal fluency test. These effects were, according to the authors, attributable to the antioxidant and neuroprotective properties of flavonoids, associated with their capacity to improve cerebral perfusion by action on NO-dependent endothelial function. Another randomized, controlled trial conducted in 2016 by Alharbi et al. evaluated the immediate cognitive effects of flavonoid consumption [117]. In this study, volunteers who consumed 240 mL of a beverage containing 272 mg of flavonoids showed a significant improvement in their verbal skills and reflexes 6 h after consumption versus placebo ($p < 0.05$). According to the authors, the cognitive benefits were also supported by the antioxidant properties of flavonoids which improve vascular functions and increase NO bioavailability, leading to an increase in cerebral vascular flow. In 2020, another randomized, controlled clinical trial demonstrated that the consumption of flavonoids, particularly flavanols, lead to increased cerebral oxygenation, resulting in a significant improvement of cognitive performance versus placebo [124]. Strength loss and neuromuscular impairment also significantly improved compared with placebo ($p < 0.05$), with a daily consumption of 1 g of quercetin for 14 days resulting in the preservation of muscle mass confirming the antireactive oxygen species (ROS) properties of flavonoids [118]. The work of Lamport et al. evaluating the effect of the consumption of 777 mg of flavonoids for 12 weeks on cognitive performance demonstrated a significant improvement in spatial memory ($p < 0.05$) as well as driving performance ($p = 0.05$) when associated with the simultaneous intake of 334 mg of proanthocyanins and 167 mg of anthocyanins [129].

Anthocyanins alone have been shown to be effective in numerous clinical studies in this area of health, with a marked effect on improving physical performance [116,122,128]. A randomized, controlled trial involving 19 healthy subjects studied the effect of daily supplementation with 35 mg of anthocyanins for one month on recovery from physical performance [128]. At the end of the supplementation period, blood levels of cellular oxidation markers such as glutathione peroxidase, superoxide dismutase, and thiobarbituric acid reactive substances were significantly decreased versus placebo ($p < 0.05$ for all). The authors concluded that anthocyanins have a protective effect on oxidative stress in red blood cells, possibly by increasing the endogenous antioxidant defense system. In 2015, another team evaluated the effect of a daily intake of 27.6 mg of anthocyanins from an açai extract on the physical performance and blood biomarkers of 14 athletes [116]. The study demonstrated that anthocyanins increased the time to exhaustion during high intensity exercise ($p = 0.045$) and decreased the metabolic stress caused by exercise ($p < 0.05$), along with decreasing the intensity of perceived exertion and improving cardiorespiratory responses ($p < 0.05$). According to the authors, these effects were due to the antioxidant properties of anthocyanins, which reduce oxidative stress during exercise. The efficacy of anthocyanins on physical performance was also demonstrated in another randomized, controlled clinical trial conducted by Cook et al. in 2017 which evaluated the effects of a 7 day supplementation of a blackcurrant extract containing 210 mg of anthocyanins [122]. The authors reported that the supplement significantly improved cardiovascular capacity by causing vasodilation and a decrease in muscle oxygen saturation, along with an increase in hemoglobin levels.

Anthocyanins have shown therapeutic benefits in the field of cognition [120,133,137,138]. A 2015 pilot study by Whyte et al. involving 14 children aged 8–10 years of age described how daily supplementation for 7 days with a blueberry drink containing 143 mg of anthocyanins improved response time assessed by the Rey Auditory Verbal Learning Test compared with placebo ($p < 0.001$), but did not improve visuospatial memory or attention [133]. Another study evaluating the effect of a 12 week supplementation with 387 mg of anthocyanidins on

the cognitive performance of elderly subjects also concluded that this family of phytonutrients had beneficial effects [120]. The results indicated a significant increase in brain activity ($p < 0.001$) and a significant increase in gray matter perfusion in the parietal ($p = 0.013$) and occipital ($p = 0.031$) lobes following supplementation. A significant improvement in working memory vs. placebo ($p = 0.05$) was also reported. The authors associated these clinical effects with an improvement in cerebral vascular function induced by anthocyanins. Beneficial effects on the maintenance of cognitive functions in elderly patients with mild cognitive impairment were also reported in a double-blind, randomized, placebo-controlled trial conducted by Do Rosario in 2021 [137]. The effects of a drink (250 mL/day) containing 201 or 47 mg of anthocyanins were evaluated on vascular functions and circulating inflammatory markers. The results suggested a significant decrease in blood TNF-α levels compared with placebo for both doses tested with no change in other parameters; these results were consistent with a decrease in the decline of cognitive function. The effect of anthocyanins on cognitive parameters in an elderly population was evaluated by Calapai et al. [138]. In this randomized, double-blind, placebo-controlled study, the effect of supplementation with 250 mg/d of anthocyanins for 12 weeks was evaluated with a battery of cognitive tests. The results showed a significant improvement in attention ($p < 0.001$), language ($p < 0.05$) and memory ($p < 0.0001$) with anthocyanins compared with placebo, as well as a decrease in anxiety ($p < 0.05$) and depression ($p < 0.0001$) scores, which could be explained by their antioxidant, anti-inflammatory, and antiapoptotic properties.

The action of phenolic acids has been studied in this area of health and this family of phytonutrients presents beneficial effects on agility [119,123,134]. A randomized, controlled study by Falcone et al. evaluated the effects of supplementation with a spearmint extract rich in phenolic acids (900 mg/d) versus placebo in 142 adults for 7–90 days [123]. The study demonstrated an overall significant effect on reactive agility ($p = 0.049$) and, more specifically, a beneficial effect on the stationary test ($p = 0.04$ at Day 30 and $p = 0.002$ at Day 90), reaction time ($p = 0.049$ at Day 30), and accuracy ($p = 0.007$ at Day 30 and $p = 0.026$ at Day 90), which was attributed to the cerebral anti-inflammatory properties of phenolic acids. Another spearmint extract has also shown beneficial effects on cognition in a study published in 2019 by the same team [134]. Young adults received 900 mg of a spearmint extract or placebo daily for 90 days and a battery of cognitive tests involving sleep, mood, and QoL were performed on Day 0 and after 7, 30, and 90 days of treatment. The results indicated a significant improvement in attention with the spearmint extract compared with placebo after 30 days ($p = 0.001$) and 90 days ($p = 0.007$) without significant improvement in the other parameters measured. Another randomized, controlled study evaluated the effect of supplementation with 300 mg of chlorogenic acid for 16 weeks on the cognitive functions of 38 subjects with memory problems [119]. The authors reported that chlorogenic acid significantly improved certain cognitive functions, particularly attention, motor speed, psychomotor speed, and executive function compared with placebo. This effect could be linked to an effect of the phytonutrient on the synthesis of apolipoprotein A1 and transthyretin, two markers associated with early cognitive decline.

Tannins have shown therapeutic interest in the area of energy and vitality as they have been shown to reduce fatigue and improve physical performance [132,136]. In a randomized, controlled study conducted in 2007, the daily intake of a tannin-rich extract (1200 mg) for 8 days resulted in a better resistance to fatigue induced by physical exercise compared with placebo ($p < 0.05$) without modification of cardiovascular parameters [136]. Other work conducted in 2010 by Trombold et al. also demonstrated that consumption of a pomegranate extract rich in ellagitannins (650 mg) for 9 days significantly increased muscle strength recovery 2 to 3 days after an eccentric elbow flexion exercise compared with placebo [132]. The authors suggested that the antioxidant properties of tannins may limit the production of free radicals and ROS during exercise and act as the source of the therapeutic benefits of these phytonutrients.

Carotenoids are also of interest in the area of health, particularly for problems related to fatigue and recovery [121,127,135]. A randomized, controlled clinical trial conducted

by Imai et al. in 2018 evaluated the benefit of the antioxidant properties of carotenoids in the management of fatigue [127]. Their work consisted of evaluating the effects of supplementation with 3 mg astaxanthin and 5 mg sesamin for 4 weeks on mental fatigue. At the end of the treatment, they observed a significant decrease in mental fatigue versus placebo ($p < 0.05$) and a lower increase in circulating levels of phosphatidylcholine hydroperoxide ($p < 0.05$), a marker of oxidative stress. The authors attribute these results to the significant antioxidant properties of the carotenoids evaluated. A double-blind, randomized, controlled trial involving 2983 subjects evaluated the long-term cognitive effects of a diet rich in carotenoids over a period of 8 years [121]. The results indicated that this type of diet was associated with an improvement in the composite cognitive score ($p = 0.002$) as well as in six neuropsychological tests: cued recall task, backward digit span task, trail making test, and semantic fluency task compared with a diet less rich in carotenoids. Once again, the antioxidant and anti-inflammatory properties of carotenoids were thought to explain these therapeutic benefits. Another study conducted by Johnson et al. in 2008 evaluated the effect of lutein supplementation on cognition and more particularly on memory and speech [135]. This study demonstrated an improvement in verbal fluency with a daily treatment of 12 mg of lutein compared with placebo ($p < 0.03$), but no positive effect on memory. Nevertheless, the authors reported a significant improvement in memory and learning level when lutein was associated with docosahexaenoic acid ($p < 0.03$), validating the therapeutic interest of combining phytonutrients.

The most well-known phytonutrient in the field of cognition and vitality is caffeine. Numerous scientific studies have reported that this phytonutrient acts on the problems of fatigue, improves concentration and physical performance and facilitates recovery [115,125,126,131].

A recent randomized, controlled trial evaluated the short-term effects on cognition and mood of caffeinated and non-caffeinated black coffee compared with placebo in 59 volunteers [126]. The results suggested that while decaffeinated coffee increased alertness versus placebo, only coffee containing 100 mg of caffeine significantly improved accuracy and reduced fatigue and headaches 30 min after ingestion; significant differences were reported versus both placebo and decaffeinated coffee. These effects could be attributed to the ability of caffeine to antagonize A1 and A2A adenosine receptors, thus increasing oxygen metabolism and increasing the synthesis of neurotransmitters such as noradrenaline, dopamine, serotonin, and GABA. Another study conducted in 2014 by Borota et al. reported beneficial effects of caffeine on memory function [115]. This randomized, controlled study in 160 subjects demonstrated an improvement in memory performance up to 24 h after coffee consumption with an inverted U-shaped dose response effect. The authors concluded that this effect was compatible with a consolidation of long-term memory and could be due to an inhibition of norepinephrine via direct blocking of adenosine by caffeine or an effect of caffeine in the CA2 area of the hippocampus.

In terms of improving physical performance, caffeine is often used by athletes as an ergogenic aid. A 2018 review evaluated 20 clinical studies involving 294 subjects to investigate the effects of caffeine on muscle strength and power [125]. The results of this analysis indicated that caffeine significantly improved strength ($p = 0.023$) and muscle power ($p = 0.047$), particularly in the upper extremities. A randomized, controlled trial by Duvnjak-Zaknich et al. evaluated the effect of caffeine 6 mg/kg or placebo administered 60 min before a team sport session (80 min duration) on performance [131]. Intermediate exercises of reactivity, agility, and decision making were conducted. The study demonstrated that caffeine significantly improved most of the parameters measured versus placebo (total time, $p = 0.001$; reactive agility time, $p = 0.001$; decision time, $p = 0.045$; movement time, $p = 0.043$). The mechanism of action involved was thought to be related to a blocking of adenosine receptors by caffeine, leading to stimulation of the CNS [131].

All the therapeutic benefits of phytonutrients are based on the same mechanisms of action described in the previous paragraphs and are mainly based on their antioxidant and anti-inflammatory activities.

4. Conclusion and Perspectives

This analysis of the literature allows highlighting of the significant benefits of phytonutrients in the prevention and management of pathologies and symptoms in the field of family health. To date, natural healthcare has primarily been based on phytotherapy, which consists of using the therapeutic properties of so-called medicinal plants to prevent or treat certain pathologies [139]. Phytonutrition is concerned with the action of molecules derived from plants that can be integrated into a balanced diet with beneficial effects on health. The phytonutritional approach is positioned at the interface between phytoaromatherapy and nutrition, constitutes an original and innovative breakthrough, and provides a source of reflection for the field of phytotherapy. Phytonutrition adds to existing data on medicinal plants and may open up new areas of therapeutic activity for some of them. In addition, phytonutrition sheds additional light on the classical mode of action of plants and their active ingredients, and could become a discipline in its own right in the near future.

This review assessed the seven largest families of phytonutrients found in food and the diet [9] and demonstrated that each of them had significant therapeutic potential in the healthcare field. Moreover, this evaluation also enabled complementary effects of the different families of phytonutrients in the same area of health to be recognized. Nevertheless, there are many other phytonutrients that were not included in this review of the literature. Similarly, our analysis focused on healthcare, but it is clear that phytonutrients also play an important role in the prevention of serious chronic diseases such as diabetes, obesity, and hypertension, along with different types of cancer or degenerative diseases [21,78,83,140]. Thus, it would be worthwhile to further investigate the mechanisms of action of phytonutrients associated with these effects in chronic diseases.

To the best of our knowledge, this review provides a deeper analysis of the potential benefits of phytonutrients in human healthcare. A phytonutrient-based approach appears to provide an innovative way to address natural health and could be a useful additional therapeutic option for physicians.

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Exposure to (Poly)phenol Metabolites after a Fruit and Vegetable Supplement Intake: A Double-Blind, Cross-Over, Randomized Trial

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Abstract: Dietary (poly)phenol intake derived from the daily consumption of five portions of fruits and vegetables could protect against the development of non-communicable diseases. However, the general population does not meet the recommended intake. Supplementation with (poly)phenol-rich ingredients, within a varied and balanced diet, could help in filling this nutritional gap. This study aimed to validate the proof-of-concept of a (poly)phenolic supplementation developed to enhance the daily consumption of potentially bioactive compounds. Oxxynea® is a (poly)phenol-rich ingredient developed to provide the quantity and the variety corresponding to five-a-day fruit and vegetable consumption. In this double-blind, randomized cross-over study, 10 participants were supplemented with 450 mg of a (poly)phenol-based supplement or a placebo. Pharmacokinetics and urinary excretion profiles were measured for 24 and 48 h, respectively, using UPHLC-MS/MS analysis. The pharmacokinetic profile displayed a triphasic absorption, indicating peaks of circulating metabolites at 1.75 ± 0.25 h, 4.50 ± 0.34 h, 9.50 ± 0.33 h and an average T_{max} (time of maximal plasma concentration) of 6.90 ± 0.96 h. Similarly, the urinary profile showed maximum metabolite excretion at 3–6 h, 6–10 h and 14–24 h after supplement consumption. Compared to individual metabolites belonging to different (poly)phenolic subfamilies, the total circulating and excreted metabolites showed a reduced coefficient of variation (CV 38%). The overall bioavailability estimated was 27.4 ± 3.4%. Oxxynea® supplementation may provide a sustained exposure to several (poly)phenolic metabolites and catabolites and reduces the inter-individual variation that could arise from supplementing only one class of (poly)phenol.

Keywords: (poly)phenol-based supplement; pharmacokinetics; urinary excretion; bioavailability; inter-individual variability

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1. Introduction

The recognized protective effect of fruit and vegetable consumption on the incidence of non-communicable diseases (NCDs) such as cardiovascular diseases, type 2 diabetes mellitus and cancer has been confirmed in several recent meta-analyses [1–3]. According to the latest Global Burden of Disease study (GBD) [4], worldwide, 3.9 million deaths and more than 95 million disability-adjusted life-years (DALYs) were attributable in 2017 to a diet low in fruits and vegetables.

The health benefits of fruit and vegetable consumption have been ascribed to different bioactive nutrient molecules, such as dietary fibers, vitamins and minerals, and more recently, to non-nutritive phytochemicals, namely phenolic compounds, which are the most prominent and investigated substances [5]. Phenolics are of particular interest, as

substantial epidemiological evidence shows inverse associations between their regular consumption and the incidence of certain NCDs [6–8].

Evidence generated through both animal and human intervention studies shows that the protective effects of phenolic compounds on NCDs may arise from several complementary mechanisms of the actions of (poly)phenols, including their capability to regulate gene expression, signaling pathways, metabolism and physiological responses [9,10].

However, it is noteworthy that despite the amount of evidence of the health benefits of phenolic compounds, which have even been discussed as "lifespan essentials" [11], and unlike vitamins and minerals, no dietary reference intake (DRI) has been set so far. The difficulty stems from the considerable diversity of phenolic compounds as, to date, more than 50,000 different phenolic structures have been identified [12]. Such structural variety is a determinant for their digestive metabolism and further absorption. Indeed, some compounds may be absorbed in the small intestine, where phase II enzymes metabolize them before entering systemic circulation [13], while the remaining unabsorbed compounds enter the large intestine, where they are degraded by the colonic microbiota, generating postbiotics of which a portion will, in turn, be absorbed and enter into the circulatory system [14]. Moreover, food matrix diversity and interindividual variabilities are also considered essential factors in pathways and the degrees of absorption of phenolics. Consequently, there is still a matter of intensive research to understand the correlation between the absorption of phenolic metabolites and their bioactivity. Thus, studying pharmacokinetics in systemic circulation and their pattern of urinary excretion is essential to have a comprehensive overview for the basis of their in vivo benefits.

Taking this complexity into account, to date, best (poly)phenol-related dietary guidelines have been established on the adequate consumption of fruits and vegetables. The World Health Organization (WHO) recommends a minimum daily intake of 400 g of fruits and vegetables [15]. Moreover, the WHO has stated that this intake must be varied at each meal to ensure an adequate intake of nutrients, including phenolic compounds [16]. In terms of (poly)phenol consumption, it is intriguing to hypothesize whether one of the rationales behind this recommendation is to allow sustained exposure to phenolic metabolites in circulation throughout the day. Based on these guidelines, the "5-a-day" fruit and vegetable campaign recommends consuming a varying number of 75–80 g servings of fruits and vegetables daily. Such guidelines are supported by several dose-response meta-analyses [1,17,18], among which the most recent included about 1.9 million participants worldwide [18]. The authors observed that the intake of five varied servings per day of fruits and vegetables was associated with lower mortality and that risk reduction plateaued at this daily amount.

It is noteworthy that 10 years after the start of the "5-a-day" promotion campaigns, the number of estimated deaths worldwide attributable to low fruit and vegetable consumption has doubled [19]. Recent reports aimed at examining the major fruit and vegetable campaigns and interventions conducted worldwide over the last 10 years concluded that the daily intake of fruits and vegetables remained well below the recommended WHO levels in both developed and developing countries [20,21].

Based on such observations, Oxxynea® has been developed based on the "5-a-day" recommendations in terms of phenolic compound intake from a diversity of fruits and vegetables, as a possible strategy to increase the daily consumption of potentially bioactive compounds in the framework of a varied and balanced diet. In this regard, the botanical extracts have been selected for (1) their phenolic content, comparable to the phenolic quantity brought by the consumption of five fruits and vegetables a day; (2) their diversity in terms of phenolic structure varieties. A key criterion was the potential of enhancing the benefit derived from consuming a complex variety of fruits and vegetables at each meal that would allow a sustained release of a plethora of (poly)phenol metabolites in circulation throughout the day, having metabolites usually appearing at different times. This study was planned to overcome the consumption of a single variety of (poly)phenols and, accordingly, support a potential multi-phasic absorption.

Thus, this study aimed to validate the proof-of-concept of a (poly)phenolic supplementation developed to enhance the daily consumption of potentially bioactive compounds. The bioavailability of phenolic compounds following Oxxynea® intake was evaluated through the absorption profiles and the urinary excretion of the supplemented (poly)phenols.

2. Materials and Methods

2.1. Development of a Nutritional Supplement Containing 5 Servings of Fruit and Vegetable (Poly)phenols

The ingredient, namely Oxxynea®, developed by FYTEXIA (France), is a food supplement that boasts a total (poly)phenol content comparable to the 5 most consumed fruits and vegetables [22], including apple, tomato, banana, orange and carrot.

The supplement was principally obtained by water/ethanol extraction of olive leaf (*Olea europea* L.), grape seed (*Vitis vinifera* L.), pomegranate (*Punica granatum* L.), grapefruit (*Citrus paradisi* Macfad.), orange (*Citrus sinensis* L. Osbeck) and by water extraction of green tea (*Camellia sinensis* L. Kuntze), bilberry (*Vaccinium myrtillus* L.) and white and red grape pomace (*Vitis vinifera* L.).

2.1.1. Total Phenolic Content

The total phenolic content of the supplement was determined using the Folin–Ciocalteu method adapted to fruit and vegetable (poly)phenol quantitation according to Brat et al. [23], using gallic acid as the standard. The total phenolic content of the 5 selected benchmark servings of fruits and vegetables was calculated based on Brat et al. quantifications [23].

2.1.2. Antioxidant Capacity

Both total antioxidant defences and total antioxidant reserves of the supplement were monitored in fresh heparinized blood using the KRL (Kit Radicaux Libres) and RESEDA (RESErves Défenses Antioxydantes) tests (Spiral Laboratories, Couternon, France), as previously described [24]. Briefly, the KRL test allows the evaluation of red blood cell resistance against 2,2′-azobis (AAPH)-induced free radicals and is measured as the time needed to haemolyze 50% of the red blood cells. The RESEDA test is based on the same principle, with a step of releasing biologically active and potentially antioxidant molecules in reserve. The KRL and RESEDA tests were also applied to the (poly)phenol fraction of a blend of 400 g of the 5 fruit and vegetable selected benchmark.

2.1.3. HPLC Analysis of Phenolic Compounds in the Supplement

Phenolic compounds within the supplement were analyzed using two different analytical methods. The first approach corresponds to an internal HPLC (High-Performance Liquid Chromatography) analysis targeting a few key compounds to check for and ensure reproducibility between different production batches (quality control). Secondly, an exhaustive characterization and quantification of phenolic compounds from the supplement provided during the clinical trial was performed through ultra-High-Performance Liquid Chromatography (uHPLC) coupled with mass spectrometry (MS).

The HPLC internal method was carried out employing an Agilent HPLC 1260 apparatus (software Openlab CDS chemstation edition) coupled with a diode array detector. Separations were carried out by means of a Zorbax Stablevond SB-C18 column (4.6 × 1.5 mm; 5 µm particle size). In order to detect different phenolic classes, three different analytical methods were adopted: one for flavan-3-ols, flavanones and secoiridoids and phenylethanoids, one for anthocyanins and one for ellagitannins.

Regarding flavan-3-ols, flavanones and secoiridoids and phenylethanoids, mobile phase A consisted of 100% water, mobile phase B was 100% acetic acid and mobile phase C was 100% acetonitrile. The linear gradient program was used as follows: (a) 0 to 5 min 94% A and 6% B; (b) 5 to 10 min 82.4% A, 5.6% B and 12% C; (c) 10 to 15 min 76.6% A, 5.4% B and 18% C; (d) 15 to 25 min 67.9% A, 5.1% B and 27% C; (e) 25 to 30 min 65% A, 5% B and 30% C; (f) 30 to 40 min 100% C; (g) 40 to 45 min 64% A and 6% B. Monitoring was performed at 280 nm at a

flow rate of 1 mL/min and injection volume of 25 µL. Flavan-3-ols were expressed as naringin or hesperidin, flavanones as catechin, epigallocatechin gallate epicatechin or epicatechin gallate equivalents and secoiridoids were expressed as oleuropein.

For anthocyanin analysis, mobile phase A was 100% water, mobile phase B consisted of 100% formic acid and mobile phase C was 100% acetonitrile. The linear gradient program was set as follows: (a) 0 to 5 min 84.2% A, 10% B and 5.8% C; (b) 5 to 20 min 77.6% A, 10% B and 12.4% C; (c) 20 to 35 min 68.2% A, 10% B and 21.8% C; (d) 35 to 40 min 58.8% A, 10% B and 31.2% C; (e) 40 to 45 min 44.7% A, 10% B and 45.3% C; (f) 45 to 50 min 44.7% A, 10% B and 45.3% C; (g) 50 to 60 min 40% A, 10% B and 50%C; (h) 60 to 65 min 84.2% A, 10% B and 5.8% C. Monitoring was performed at 520 nm at a flow rate of 0.8 mL/min and injection volume of 10 µL. Anthocyanins were expressed as kuromanin equivalent.

Concerning ellagitannins, mobile phase A was 100% water, mobile phase B was 100% phosphoric acid and mobile phase C was 100% methanol. The linear gradient program was: (a) 0 to 5 min 94.9% A, 0.1% B and 5% C; (b) 5 to 15 min 89.9% A, 0.1% B and 10% C; (c) 15 to 30 min 69.9% A, 0.1% B and 30% C. Monitoring was performed at 260 nm at a flow rate of 0.8 mL/min and injection volume of 5 µL. Ellagitannins were expressed as punicalagin equivalent.

Regarding exhaustive characterization of phenolic compounds from the supplement, the dried powder was firstly extracted in triplicate, as reported by Mena et al. [25]. Briefly, 200 mg of powder was extracted with 1 mL of 80% aqueous methanol acidified with formic acid (1%) and sonicated for 25 min. The mixture was centrifugated at 12,000 rpm for 10 min at room temperature, and the supernatant was collected. Two additional extractions were performed for each sample with an extra 0.5 mL of the same solvent, as described above, after which they were centrifugated. The three supernatants were pooled before uHPLC-MSn analysis.

The methanolic extract was analyzed using an Accela uHPLC 1250 apparatus equipped with a linear ion trap (LIT) MS (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated ESI (H-ESI-II) probe (Thermo Fisher Scientific Inc.). Separation was carried out by means of a Restek C18 (100 × 2.1 mm) column, 3 µm particle size (Restek Corporation, Bellefonte, PA, USA).

Based on previous works [26,27], anthocyanins were detected in positive ionization mode, with mobile phase, pumped at a flow rate of 0.3 mL/min, consisting of a mixture of acidified acetonitrile (0.1% formic acid) (solvent A) and 0.1% aqueous formic acid (solvent B). Following 0.5 min of 5% solvent A in B, the proportion of A was increased linearly to 51% over a period of 8.5 min. Solvent A was increased to 80% in 0.5 min, maintained for 2 min, then the start condition was re-established in 0.5 min and maintained for 5 min to re-equilibrate the column (total run time: 17 min). The H-ESI-II interface was set to a capillary temperature of 275 °C, and the source heater temperature was 300 °C. The sheath gas (N2) flow rate was set at 40 (arbitrary units), and the auxiliary gas (N2) flow rate was at 5. During anthocyanin analysis, the source voltage was 4.5 kV, and the capillary and tube lens voltage were 20 and 95 V, respectively.

For all other phenolic classes, spectrometric analyses were performed in negative ionization mode. Maintaining the same chromatographic gradient, the H-ESI-II interface was set to a capillary temperature of 275 °C, and the source heater temperature was 250 °C. The sheath gas (N2) flow rate was set at 60 (arbitrary units), and the auxiliary gas (N2) flow rate at 15. The source voltage was 4 kV, the capillary voltage was −49 V and the tube lens voltage was −153 V. A preliminary analysis of 5 µL of samples was carried out using full-scan, data-dependent MS3 analysis, to unequivocally identify the aglycones, scanning from a mass to charge (m/z) range of 100–900 for anthocyanins and from m/z range 100–2000 for the negative analysis, using a collision-induced dissociation (CID) equal to 35 (arbitrary units) to obtain fragmentation.

Data processing was performed using Xcalibur software from Thermo Scientific (Thermo Fisher Scientific Inc.). All compounds were quantified through external cali-

bration with commercial standards, when available, or with the most structurally similar reference compound (Supplemental Table S1).

Chemicals and reagents used for characterization are available in Supplemental material.

2.2. Pharmacokinetics and Urinary Excretion

2.2.1. Subjects

Ten healthy participants were recruited from the region of Murcia in southern Spain to participate in the study, in February 2018. Both men and women, 18 years old or older, having a normal body mass index (BMI) range (18.50–24.99 kg/m^2), were included in the study. Subjects were excluded if they had a metabolic disorder or any kind of disease, were using medication or food supplements, were pregnant or breastfeeding, were smokers or had an allergy to any component of the supplement.

The study was approved by the UCAM (Universidad Católica San Antonio de Murcia, Spain) Ethics Committee (EC-number: CE111701) and conducted per guidelines laid out in the Declaration of Helsinki [28] and in compliance with Good Clinical Practices defined in the ICH Harmonized Tripartite Guideline [29]. All participants were informed about the study procedures, agreed to adhere to lifestyle modifications throughout the course of the study and signed written informed consent before entering the study. This trial was registered at clinicaltrials.gov as NCT03432104.

2.2.2. Study Design

This study was designed as a 3-week randomized, double-blind, cross-over clinical trial. Eligible participants were randomized using simple block randomization of 1:1. Once enrolled, subjects received either one 450 mg capsule of the test supplement or one 450 mg capsule of placebo on the first day of the study (Phase I: D1). After three days of sampling, volunteers started a wash-out period of 1 week and were then enrolled for the second test Phase with ingestion of the opposite supplement (Phase II: D1) (Figure 1). The placebo product was 100% maltodextrin, which was (poly)phenol-free.

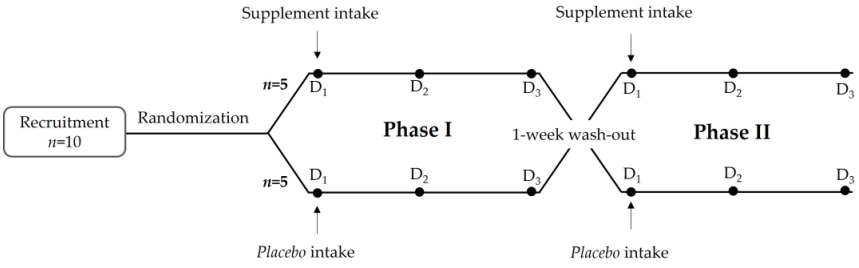

Figure 1. General flow diagram of the study.

Both the supplement and the placebo were supplied in a 450 mg capsule of identical opaque appearance and flavor.

Two days before the supplementation and during both Phase I and Phase II, volunteers were asked to consume a (poly)phenol-free diet. In order to facilitate participant adherence to this dietary restriction, the Research Center provided meals and snacks for the whole period, as well as a list of permitted and forbidden foods in order to avoid any deviations. Additionally, volunteers were asked to abstain from physical exercise for four days before the beginning of the study and throughout the study. Physical activity abstinence was monitored by an accelerometry device, the Fitbit® activity tracker (Fitbit Inc., San Francisco, CA, USA).

2.2.3. Plasma Pharmacokinetics

On the day of the test (D1), volunteers visited the Research Center after an overnight fast. Blood was collected at T0 to determine baseline levels of plasma phenolic metabolites. Then, volunteers were invited to take the 450 mg capsule of the supplement or the placebo

with 250 mL of still water (Figure 2). Additional blood samples were then collected at 1 h (D1, T1), 2 h (D1, T2), 3 h (D1, T3), 4 h (D1, T4), 5 h (D1, T5), 6 h (D1, T6), 8 h (D1, T8), 10 h (D1, T10) and 24 h (D2, T24) after capsule intake. Blood was taken from the basilica vein and centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was immediately extracted and frozen at −80 °C for further analysis.

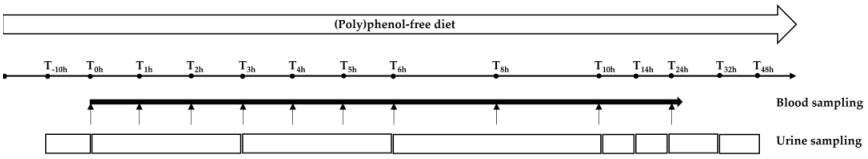

Figure 2. Blood and urine collection time during the 48 h period of both phase I and phase II.

Plasma samples were then extracted using a solid phase extraction (SPE) method, as previously reported by Castello and colleagues [30]. Briefly, 350 µL of plasma samples was diluted (1:1) with phosphoric acid 4% (*v*/*v*). After plate activation, 600 µL of the diluted plasma samples was loaded on a 96 well µ-SPE HLB (Oasis® HLB µElution Plate 30 µm, Waters, Milford, Massachusetts, MA, USA). Samples were washed with 200 µL of water and 200 µL of 0.2% (*v*/*v*) acetic acid. Finally, samples were eluted with 60 µL of methanol for UHPLC-ESI-MS/MS analysis.

2.2.4. Urinary Excretion

Urine samples were collected the day before (baseline) and during the 48 h period following the supplementation (T0-T3, T3-T6, T6-T10, T10-T14, T14-T24, T24-T32 and T32-T48) (Figure 2). Urine volumes at each collection period were registered, and samples were aliquoted and stored at −80 °C until further processing.

Then, urine samples were prepared as previously reported by Brindani and colleagues [31]. Briefly, urine samples were defrosted, vortexed, diluted in 0.1% formic acid in water (1:4, *v*/*v*) and centrifuged at 12,000 rpm for 5 min. Finally, urine samples were filtered (0.45 µm nylon filter) prior to UHPLC-ESI-MS/MS analysis.

2.2.5. Plasma and Urine UHPLC-ESI-MS/MS Analysis

Plasma and urine samples were analyzed by a UHPLC DIONEX Ultimate 3000 equipped with a triple quadrupole TSQ Vantage (Thermo Fisher Scientific Inc) fitted with a heated ESI (H-ESI) (Thermo Fisher Scientific Inc) probe, as previously reported [31]. Separations were carried out by means of a Kinetex EVO C18 (100 × 2.1 mm) column, 2.6 µm particle size (Phenomenex, Torrance, CA, USA), installed with a pre-column (Phenomenex). Mobile phase A was 0.2% formic acid in water, and mobile phase B was acetonitrile containing 0.2% formic acid. The gradient started with 5% B, and isocratic conditions were maintained for 0.5 min, reaching 95% B after 6.5 min, followed by 1 min at 95%. The starting gradient was then immediately re-established and maintained for 5 min to re-equilibrate the column. The flow rate was 0.4 mL/min, the injection volume was 5 µL and the column temperature was set at 40 °C.

The applied MS method consisted of the selective determination of each target precursor ion by the acquisition of characteristic product ion in selective reaction monitoring (SRM) mode (Supplemental Table S2). For all the analyses, the spray voltage was set at 3 kV, the vaporizer temperature at 300 °C and the capillary temperature operated at 270 °C. The sheath gas flow was 50 units, and auxiliary gas pressure was set to 10 units. Ultra-high purity argon gas was used for collision-induced dissociation (CID). The S-lens values were defined for each compound based on infusion parameter optimization. Conversely, for those compounds not available for infusion, the S-lens values were set using the values obtained for the chemically closest available standards. Quantification was performed with calibration curves of standards when available or using the most structurally similar

compound. Data processing was performed using Xcalibur software (Thermo Scientific Inc., San Jose, CA, USA).

Chemicals and reagents used for metabolite and catabolite quantification are available in Supplemental material.

2.2.6. Data Analysis

All data were expressed as mean values ± standard deviation (SD) and mean values ± standard error to the mean (SEM) for metabolites in blood and urine samples. PKSolver add-on program [32] in Microsoft Excel was used to determine pharmacokinetic parameters of blood phenolic metabolites, including time to reach the maximum plasma concentration (Tmax, h), maximum plasma concentration (Cmax, nmol/L), area under the curve (AUC0-24, nmol/L·h), and time of presence of metabolites in circulation (Tpres, h). Tpres was calculated by taking the time interval between the first appearance and the last appearance of the considered metabolite. Coefficient of variation (CV) was evaluated for each metabolite and multivariate principal component analysis (PCA) was applied to explore the inter-individual variation in the urinary metabolites among the participants. Principal component analysis was performed in R software (v 4.1) using the package mixomics (v 6.16.3) [33].

3. Results

3.1. Total Phenolic Content and Antioxidant Capacity

In the selected 400 g of the five most consumed fruits and vegetables in France, the total phenolic content was 228 mg gallic acid equivalent (GAE). Therefore, the supplement was developed to provide the same quantity of total (poly)phenols, which was confirmed by the Folin–Ciocalteu analysis. One daily dose of 450 mg of the test supplement provided 230 mg of GAE total phenolic compounds (Table 1). Following the same approach, the antioxidant capacity of the supplement was similar to the value calculated for 400 g of the five fruit and vegetable references, corresponding to 383 mg GAE and 396 mg GAE, respectively (Table 1).

Table 1. Comparison of total (poly)phenol content (mg GAE) and antioxidant capacity between 400 g of five servings of fruits and vegetables and a daily dose of 450 mg of the test supplement. Values are reported as mean ± SD (*n* = four different production batches of the supplement).

	Five Fruits and Vegetables—400 g	**Supplement—450 mg**
Total (poly)phenol content (mg GAE)	228 ± 23	230 ± 23
Total antioxidant capacity (mg GAE)	396 ± 20	383 ± 19

3.2. HPLC Analysis If Phenolic Compounds in the Supplement

The HPLC internal analysis of the supplement revealed five main families of phenolic compounds found in the supplement, including, in decreasing order, flavan-3-ols > flavanones > ellagitannins > secoiridoids and phenylethanoids > anthocyanins. The total phenolic content of the supplement was established at 144.8 ± 9.9 mg (Table 2).

Table 2. Internal HPLC analysis and quantification of the main (poly)phenolic families occurring in the supplement. Values are reported as mean (mg/450 mg) ± SD (*n* = four different production batches of the supplement).

Family	**Mean (mg/450 mg)**
Flavan-3-ols	50.7 ± 3.2
Flavanones	39.1 ± 2.9
Ellagitannins	26.7 ± 2.0
Secoiridois and Phenylethanoids	22.2 ± 0.9
Anthocyanins	6.1 ± 1.0
TOTAL (poly)phenols	**144.8 ± 9.9**

The more comprehensive analysis, performed through uHPLC-ESI-MSn, allowed us to identify or tentatively identify 136 phenolic compounds, among which 124 were quantified (Table 3). Phenolic compounds belonged to different phenolic families, i.e., in a decreasing order, flavan-3-ols (35.2%) > ellagitannins (19.2%) > phenylethanoids (14.8%) > flavanones (12.6%) > secoiridoids (9.0%) > flavones (3.4%) > flavonols (2.6%) > hydroxybenzoic acids (1.7%) > anthocyanins (0.8%) > hydroxycinnamic acids (0.3%) > stilbenoids (0.2%) > gallotannins (0.1%) > dihydrochalcones (<0.1%). The total phenolic content of the supplement was established at 221.1 ± 9.5 mg, corresponding to 594.9 ± 14.5 μmol (Table 3).

Table 3. Quantification of detected (poly)phenols occurring in the supplement. Values are expressed as mean ± SD (n = 3).

Compounds	Mean (μg/450 mg)
Flavan-3-ols	
(+)-Catechin	4100.4 ± 169.5
(−)-Epicatechin	4737.9 ± 69.0
(+)-Gallocatechin	399.9 ± 5.9
(−)-Epigallocatechin	1989.7 ± 67.6
(Epi)catechin gallate	28,879.6 ± 639.5
(Epi)gallocatechin-methylgallate	294.6 ± 3.6
(−)-Epigallocatechin gallate	27,109.5 ± 587.8
(Epi)gallocatechin gallate	1516.6 ± 76.1
Procyanidin dimer A-type	4441.0 ± 363.0
Procyanidin dimer B-type	571.7 ± 33.7
Procyanidin dimer B-type	914.9 ± 47.7
Procyanidin dimer B-type	432.5 ± 25.9
Procyanidin dimer B-type	276.0 ± 5.1
Prodelphinidin dimer B-type (1 unit of (epi)GC + 1(epi)C)	70.7 ± 2.2
Prodelphinidin dimer B-type (1 unit of (epi)GC + 1(epi)C)	115.7 ± 3.7
Prodelphinidin dimer B-type gallate (1 unit of (epi)GC + 1(epi)C)	1339.0 ± 96.7
Prodelphinidin tetramer B-type gallate (2 unit of (epi)GC + 2(epi)C)	303.2 ± 14.9
Prodelphinidin tetramer B-type gallate (2 unit of (epi)GC + 2(epi)C)	257.0 ± 48.6
Prodelphinidin dimer B-type gallate (2 units (epi)GC)	83.6 ± 3.6
Procyanidin trimer B-type	n.q.
Procyanidin trimer B-type	n.q.
Procyanidin trimer B-type	n.q.
(Epi)catechin-gallate dimer	n.q.
Sum of Flavan-3-ols (mg/450 mg)	**77.83 ± 0.91**
Ellagitannins	
Ellagic acid	293.9 ± 8.5
Galloyl-hexoside	1.7 ± 0.3
Galloyl-hexoside	10.9 ± 0.4
Galloyl-hexoside	27.2 ± 1.2
Galloyl-hexoside	21.6 ± 1.5
Galloyl-hexoside	22.3 ± 0.8
Ellagic acid hexoside	116.6 ± 9.1
Ellagic acid dimethyl ether glucurionide	5.3 ± 1.0
Galloyl-HHDP-hexoside	69.9 ± 2.2
Galloyl-HHDP-hexoside	4.5 ± 0.6
Galloyl-HHDP-hexoside	49.5 ± 3.0
Galloyl-HHDP-hexoside	55.4 ± 4.6
Galloyl-HHDP-hexoside	88.2 ± 1.6
Galloyl-HHDP-hexoside	360.7 ± 27.8
Galloyl-HHDP-hexoside	252.0 ± 9.3
Gallagyl-hexoside (Punicalin α/A)	93.1 ± 2.3
Gallagyl-hexoside (Punicalin β/B)	1266.9 ± 156.5

Table 3. *Cont.*

Compounds	Mean (μg/450 mg)
Bis-HHDP-hexoside (Pedunculagin I isomer)	41.0 ± 0.5
Bis-HHDP-hexoside (Pedunculagin I isomer)	249.7 ± 10.2
Bis-HHDP-hexoside (Pedunculagin I isomer)	238.7 ± 11.7
Galloyl-bis-HHDP-hexoside (Casuarinin)	145.0 ± 9.3
Punicalagin isomer	1026.7 ± 59.8
HHDP-gallagyl-hexoside (Punicalagin α)	10,935.5 ± 1237.4
HHDP-gallagyl-hexoside (Punicalagin β)	24,246.1 ± 1017.9
Di(HHDP-galloylglucose)-pentoside	2029.8 ± 182.0
Di(HHDP-galloylglucose)-pentoside	181.8 ± 12.0
Di(HHDP-galloylglucose)-pentoside	662.2 ± 42.6
Sum of Ellagitannins (mg/450 mg)	**42.50 ± 2.22**
Phenylethanoids	
Tyrosol (tentative identification)	159.0 ± 8.7
Hydroxytyrosol	21,065.2 ± 865.2
Hydroxytyrosol-*O*-hexoside	10,467.2 ± 367.0
Oleoside	517.1 ± 12.0
Verbascoside (Caffeoyl-phenylethanoid glycoside)	488.9 ± 15.0
Verbascoside (Caffeoyl-phenylethanoid glycoside)	81.8 ± 4.1
Sum of Phenylethanoids (mg/450 mg)	**32.78 ± 1.25**
Flavanones	
Naringenin	56.6 ± 2.2
Eriodyctiol	9.0 ± 0.5
Tetrahydroxyflavanone	6.6 ± 0.2
Naringenin-*O*-glucoside	7.4 ± 0.8
Tetrahydroxyflavanone-*O*-rhamnoside (tentative identification)	22.2 ± 0.3
Hesperetin-*O*-hexoside	681.5 ± 19.9
Naringenin-7-*O*-rutinoside (Narirutin)	427.8 ± 4.2
Naringenin-7-*O*-neohesperidoside (Naringin)	17,923.8 ± 297.3
Naringenin-*O*-neohesperidoside	69.9 ± 1.3
Isosakuranetin-*O*-rutinoside (Didymin)	739.6 ± 6.9
Eriocitrin or Neoeriocitrin	n.q.
Naringenin-*C*-dihexoside	37.9 ± 1.2
Hesperetin-7-*O*-rutinoside (Hesperidin)	7734.6 ± 635.6
Hesperetin-7-*O*-neohesperidoside (Neohesperidin)	90.6 ± 7.3
Sum of Flavanones (mg/450 mg)	**27.81 ± 0.95**
Seco-iridoids	
Oleuropein aglycone	n.q.
Oleuropein aglycone	n.q.
Oleuropein aglycone	n.q.
Oleuropein aglycone	n.q.
Oleuropein aglycone	n.q.
Oleuropein	15,980.1 ± 1067.3
Oleuropein	3930.0 ± 200.7
Sum of Seco-iridoids (mg/450 mg)	**19.91 ± 1.27**
Flavones	
Apigenin	n.q.
Trihydroxyflavone	221.7 ± 2.7
Apigenin-*O*-hexoside	100.4 ± 2.8
Luteolin-*O*-hexoside	597.1 ± 18.5
Luteolin-*O*-hexoside	3465.2 ± 142.5
Luteolin-*O*-hexoside	1420.6 ± 62.3
Luteolin-*O*-hexoside	509.2 ± 4.3
Dihydroluteolin-*O*-hexoside	264.7 ± 3.3
Apigenin-*O*-rutinoside	239.2 ± 2.5
Luteolin-*O*-rutinoside	297.7 ± 4.5
Luteolin-*O*-rutinoside	207.9 ± 8.2

Table 3. *Cont.*

Compounds	Mean (μg/450 mg)
Chrysoeriol-*O*-rutinoside or Diosmetin-*O*-rutinoside	35.3 ± 0.4
Luteolin-*O*-dihexoside (tentative identification)	50.5 ± 7.4
Luteolin-*O*-dihexoside	32.0 ± 1.1
Sum of Flavones (mg/450 mg)	**7.44 ± 0.10**
Flavonols	
Quercetin	3448.8 ± 256.9
Rhamnetin	1.7 ± 0.1
Isorhamnetin	8.0 ± 0.4
Myricetin	7.5 ± 0.4
Quercetin-*O*-pentoside	67.8 ± 2.5
Myricetin-*O*-rhamnoside	115.4 ± 11.3
Quercetin-*O*-hexoside	428.0 ± 10.2
Quercetin-*O*-glucuronide	706.1 ± 16.5
Myricetin-*O*-hexoside	330.5 ± 5.9
Kaempferol-*O*-rutinoside	86.4 ± 4.2
Tetrahydroxy-dimethoxyflavone-*O*- hexoside (Syringetin-*O*-hexoside)	70.6 ± 1.6
Isorhamnetin-*O*- dirhamnoside	225.6 ± 6.5
Quercetin-*O*-rutinoside (Rutin)	187.4 ± 8.6
Isorhamnetin-*O*-rutinoside	106.6 ± 1.6
Sum of Flavonols (mg/450 mg)	**5.79 ± 0.25**
Hydroxybenzoic acids	
3-Hydroxybenzoic acid	365.5 ± 25.9
4-Hydroxybenzoic acid	191.7 ± 4.5
Hydroxybenzoic acid	349.5 ± 12.1
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	152.9 ± 7.3
Dihydroxybenzoic acid	98.9 ± 7.7
Dihydroxyphenylacetic acid	110.1 ± 34.6
Dihydroxyphenylacetic acid	186.3 ± 26.8
Dihydroxyphenylacetic acid	183.3 ± 9.0
Gallic acid	1804.3 ± 38.2
Hydroxyphenyllactic acid	n.q.
Ethyl-gallate	415.5 ± 11.2
Sum of Hydroxybenzoic acids (mg/450 mg)	**3.81 ± 0.01**
Anthocyanins	
Cyanidin-3-*O*-hexoside	392.9 ± 5.1
Malvidin-3-*O*-arabinoside	39.8 ± 0.3
Peonidin-3-*O*-hexoside	156.6 ± 7.1
Delphinidin 3-*O*-hexoside	370.6 ± 24.6
Petunidin-3-*O*-hexoside	251.6 ± 5.4
Malvidin-3-*O*-hexoside	285.4 ± 11.4
Cyanidin-3-*O*-rutinoside	87.3 ± 0.9
Delphinidin 3-*O*-rutinoside	106.5 ± 3.4
Sum of Anthocyanins (mg/450 mg)	**1.69 ± 0.05**
Hydroxycinnamic acids	
Caffeic acid	206.6 ± 2.0
5-Caffeoylquinic acid	382.7 ± 8.6
Ferulic acid-*O*-hexoside	26.4 ± 1.5
Sum of Hydroxycinnamic acids (mg/450 mg)	**0.62 ± 0.01**
Stilbenoids	
Piceid (Resveratrol-*O*-glucoside)	514.2 ± 13.6
Sum of Stilbenoids (mg/450 mg)	**0.51 ± 0.01**
Gallotannins	
Digalloylglucose	82.9 ± 1.8
Digalloylglucose	6.5 ± 0.1
Digalloylglucose	27.5 ± 0.9

Table 3. *Cont.*

Compounds	Mean (µg/450 mg)
Digalloylglucose	49.3 ± 4.9
Digalloylglucose	33.1 ± 0.3
Trigalloylglucose	112.7 ± 1.4
Sum of Gallotannins (mg/450 mg)	**0.31**
Dihydrochalcones	
Phloretin	32.0 ± 0.3
Sum of Dihydrochalcones (mg/450 mg)	**0.03 ± 0.01**
Coumarin	
Scopoletin-*O*-hexoside	n.q.
Sum of Coumarin (mg/450 mg)	**n.q.**
TOTAL (POLY)PHENOL	**221.1 ± 9.5 (mg/450 mg)** **594.9 ± 14.5 (µmol/450 mg)**

HHDP: means hexahydroxydiphenoyl; (epi)GC means (epi)gallocatechin; (epi)C means (epi)catechin; n.q means compounds that were identified but not quantified because <LOQ.

3.3. Characteristics of the Subjects

The selected participants were six males and four females with the following characteristics: age 27.8 ± 5.5 years, height 173.1 ± 9.0 cm, body weight 69.0 ± 11.0 kg and BMI 22.9 ± 1.8 kg/m^2. All participants followed the recommendations in terms of abstinence from physical activity as assessed with the Fitbit® activity tracker (data not shown).

3.4. Plasma Pharmacokinetics

The absorption pattern of total plasma metabolites in the supplemented group revealed a 24 h higher presence of circulating phenolic metabolites and clearly displayed three distinct absorption phases throughout the gastrointestinal tract, showing a first absorption peak occurring 2 h after taking the supplement, a second peak arising 5 h post-consumption and a last peak observed 10 h after supplement consumption. Out of the 47 monitored phenolic metabolites, 28 different plasma phenolic metabolites were identified and quantified (Table 4 and Supplemental Table S2), belonging to 7 different classes of phenolic metabolites, according to the (poly)phenol classes identified in the food supplement. All individual metabolites were mainly found as conjugated forms with sulfate, glucuronide, methyl or glycine moieties.

Table 4. Pharmacokinetic parameters of phenolic metabolites detected in plasma of the supplemented volunteers (n = 10). Values are expressed as mean ± SEM. T_{max}: time to reach the maximum plasma concentration; C_{max}: maximum plasma concentration; AUC0-24: area under the curve; T_{pres}: time of presence of metabolites in circulation (calculated by taking the time interval between the first appearance and the last appearance of the considered metabolite); CV: Coefficient of variation, expressed in percentage.

Id.	Phenolic Metabolites	AUC0-24 (CV) (nmol/L·h)	T_{pres} (h)	T_{max} (h)	C_{max} (nmol/L)
	(Epi)catechin derivatives				
5	(Epi)catechin-sulfate_isomer 1	18.41 ± 4.09 (70)	6.65 ± 2.03	8.15 ± 2.69	4.72 ± 0.96
4	(Epi)catechin-glucuronide_isomer 2	43.70 ± 6.66 (48)	10.30 ± 2.29	3.60 ± 0.91	10.88 ± 2.44
3	Methoxy(epi)gallocatechin glucuronide	9.57 ± 2.70 (89)	4.86 ± 1.64	3.14 ± 1.20	4.72 ± 0.62
	Sum of (Epi)catechin derivatives	**71.68 ± 9.08 (40)**	**13.35 ± 2.53**	**3.10 ± 0.87**	**16.37 ± 2.13**
	Flavanone derivatives				
26	Hesperetin-diglucuronide	5.09 ± 1.32 (82)	6.17 ± 2.04	10.78 ± 2.55	1.49 ± 0.37
27	Naringenin-glucuronide	339.74 ± 75.66 (70)	14.70 ± 2.57	5.30 ± 0.98	95.42 ± 29.73
28	Hesperetin-7-glucuronide	152.05 ± 27.84 (58)	17.83 ± 1.54	7.11 ± 0.90	19.95 ± 2.31
29	Hesperetin-sulfate	173.05 ± 55.23 (101)	15.35 ± 1.92	6.50 ± 0.86	31.62 ± 11.38
	Sum of flavanone derivatives	**669.93 ± 98.61 (47)**	**21.65 ± 0.42**	**5.30 ± 1.17**	**124.75 ± 39.01**

Table 4. *Cont.*

Id.	Phenolic Metabolites	AUC0-24 (CV) (nmol/L·h)	T_{pres} (h)	T_{max} (h)	C_{max} (nmol/L)
	Other flavonoid derivatives				
48	Luteolin-sulfate	3.41 ± 0.67 (62)	7.60 ± 2.19	1.55 ± 0.24	0.44 ± 0.14
	Phenylethanoid derivatives				
33	2-(Phenyl)ethanol-3′-glucuronide (Hydroxytyrosol-glucuronide)	50.92 ± 5.85 (36)	8.45 ± 2.19	1.45 ± 0.32	24.94 ± 5.63
	Phenyl-y-valerolactones and phenyl valeric acids				
20	5-(Methoxyhydroxyphenyl)-γ-valerolactone-sulfate	31.12 ± 8.29 (84)	16.25 ± 1.44	8.25 ± 0.80	7.75 ± 2.52
9	5-(Methoxy-hydroxyphenyl)-γ-valerolactone- glucuronide	7.63 ± 2.52 (105)	9.00 ± 3.06	10.00 ± 3.15	1.54 ± 0.35
16	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate	30.46 ± 11.85 (123)	10.57 ± 2.72	8.88 ± 2.41	9.97 ± 4.31
21	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-sulfate	141.70 ± 50.37 (112)	12.15 ± 1.84	7.00 ± 0.75	40.05 ± 16.85
10	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	125.55 ± 47.68 (120)	14.44 ± 2.42	12.78 ± 2.85	14.96 ± 5.61
13	5-(Phenyl)-γ-valerolactone-sulfate-glucuronide	10.24 ± 3.34 (103)	10.17 ± 2.42	8.67 ± 2.17	1.55 ± 0.29
22	5-(Methoxyphenyl)-γ-valerolactone-sulfate	18.59 ± 8.47 (144)	7.13 ± 2.33	6.50 ± 1.28	7.34 ± 4.47
23	5-Phenyl-γ-valerolactone-3′-sulfate	10.11 ± 3.89 (122)	9.33 ± 2.45	8.22 ± 2.13	2.15 ± 0.48
17	5-(Phenyl)-γ-valerolactone-3′-glucuronide	22.00 ± 6.41 (92)	7.13 ± 2.27	5.88 ± 0.95	6.34 ± 1.66
15	4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate	61.35 ± 23.81 (123)	13.06 ± 2.97	6.19 ± 1.06	13.10 ± 3.94
18	4-Hydroxy-5-(phenyl)valeric acid-sulfate	47.47 ± 18.41 (123)	10.30 ± 2.28	5.60 ± 0.88	11.73 ± 2.95
14	5-(Methoxyphenyl)valeric acid-glucuronide	39.35 ± 14.74 (118)	12.06 ± 2.47	7.22 ± 0.91	8.80 ± 3.60
	Sum of Phenyl-y-valerolactones and phenyl valeric acids	**545.58 ± 155.32 (90)**	**21.60 ± 0.72**	**8.50 ± 2.30**	**84.55 ± 36.40**
	(Hydroxyphenyl)propanoic acids				
41	3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate (Dihydrocaffeic acid-sulfate)	27.41 ± 11.21 (129)	9.06 ± 2.56	4.56 ± 1.13	18.24 ± 11.27
46	3-(3′-Methoxyphenyl)propanoic acid-4′-sulfate (Dihydroferulic acid-sulfate)	558.28 ± 531.21 (301)	7.14 ± 3.61	6.43 ± 2.97	168.73 ± 156.13
40	3-(3′-Hydroxyphenyl)propanoic acid (3-(3-Hydroxyphenyl)propionic acid)	1554.29 ± 644.33 (131)	7.50 ± 2.38	7.13 ± 0.77	443.09 ± 99.28
	Sum of (hydroxyphenyl)propanoic acids	**2139.98 ± 953.73 (141)**	**12.60 ± 2.76**	**5.50 ± 1.01**	**425.53 ± 122.90**
	Hydroxybenzoic acids and simple benzenes				
45	4-Hydroxybenzoic acid-3-sulfate (Protocatechuic acid-3-sulfate)	196.06 ± 32.72 (53)	13.45 ± 2.80	2.10 ± 0.46	43.60 ± 7.34
47	4′-Hydroxyhippuric acid	5719.44 ± 1327.87 (73)	21.55 ± 0.74	6.50 ± 1.28	816.10 ± 269.47
36	Dihydroxybenzene-sulfate (Pyrogallol-sulfate)	1677.03 ± 322.08 (61)	23.30 ± 0.27	7.10 ± 0.86	185.03 ± 34.67
37	Methoxyhydroxybenzene-sulfate (Pyrogallol-methoxy-sulfate)	885.81 ± 266.69 (95)	21.35 ± 1.41	7.00 ± 0.93	108.13 ± 24.62
	Sum of hydroxybenzoic acids and simple benzenes	**8478.34 ± 1448.99 (54)**	**23.60 ± 0.22**	**7.70 ± 0.86**	**958.18 ± 269.69**
	SUM of all metabolites	**11,959.85 ± 2200.65 (58)**	**23.80 ± 0.24**	**6.90 ± 0.96**	**1271.11 ± 281.33**

The relative contribution of the total AUC0-24 of each class of phenolic derivatives is presented in Figure 3. 4′-Hydroxyhippuric acid was not included in the analysis as this compound may originate from both phenolic metabolism and endogenous precursors [34].

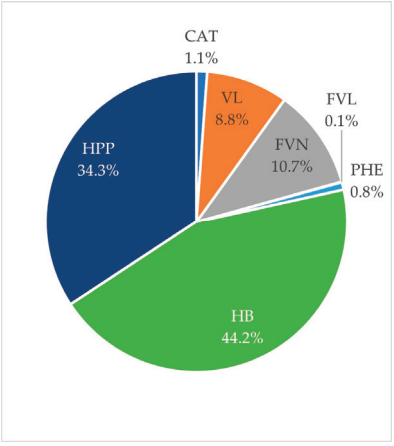

Figure 3. Relative plasma AUC0-24 for each class of phenolic metabolite. HB: Hydroxybenzoic acids and simple benzenes; HPP: Hydroxyphenylpropanoic acids; FVN: flavanone derivatives; VL: Phenyl y-valerolactones and phenyl valeric acids; CAT: (Epi)catechin derivatives; PHE: Phenylethanoid derivatives; FVL: Flavonol derivatives. 4′-hydroxyhippuric acid was not included.

Two main subclasses of flavan-3-ol derivatives were identified: (epi)catechin phase-II conjugates and phenyl-y-valerolactone and phenylvaleric acid derivatives. Among them, the second subclass was predominant in blood with a relative AUC0-24 eight times more abundant than the (epi)catechin derivatives.

The three identified and quantified (epi)catechin conjugates (Id. 3–5) started appearing in the blood in the first two hours post-consumption (average Tmax = 3.1 ± 0.37 h), indicating an absorption starting in the upper part of the gastrointestinal tract. We observed inter-individual variability in both the absorption time and plasma concentration (AUC0-24) of these compounds. The variation of AUC0-24, expressed as CV, ranged from 48% for (epi)catechin-glucuronide (isomer 2) to 89% for methoxy-(epi)gallocatechin-glucuronide. Considering the total AUC0-24 of (epi)catechin derivatives, the variation in AUC dropped to 40%. The 12 detected phenyl-y-valerolactone and phenylvaleric acid derivatives (Id. 9, 10, 13–18, 20–23) appeared later in the circulatory system, peaking between 6 and 24 h for most volunteers. The average Tmax was 8.50 ± 2.30 h post-intake, confirming the predominant colonic origin of these compounds. Consequently, the presence of these compounds was extended to 21.6 ± 0.72 h. It is noteworthy that the inter-individual variability was much higher for these compounds, as AUC0-24 varied from 84% to 144%; however, considering the sum of phenyl-y-valerolactone and phenylvaleric acids, the CV of total AUC0-24 was reduced to 90%.

Flavanone derivatives accounted for more than 10% of the total AUC0-24 and displayed a biphasic absorption pattern. Indeed, naringenin and hesperetin derivatives (Id. 26–29) started appearing in the bloodstream in the first two hours post-ingestion of the supplement, indicating an absorption in the upper gastrointestinal tract. Nevertheless, the absorption pattern also demonstrated more important secondary peaks between 3 and 10 h post-consumption, involving a significant implication of colonic microbiota in the absorption and metabolism of the flavanone derivatives. Such a biphasic absorption promoted an average presence time in the bloodstream of 21.65 ± 0.42 h.

Only one phenylethanoid derivative, namely 2-(phenyl)ethanol-3′-glucuronide (Id. 33), was quantified in the plasma with the analytical method employed. This compound

was rapidly absorbed (Tmax 1.45 ± 0.32 h) with the highest AUC in the first two hours post-intake. The inter-individual variability in AUC0-24 of this metabolite was 36%.

Hydroxybenzoic acids and simple benzenes (Id. 36, 37, 45, 47) were the most abundant class of phenolic metabolites, accounting for nearly 45% of total AUC0-24. Excluding 4-hydroxyhippuric acid, dihydroxybenzene-sulfate (Id. 36) presented the highest Cmax, AUC0-24 and persistence in the blood for this class of catabolite. This compound derives from gut microbiota metabolism, as confirmed by Tmax 7.10 ± 0.86 h. 4-Hydroxybenzoic acid-3-sulfate (Id. 45) also presented a biphasic absorption, with a first major peak occurring at 1.38 ± 0.18 h post-absorption, and a second minor peak between 3 and 10 h post-intake. The inter-individual variability of total AUC0-24 for this family of compounds was 54%.

Finally, hydroxyphenylpropanoic acid derivatives (Id. 40, 41, 46) represented 34.3% of the total AUC0-24. Three different derivatives of this class were identified and quantified, with 3-(3′-hydroxyphenyl)propanoic acid being the most abundant. These derivatives were microbial-derived metabolites with a Tmax arising between 4 and 7 h post-intake of the supplement. Also, AUC0-24 CV ranged from 129 to 301%, indicating high inter-individual variability in the production of these compounds.

Finally, the total metabolite concentration reached within the 24 h blood-sampling period is presented in Figure 4. The placebo group, as compared to the supplemented group, displayed a minimal absorption rate, thus confirming a good adherence to the (poly)phenol-free diet restriction. Compliance with this diet is also validated through the baseline quantification of metabolites (T0 h) that is <200 nmol/L within the two groups. It must be highlighted that mammalian metabolic pathways, including the hepatic metabolism of the surplus aromatic amino acids, exist and lead to low molecular weight catabolites, interfering with the quantified metabolites [35]. Considering the whole (poly)phenol plasma concentration quantified after supplement consumption, a triphasic absorption pattern can be outlined, including a first plasma concentration peak at 1.75 ± 0.25 h, accounting for phase II human metabolites, a second peak at 4.50 ± 0.34 h, accounting for the earlier microbial catabolites, and a last plasma concentration peak at 9.50 ± 0.33 h, described by those catabolites derived from a later microbiota activity (Figure 4). In general, the presence time (Tpres) of metabolites in the circulation was equal to 23.80 ± 0.24 h, the mean Tmax was 6.90 ± 0.96 h, and the total AUC0-24 was more than 10 μmol/L·h, with a CV of 58%.

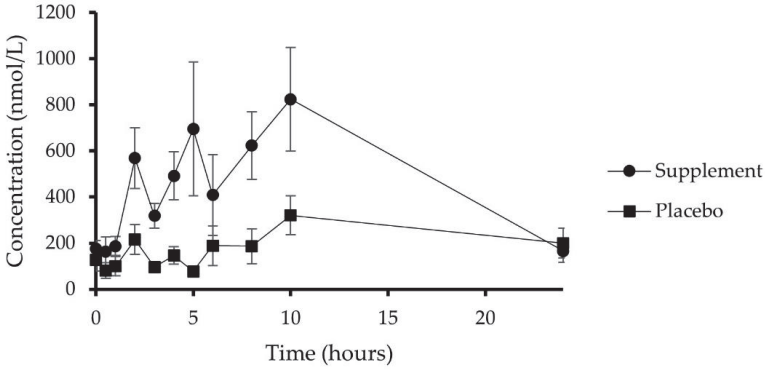

Figure 4. Sum of all (poly)phenol metabolites detected in plasma in placebo (n = 10) and supplemented subjects (n = 10) over a 24 h period. Data are expressed as means ± SEM.

3.5. Urinary Excretion

In urine, eight different classes of phenolic compounds were represented by forty-four different conjugated metabolites that were identified and quantified (Table 5 and Supplemental Table S2). The most abundant class identified was phenyl-y-valerolactone and phenylvaleric acid derivatives, accounting for more than 66% of total excretion, followed by hydroxybenzoic acids and simple benzenes (23.2%), hydroxyphenylpropanoic acids (6.1%),

flavanones (1.9%), ellagitannins (1.5%), (epi)catechin derivatives (0.8%), phenylethanoid derivatives (0.4%) and other minor flavonoids (0.1%) (Figure 5).

Table 5. Phenolic metabolites detected in urine of the supplemented volunteers (n = 10). Values are expressed as μg of compound, and data are reported as mean ± SEM. nd means not detected.

Id.	Phenolic Metabolites	0 (h)	0–3 (h)	3–6 (h)	6–10 (h)	10–14 (h)	14–24 (h)	24–32 (h)	32–48 (h)	Total (0–48 h)	CV (%)
	(Epi)catechin derivatives										
5	(Epi)catechin-sulfate_isomer 1	nd	14.26 ± 7.60	10.63 ± 3.02	0.49 ± 0.49	nd	nd	nd	2.28 ± 2.28	27.65 ± 8.42	96
6	(Epi)catechin-sulfate_isomer 2	nd	36.14 ± 15.62	29.95 ± 11.99	0.00 ± 0.00	1.66 ± 1.20	4.12 ± 2.29	2.06 ± 2.06	9.52 ± 6.72	83.45 ± 19.91	75
2	(Epi)catechin-glucuronide_isomer 1	nd	0.17 ± 0.17	2.60 ± 1.15	9.09 ± 3.87	3.10 ± 1.10	5.67 ± 1.80	1.72 ± 0.92	nd	22.35 ± 5.67	80
4	(Epi)catechin-glucuronide_isomer 2	nd	0.59 ± 0.59	14.00 ± 7.73	53.64 ± 44.88	3.19 ± 1.65	3.65 ± 2.21	1.22 ± 1.22	nd	76.29 ± 45.22	187
7	Methoxy(epi)catechin-sulfate	nd	39.90 ± 19.16	48.81 ± 12.64	7.02 ± 4.78	4.33 ± 2.25	5.00 ± 3.44	2.12 ± 2.12	nd	107.19 ± 26.64	79
1	Methoxy-(epi)catechin-glucuronide	nd	nd	0.59 ± 0.52	1.29 ± 0.94	0.74 ± 0.36	0.24 ± 0.22	nd	nd	2.86 ± 1.48	164
3	Methoxy-(epi)gallocatechin-glucuronide	nd	1.91 ± 0.99	3.38 ± 0.81	2.36 ± 1.11	0.78 ± 0.33	1.33 ± 0.61	0.18 ± 0.18	nd	9.94 ± 2.24	71
	Sum of (Epi)catechin derivatives	**nd**	**92.97 ± 38.65**	**109.96 ± 27.97**	**73.89 ± 48.67**	**13.80 ± 5.01**	**20.00 ± 4.58**	**7.30 ± 3.54**	**11.80 ± 8.79**	**329.72 ± 86.05**	**83**
	Flavanone derivatives										
29	Hesperetin-sulfate	nd	2.03 ± 2.03	1.19 ± 0.81	0.23 ± 0.23	nd	1.22 ± 0.90	0.33 ± 0.33	2.21 ± 2.21	7.21 ± 5.41	237
28	Hesperetin-7-glucuronide	14.48 ± 5.94	4.02 ± 2.90	20.07 ± 4.27	28.56 ± 5.46	9.57 ± 2.19	30.72 ± 8.04	19.07 ± 5.57	22.33 ± 5.83	148.81 ± 14.88	32
26	Hesperetin-diglucuronide	nd	0.05 ± 0.05	0.16 ± 0.08	0.12 ± 0.07	0.10 ± 0.05	0.45 ± 0.26	0.16 ± 0.07	0.04 ± 0.04	1.09 ± 0.36	104
27	Naringenin-glucuronide	16.31 ± 16.31	65.92 ± 53.49	187.75 ± 72.43	237.98 ± 90.88	39.21 ± 11.90	78.88 ± 45.12	30.40 ± 16.54	9.13 ± 9.13	665.58 ± 148.66	71
25	Naringenin-diglucuronide	0.17 ± 0.17	0.56 ± 0.38	2.47 ± 1.02	1.66 ± 1.01	0.46 ± 0.21	0.54 ± 0.28	0.09 ± 0.09	nd	5.95 ± 1.56	83
	Sum of flavanone derivatives	**30.96 ± 15.78**	**72.59 ± 58.76**	**211.64 ± 77.44**	**268.55 ± 93.64**	**49.33 ± 12.85**	**111.81 ± 50.11**	**50.04 ± 19.22**	**33.70 ± 15.01**	**828.64 ± 159.16**	**61**
	Other flavonoid derivatives										
30	Luteolin-glucuronide	nd	1.84 ± 1.25	2.67 ± 0.96	3.39 ± 2.32	1.80 ± 0.84	5.97 ± 2.77	3.07 ± 2.06	6.12 ± 2.55	24.88 ± 7.86	100
31	Myricetin-glucuronide	nd	0.00 ± 0.00	0.72 ± 0.49	2.78 ± 1.05	1.81 ± 0.68	3.68 ± 1.04	1.30 ± 0.56	nd	10.29 ± 2.19	67
32	Quercetin-diglucuronide	nd	0.04 ± 0.04	0.39 ± 0.19	0.50 ± 0.25	0.21 ± 0.19	0.42 ± 0.25	0.30 ± 0.23	nd	1.86 ± 1.01	171
	Sum of other flavonoid derivatives	**nd**	**1.88 ± 1.29**	**3.78 ± 1.42**	**6.68 ± 2.59**	**3.83 ± 1.14**	**10.07 ± 2.88**	**4.67 ± 2.16**	**6.12 ± 2.55**	**37.03 ± 7.60**	**65**
	Phenylethanoid derivatives										
34	Oleuropein-sulfate	0.02 ± 0.02	0.01 ± 0.01	0.03 ± 0.01	nd	nd	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.02	0.10 ± 0.03	106
33	2-(Phenyl)ethanol-3′-glucuronide (Hydroxytyrosol-glucuronide)	nd	53.75 ± 15.51	69.04 ± 13.20	38.02 ± 8.17	10.90 ± 3.90	10.23 ± 3.71	0.75 ± 0.75	nd	182.69 ± 19.99	35
	Sum of phenylethanoid derivatives	**0.02 ± 0.02**	**53.76 ± 15.51**	**69.06 ± 13.20**	**38.02 ± 8.17**	**10.90 ± 3.90**	**10.24 ± 3.72**	**0.77 ± 0.74**	**0.02 ± 0.02**	**182.79 ± 19.98**	**35**
	Phenyl-γ-valerolactones and phenylvaleric acids										
8	5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide	nd	nd	23.24 ± 17.36	26.86 ± 11.59	6.17 ± 3.84	5.33 ± 2.23	nd	nd	61.61 ± 29.59	152
20	5-(Methoxy-hydroxyphenyl)-γ-valerolactone–sulfate	nd	1.15 ± 0.85	123.73 ± 64.97	287.71 ± 81.19	98.28 ± 40.32	116.82 ± 44.46	21.40 ± 9.99	2.02 ± 2.02	651.12 ± 170.13	83
9	5-(Methoxyhydroxyphenyl)-γ-valerolactone-glucuronide	nd	nd	9.83 ± 6.55	18.07 ± 6.62	9.28 ± 2.54	16.44 ± 4.84	4.47 ± 2.31	nd	58.09 ± 11.33	62
16	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate	18.25 ± 10.84	4.64 ± 3.47	439.69 ± 219.42	1256.55 ± 516.93	944.18 ± 474.02	3986.28 ± 711.64	1721.60 ± 393.97	586.15 ± 240.93	8957.33 ± 1361.49	48
21	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-sulfate	344.60 ± 186.50	166.29 ± 126.77	2548.76 ± 1207.06	2277.16 ± 795.52	919.49 ± 314.48	3618.13 ± 985.44	1346.37 ± 481.02	389.78 ± 215.48	11,610.60 ± 1923.81	52
10	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	5.34 ± 5.34	0.00 ± 0.00	101.70 ± 51.36	316.86 ± 132.04	199.53 ± 88.98	624.93 ± 108.36	281.58 ± 60.75	65.29 ± 25.62	1595.23 ± 260.71	52
13	5-(Phenyl)-γ-valerolactone-sulfate-glucuronide	0.34 ± 0.34	0.13 ± 0.13	9.26 ± 4.10	23.29 ± 7.81	10.14 ± 3.00	20.29 ± 4.37	7.77 ± 2.43	1.43 ± 0.97	72.65 ± 15.10	66

Table 5. *Cont.*

Id.	Phenolic Metabolites	0 (h)	0–3 (h)	3–6 (h)	6–10 (h)	10–14 (h)	14–24 (h)	24–32 (h)	32–48 (h)	Total (0–48 h)	CV (%)
22	5-(Methoxy-phenyl)-γ-valerolactone-sulfate	5.66 ± 3.78	2.16 ± 1.13	7.50 ± 2.92	18.92 ± 9.27	12.82 ± 4.86	22.47 ± 5.87	9.17 ± 3.61	4.24 ± 2.18	82.94 ± 15.05	57
23	5-(Phenyl)-γ-valerolactone-3′-sulfate	41.07 ± 30.35	4.29 ± 2.19	52.98 ± 22.54	175.24 ± 98.80	76.01 ± 39.48	345.14 ± 198.47	100.92 ± 46.88	34.61 ± 16.03	830.26 ± 275.43	105
17	5-(Phenyl)-γ-valerolactone-3′-glucuronide	15.01 ± 15.01	15.42 ± 15.42	196.48 ± 138.99	169.91 ± 105.95	65.96 ± 36.27	468.44 ± 330.34	81.71 ± 28.26	16.56 ± 16.56	1029.49 ± 394.77	121
15	4-Hydroxy-5-(Hydroxyphenyl)valeric acid-sulfate	31.48 ± 18.11	9.56 ± 5.01	268.65 ± 125.49	432.54 ± 133.32	216.01 ± 58.49	571.58 ± 137.24	216.26 ± 60.42	30.47 ± 14.26	1776.56 ± 310.38	55
19	4-Hydroxy-5-(methoxy-phenyl)valeric acid–sulfate	nd	0.30 ± 0.21	3.34 ± 1.93	6.42 ± 1.94	1.84 ± 0.84	4.02 ± 1.53	0.29 ± 0.15	nd	16.20 ± 4.31	84
11	4-Hydroxy-5-(methoxyphenyl)valeric acid-glucuronide (4-Hydroxy-5-phenylvaleric acid-methoxy-glucuronide)	nd	nd	4.79 ± 2.48	6.48 ± 4.24	8.52 ±3.93	18.97 ± 6.71	1.78 ±1.78	nd	40.54 ± 10.11	79
18	4-Hydroxy-5-(phenyl)valeric acid-sulfate	0.81 ± 0.61	0.23 ± 0.11	2.28 ± 1.17	4.40 ± 2.19	2.01 ± 0.97	10.89 ± 6.29	3.86 ± 1.36	1.41 ± 0.57	25.89 ± 8.19	100
24	5-(Hydroxyphenyl)valeric acid-sulfate	5.68 ± 5.68	0.58 ± 0.42	5.06 ± 5.06	7.41 ± 4.74	4.35 ± 2.69	34.54 ± 13.80	31.20 ± 5.27	29.85 ± 15.45	118.67 ± 27.65	74
12	5-(Methoxyphenylvaleric) acid-sulfate	nd	1.90 ± 1.90	374.69 ± 221.67	543.24 ± 149.94	169.00 ± 78.02	277.94 ± 104.02	42.67 ± 21.21	5.27 ± 3.42	1414.71 ± 421.96	94
14	5-(Methoxy-phenylvaleric) acid–glucuronide	8.97 ± 6.01	nd	59.80 ± 41.17	90.37 ± 34.08	22.28 ± 11.86	14.60 ± 7.48	nd	3.07 ± 3.07	199.09 ± 78.07	124
	Sum of phenyl-γ-valerolactones and phenylvaleric acids (Hydroxyphenyl)propanoic acids	**477.22 ± 266.28**	**206.65 ± 148.90**	**4231.78 ± 1978.16**	**5661.43 ± 1887.41**	**2765.88 ± 963.32**	**10,156.83 ± 1864.13**	**3871.06 ± 972.99**	**1170.14 ± 510.79**	**28,540.99 ± 3805.96**	**42**
41	3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate (Dihydrocaffeic acid-sulfate)	27.49 ± 27.49	101.08 ± 52.47	103.59 ± 54.34	41.08 ± 16.51	56.67 ± 24.78	389.70 ± 95.00	208.69 ± 73.47	289.45 ± 118.16	1217.76 ± 256.95	67
40	3-(3′-Hydroxyphenyl)propanoic acid (3-(3-Hydroxyphenyl)propionic acid)	96.20 ± 96.20	85.67 ± 85.67	nd	nd	287.94 ± 287.94	262.06 ± 138.26	nd	676.70 ± 383.40	1408.57 ± 544.97	122
	Sum of (Hydroxyphenyl)propanoic acids	**123.69 ± 97.07**	**186.75 ± 112.79**	**103.59 ± 54.34**	**41.08 ± 16.51**	**344.61 ± 305.36**	**651.76 ± 199.89**	**208.69 ± 73.47**	**966.15 ± 399.30**	**2626.33 ± 618.94**	**75**
	Hydroxybenzoic acids and simple benzenes										
39	3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	0.71 ± 0.46	8.71 ± 8.71	3.89 ± 3.81	3.57 ± 2.82	1.42 ± 1.38	3.63 ± 2.95	3.33 ± 2.94	8.73 ± 8.35	33.92 ± 30.72	286
38	Methoxy-hydroxybenzoic acid-sulfate (Gallic acid-methoxy-sulfate)	1.18 ± 1.18	18.56 ± 8.39	26.07 ± 5.72	2.89 ± 1.25	1.18 ± 1.18	2.43 ± 1.72	2.23 ± 1.49	2.34 ± 2.34	56.87 ± 13.94	78
35	4-Hydroxybenzoic acid-3-glucuronide (Protocatechuic acid-3-glucuronide)	1.13 ± 1.13	14.20 ± 4.88	24.68 ± 5.82	12.53 ± 4.10	3.10 ± 1.12	4.51 ± 1.34	nd	nd	60.16 ± 9.86	52
36	Dihydroxybenzene-sulfate (Pyrogallol-sulfate)	747.18 ± 254.96	125.75 ± 42.49	549.56 ± 129.84	948.39 ± 201.27	504.78 ± 150.35	1676.23 ± 346.95	721.74 ± 169.19	541.99 ± 169.88	5815.62 ± 765.07	42
37	Methoxyhydroxybenzene-sulfate (Pyrogallol-methoxy-sulfate)	311.66 ± 96.06	104.78 ± 28.89	425.64 ± 95.78	783.20 ± 204.13	409.48 ± 127.88	1211.89 ± 191.45	538.08 ± 118.63	284.32 ± 101.86	4069.06 ± 502.55	39
	Sum of hydroxybenzoic acids and simple benzenes	**1061.79 ± 329.42**	**272.00 ± 85.28**	**1029.84 ± 219.14**	**1750.58 ± 408.15**	**919.95 ± 279.48**	**2898.70 ± 524.69**	**1265.38 ± 274.37**	**837.38 ± 246.45**	**10,035.62 ± 1234.49**	**39**
	Ellagitannin derivatives										
42	8-Hydroxy-urolithin-3-glucuronide (Urolithin A-glucuronide)	nd	8.35 ± 7.92	4.88 ± 4.63	2.42 ± 2.30	18.31 ± 14.57	68.99 ± 33.01	69.22 ± 21.30	96.89 ± 37.45	269.07 ± 84.25	99
43	9-Hydroxy-urolithin-3-glucuronide (Isourolithin A-glucuronide)	nd	nd	nd	6.91 ± 6.55	5.49 ± 5.21	36.41 ± 34.54	14.38 ± 9.83	5.33 ± 5.05	68.53 ± 55.19	255

Table 5. *Cont.*

Id.	Phenolic Metabolites	0 (h)	0–3 (h)	3–6 (h)	6–10 (h)	10–14 (h)	14–24 (h)	24–32 (h)	32–48 (h)	Total (0–48 h)	CV (%)
44	Urolithin-3-glucuronide (Urolithin B-glucuronide)	nd	nd	nd	5.31 ± 5.04	39.56 ± 37.53	144.45 ± 90.90	74.85 ± 48.79	30.99 ± 29.40	295.17 ± 185.26	198
	Sum of ellagitannin derivatives	**nd**	**8.35 ± 7.92**	**4.88 ± 4.63**	**14.64 ± 11.53**	**63.37 ± 57.13**	**249.86 ± 135.01**	**158.45 ± 58.48**	**133.21 ± 42.40**	**632.76 ± 251.20**	**126**
	SUM of all metabolites	**1693.68 ± 579.94**	**894.12 ± 325.73**	**5764.04 ± 2219.80**	**7853.42 ± 2304.78**	**4165.34 ± 1501.54**	**14,084.29 ± 2394.88**	**5550.52 ± 1194.80**	**3145.19 ± 1052.77**	**43,150.60 ± 5117.69**	**38**

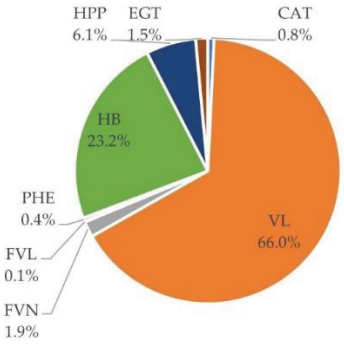

Figure 5. 48 h urinary excretion of phenolic metabolites. HB: Hydroxybenzoic acids and simple phenols; HPP: Hydroxyphenylpropanoic acids; FVN: Flavanone derivatives; VL: Phenyl- y-valerolactones and phenyl valeric acids; CAT: (Epi)catechin derivatives; PHE: Phenylethanoid derivatives; FVL: Flavonol derivatives. 4′-hydroxyhippuric acid was not included.

Flavan-3-ol derivatives were the most abundant compounds quantified, with the class of phenyl-y-valerolactone and phenylvaleric acid derivatives being predominant. Three (epi)catechin derivatives identified in plasma were also recovered in urine with an excretion peak of 3 and 6 h post-consumption, confirming a predominant absorption in the upper gastrointestinal tract (Figure 6A). Inter-individual variability in the excretion of these derivatives was high, as CV ranged from 71% to 164%. A total of 17 different phenyl-y-valerolactone and phenylvaleric acid derivatives were identified in urine, and among them 12 were also detected in plasma (Id. 9, 10, 13–18, 20–23). Many of these compounds presented a double excretion peak, the first one between 6 and 10 h and the second one appearing at 14–24 h, indicating a preferential microbiota-derived metabolism along the large intestine (Figure 6B). Inter-individual variability, reported as CV, was, averagely, 42%.

Regarding flavanones, up to five different compounds were found in urine; naringin-diglucuronide (Id. 25) was the only flavanone derivative not detected in plasma. The excretion pattern of these derivatives confirms the biphasic absorption, showing, for most compounds, two distinct excretion peaks corresponding to 6–10 h and 14–24 h post-supplement consumption (Figure 6C). Also, for flavanone metabolites, a high inter-individual variability can be suggested (61%).

In contrast to plasma, where only one phenylethanoid derivative was identified, in urine both 2-(phenyl)ethanol-3′-glucuronide (Id. 33) and oleuropein-sulfate (Id. 34) were quantified. However, the excretion rate of the last one did not exceed 0.10 μg in 48 h. In general, both phenylethanoid derivatives were rapidly excreted with a peak occurring 3–6 h post-absorption, and their excretion was completed in 32 h (Figure 6D). In contrast to other metabolites, the inter-individual variability of these compounds was comparatively low (35%).

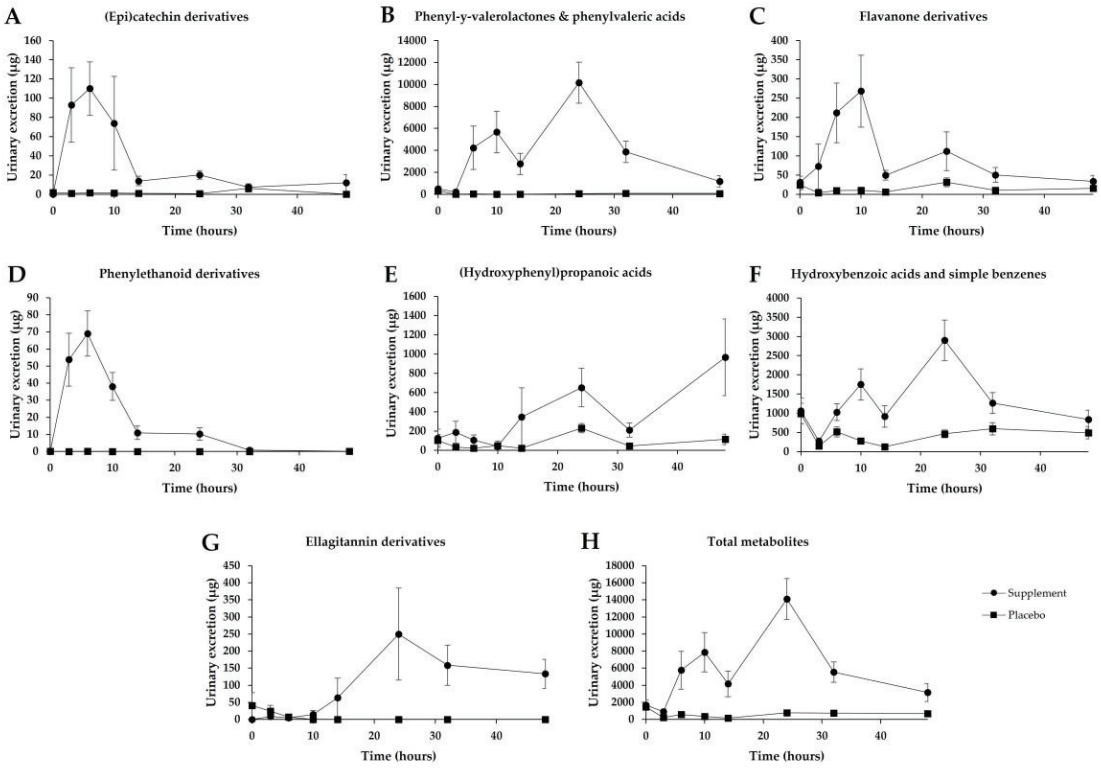

Figure 6. Urinary excretion in placebo (n = 10) and supplemented subjects (n = 10) over a 48 h period. (**A**) (Epi)catechin derivatives; (**B**) Phenyl-γ-valerolactones and phenylvaleric acids; (**C**) Flavanone derivatives; (**D**) Phenylethanoid derivatives; (**E**) (Hydroxyphenyl)propanoic acids; (**F**) Hydroxybenzoic acids and simple benzenes; (**G**) Ellagitannin derivatives; (**H**) Total metabolites. Data are means ± SEM.

Hydroxyphenylpropanoic acids were the third most important class of compounds excreted. Two derivatives were quantified (Id. 40, 41) in urine samples, displaying a biphasic excretion pattern (Figure 6E) and a high inter-individual variability with a CV of 75%.

Hydroxybenzoic acids and simple benzenes were the second most abundant classes of urinary metabolites, mainly represented by trihydroxybenzene derivatives (Id. 36, 37), for which excreted concentration reached more than 4 mg after 48 h. These catabolites reached their maximum excretion rate between 14 and 24 h post-consumption, confirming their microbial origin (Figure 6F). The coefficient of variation for the excretion of this class of compounds was 39%.

Finally, three ellagitannin catabolites were quantified in urine, including 8-hydroxy-urolithin-3-glucuronide (Id. 42), 9-hydroxy-urolithin-3-glucuronide (Id. 43) and urolithin-3-glucuronide (Id. 44). It is noteworthy that six volunteers of nine excreted 8-hydroxy-urolithin-3-glucuronide, and, among them, two excreted urolithin-3-glucuronide as well (data not shown). The peak excretion of these ellagitannin catabolites resulted between 14 and 24 h, confirming their microbial origin (Figure 6G).

The total amount of urinary excreted metabolites over a 48 h period after supplement consumption is presented in Figure 6H. The placebo group, as compared to the supplemented group, displayed a minimal excretion rate. On the contrary, after supplement consumption, the urinary excretion pattern of (poly)phenol metabolites displayed two main peaks, confirming the different absorption steps within the gastrointestinal tract. After 48 h of supplement ingestion, the metabolite excretion result was four times higher

than the curve recorded in the placebo group, indicating a sustained and more protracted release of various phenolic catabolites. The total urinary excretion of metabolites derived from supplement consumption was 43.0 mg, corresponding to 163.1 ± 20.1 µmol. Thus, based on the excreted µmols of quantified metabolites, the total bioavailability of phenolic compounds was evaluated to be 27.4 ± 3.4%.

3.6. Inter-Individual Variability

A high inter-individual variability was highlighted both in circulating plasma phenolic metabolites and in their urinary excretion (Tables 4 and 5). The coefficient of variation of excreted (poly)phenol subfamilies ranged from 34.5% for phenylethanoid derivatives to 126% for ellagitannin derivatives. Considering the total excreted metabolites, the mean CV was 38%.

Unsupervised multivariate analysis (PCA) was applied to urinary metabolites measured at different collection points (from 0 to 48 h). Three principal components (PCs) explained up to 52% of the total variance. PC1 explained 29% of total variability and was mainly loaded by dihydroxyphenyl-y-valerolactone and dihydroxyphenylvaleric acid derivatives (Figure 7D). PC2 and PC3 explained 14% and 9%, respectively, of the total variability. PC2 presented positive loads for (epi)catechin derivatives and negative ones for urolithin derivatives. Contrary to PC1, PC3 was positively loaded mainly by monohydroxypehnyl-valerolactone, monohydroxyphenyl valeric acid derivatives, hydroxyphenylpropanoic acid derivatives and hydroxybenzoic acid derivatives (Figure 7D). Urine samples collected 10 h or more post-intake presented positive PC1 score values (Figure 7A) (Id. 21, 15, 13, 9). Samples collected during the 3 to 10 h urinary collection period had the highest positive score values for both PC2 and PC3 (Figure 7B). In general, the large inter-subject variability observed for metabolite production is well represented by PCA plots (Figure 7), the area covered by the surface delimited by time points being quite different among subjects.

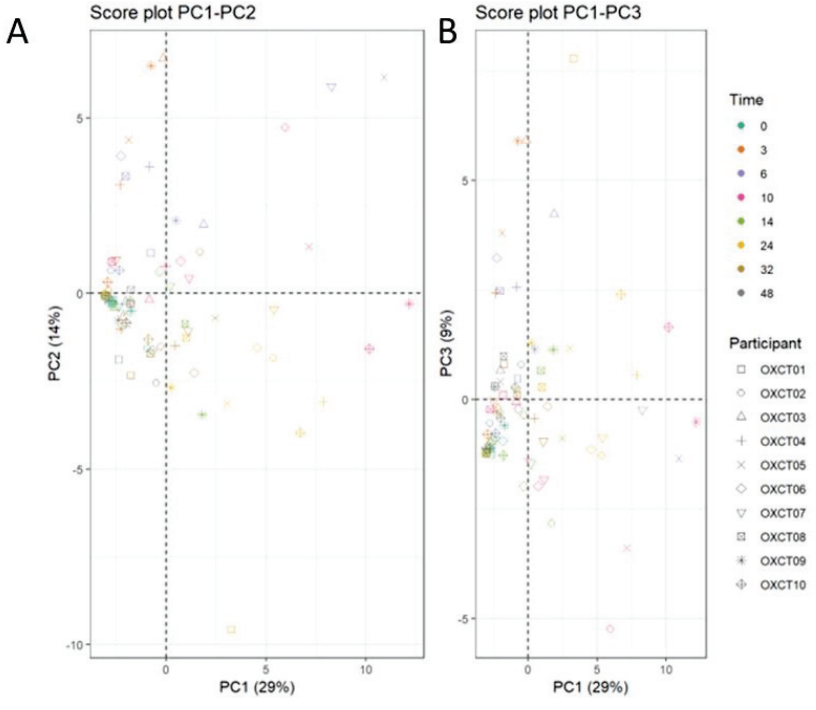

Figure 7. *Cont.*

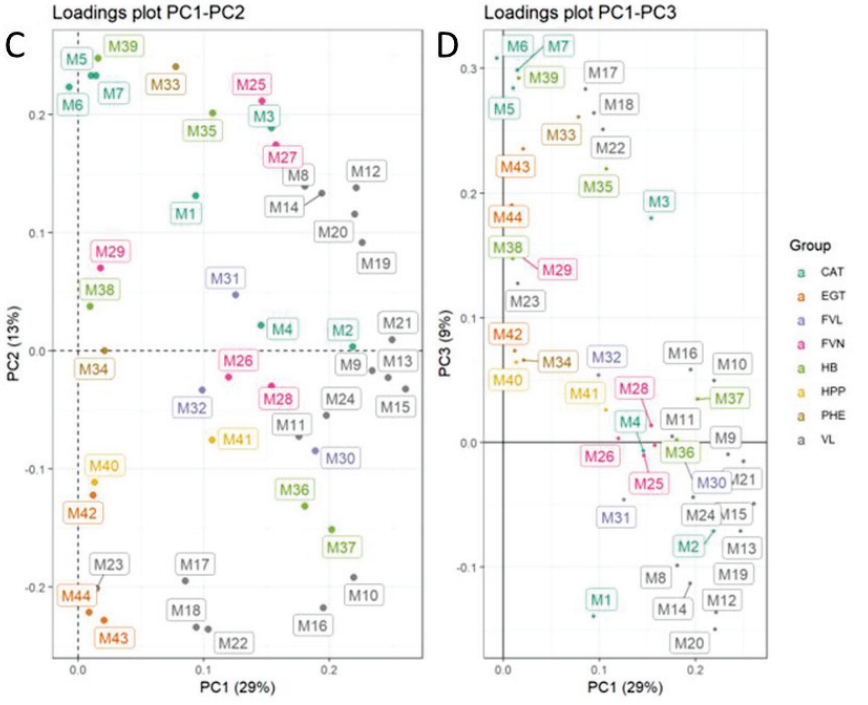

Figure 7. Inter-individual variability for urinary metabolites illustrated through principal component analysis. (PCA). (**A**) Score plots of PC1 versus PC2 for all the volunteers and (**B**) Score plots of PC1 versus PC3; (**C**) Loading plots of PC2 versus PC1 for all urinary metabolites and (**D**) PC1 versus PC3.

4. Discussion

Strong evidence recommends increasing fruit and vegetable intake for health benefits. However, their consumption has not risen in recent years. Unarguably, nutritional supplements cannot replace the consumption of fruits and vegetables, which must remain the first option because of their higher nutritional complexity, which includes fiber, vitamins and minerals, besides bioactive compounds. However, a formulated food supplement rich in (poly)phenol compounds could aid in filling this nutritional gap. In this context, supplementation, inserted in a varied and balanced diet, represents a possible solution to improve the daily consumption of dietary (poly)phenols to respond to consumer demand, enhancing the well-known benefits of regular and varied fruit and vegetable consumption.

Based on the modified Folin–Ciocalteu method [23], the supplement provided the same amount of phenolic compounds as the selected references, i.e., the five most consumed fruits and vegetables in France. Our exhaustive analysis confirmed that the main (poly)phenolic families were represented, covering up to 124 different phenolic compounds. The most abundant subfamily in the supplement was flavan-3-ols, being that these phenolics are the most abundant both in grape and green tea extracts [36,37]. Ellagitannins, whose main compounds were ellagic acid and punicalagin derivatives, are characteristic of pomegranate extracts [38], whereas citrus extracts provided mainly flavanones comprising naringenin and hesperetin derivatives [39]. Secoiridoids and phenylethanoids are common phenolics of olive leaf extract [40], and anthocyanins mainly result from bilberry, grape and pomegranate extracts [38,41,42].

From the pharmacokinetic study, a triphasic absorption profile was obtained for circulating plasma metabolites, indicating that some compounds are rapidly absorbed in the upper part of the gastrointestinal tract, although the most extensive part necessitates being firstly metabolized by the colonic microbiota before absorption. Such a pattern

validates that the intake of the supplement helps provide a continuous supply of various metabolites over a time period of 24 h that corresponds to a continuous absorption. The global pattern of the urinary excretion profile showed that excreted phenolic metabolites had not yet reached back baseline level 48 h post-consumption, resulting in a long-lasting phenolic presence in the bloodstream. This supports the hypothesis that mimicking the main meal intake of five fruits and vegetables would deliver a sustained and continuous release of metabolites. However, there are limited studies that have evaluated the circulating metabolites after the recommended intake of fruits and vegetables over a 24 h period [43]. This gap in the literature has been noted in a few reviews, which have indicated the need for such studies in the future [44,45], especially focusing on understanding the time-dependent distribution of metabolites in the systemic circulation, as has been recently reported for coffee consumption [46], for instance.

A total of 28 different circulating metabolites and up to 44 urine-excreted compounds were identified and quantified, among which flavan-3-ol-derived metabolites covered more than half. Among the last, 3 phase II (epi)catechin derivatives were identified early in plasma, indicating an absorption starting in the first part of the gastrointestinal tract, in accordance with previous reports [47]. These metabolites arise from the conjugation of parent compounds at both the enterocyte and hepatic levels, generating methylated, glucuronidated and sulfated (epi)catechin derivatives in the bloodstream [48]. However, the main flavan-3-ol parent compounds are metabolized by the gut microbiota, generating characteristic flavan-3-ols colonic catabolites, phenyl-y-valerolactones and, by breaking the valerolactone ring, phenylvaleric acids [49,50]. Here, 12 different phenyl-y-valerolactones and phenylvaleric acids were quantified in plasma with an average Tmax of 8.50 $\pm$ 2.30 h, confirming the colonic origin of these compounds. It is noteworthy that further metabolizing these compounds through β-oxidation of the side chain of the phenylvaleric acids also gives rise to lower molecular weight phenolic acids such as phenylpropanoic and hydroxybenzoic acid catabolites [50], which were both identified with relatively high AUC in the present pharmacokinetic study. However, these phenolic acids are also gut microbial metabolites of other flavonoids [49,51], as well as from the hepatic metabolism of the surplus aromatic amino acids [35], thus explaining their high concentration in the blood. The excretion pattern of flavan-3-ols metabolites confirmed the extensive bioconversion of the parent compounds at both intestinal and colonic levels.

The metabolism of flavanones demonstrated a typical pattern of absorption and excretion depicted by a biphasic curve, as previously shown [34,51,52]. It is noteworthy that colonic metabolism is, however, predominant in the formation of flavanone metabolites, as demonstrated by an average Tmax of 5.30 $\pm$ 1.17 h and confirmed by a delayed urinary excretion time. On the contrary, olive-derived phenolic compounds were rapidly absorbed and excreted, indicating a predominant role of the small intestine in their metabolism, and confirming that oleuropein is an effective source of hydroxytyrosol [53]. Finally, three ellagitannin derivatives were quantified in urine, namely 8-hydroxy-urolithin-3-glucuronide, 9-hydroxy-urolithin-3-glucuronide and urolithin-3-glucuronide, typical metabolites derived from ellagic acid lactone ring cleavage, decarboxylation and dehydroxylation reactions [54].

A key point to be considered in this study is the high inter-individual variability in both the absorption and excretion of phenolic compounds. Such variability may be driven by several factors, including genetic makeup, gut microbiota composition and functionality, age, gender and physiological status [55]. As an example, genetic variants in the catechol methyl transferase (COMT) [56] and the sulfotransferase (SULT) [57] enzymes may explain, at least in part, the variability illustrated in the excretion of native forms of metabolites, such as (epi)catechin derivatives (Id. 5, 6, 7). It is noteworthy that colonic metabolites displayed an even higher inter-individual variability, emphasizing the key role of the gut microbiota in (poly)phenol catabolism and bioavailability. Emerging research in this field describes the existence of different (poly)phenol-metabolizing phenotypes, namely metabotypes that have been so far clearly described for soya isoflavones and ellagitannins [58–60]. The latter is a good illustration of the impact of the microbiota; indeed, individual data

(not shown) demonstrated that six volunteers (amongst nine) in our study were able to produce 8-hydroxy-urolithin-3-glucuronide, and that, among them, only two were able to additionally produce 9-hydroxy-urolithin-3-glucuronide and urolithin3-glucuronide. This is in agreement with the distribution of the three urolithin metabotypes previously defined [61]. Moreover, the inter-individual variability in flavan-3-ol production based on quali-quantitative differences, instead of the production or non-production of specific catabolites, which emerged in the present work, is in accordance with recent evidence on the existence of metabotypes of flavan-3-ol colonic metabolites [50,62].

Obviously, depending on the metabotype, the inherent nutritional benefits attributable to the resulting bioactive phenolic metabolites or catabolites may greatly vary between subjects [63]. Considering this main observation, this study aimed at counterbalancing the inter-individual variability by providing a sufficiently varied source of phenolic compounds from several plant origins to compensate for and balance individual gaps. Indeed, as hypothesized earlier, a low CV for total metabolites was observed after supplement consumption, indicating the ability of various participants to utilize distinct substrates differently. Potential individual benefits may change among individuals, of course, but considering the pleiotropic effects of (poly)phenols in disease prevention this should not be a major issue. The observation of low inter-individual variability demonstrates that eating a diversity of (poly)phenols from several fruit and vegetable sources, as should be the case if the five-a-day recommendation is followed, must help to improve the occurrence and absorption of diverse bioactive metabolites providing nutritional benefits. This is in contrast to the consumption of individual (poly)phenols or not-well-characterized botanical sources, for which some individuals may be unable or limited in metabolizing parent compounds, resulting in "non-responder" putative nutritional benefits. However, a limit of the study is the restricted controlled diet followed by the volunteers. Although a free-(poly)phenol diet is needed to establish the metabolism, absorption and bioavailability of the (poly)phenol supplement, it would be reasonable to evaluate the actual influence of supplement consumption in daily (poly)phenol intake and in circulating bioactive metabolites in the context of a varied and balanced diet.

Finally, the bi-directional relationship existing between (poly)phenols and the gut microbiota must also be taken into consideration [57]. Indeed, chronic supplementation with phenolic compounds can modulate the microbiota composition of individuals, impacting the inter-individual variability in their absorption, metabolism and bioavailability, resulting in modified and hopefully enhanced nutritional benefits.

The question of the diversification of the source of fruits and vegetables has been set as a better predictive factor for nutritional benefits. Accordingly, future investigations should focus on nutritional endpoints linked to the well-known benefits associated with a regular consumption of varied (poly)phenolic compounds from fruits and vegetables. This would include an investigation of the possible links related to the inter-individual variation in biomarkers of the physiological or biological parameters of interest.

5. Conclusions

In conclusion, this randomized, double-blind, cross-over clinical trial study demonstrated that the supplementation of a complex (poly)phenolic ingredient could overcome the issue of inter-individual variation in bioavailability, linked to the ability of subjects to metabolize substrates differently. Contemporary consumption of a (poly)phenol-rich supplement, in the context of a varied and balanced diet, may benefit individuals in improving the daily consumption of bioactive compounds and, consequently, the occurrence and absorption of diverse bioactive metabolites, providing long exposure to bioactive circulating molecules. Future studies are warranted to understand the chronic effects of complex (poly)phenolic ingredient supplementation on biological biomarkers and its bioactivity and bioefficacy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14224913/s1, Chemicals and reagents; Table S1: Retention

time (RT), spectrometric characteristics and standards used for quantification of detected native (poly)phenols occurring in the supplement.; Table S2: Spectrometric characteristics of quantified metabolites in plasma and urine samples [31,64].

Author Contributions: Conceptualization, C.R. and J.C.; management of human trial (recruitment, blood sampling), L.H.C. and P.E.A.; methodology, L.B., P.M. and D.D.R.; investigation, L.B. and P.M.; data analysis, L.B., C.R. and J.M.; writing—original draft preparation, C.R.; writing—review and editing, C.R., L.B., J.M., P.M., D.D.R. and J.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author, due to privacy restriction.

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Review

Low Tissue Creatine: A Therapeutic Target in Clinical Nutrition

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Abstract: Low tissue creatine characterizes many conditions, including neurodegenerative, cardiopulmonary, and metabolic diseases, with a magnitude of creatine shortfall often corresponds well to a disorder's severity. A non-invasive monitoring of tissue metabolism with magnetic resonance spectroscopy (MRS) might be a feasible tool to evaluate suboptimal levels of creatine for both predictive, diagnostic, and therapeutic purposes. This mini review paper summarizes disorders with deficient creatine levels and provides arguments for assessing and employing tissue creatine as a relevant target in clinical nutrition.

Keywords: creatine; magnetic resonance spectroscopy; cost-effectiveness; brain; muscle; nutrition

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1. Introduction

Phosphorylated creatine (2-[carbamimidoyl(methyl)amino]acetic acid) is the main high-energy phosphate-storage compound able to promptly regenerate adenosine triphosphate (ATP) by the action of creatine kinase, with ATP being the critical energy-containing molecule found in all living cells. Creatine-phosphocreatine system serves both as a temporal and spatial energy buffer of ATP levels, and intracellular energy transport carrier connecting sites of energy production with sites of energy utilization [1,2]. Humans can obtain creatine via endogenous synthesis (from amino acids glycine and *L*-arginine) and through different omnivorous foods (especially red meat and fish), accounting for a total daily creatine output of approximately 2 g for a 70 kg young adult man [3–5]. Creatine homeostasis appears to be finely tuned to the dynamics of its endogenous–exogenous provision and utilization as an energy facilitator. This is illustrated by the relatively stable intracellular levels of total creatine (creatine plus phosphocreatine) across various energy-demanding tissues in normal conditions, including the brain (4–5 mM) [6], skeletal muscle (25–30 mM) [7], and myocardium (25–30 mM) [8]. On the other hand, a reduction in intracellular creatine concentrations could result in a hypo-energetic state that occurs in a broad spectrum of pathophysiological situations, with a degree of creatine deficit often correlates with a disorder severity. This mini review summarizes disorders with low creatine levels and provides arguments for assessing and employing tissue creatine as a relevant target in clinical nutrition.

2. Creatine Shortfall in Clinical Medicine

The reduced levels of intracellular creatine complement many inherited and acquired disorders, with cerebral creatine deficiency syndromes (CCDS) often considered a clinical paradigm of creatine shortfall. CCDS are rare inborn errors of creatine synthesis machinery and transport, being autosomal recessive or X-linked conditions [9]. CCDS patients typically show clinical features of low bioenergetics (e.g., muscle hypotonia, speech delay, seizures, intellectual impairment, behavioral abnormalities), with low levels of creatine found in the brain, skeletal muscle, and biofluids considered an essential element of preliminary and confirmatory diagnosis of these conditions [10]. However, CCDS often remain underdiagnosed since the assessment of creatine metabolism is not routinely included in

standard diagnostic workup [11]. Other neurological pathologies accompanied by insufficient cerebral creatine levels (or impaired creatine-related metabolic ratios) include autism spectrum disorder (ASD) [12], traumatic brain injury [13], multiple sclerosis [14], gyrate atrophy of the choroid and retina [15], post-viral fatigue syndrome [16], and brain malignancies [17,18], while creatine levels were suboptimal in the skeletal muscle of neurological patients suffering from muscular dystrophy and neuromuscular diseases [19,20]. Several cardiovascular conditions exhibit a decrease in myocardial or subendocardial creatine, such as dilated cardiomyopathy [21], aortic valve disease [22], and transplanted heart [23]. Interestingly, it appears that a reduction in myocardial creatine significantly correlates with the clinical severity of heart failure [24], with tissue creatine put forward as an independent multivariate predictor of cardiovascular mortality in patients with failing hearts [25,26]. Tissue creatine levels were also diminished in the skeletal muscle and brain of patients with chronic respiratory failure [27,28], extracts from lung cancer patients [29], pancreatic parenchyma in patients with adenocarcinoma [30], in the brain of patients with hepatitis C and chronic HIV infection [31,32], and in the thalamus of infants with malnutrition [33], while suboptimal creatine levels are suggested in chronic kidney disease [34], and preterm birth [35]. Although the causes of the above conditions are different, they all share a disease-associated creatine deficit. The lack of creatine might not be necessarily the underlying cause for all conditions listed above but perhaps a possible side effect of these diseases. Nevertheless, patients with lower levels appear to be at a higher risk of developing more severe illness [10,24], implying low tissue creatine as a possible prognostic indicator of disease severity. In addition, it might be used as a predictive biomarker to identify individuals with specific conditions who are more likely to experience a favorable effect from the exposure to supplemental creatine. Cut-off points for low tissue creatine are not established so far for specific organs and pathologies, yet various degrees of deficiency of the cerebral creatine pool are seen in neurology, from almost complete depletion of the cerebral creatine pool in CCDS [36,37] to a partial reduction (~10%) seen in other conditions [38,39] (Table 1).

Table 1. Summary of human studies (excluding CCDS) describing low tissue creatine levels.

Pathology	Refs.
Autism spectrum disorder	[40–45]
Concussion and mild traumatic brain injury	[13,46]
Multiple sclerosis	[47–51]
Gyrate atrophy of the choroid and retina	[15,52]
Post-viral fatigue syndrome	[53,54]
Primary and secondary brain tumors	[17,18]
Neuromuscular disease	[19]
Facioscapulohumeral muscular dystrophy	[20]
Dilated cardiomyopathy	[21,24,25]
Aortic valve disease	[22]
Heart transplantation	[23]
Coronary disease	[26]
Chronic obstructive pulmonary disease	[27,28]
Lung cancer	[29]
Pancreatic cancer	[30]
Hepatitis C	[31]
Chronic HIV infection	[32]
Infant malnutrition	[33]

3. Methods for Tissue Creatine Evaluation

A breakthrough in monitoring creatine levels has occurred with magnetic resonance spectroscopy (MRS), a state-of-the-art analytical technique that can be used to non-invasively measure biochemical changes across different tissues and organs. By observing local magnetic fields around atomic nuclei, MRS allows the measurement of in vivo chemical information in a specific volume of interest, with creatine included in routine MRS profil-

ing [55]. Each metabolite has a different peak in the spectrum, which appears at a known frequency (e.g., creatine at 3.03 ppm). Both proton and non-proton MRS techniques are available, with ^{1}H MRS appearing to be more convenient than other methods to evaluate creatine concentrations. More accurate MRS methods of creatine assessment involve absolute quantification of creatine levels instead of resonance amplitudes presented as ratios [56]. The experts' working group on reporting standards for MRS recently provided a set of rules required for the acquisition, post-processing, and analysis required for appropriate interpretation of results [57], facilitating MRS use in everyday practice. MRS is a relatively expensive technique and requires an educated technician, yet it has gained popularity in recent years, as illustrated by more studies employing MRS in nutritional research [58]. The fact that MRS systems are now available in most medical centers allows the routine evaluation of creatine metabolism in many pathologies, at least those that affect energy-demanding organs. Alternatively, tissue biopsy might provide a means to assess creatine levels in specific tissues (predominantly the skeletal muscle), yet this procedure's invasiveness and possible complications prevent its ample applicability in regular practice. Possible surrogate indices of tissue creatine, including serum or salivary creatine, liquid biopsy, and creatine in cerebrospinal fluid, require more evidence to support or prove its use as MRS substitutes.

4. Transition of Tissue Creatine Evaluation from Research to Routine Practice

A meaningful application of any biomarker holds a considerable value in nutrition and medicine, yet a quest from its recognition to clinical use is often slow and arduous [59]. In the case of tissue creatine quantified by MRS, a number of requirements for a successful transition from the research environment to routine clinical practice appear to be nearing completion, with its possible uses potentially encompassing both predictive and diagnostic purposes. The analytical validity of MRS suggests relatively high reproducibility for tissue metabolites measurements (including total creatine) in various tissues [60,61]. MRS employing creatine-related metabolic ratios has high diagnostic accuracy and clinical validity in differentiating neoplasms from non-neoplastic brain tumors [62], brain metastases and primary high-grade gliomas [63], tremor-dominant Parkinson's disease (PD) and non-PD tremor [64], various parkinsonian syndromes [65], high-functioning adults with ASD and typically developing peers [66], and bone and soft tissue tumors versus normal muscle [67]. In addition, MRS sensitivity (92%) and specificity (76%) were equivalent to other superior methods in aiding the localization of prostate metabolic abnormalities that might include creatine [68]. Evaluating the clinical utility of tissue creatine is still under critical observation. Early studies suggest that MRS-driven creatine indices can help distinguish responders and non-responders to drug treatment in insomnia patients [69], with the test likely improving healthcare and changing outcomes. Although no extensive cost-effectiveness trials are available at this moment, a study suggests that abnormal creatine levels consistent with myocardial ischemia predicted cardiovascular outcomes, higher rates of anginal hospitalization, repeat catheterization, and more significant treatment costs [26]. Another trial suggests that the use of MRS may be cost-effective in specific contexts, for example, in settings where the cost of transrectal ultrasound-guided prostate biopsy exceeds the cost of obtaining an MRS sequence by ~£115 [68]. The above studies suggest added value and costs saved by knowing a patient's tissue creatine levels.

5. Creatine in Clinical Nutrition

A lack of cellular energy as a cause of disease has recently regained attention [70,71], and tissue creatine shortage might be a suitable proxy that characterizes poor bioenergetics seen in conditions that impact top energy-consuming organs. Low creatine levels perhaps indicate a high-scale utilization of this energy-facilitating compound for replenishing in-demand ATP for dysfunctional cells and/or an impaired internal production and intra- and extra-cellular transport of creatine (at least in CCDS). Whether low creatine in many acquired conditions is a consequence of a disease or a causative factor (or both) remains

poorly addressed. However, supplemental creatine or creatine analogs appear to partially or totally restore tissue creatine levels and attenuate clinical features of many maladies, including CCDS [36,72–74], mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS) syndrome [75], acute oxygen deprivation [76], chronic fatigue syndrome [77], Huntington disease [78], treatment-resistant major depressive disorder [79,80], methamphetamine-complemented depression [81], chronic heart failure [82], and rheumatoid arthritis [83], to name just a few. How much creatine needs to be compensated likely depends on the degree and type of creatine shortage; some conditions may entail covering amounts one magnitude over average dietary requirements (for a detailed review, see Ref. [84]). A vast majority of pharmacovigilance studies demonstrated favorable safety of supplemental creatine, with creatine posing no adverse health risks in humans across various life stages and conditions, at dosages ranging from 0.03 to 0.8 g per kilogram of body weight per day for up to 5 years, and dosages commonly prescribed for CCDS and other diseases/syndromes 2 to 20 g/day (for a detailed review, see Refs. [39,84]). In line with affirmative evidence from safety trials, the U.S. Food and Drug Administration (FDA) recently recognized creatine monohydrate as a safe ingredient (Generally Recognized as Safe, GRAS) [85]. This labels creatine as a non-toxic food substance under the conditions of its intended use, and perhaps expands its use across various nutritional domains. Still, determining the most bioactive chemical formulation of creatine remains a challenge [86].

6. Conclusions

A drop in creatine levels accompanies various hereditary and non-hereditary diseases, and the human brain, skeletal muscle, and heart appear to be affected rather badly by creatine shortage. Being either an etiological factor or a secondary finding, lower tissue creatine concentrations almost always refer to a more severe phenotype, implying a key role of creatine in normal homeostasis and health protection. Evaluating tissue creatine (predominantly by non-invasive techniques) might thus assist in an appropriate diagnosis, prediction, and even a nutritional treatment of a specific disease characterized by creatine shortfall, with the assay thus providing an additional value in terms of health care (Figure 1). Still, to recognize tissue creatine as a potential novel biomarker candidate, additional studies are highly warranted to substantiate its biological plausibility, practicability in real-life situations, sensitivity and specificity, and cost-effectiveness, along with its responsiveness to treatment (such as exogenous creatine) in various clinical populations.

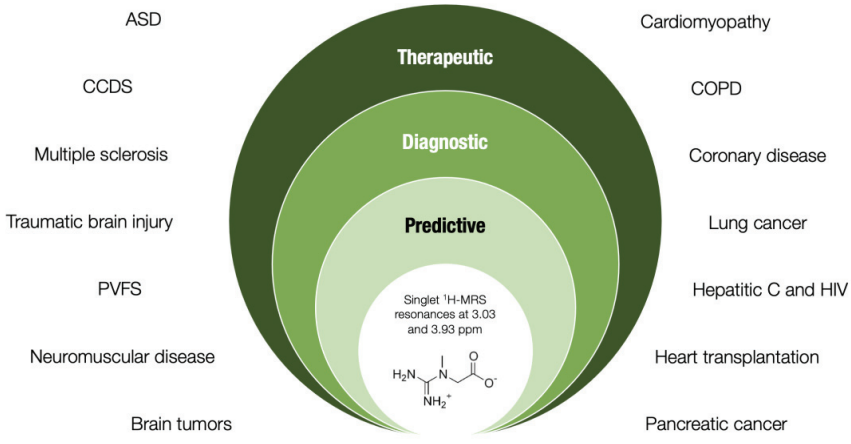

Figure 1. Theoretical framework of tissue creatine assessment via proton magnetic resonance spectroscopy (MRS) in clinical science. *Abbreviations*: ASD, autism spectrum disorder; CCDS, cerebral creatine deficiency syndromes; PFVS, post-viral fatigue syndrome; COPD, chronic obstructive pulmonary disease.

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Short-Term Ingestion of Medium-Chain Triglycerides Could Enhance Postprandial Consumption of Ingested Fat in Individuals with a Body Mass Index from 25 to Less than 30: A Randomized, Placebo-Controlled, Double-Blind Crossover Study

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Abstract: The elimination of obesity is essential to maintaining good health. Medium-chain triglycerides (MCTs) inhibit fat accumulation. However, studies examining energy expenditure and fat oxidation with continuous ingestion of MCTs show little association with the elimination of obesity. In this study, we conducted a randomized, double-blind crossover clinical trial to investigate the effects of continuous ingestion of MCTs on postprandial energy expenditure and ingested long-chain triglycerides (LCTs) oxidation. A daily 2 g of MCTs were ingested for two weeks by sedentary participants with a body mass index (BMI) from 25 (kg/m^2) to less than 30. Ingestion of a meal containing MCTs and isotopic carbon-13-labeled (13C) LCTs increased energy expenditure and consumption of diet-derived LCTs, as determined by postprandial 13C carbon dioxide excretion, compared to canola oil as the placebo control. These results indicate that continuous ingestion of MCTs could enhance postprandial degradation of diet-derived fat and energy expenditure in sedentary, overweight individuals.

Keywords: medium-chain triglycerides (MCTs); obesity; energy expenditure; diet-derived fat; postprandial resting metabolism; sedentary; octanoic acid; decanoic acid

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1. Introduction

Obesity is well-known as a risk factor for metabolic diseases such as hypertension, dyslipidemia, and type 2 diabetes [1–3]. Its elimination is essential to maintaining good health by reducing the occurrence and extension of health problems caused by metabolic diseases [4]. The basic strategy to eliminate obesity is to increase energy expenditure and limit energy intake [4,5]. Lipids, one of the energy-producing nutrients, have been shown to have varying effects on obesity, depending on the types of fatty acids [6]. It is crucial to examine the effects of fatty acids in lipids on energy and lipid metabolism relating to obesity.

Medium-chain fatty acids (MCFAs) are widely defined as straight-chain saturated fatty acids with a chain length ranging from 6 to 12 carbon atoms [7,8]. In a nutritional study, research on the alternative use of long-chain fatty acids started mainly on fatty acids with a chain length of 8 carbon atoms (octanoic acid, C8) and 10 carbon atoms (decanoic acid, C10) [7,9]. This was followed by research on biological regulatory functions different from those of long-chain fatty acids, one of which was research on the elimination of obesity [10,11].

Continuous ingestion of medium-chain triglycerides (MCTs) or medium- and long-chain triglycerides (MLCTs) to suppress fat accumulation has been compared to ingestion of long-chain triglycerides (LCTs) [12–16]. Continuous ingestion of MCTs to enhance fat

oxidation during low-intensity physical activity was reported over the effect of LCTs [7,17]. However, there are few results on the effects of short-term MCT ingestion on postprandial energy and lipid metabolism, which can be associated with the elimination of obesity. Studies examining postprandial enhancement of energy expenditure with continuous ingestion of MCTs have not reached a consensus; some reports [18–20] show a significant increase while others [21] find no difference. Studies examining the increase in fat oxidation after meals have reported either positive [19,20] or negative [18] effects. Ingested MCTs are oxidized more than LCTs [22,23]. However, little is known about the enhanced degradation of dietary LCTs when MCTs are ingested continuously.

In this study, we conducted a randomized, double-blind crossover clinical trial, using canola oil as a placebo control, to examine the effects of continuous ingestion of MCTs on postprandial ingested LCTs oxidation, energy expenditure, and fat oxidation. Individuals with a body mass index (BMI) over or equal to 25 (kg/m^2) and less than 30 were given 2 g of MCTs daily for two weeks, followed by a meal containing MCTs and carbon 13 isotope-labeled (13C) LCTs. To measure 13C carbon dioxide, expired air was collected postprandially. Oxygen consumption and carbon dioxide production were measured to examine energy expenditure and fat oxidation after a meal.

2. Materials and Methods

Ethical considerations, setting the target number of participants, eligibility and exclusion criteria, the oils and fats studied, study design, participant compliance matters during the intervention period, and dietary surveys during the intervention period were briefly described. These details were detailed previously [7].

2.1. Ethical Considerations

The present study was conducted in compliance with the Declaration of Helsinki (revised in 2013), the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects, and the Japanese Act on the Protection of Personal Information [24,25]. We obtained approval from the ethics committees of Yoga Allergy Clinic (approval number 21000023). We registered this clinical trial in UMIN-CTR before the recruitment of participants (UMIN000043022, URL: https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&recptno=R000049074&type=summary&language=E, accessed on 24 January 2022).

2.2. The Number of Participants

The target number of participants was set at 30. The number of participants required was estimated to be a minimum of 18 or 23 based on the evaluation indicators of a previous study [17]. The number of participants was determined by comparing the MCT intake and measuring instrument of a previous study with the present study.

2.3. Target Participants

The target participants were those who met the following criteria: healthy Japanese males and females aged between 35 and 64 years (at the time of obtaining written consent); a BMI over or equal to 25 and less than 30; non-smokers; consumed less than 30 g/day of alcohol; those who received a sufficient explanation of the purpose and content of the research, had the capacity to consent, had volunteered willingly to participate in the study, had a good understanding of the study, and agreed to participate in the study in writing.

Those who met the following criteria were excluded from the participants: persons with serious diseases or histories (cardiac, hepatic, renal, cardiovascular, or hematological diseases); who had experienced chest pain or abnormal pulse while at rest; who were currently undergoing exercise or diet therapy under the medical supervision of a physician; who frequently experienced shortness of breath, dizziness, vertigo, or loss of consciousness; who were allergic to drugs, food, raw materials of test meal (soybeans, milk protein), or had a history of allergy; who had a current or past history of drug or alcohol dependence; who take

any health foods, supplements, or drugs that may affect fatigue reduction, fat burning, or obesity control; who exercised to maintain or improve physical fitness for a total of 60 min or more per week; whose weight fluctuated by ±5 kg or more over two months.

2.4. Test Diets

MCTs (The Nisshin OilliO Group, Ltd., Tokyo, Japan) (C8:C10 ratio = 75:25) was used as the test oil (TO). The control oil (CO) was canola oil, a rapeseed oil with low erucic acid (The Nisshin OilliO Group, Ltd., Tokyo, Japan). The participants consumed the TO or the CO at 2 g per day for the 13-day intervention periods.

2.5. Management during the Intervention Period

The present study consisted of two 13-day interventions and a one-day measurement, separated by a 14-day washout period. We asked the participants to maintain body weight; consume the TO or CO; weigh themselves daily; make as few changes as possible to their lifestyle and environment (such as meals, alcohol consumption [less than 30 g/day], exercise, sleep, and work); not ingest any medicines, supplements, health foods (including oil for health), or functional foods that may affect the reduction of fatigue, fat burning, or obesity, and report the results daily for electronic recording; refrain from strenuous exercise for three days before the measurement; and refrain from drinking alcohol on the day before that.

2.6. Dietary Survey

For three days, the 11th to 13th days after TO or CO ingestion, we asked the participants to take photographs of their meals and complete record sheets. We calculated nutritional values from the photos and record sheets, and determined the daily intake of energy, protein, fat, carbohydrate, C8, and C10. In calculating nutritional values, we used the Standard Tables of Food Composition in Japan 2015 (seventh revised version) [26].

2.7. Measurements

We used an ordinary pressure-type human calorimeter (Fuji Human Calorimeter FHC-30S, Fuji Medical Science Co., Ltd., Chiba, Japan) installed in the metabolic measurement room. The specification of the human calorimeter was described previously [7]. The inflow rate of air into the room was set at 50 L/min, and the air supply and exhaust were controlled at the same rate with an accuracy of ±0.5%. The room temperature and humidity were controlled at 25 °C ± 0.1 °C and 50% ± 1.0%, respectively. The accuracy of an analyzer (Thermo Scientific Prima Pro, Process Mass Spectrometer, Thermo Fisher Scientific, Inc., Waltham, MA, USA) installed in the human calorimeter for the exhausted oxygen and carbon dioxide was ±0.002%. The accuracy of the measurements during this study was confirmed by conducting three alcohol combustion tests before, during, and after the study. The theoretical values of oxygen consumption and carbon dioxide production were calculated from the weight of alcohol burned. These measured values were obtained by analyzing the measured data using Henning's formula [27]. The recovery rate (formula: (measured value/theoretical value) × 100; unit: %), which is the measurement accuracy expressed from the theoretical and measured values, was within 100% ± 2% for both oxygen consumption and carbon dioxide production.

The participants visited the metabolic measurement room in an overnight fasted state and consumed a meal (energy 483 kcal, protein 15.5 g, fat 15.2 g, carbohydrate 75.1 g) containing 2 g of TO or CO and 400 mg of 13C triolein (TRIOLEIN, 1,1,1-13C3, purity 99%, CLM-163-PK, Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA).

The resting oxygen uptake rate and carbon dioxide production rate were measured with participants in a sitting position, over a period of time from before meal ingestion to four hours afterwards. The measured values were analyzed using Henning's formula. Expired gas was collected every hour, using a gas bag (Tedlar bag, 1-2711-04, As One Corporation, Osaka, Japan). The collected expired gas was used to measure the carbon 13/carbon 12 ratio in carbon dioxide, using the gas analyzer installed in the human calorimeter.

2.8. Calculation of the Consumption Rate of Diet-Derived LCTs

To determine the consumption rate of diet-derived LCTs, we used the following equation to calculate the consumption rate of 13C triolein, based on the carbon 13/carbon 12 ratio in carbon dioxide of the collected expired gas, the carbon dioxide production rate obtained from the human calorimeter, and the ingested 13C triolein weight [28].

Consumption rate of diet-derived LCTs (%) = {(carbon 13/carbon 12 ratio in carbon dioxide after ingestion − carbon 13/carbon 12 ratio in carbon dioxide before ingestion) × carbon dioxide production rate after ingestion × 100} /{ingested weight of 13C triolein × (number of carbon 13 labels in 13C triolein/molecular weight of 13C triolein) × 22.4}.

To determine the cumulative consumption rate of postprandial diet-derived LCTs from the obtained consumption rate of diet-derived LCTs over time, the area under the curve (AUC) was determined using the trapezoidal method.

2.9. Calculation of Postprandial Energy Expenditure Rate, Respiratory Quotient, and Fat and Carbohydrate Oxidation Rates

We calculated the respiratory quotient (RQ), fat oxidation rate, carbohydrate oxidation rate, and energy expenditure rate from the oxygen uptake rate and carbon dioxide production rate obtained from the human calorimeter, using the following equations [29].

$$RQ = \text{carbon dioxide production rate}/\text{oxygen uptake rate}.$$

$$\text{Fat oxidation rate (mg/min)} = 1.67 \times \text{oxygen uptake rate} - 1.67 \times \text{carbon dioxide production rate}.$$

$$\text{Carbohydrate oxidation rate (mg/min)} = 4.55 \times \text{carbon dioxide production rate} - 3.21 \times \text{oxygen uptake rate}.$$

$$\text{Energy expenditure rate (kcal/min)} = 9.75 \times \text{fat oxidation rate} + 3.74 \times \text{carbohydrate oxidation rate}.$$

We calculated the AUC using the trapezoidal method to determine the cumulative value of energy expenditure, fat and carbohydrate oxidation from the energy expenditure rate, and rates of fat and carbohydrate oxidation measured over time.

2.10. Primary and Secondary Outcomes

The primary outcome was the cumulative consumption rate of diet-derived LCTs. Secondary outcomes were cumulative values of energy expenditure, fat and carbohydrate oxidation, energy expenditure rate, and RQ.

2.11. Statistical Analysis

The cumulative consumption rate of diet-derived LCTs and the cumulative values of energy expenditure and fat and carbohydrate oxidation during a four-hour postprandial period were checked for normality by a Shapiro–Wilk test, to compare between the TO and CO intervention groups. If there was no normality, we performed a Mann–Whitney U test. If there was normality, we used the F test to check for equivariance. We conducted a Student's *t*-test if there was an equivariance, and a Welch's *t*-test if equivariance was absent. We analyzed for significance the intervention effect (the value of the TO group minus that of the CO group) of the cumulative consumption rate of diet-derived LCTs and cumulative values of energy expenditure and fat and carbohydrate oxidation during the four-hour postprandial period, using the Mann–Whitney U test.

In the difference of postprandial longitudinal energy expenditure and RQ, and their intervention effect in the TO and CO groups, we compared data using a linear mixed model. We set the intervention group, time, and ingestion order as a fixed effect, and the participant as a random effect. We estimated the values by the restricted maximum likelihood method with a random intercept model equation. We analyzed the fixed effect for significance and determined estimates and 95% confidence intervals. If the estimated values for the intervention group were significant, normality was checked with the Shapiro–Wilk test to compare the values for each time between the two intervention groups. If there was no

normality, a Mann–Whitney U test was conducted. If there was normality, we conducted an F test to check for equivariance. We performed a Student's *t*-test if equivariance was present, and a Welch's *t*-test if equivariance was not present.

When measurement indices showed significant differences, we analyzed carryover effects. If we found them to be of significance, we withheld the results.

We used Microsoft Excel for Office365 MSO (Microsoft Japan Co., Ltd., Tokyo, Japan) to calculate the basic statistics of the analyzed data. We used the statistical package R (Version 3.4.3, R Core Team, Vienna, Austria) for statistical processing. For all tests, p values of less than 5% were considered to indicate a significant difference.

3. Results

Some of the data (metabolic data during low-intensity physical activity) obtained in this study has been published previously [7]. An analysis of metabolic data during resting was conducted in this study.

3.1. Participants

Seventy-two participants who obtained written informed consent were screened by interviews, physical measurements, and biochemical and hematological tests. Thirty eligible participants were randomly assigned to two groups (14 participants and 16 participants)—they ingested the test diets and were measured. One participant discontinued the study before the second measurement. Twenty-nine participants completed the study. A flowchart of the participants was provided previously [7]. Those 29 participants were used as the study participants, for analysis. The 29 analysis participants (17 males and 13 females) at the screening were aged 50.3 ± 9.1 (mean ± standard deviation), 165.4 ± 8.9 cm height, 73.9 ± 9.5 kg weight, and BMI 26.8 ± 1.3 kg/m^2.

3.2. Dietary Intake

There were no significant differences in energy (CO: 1798.2 ± 316.1; TO: 1974.8 ± 359.4 kcal, mean ± standard deviation), protein (CO: 67.2 ± 14.2; TO: 71.3 ± 16.5 g), carbohydrate (CO: 226.5 ± 61.8; TO: 246.2 ± 57.3 g), and fat (CO: 64.8 ± 15.6; TO: 73.7 ± 22.6 g) intakes between the two groups. Intakes of MCFAs (C8, C10) were significantly greater in the TO group (C8: 2.0 ± 0.1; C10: 0.9 ± 0.2 g) than in the CO group (C8: 0.1 ± 0.1; C10: 0.2 ± 0.2 g).

3.3. Measurement Result of Primary Outcome

The intervention effect value in the cumulative consumption rate of diet-derived LCTs was 0.7% ± 0.4% (mean ± standard error), which indicated a significantly greater consumption rate in the TO group than in the CO group (Table 1).

Table 1. Cumulative values for four hours after a meal [1] Cumulative consumption rate of diet-derived LCTs and cumulative values of energy expenditure and fat and carbohydrate oxidation in the postprandial measurement.

	CO Group	TO Group	Intervention Effect Value (TO–CO)
Rate of diet-derived LCTs, %	3.8 ± 0.5	4.5 ± 0.5	0.7 ± 0.4 [#]
Energy expenditure, kcal	18.6 ± 2.1	26.1 ± 2.6 *	7.5 ± 3.3
Fat oxidation, g	0.6 ± 0.3	0.7 ± 0.3	0.1 ± 0.2
Carbohydrate oxidation, g	19.1 ± 1.9	18.5 ± 1.8	−0.7 ± 2.3

[1] Values are expressed as means ± standard errors. n = 29. * Significant difference compared to control group ($p < 0.05$, Mann–Whitney U test). [#] Significant mean difference was detected ($p < 0.05$, Mann–Whitney U test).

3.4. Measurement Results of Secondary Outcomes

The cumulative value of energy expenditure in the TO group was 27.0 ± 2.5 kcal, which was significantly greater than that in the CO group (19.5 ± 2.0 kcal). As for the

cumulative values of fat and carbohydrate oxidation, there were no significant differences between the two groups (Table 1).

Changes in measurement values of energy expenditure rate and RQ were not significantly different between the two groups (Table 2).

Table 2. Changes in measurement values over time for four hours after a meal [1]; energy expenditure rate and respiratory quotient in the postprandial measurement.

	CO Group	TO Group	Intervention Effect Value (TO–CO)
Energy expenditure rate, kcal/min			
Baseline	1.12 ± 0.03	1.10 ± 0.03	−0.021 ± 0.014
1 h after	1.25 ± 0.04	1.26 ± 0.04	0.016 ± 0.015
2 h after	1.23 ± 0.03	1.25 ± 0.03	0.021 ± 0.017
3 h after	1.14 ± 0.03	1.15 ± 0.03	0.014 ± 0.012
4 h after	1.09 ± 0.03	1.10 ± 0.03	0.005 ± 0.013
Respiratory quotient			
Baseline	0.81 ± 0.01	0.82 ± 0.01	0.013 ± 0.011
1 h after	0.87 ± 0.01	0.88 ± 0.01	0.008 ± 0.007
2 h after	0.89 ± 0.01	0.89 ± 0.01	−0.001 ± 0.012
3 h after	0.90 ± 0.01	0.89 ± 0.01	−0.010 ± 0.011
4 h after	0.85 ± 0.01	0.86 ± 0.01	0.014 ± 0.009

[1] Values are expressed as means ± standard errors. n = 29. There were no significant differences between the groups.

3.5. Carryover Effect of Measurement Outcomes

There were no significant carryover effects in the measurement outcomes.

4. Discussion

In a survey of the Japanese population, approximately 30% of men and 20% of women had a BMI of 25 or higher [30]. The overweight participants in the present study, with a BMI over or equal to 25 and less than 30, are classified as pre-obese by the World Health Organization [31] and obese level I in Japan.

In this study, we investigated the beneficial effect of MCTs on postprandial ingested fat consumption, which could be involved in the elimination of obesity. Stable isotope-labeled LCTs were fed to the participants. We measured the isotope-labeled carbon dioxide production associated with the increased degradation of diet-derived LCTs. The results showed that the continuous ingestion of MCTs significantly enhanced postprandial consumption of diet-derived fat, compared to canola oil. The intake of MCFAs (C8 and C10) by the CO group during the two-week intervention period was equivalent to approximately one-tenth that of the TO group, which approximates to the average intake of Japanese people. Estimating from the report of the National Health and Nutrition Examination Survey in Japan [30], MEXT meeting materials [32], and the Standard Tables of Food Composition in Japan 2015 (seventh revised edition) [26], the average total C8 and C10 intake by Japanese people is 0.2–0.3 g (2003: 0.252 g; 2008: 0.230 g; 2013: 0.249 g; 2018: 0.265 g; 2019: 0.271 g), and the percentage of total fat intake is approximately 0.5% (2003: 0.528%; 2008: 0.499%; 2013: 0.512%; 2018: 0.496%; 2019: 0.499%). The present study shows that continuous ingestion of 10 times more MCFAs (C8 and C10) than the daily intake by sedentary, overweight individuals may enhance the degradation of exogenous fat associated with the elimination of obesity to a greater extent than routinely consumed LCTs.

A previous study using isotope-labeled MCTs and LCTs showed that ingested MCTs are more degraded postprandially than ingested LCTs [22,23]. However, no studies seem to have reported accelerated degradation of ingested LCTs after a meal following continuous ingestion of MCTs. A previous study [33] examined the increased degradation of exogenous LCTs when continuously ingesting coconut oil (mainly lauric acid (C12) and myristic acid (C14)) as MCTs, which differs from the MCTs (C8:C10 = 75:25) in the present study. It

reported that there was no difference or a decrease in the consumption of diet-derived LCTs, compared to continuous ingestion of beef tallow as the control diet.

Two previous studies examining the degradation of diet-derived 13C-triolein showed that it did not change in normal weight subjects (in a study examining differences in exercise habits [34]), and decreased in obese subjects, compared to normal weight subjects (in a study examining differences in a BMI [23]). Although a previous study suggested that the degradation of dietary triolein decreases with obesity, as shown in the present study, a two-week ingestion of MCTs significantly increased the consumption of diet-derived triolein in sedentary, overweight individuals, and short-term ingestion of MCTs could increase diet-derived fat degradation.

Multiple factors are involved in the degradation of LCTs after a meal: degradation by digestive enzymes in the intestinal tract, absorption from the intestinal tract, re-esterification and lipoprotein synthesis in small intestinal cells, lymphatic transport, lipoprotein metabolism in the blood, binding of free fatty acids to albumin, and metabolism of fatty acids taken up by organs [35]. Among these factors, fatty acid metabolism in the liver is considered possibly to affect the accelerated degradation of ingested LCTs by continuous ingestion of MCTs. LCTs in lipoproteins released into the blood after a meal are converted into fatty acids by lipoprotein lipase in the blood and distributed to adipose tissue, muscle tissue, and liver, which are the major organs that use fatty acids in the body [35,36]. Studies of animals have shown that consumption of MCFAs (C8 and C10) increases the degradation of fatty acids, including beta-oxidation, and increases the degradation of stored fat and the release of fatty acids into the bloodstream in adipose tissues [37–39]. Of these organs, the liver actively consumes energy even during resting [40], and ingested long-chain fatty acids from the diet that reach the liver after a meal may also accelerate their degradation.

In this study, a two-week ingestion of MCTs increased postprandial energy expenditure more than canola oil ingestion, in participants with an average BMI of 26.8. This suggests that continuous ingestion of MCTs by overweight individuals may increase postprandial energy expenditure, contributing to the resolution of obesity. Many clinical studies have reported increases in postprandial energy expenditure with single or multiple ingestions per day of MCTs [19,41–43] or MLCTs [44,45]. In a study of lean and obese subjects, postprandial energy expenditure was significantly higher when they consumed MCTs than when they consumed LCTs [46]. A negative result [21] in subjects with a mean BMI of 21.4 and a positive result [18] in subjects with a mean BMI of 22.6 were reported, for increased energy expenditure after a meal containing MCTs and after continuous ingestion of MCTs, with inconsistent results shown in lean individuals. On the other hand, positive results in subjects with mean BMIs of 26 and 31.8 have been reported [19,20]. In studies of pre-obese and obese individuals, the increase in energy expenditure after the meal containing MCTs and with continuously consumed MCTs was consistent, supporting the results of the present study.

White et al. [21] showed negative results in enhancing the effect of continuous ingestion of MCTs on energy expenditure, pointing out that a higher proportion of MCTs in the fat ingested to evaluate energy expenditure may increase energy expenditure. They used butter and coconut oil as sources of MCFAs, and the fats in the evaluation had 7.9% MCFAs (C8 and C10) in total fatty acids. Compared to the control, C12 and C14 were approximately 16% and 9% higher, and oleic acid was approximately 15% lower. In the evaluation diet of the present study, MCTs were approximately 13% in total fat and did not differ, except for the 2 g of TO and CO. In previous single-dose studies with relatively low intakes of MCFAs (C8 and C10), the C8 and C10 in the fat were approximately 4% when the results were negative for effects on postprandial energy expenditure [47], whereas the C8 and C10 in the diet were approximately 12% when the results were positive [45]. Although the proportion of MCTs in fat intake that shows an effect of increased energy expenditure after a meal is not sufficiently clear, the ratio may affect energy expenditure after a meal.

In this study, fat and carbohydrate oxidation did not differ from the CO group in substrate utilization, when the significant increase in postprandial energy expenditure occurred after consumption of MCTs in the TO group. There was no significant change in body weight during the intervention period. The amount of energy intake was within the range of the daily amount of the participants, and the fat energy intake was approximately 33%, which is similar to the daily diet of Japanese people. A previous human study reported that fat oxidation did not increase when the subjects consumed 15 g of MCTs daily, within the daily energy intake range, and less than 30% of fat energy [18]. Studies of animals have reported that consumption of MCTs enhances carbohydrate utilization. Continuous ingestion of MCTs resulted in increased glucose uptake in muscle tissue and de novo fatty acid synthesis, resulting in increased glucose uptake in the liver [48,49]. These studies indicate that continuous ingestion of MCTs may enhance the utilization of both carbohydrate and fat when postprandial energy expenditure is increased. On the other hand, one human study with 150% of energy intake of daily energy [19] and another with 40% of fat energy during the interventions [20] reported that fat oxidation during continuous ingestion of MCTs is higher than for ingestion of LCTs. One possible cause of this difference in results is an increase in the energy proportion of fat to the total energy. In a study that measured 24 h RQ after two days of ingesting diets with 20% and 50% fat energy, RQ was significantly lower and fat oxidation was higher in both lean and obese subjects after consuming a diet with 50% fat energy [34]. In a study of individuals with an average BMI of 24, a three-day high-fat diet (40% fat energy) resulted in more degradation of the ingested fat than a high-carbohydrate diet (10% fat energy) [50]. Even in a single-dose study, the rate of fat oxidation after a fat-rich diet increased significantly, compared to a carbohydrate-rich diet, in both lean and obese individuals [51]. These studies suggest that postprandial fat oxidation may have more to do with the ratio of fat energy in total energy than with differences in fatty acids of the lipid (MCTs or LCTs). In the present study, RQ did not decrease after a meal containing MCTs when the ratio of fat energy to total energy was approximately 33%. Continuous ingestion of MCTs enhances postprandial energy expenditure and may lead to the elimination of obesity, but the substrates used for energy expenditure may depend on the balance between fat and carbohydrate intakes.

On the other hand, continuous ingestion of MCTs could enhance lipolysis when there is increased energy expenditure in the skeletal muscle and may contribute to the resolution of obesity. In reported human studies, continuous ingestion of MCTs increased fat oxidation during aerobic exercise in recreational athletes [52] and people without exercise habits [7,17], compared to ingestion of LCTs. Studies of animals have reported explainable action mechanisms for increased fat oxidation during aerobic exercise, including increased mitochondrial biosynthesis in skeletal muscle, increased fat transport, and increased ketone body metabolism [49,53,54]. In addition, continuous ingestion of MCTs and MLCTs has been reported to increase fat metabolism, which may contribute to the resolution of obesity. Studies of animals have shown increased expression of uncoupling protein 1 (in adipose tissue) [39] and uncoupling protein 3 (in the mitochondria of skeletal muscle) [38], and increased enzyme activities and expression of genes involved in fatty acid degradation in the liver [37]. It has been shown that continuous ingestion of MCTs may lead to the elimination of obesity, with various responses that differ from those of ingestion of LCTs when the metabolism of energy-producing nutrients is dramatically changed, such as through diet or physical activity.

The limitations of the present study are the following. First, although we observed a positive effect on postprandial ingested fat consumption after the two-week intervention period, the direct effect on the accumulation of body fat is unknown. Second, although we observed a significant increase in energy expenditure in the four hours after a meal, the daily energy expenditure is unknown. Third, although we observed a positive effect of MCTs on the daily diet of the Japanese, the effects of MCTs when consuming diets of different compositions of macro-nutrients (protein, fat, and carbohydrate) is unknown.

5. Conclusions

From the results of the present study, continuous ingestion of 2 g/day of MCTs by individuals with a BMI over or equal to 25 and less than 30 kg/m^2 could enhance the oxidation of diet-derived LCTs. The mechanism of action may be enhanced fatty acid degradation in the liver, caused by the continuous ingestion of MCTs. In addition, continuous ingestion of MCTs could increase postprandial energy expenditure (compared to canola oil) in sedentary, overweight individuals, but the substrates consumed for energy may depend on the ratios of fat and carbohydrate to total energy intake.

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Institutional Review Board Statement: The present study was conducted according to the guidelines of the Declaration of Helsinki (2013) and was approved by the ethics committees of Yoga Allergy Clinic (approval number: 21000023). This trial was registered at https://www.umin.ac.jp/ctr/index.htm as UMIN000043022.

Informed Consent Statement: Informed consent was obtained from all participants involved in the present study.

Data Availability Statement: Data not available due to commercial restrictions.

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Conflicts of Interest: The authors N.N. and S.T. are employees of The Nisshin OilliO Group, Ltd. The company is engaged in the manufacture and sale of products related to MCTs. K.K. is the physician who supervised the present study, and had no conflict of interest.

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Changes in Biomarkers of Non-Alcoholic Fatty Liver Disease (NAFLD) upon Access to Avocados in Hispanic/Latino Adults: Secondary Data Analysis of a Cluster Randomized Controlled Trial

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a public health concern and Hispanic/Latinos are disproportionately affected. There is evidence for favorable effects of dietary intake of monounsaturated fatty acids (MUFA) on NAFLD, yet studies examining avocados as a source of MUFA on hepatic function have not been assessed. We investigated the effects of low (3) vs. high (14) avocado allotment on biomarkers of NAFLD, oxidative stress, and NAFLD fibrosis score in a sample of Hispanic/Latino adults. Primary outcomes include hepatic function biomarkers [gamma glutamyltransferase (GGT), high-sensitivity c-reactive protein (hsCRP), and NAFLD fibrosis score]. Unpaired, two-sided *t*-tests were used to assess mean differences between intervention groups at 6 months and analysis of covariance models were used to adjust for diet quality and change in avocado intake from baseline to 6 months. Multivariable linear regression models evaluated the baseline and post-intervention association between avocado allotment group and outcomes, adjusting for covariates and stratifying by prediabetes status. No statistically significant differences were observed between low and high avocado allotment groups in liver enzymes, GGT, hsCRP or NAFLD fibrosis score. Findings persisted after stratifying by prediabetes status. Varied intake of avocados resulted in no effects on biomarkers of NAFLD in healthy adults, free of severe chronic disease.

Keywords: *Persea americana*; non-alcoholic liver disease; liver enzymes; inflammation; oxidative stress

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver disease in the United States (US) and the most common cause of abnormal liver functions tests [1]. Steatohepatitis and hepatic fibrosis, both features of NAFLD, are prognostic indicators of an increased risk for morbidity and mortality, including that from cardiovascular disease (CVD) [2,3]. Notably, the majority of deaths in individuals with NAFLD are cardiovascular events, followed by hepatocellular carcinoma and end-stage liver disease [4,5]. Therefore, NAFLD is a serious public health concern affecting almost one third of the US population [6,7].

There is a significant racial and ethnic disparity in the prevalence of NAFLD in the US, with a disproportionately higher burden in Hispanic/Latinos [8]. In this regard, Shaheen et al., showed that Mexican-Americans had the highest prevalence of severe NAFLD (46%)

relative to the other racial/ethnic groups (other Hispanic 30%, non-Hispanic Black 23%, non-Hispanic White 32%, and other race 28%); yet, only male Mexican-Americans, but not females, had a higher likelihood of both moderate and severe NAFLD relative to non-Hispanic whites [8]. Moreover, in a recent systematic review and meta-analysis, Rich et al. [9] reported significant racial disparities in NAFLD prevalence and severity in the US, with the highest proportion in Hispanic/Latinos (22.9%, 95% CI 21.6–24.1%), compared to non-Hispanic Whites (14.4%, 95% CI 14.0–14.8%), among participants in population-based studies. Regarding severity, ten studies evaluated NASH (nonalcoholic steatohepatitis) among NAFLD patients, and the pooled NASH prevalence was 31.4% (95% CI 30.1–32.7%). However, NASH prevalence was highest in Hispanics (45.4%, 95% CI 40.7–50.2%) and intermediate in Whites (32.2%, 95% CI 30.7–33.7%) [9]. In addition, a recent epidemiologic study showed that the mortality rates for NAFLD-cirrhosis and hepatocellular carcinoma have increased in non-Hispanic Whites followed by Hispanics [10].

Furthermore, the findings of a recent prospective study of 400 patients reported the prevalence of NAFLD among adult Hispanic/Latinos to be 19.4% [11], which differs among the Hispanic/Latino sub-populations. Particularly, Hispanic/Latinos of Mexican origin have the highest prevalence of NAFLD (33%), followed by Hispanic/Latinos of Puerto Rican origin (18%), and Hispanic/Latinos of Dominican origin (16%) [12]. Additionally, Le et al. [7] analyzed 1999–2012 data from the National Health and Nutrition Examination Survey (NHANES) to determine NAFLD prevalence and risk factors using two noninvasive techniques: the United States fatty liver index (USFLI) to ascertain NAFLD and NAFLD fibrosis score (NFS) to ascertain advance fibrosis. They reported that among 6000 individuals, 30.0% had NAFLD and 10.3% of these had advanced fibrosis. Some of the risk factors associated with NAFLD were male sex, slightly older in age (mean $\pm$ standard deviation, 53.2 $\pm$ 16.6 years among those with NAFLD vs. 47.3 $\pm$ 16.9 years among those free of NAFLD), Mexican-American ethnicity, lower income, and a lower education level. Similarly, and compared to their non-NAFLD counterparts, those with NAFLD were more likely to have cardiometabolic conditions such as metabolic syndrome, diabetes, ischemic heart disease, congestive heart failure, and a history of stroke, as well as a higher body mass index (BMI) and waist circumference. Conversely, in those with NAFLD, male sex and Mexican-American ethnicity were shown to be protective factors against advanced fibrosis [7].

Recent evidence suggests adherence to a dietary pattern high in monounsaturated fatty acids (MUFAs) is significantly associated with improved NAFLD-related markers including liver enzyme levels, liver fat content, fatty liver index, and steatosis [13]. MUFAs have been shown to enhance lipid oxidation and hinder lipogenesis, lowering hepatic steatosis [14]. Furthermore, other suggested favorable effects of MUFAs on NAFLD are associated with improved blood lipid profiles while stimulating removal of adverse circulating triglycerides [15,16]. However, trials thus far have been limited to examining the effects of olive oil consumption as main source of MUFAs [17–19], and have not investigated other foods sources. Therefore, examination of the impact of other MUFA-rich foods on hepatic health is warranted.

Avocados are a nutrient-dense food, high in MUFAs, and a rich source of antioxidants and polyphenolic compounds [20]. Although the effects of avocado on blood lipids have been previously studied [21], less is known about its effects on hepatic health. In this respect, there has only been one randomized controlled trial that has examined the impact of avocado consumption on CVD-related biomarkers of oxidative stress [22], which showed that a moderate-fat diet with 1 avocado/day for 5 weeks decreased circulating oxidized low-density lipoprotein (oxLDL) by 8.8% in adults with overweight and obesity, compared to baseline, after a run-in with the average American diet. Of note, none of the diets significantly affected plasma F2-isoprostane; a biomarker specific to lipid peroxidation. While the aforementioned study is the first to assess the effects of avocado on specific CVD-related oxidative stress measures, the effects of avocado on NAFLD biomarkers in healthy individuals free of chronic disease has not been reported.

In secondary analysis of data from the *Effects of Different Allotments of Avocados on the Nutritional Status of Families: A Cluster Randomized Controlled Trial* [23], we sought to determine the effects of high vs. low avocado intake on biomarkers of NAFLD and hepatic oxidative stress, with and without adjustment for adherence and other dietary adjustments. We hypothesized compared to low avocado intake, high avocado intake would have a beneficial effect on oxidative stress and hepatic health indicated by greater reductions in liver function tests and NAFLD fibrosis score.

2. Materials and Methods

2.1. Study Design and Population

The Effects of Different Allotments of Avocados on the Nutritional Status of Families: A Cluster Randomized Controlled Trial (i.e., the parent trial) was a cluster, randomized controlled trial of Hispanic/Latino families residing in San Diego County, California, and examined the impact of two levels of avocado allotment (i.e., 3 avocados/week/family vs. 14 avocados/week/family), plus a standard nutrition education intervention, on the nutritional status of Hispanic/Latino families. The primary outcomes of the parent trial included change in a family's self-reported total energy and macro- and micronutrient intakes.

Details on the methodology of the parent trial have been described elsewhere [23]. In brief, recruitment included a query search for potential participants of electronic medical records from a comprehensive health care system in South and Central San Diego County that primarily serves Hispanic/Latinos. Other recruitment strategies included telephone calls, flyers, and in-person contacts during clinic health fairs. A 14-day run period further evaluated and confirmed eligibility, as well as assessed commitment and adherence to study procedures by a study *promotora* (community health worker). Baseline measures were taken at this time, including a fasting blood draw at a local LabCorp clinic, and the head of household was self-identified by each family (one per family). After this, a computer-generated blocked, randomization sequence randomized the confirmed families.

Seventy-two families with at least 3 members of $\geq$5 years of age and residing in the same home, free of severe chronic disease, not on specific diets, and self-identified of Hispanic/Latino heritage, were randomized to one of two levels of avocado allotment (Low = 3/week/family or High = 14/week/family) for 6 months plus 12 bi-weekly standard nutrition education sessions led by *promotoras* (Figure 1 and Table 1). For each family randomized in the parent trial, a "head of household", self-identified as the family member responsible for grocery shopping and meal preparation (i.e., in the Hispanic/Latino culture is commonly female, usually the mother, grandmother, or aunt). These heads of household completed all assessments and measurements in this secondary analysis. There were 71 women and 1 man who identified as the head of household.

The pre-planned ancillary study reported here was limited to head of household participants with complete hepatic function, inflammation, and oxidative stress biomarker values as part of the comprehensive metabolic panel of the original trial's laboratory measurement at baseline and 6 months (Figure 1).

The Institutional Review Boards at the University of California San Diego and San Diego State University approved the original study protocol including subsequent analyses. Written, informed consent was provided by all participants. The original clinical trial was registered under clinicaltrials.gov study identifier NCT02903433.

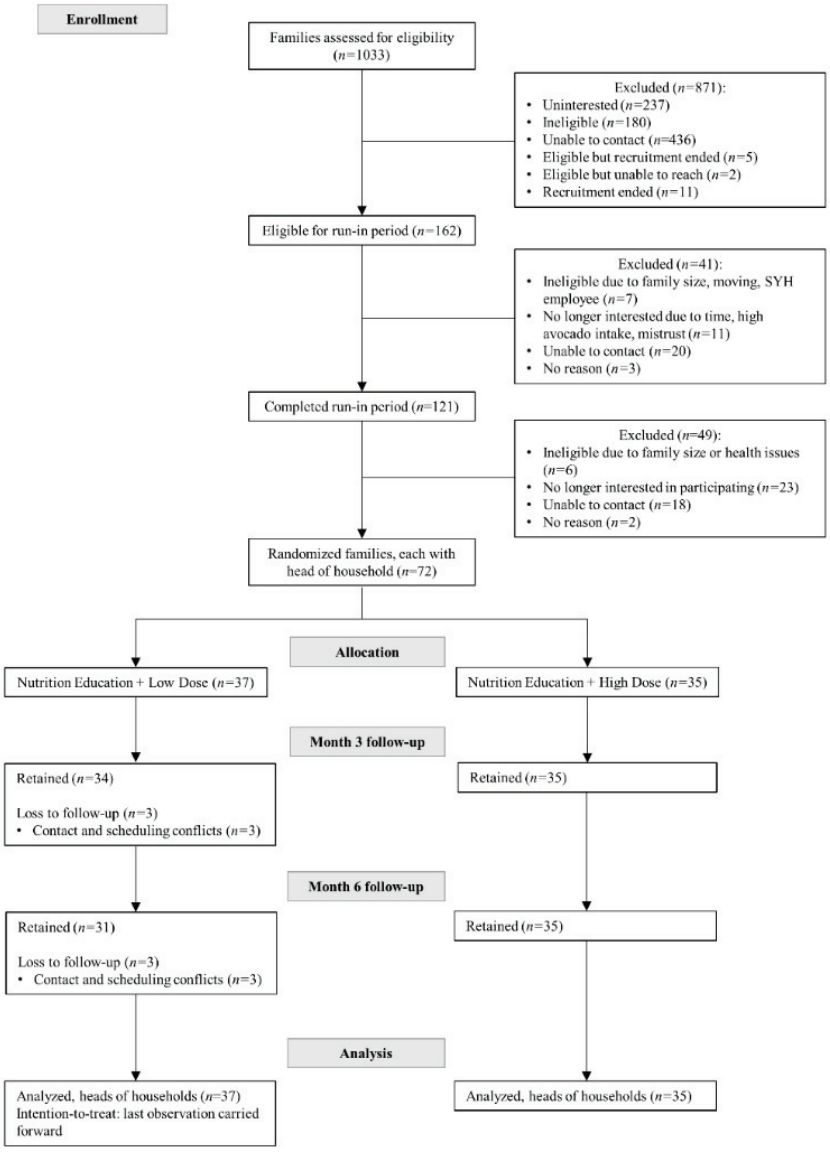

Figure 1. Consolidated standards of reporting trials (CONSORT) flow diagram of the Changes in Biomarkers of Non-Alcoholic Fatty Liver Disease (NAFLD) upon Access to Avocados in Hispanic/Latino Adults: Secondary Data Analysis of a Cluster Randomized Controlled Trial, ancillary study of the Effects of Avocado Intake on the Nutritional Status of Families Trial. This figure has been previously published in the parent trial [23].

Table 1. Baseline characteristics of randomized participants [1].

	Low Avocado Allotment (*n* = 37)	High Avocado Allotment (*n* = 35)
Characteristic		
Age, years	46.5 ± 11.3	44.5 ± 8.4
Female, %	97.3	100
Years lived in the United States	16.5 ± 12.6	17.7 ± 12.9
Country of birth, %		
Mexico	86.5	80.0
United States or Other	13.5	20.0
Heritage, %		
Central America	2.7	2.9
Mexico	97.3	97.1
Marital status, %		
Married or cohabitation	73.0	71.5
Separated, divorced, or widowed	18.9	20.1
Single	8.1	8.6
Highest level of education achieved, %		
High school	24.3	28.6
Trade school or Associate's degree	23.6	31.4
Bachelor's degree	18.2	17.1
Master's degree or above	2.7	5.8
No diploma	10.8	8.6
Other	18.2	8.6
Employment status, %		
Employed for wages	24.3	28.6
Self-employed	10.8	8.6
Homemaker	48.7	40.0
Other	16.2	22.9
Total family income in US dollars/year, %		
Less than $30,000	40.5	54.3
Greater than $30,000	40.5	37.1
Unknow	18.9	8.6
Moderate vigorous physical activity, minutes/week	593.9 ± 672.9	582.9 ± 649.8
BMI, kg/m-squared	30.6 ± 6.1	30.5 ± 6.2
Waist circumference, cm	93.2 ± 13.4	95.0 ± 12.7
Systolic blood pressure, mmHg	116.9 ± 15.8	111.5 ± 15.7
Diastolic blood pressure, mmHg	73.4 ± 12.3	69.4 ± 8.8
Diabetes/prediabetes status [2], %	54.2	45.8
Glucose, mg/dL	102.3 ± 38.3	103.5 ± 30.4
Hemoglobin A1c%	5.9 ± 1.2	5.7 ± 1.0
hsCRP, mg/L	3.9 ± 4.5	2.9 ± 2.8
GGT, IU/L	25.1 ± 33.0	21.9 ± 15.0
AST, IU/L	21.0 ± 10.3	21.8 ± 12.0
ALT, IU/L	25.5 ± 21.8	23.3 ± 18.3
Alkaline phosphatase, IU/L	76.2 ± 21.8	71.3 ± 19.1
NAFLD fibrosis score [3]	−2.2 ± 1.2	−2.1 ± 1.4

[1] Mean ± SD (all such values). [2] Defined as a fasting glucose level ≥ 100 mg/dL or glycosylated hemoglobin ≥ 5.7% and/or reported use of glucose-lowering medication, at baseline. [3] <−1.455: predictor of absence of significant fibrosis (F0–F2 fibrosis), −1.455 to 0.676: indeterminate score, >0.676: predictor of presence of significant fibrosis (F3-F4 fibrosis). ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease; US, United States.

2.2. Intervention

Heads of households and respective families in both intervention groups of the parent trial received nutrition education and avocados over a 6-month period. The nutrition education was identical for both groups and consisted of 12 bi-weekly culturally and language appropriate nutrition education standardized sessions derived from the United

States Department of Agriculture MyPlate/MiPlato (http://www.choosemyplate.gov/ (accessed on 11 March 2016).) and aligned with the Dietary Guidelines for Americans [24]. These sessions were delivered by *promotoras* in participants' homes with the goal of providing families with knowledge and tips to improve diet quality and meet nutritional goals, without individually counseling on energy restriction or elimination of any foods. Families were also provided with a recipe booklet to encourage inclusion of avocados in new ways.

The dose of avocados in the low intake allocation group was 3 avocados/week/family, based on the average reported intake in a pilot study survey of selected individuals in the target population (data not shown). The rationale for this allotment was to standardize the control arm and reduce potential variability. The high intake allocation group was provided 14 avocados/week/family to allow for a substantial increase in daily intake (by allowing for up to 2 avocados/family/day). Avocados were delivered to families each week by the *promotoras* throughout the 6-month intervention period. Participating families were encouraged to not purchase additional avocados.

2.3. Avocado Intake and Intervention Adherence

Avocado intake was assessed using a validated [25], self-administered, web-based, VioScreen (VioCare, Inc., Princeton, NJ, USA) food frequency questionnaire (FFQ) [26] at baseline and 6 months. A continuous intake value was derived based on the number of avocados consumed by the head of household at 6 months since we are only examining the baseline and 6-month measurements. This variable was used as an adherence measure in the statistical analysis.

2.4. Hepatic Function, Inflammation, and Oxidative Stress

Heads of households completed a fasting blood draw at a local Laboratory Corporation of America Holdings (LabCorp, Burlington, North Carolina, USA) site at baseline and 6 months. This visit was scheduled within 5 days before or after their clinic visit (per the parent trial protocol). Research assistants helped coordinate the LabCorp appointment early in the morning after a 10–12-h overnight fast (no food or drink) for all study participants. In addition to a comprehensive metabolic panel measured as part of the clinical assessments in the parent trial, this ancillary study measured liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase; inflammation biomarker high sensitivity C-reactive protein (hsCRP); oxidative stress indicator gamma-glutamyl transferase (GGT); platelet count and serum albumin.

The NAFLD fibrosis score was calculated with the formula constructed and validated by Angulo et al., and includes age, AST, ALT, albumin, platelet count, BMI, and presence/absence of impaired fasting glucose or diabetes [27]. The score formula = $-1.675 + 0.037 -$ age (years) $+ 0.094 -$ BMI (kg/m^2) $+ 1.13 \times$ impaired fasting glucose (IFG)/diabetes (yes = 1, no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet count ($\times$109/l) $- 0.66 \times$ albumin (g/dl).

2.5. Covariate Assessment

Participant's socio-demographic characteristics and lifestyle factors and behaviors were collected. Additional measures consisted of anthropometric measurements and physical activity. Anthropometric measurements included weight and height measured by a calibrated balance beam scale and stadiometer, respectively; and waist and hip circumference, which were measured with a semi-flexible tape measure, 2 cm above the iliac crest for waist and at the level of the widest circumference over the greater trochanters for the hip. Physical activity was measured via the global physical activity questionnaire (GPAQ) [28]. The dietary variables used in this study as covariates included total energy intake; intakes of fruits, vegetables and alcohol; and the healthy eating index (HEI)−2015, derived from the VioScreen FFQ [26].

2.6. Statistical Analysis

Descriptive statistics were used to characterize the study population by intervention group. Continuous variables were expressed as mean and standard deviation (SD), while categorical variables were expressed as frequencies and percentages. Normality was evaluated for all continuous variables. Unpaired, 2-sided *t*-tests were used to assess mean differences between intervention groups at 6 months for the biomarker outcomes: liver enzymes, GGT, hsCRP and NAFLD fibrosis score. These analyses were additionally adjusted for adherence to the HEI−2015 score in separate analysis of covariance (ANCOVA) models. ANCOVA models were also used to adjust for change in avocado intake from baseline to 6 months separately.

In multivariable analysis, four serial multivariable linear regression models evaluated the baseline and post-intervention association between avocado allotment group and biomarker outcomes adjusting for potential confounders, and mean between-group differences were determined. Model 1 adjusted for: age (years), moderate-vigorous physical activity (minutes/week), total energy intake (kcal/day), and alcohol intake (grams/day). Model 2 additionally adjusted for: change (from baseline) in fruit and vegetable intake (servings/day). Model 3 further adjusted for: change (from baseline) in waist circumference. Finally, model 4 adjusted for all variables in Model 3 as well as the diet quality score HEI−2015 at 6 months.

All analyses were conducted in the full sample as well as in subgroups defined by prediabetes status, which was defined as having a fasting glucose of $\geq$100 mg/dL or hemoglobin A1c % of $\geq$5.7 or use of antidiabetic medications at baseline. Mean difference and standard error of the mean (SE) or 95% confidence intervals (CI) are presented, where appropriate. Primary analyses were conducted as intention-to-treat without adjustment for intervention adherence (avocado intake at month 6). We also performed per protocol adherence analysis which considered intervention adherence and limited to participants who completed the study. All p values presented are from 2-tailed analyses; p values of <0.05 were considered statistically significant. Analyses were conducted with SAS version 9.4 (SAS Institute Inc, Cary, NC, USA).

3. Results

Of all heads of household trial participants, 83% were born in Mexico and, on average, had lived in the US for an average of 17.3 $\pm$ 12.6 years. The majority were married or cohabitating, homemakers, and their highest educational attainment was an Associate's degree. Study participants had a mean age of 45.5 $\pm$ 9.9 years, a BMI of 30.5 $\pm$ 6.1 kg/m^2. Less than a quarter of participants used medications, with 22.2% exclusively taking nonsteroidal anti-inflammatory drug (NSAIDs) (21.6% in the low avocado allotment group vs. 11.4% in the high avocado allotment group, p-value = 0.25). At baseline, the mean avocado intake in heads of household was 1.5 $\pm$ 1.4 and 1.5 $\pm$ 1.7 per week in the low and high avocado allotment groups, respectively. At 6 months, the mean intake of avocado was 1.7 $\pm$ 1.7 and 5.4 $\pm$ 3.3 per week in the low and high allotment groups, respectively. Blood pressure, lipid profile and other cardiometabolic risk measures are detailed in Table 1. Mean liver enzyme and GGT values were within normal range, and participants had a mean NAFLD fibrosis score of −2.2 $\pm$ 1.3. Changes in hepatic function measures at 6 months are shown in Table 2. Per intention-to-treat and per protocol analyses, and after adjusting for diet quality, there were no differences between low and high avocado allotment groups in liver enzymes, GGT, hsCRP or NAFLD fibrosis score. When stratifying by prediabetes status, participants with prediabetes randomized to the high avocado allotment study group showed a reduction in GGT at 6 months in per protocol analysis (Table 3). The mean 6-month change in GGT was +17.3 IU/L (95% CI: −2.1 to 36.8) for the low avocado allotment group and −10.3 IU/L (95% CI: −29.7 to 9.2) for the high avocado allotment group. The between-group mean difference was of borderline statistical significance (p = 0.06).

Changes in hepatic function outcomes at 6 months, after accounting for change in avocado intake, are shown in Table 4. We found no differences between low and high

avocado allotment groups in hsCRP, GGT, liver enzymes, and NAFLD fibrosis score, per intention-to-treat or per protocol analyses. These results persisted after stratifying by prediabetes status (Table 5). As in the analyses not accounting for change in individual-level participant avocado intake, participants with prediabetes randomized to the high avocado allotment study group showed a reduction in GGT at 6 months in per protocol analysis. The mean 6-month change in GGT was +13.8 IU/L (95% CI: −5.3 to 32.7) for the low avocado allotment group and −6.6 IU/L (95% CI: −25.6 to 12.4) for the high avocado allotment group. However, the between-group mean difference did not reach statistical significance ($p = 0.14$).

The multivariable linear regression models assessing 6-month changes in hepatic function markers related to avocado allotment in all study participants, with and without stratification by prediabetes status, are shown in Tables 6 and 7, respectively. After adjusting for intervention group, change in waist circumference and diet quality, there were reductions in GGT and alkaline phosphatase at 6 months. However, the associations did not differ ($p > 0.05$) (Table 6). In adjusted analyses, when stratifying by prediabetes status, participants without prediabetes had a −4.4 IU/L (95% CI: −11.5 to 2.6) and −5.6 (95% CI: −11.5 to 0.4) IU/L reduction in GGT and alkaline phosphatase, respectively, in per protocol analysis. These reductions did not differ ($p > 0.05$ for both) (Table 7).

Table 2. Changes in hepatic function outcomes at 6 months, per intention-to-treat and protocol adherence analyses.

Outcome	Within-Group Differences		Mean between-Group [1] Difference (95% CI)	*p*-Value [2]
	Low Avocado Allotment Mean (95% CI) (*n* = 37)	High Avocado Allotment Mean (95% CI) (*n* = 35)		
hsCRP, mg/L				
Intention-to-treat	−0.2 (−0.9 to 0.5)	−0.2 (−0.6 to 0.3)	0.0 (−0.9 to 0.8)	0.95
Intention-to-treat [3]	−0.3 (−0.8 to 0.3)	−0.1 (−0.7 to 0.5)	−0.1 (−1.0 to 0.7)	0.78
Protocol adherence [4,5]	0.0 (−0.8 to 0.7)	−0.4 (−1.1 to 0.3)	0.4 (−0.7 to 1.4)	0.53
Protocol adherence [3–5]	−0.1 (−0.8 to 0.7)	−0.3 (−1.0 to 0.4)	0.2 (−0.9 to 1.3)	0.70
GGT, IU/L				
Intention-to-treat	3.5 (−7.3 to 14.2)	0.0 (−3.8 to 3.7)	3.5 (−7.8 to 14.8)	0.54
Intention-to-treat [3]	2.9 (−5.2 to 11.0)	0.5 (−7.8 to 8.9)	2.4 (−9.4 to 14.2)	0.68
Protocol adherence [4,5]	7.1 (−2.9 to 17.1)	−2.7 (−12.0 to 6.6)	9.8 (−4.9 to 24.5)	0.19
Protocol adherence [3–5]	6.4 (−3.7 to 16.6)	−2.1 (−11.5 to 7.4)	8.5 (−6.6 to 23.7)	0.27
AST, IU/L				
Intention-to-treat	−0.4 (−3.1 to 2.4)	−1.6 (−5.1 to 1.8)	1.3 (−3.0 to 5.6)	0.56
Intention-to-treat [3]	−0.4 (−3.5 to 2.7)	−1.6 (−4.7 to 1.6)	1.2 (−3.3 to 5.6)	0.60
Protocol adherence [4,5]	−0.3 (−4.1 to 3.5)	−1.7 (−5.3 to 1.8)	1.4 (−4.2 to 7.1)	0.61
Protocol adherence [3–5]	−0.4 (−4.3 to 3.5)	−1.7 (−5.3 to 2.0)	1.3 (−4.5 to 7.1)	0.66
ALT, IU/L				
Intention-to-treat	−0.2 (−5.2 to 4.7)	−1.6 (−5.9 to 2.7)	1.4 (−5.1 to 7.8)	0.67
Intention-to-treat[3]	−0.3 (−4.9 to 4.2)	−1.5 (−6.2 to 3.2)	1.2 (−5.5 to 7.8)	0.73
Protocol adherence [4,5]	0.1 (−5.6 to 5.8)	−2.0 (−7.3 to 3.3)	2.1 (−6.3 to 10.5)	0.62
Protocol adherence [3–5]	0.0 (−5.8 to 5.8)	−1.9 (−7.3 to 3.6)	1.8 (−6.8 to 10.5)	0.67
Alkaline phosphatase, IU/L				
Intention-to-treat	−2.7 (−6.6 to 1.2)	−1.9 (−4.8 to 0.9)	−0.8 (−5.6 to 3.9)	0.73
Intention-to-treat [3]	−2.8 (−6.2 to 0.6)	−1.8 (−5.3 to 1.7)	−1.0 (−5.9 to 4.0)	0.69
Protocol adherence [4,5]	−2.2 (−6.4 to 2.0)	−2.8 (−6.7 to 1.1)	0.6 (−5.6 to 6.8)	0.85
Protocol adherence [3–5]	−2.3 (−6.6 to 1.9)	−2.7 (−6.7 to 1.3)	0.4 (−6.0 to 6.8)	0.91
NAFLD fibrosis score				
Intention-to-treat	0.1 (−0.1 to 0.2)	0.2 (−0.1 to 0.4)	−0.1 (−0.4 to 0.2)	0.43
Intention-to-treat [3]	0.1 (−0.1 to 0.2)	0.1 (−0.1 to 0.3)	−0.1 (−0.4 to 0.2)	0.51
Protocol adherence [4,5]	0.1 (−0.1 to 0.3)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.3)	0.99
Protocol adherence [3–5]	0.1 (−0.1 to 0.4)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.4)	0.91

[1] Mean difference is low-high avocado allotment group. [2] From unpaired *t*-test or ANCOVA model (adjusted for Healthy Eating Index 2015 score at Month 6 and/or intervention adherence), where appropriate. [3] Adjusted for Healthy Eating Index 2015 score at Month 6. [4] Adjusted for intervention adherence. [5] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 31/35 at month 6. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

Table 3. Changes in hepatic function outcomes at 6 months, stratified by prediabetes status [1], per intention-to-treat and protocol adherence analyses.

Outcome	Within-Group Differences				Mean between-Group Difference [2] (95% CI)			
	Low Avocado Allotment (n = 37)		High Avocado Allotment (n = 35)					
	Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes	p-Value [3]	Mean (95% CI) Prediabetes	p-Value [3]
	Yes (n = 18)	No (n = 19)	Yes (n = 15)	No (n = 20)	Yes		No	
hsCRP, mg/L								
Intention-to-treat	−0.2 (−1.7 to 1.2)	−0.2 (−0.7 to 0.4)	−0.2 (−1.1 to 0.7)	−0.2 (−0.7 to 0.3)	−0.0 (−1.7 to 1.6)	0.95	0.0 (−0.7 to 0.7)	0.99
Intention-to-treat + HEI [4]	−0.3 (−1.4 to 0.8)	−0.1 (−0.7 to 0.4)	−0.1 (−1.4 to 1.1)	−0.2 (−0.8 to 0.3)	−0.2 (−1.9 to 1.5)	0.82	0.1 (−0.7 to 0.9)	0.77
Protocol adherence [5–7]	−0.2 (−1.6 to 1.3)	0.1 (−0.6 to 0.8)	−0.3 (−1.8 to 1.1)	−0.4 (−1.0 to 0.2)	0.2 (−2.0 to 2.3)	0.86	0.5 (−0.4 to 1.5)	0.29
Protocol adherence + HEI [4–7]	−0.2 (−1.6 to 1.2)	0.2 (−0.5 to 0.9)	−0.3 (−1.7 to 1.2)	−0.5 (−1.1 to 0.1)	−0.1 (−2.1 to 2.2)	0.98	0.7 (−0.3 to 1.7)	0.16
GGT, IU/L								
Intention-to-treat	8.1 (−15.0 to 31.1)	−0.9 (−2.5 to 0.7)	−2.6 (−8.0 to 2.8)	1.9 (−3.5 to 7.3)	10.7 (−12.8 to 34.1)	0.35	−2.8 (−8.3 to 2.8)	0.31
Intention-to-treat + HEI [4]	7.4 (−9.5 to 24.2)	−0.4 (−4.5 to 3.7)	−1.8 (−20.3 to 16.7)	1.5 (−2.5 to 5.5)	9.1 (−16.0 to 34.3)	0.46	−1.9 (−7.8 to 4.0)	0.52
Protocol adherence [5–7]	17.3 (−2.1 to 36.8)	−2.4 (−7.5 to 2.7)	−10.3 (−29.7 to 9.2)	3.0 (−1.5 to 7.5)	27.6 (−1.6 to 56.8)	0.06	−5.4 (−12.8 to 2.1)	0.16
Protocol adherence + HEI [4–7]	16.5 (−3.0 to 35.9)	−1.8 (−7.1 to 3.6)	−9.4 (−28.8 to 10.0)	2.5 (−2.2 to 7.1)	25.9 (−3.4 to 55.1)	0.09	−4.2 (−12.2 to 3.8)	0.29
AST, IU/L								
Intention-to-treat	0.0 (−4.7 to 4.7)	−0.7 (−4.2 to 2.9)	−5.3 (−12.2 to 1.6)	1.1 (−2.1 to 4.3)	5.3 (−2.5 to 13.0)	0.18	−1.8 (−6.4 to 2.8)	0.44
Intention-to-treat + HEI [4]	−0.3 (−5.5 to 4.9)	0.0 (−3.4 to 3.3)	−4.9 (−10.6 to 0.8)	0.5 (−2.7 to 3.7)	4.7 (−3.1 to 12.4)	0.23	−0.5 (−5.3 to 4.2)	0.82
Protocol adherence [5–7]	−1.0 (−7.6 to 5.5)	−0.5 (−4.8 to 3.8)	−4.2 (−10.8 to 2.3)	0.8 (−2.9 to 4.6)	3.2 (−6.6 to 13.0)	0.51	−1.3 (−7.6 to 5.0)	0.67
Protocol adherence + HEI [4–7]	−1.3 (−7.8 to 5.2)	0.6 (−3.8 to 4.9)	−3.9 (−10.4 to 2.6)	0.0 (−3.8 to 3.8)	2.6 (−7.2 to 12.4)	0.59	0.6 (−6.0 to 7.1)	0.86
ALT, IU/L								
Intention-to-treat	0.5 (−9.1 to 10.1)	−0.9 (−5.4 to 3.5)	−6.3 (−15.3 to 2.8)	1.9 (−1.4 to 5.2)	6.8 (−6.0 to 19.6)	0.29	−2.8 (−8.2 to 2.5)	0.28
Intention-to-treat + HEI[4]	0.2 (−8.5 to 8.8)	−0.3 (−4.2 to 3.6)	−5.9 (−15.4 to 3.7)	1.3 (−2.5 to 5.0)	6.0 (−6.9 to 19.0)	0.35	−1.6 (−7.1 to 3.9)	0.56
Protocol adherence [5–7]	−0.4 (−11.2 to 10.5)	−0.5 (−5.5 to 4.5)	−5.3 (−16.2 to 5.6)	1.4 (−2.9 to 5.8)	5.0 (−11.4 to 21.3)	0.54	−1.9 (−9.2 to 5.4)	0.60
Protocol adherence + HEI [4–7]	−0.7 (−11.7 to 10.2)	0.6 (−4.5 to 5.7)	−4.9 (−15.9 to 6.0)	0.5 (−3.9 to 5.0)	4.2 (−12.3 to 20.7)	0.61	0.1 (−7.6 to 7.6)	0.99
Alkaline phosphatase, IU/L								
Intention-to-treat	−1.9 (−9.7 to 5.8)	−3.5 (−6.6 to −0.3)	−4.6 (−9.2 to 0.0)	0.1 (−3.6 to 3.8)	2.7 (−6.1 to 11.4)	0.54	−3.6 (−8.3 to 1.1)	0.13
Intention-to-treat + HEI [4]	−2.0 (−8.3 to 4.3)	−3.8 (−7.3 to −0.4)	−4.6 (−11.5 to 2.3)	0.4 (−2.9 to 3.8)	2.6 (−6.8 to 12.0)	0.57	−4.3 (−9.2 to 0.7)	0.09
Protocol adherence [5–7]	0.1 (−7.2 to 7.4)	−4.7 (−9.1 to −0.4)	−7.0 (−14.3 to 0.3)	0.6 (−3.2 to 4.4)	7.1 (−3.9 to 18.1)	0.20	−5.3 (−11.7 to 1.0)	0.10
Protocol adherence + HEI [4–7]	0.0 (−7.5 to 7.6)	−5.4 (−9.9 to −0.9)	−7.0 (−14.5 to 0.5)	1.1 (−2.8 to 5.1)	7.0 (−4.3 to 18.3)	0.21	−6.5 (−13.3 to 0.2)	0.06
NAFLD fibrosis score								
Intention-to-treat	−0.1 (−0.4 to 0.1)	0.2 (−0.1 to 0.5)	−0.1 (−0.4 to 0.2)	0.4 (0.1 to 0.6)	0.0 (−0.4 to 0.4)	0.98	−0.2 (−0.5 to 0.2)	0.38
Intention-to-treat + HEI [4]	−0.1 (−0.4 to 0.1)	0.2 (0.0 to 0.5)	−0.1 (−0.4 to 0.2)	0.4 (0.1 to 0.6)	0.0 (−0.4 to 0.4)	0.97	−0.1 (−0.5 to 0.2)	0.49
Protocol adherence [5–7]	−0.2 (−0.5 to 0.2)	0.3 (0.0 to 0.6)	−0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.6)	0.0 (−0.5 to 0.4)	0.83	0.0 (−0.5 to 0.5)	0.98
Protocol adherence + HEI [4–7]	−0.2 (−0.5 to 0.2)	0.3 (0.0 to 0.7)	−0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.6)	0.0 (−0.5 to 0.4)	0.84	0.0 (−0.5 to 0.6)	0.87

[1] Defined as a fasting glucose level ≥100 mg/dL or glycosylated hemoglobin ≥5.7% and/or reported use of glucose-lowering medication, at baseline. [2] Mean difference is low-high avocado allotment group. [3] From unpaired t-test or ANCOVA model (adjusted for Healthy Eating Index 2015 score at Month 6 and/or intervention adherence), where appropriate. [4] Adjusted for Healthy Eating Index 2015 score at Month 6. [5] Adjusted for intervention adherence. [6] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 31/35 at month 6. [7] Sample size by prediabetes/diabetes status (yes/no) for low avocado allotment: 15/16 and for high avocado allotment: 15/20 at month 6. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

Table 4. Changes in hepatic function outcomes at 6 months after accounting for change in avocado intake [1], per intention-to-treat and protocol adherence analyses.

Outcome	Within-Group Differences		Mean between-Group Difference [2] (95% CI)	*p*-Value [3]
	Low Avocado Allotment Mean (95% CI) (*n* = 37)	High Avocado Allotment Mean (95% CI) (*n* = 35)		
hsCRP, mg/L				
Intention-to-treat	−0.1 (−0.7 to 0.5)	−0.3 (−0.9 to 0.4)	0.2 (−0.8 to 1.2)	0.73
Intention-to-treat + HEI [4]	−0.2 (−0.8 to 0.5)	−0.2 (−0.9 to 0.4)	0.1 (−0.9 to 1.1)	0.91
Protocol adherence [5,6]	−0.1 (−0.9 to 0.6)	−0.3 (−0.9 to 0.4)	0.2 (−0.9 to 1.2)	0.80
Protocol adherence + HEI [4–6]	−0.2 (−0.9 to 0.5)	−0.2 (−0.9 to 0.5)	0.0 (−1.1 to 1.1)	0.99
GGT, IU/L				
Intention-to-treat	6.0 (−2.6 to 14.6)	−2.7 (−11.6 to 6.1)	8.7 (−4.5 to 21.9)	0.19
Intention-to-treat + HEI [4]	5.5 (−3.3 to 14.2)	−2.1 (−11.2 to 6.9)	7.6 (−6.0 to 21.2)	0.27
Protocol adherence [5,6]	6.7 (−3.0 to 16.4)	−2.3 (−11.3 to 6.8)	8.9 (−5.1 to 23.0)	0.21
Protocol adherence + HEI [4–6]	6.0 (−3.9 to 15.9)	−1.7 (−10.9 to 7.6)	7.6 (−6.9 to 22.2)	0.30
AST, IU/L				
Intention-to-treat	0.0 (−3.3 to 3.2)	−1.9 (−5.3 to 1.4)	1.9 (−3.2 to 6.9)	0.46
Intention-to-treat + HEI [4]	−0.1 (−3.4 to 3.3)	−1.9 (−5.4 to 1.6)	1.8 (−3.4 to 7.0)	0.49
Protocol adherence [5,6]	−0.1 (−3.8 to 3.6)	−1.9 (−5.4 to 1.5)	1.8 (−3.6 to 7.2)	0.50
Protocol adherence + HEI [4–6]	−0.2 (−4.0 to 3.6)	−1.9 (−5.4 to 1.7)	1.7 (−3.9 to 7.3)	0.54
ALT, IU/L				
Intention-to-treat	0.3 (−4.6 to 5.1)	−2.1 (−7.2 to 2.9)	2.4 (−5.1 to 9.9)	0.53
Intention-to-treat + HEI [4]	0.2 (−4.8 to 5.1)	−2.0 (−7.2 to 3.1)	2.2 (−5.6 to 10.0)	0.58
Protocol adherence [5,6]	0.2 (−5.3 to 5.8)	−2.1 (−7.2 to 3.1)	2.3 (−5.7 to 10.3)	0.57
Protocol adherence + HEI [4–6]	0.1 (−5.5 to 5.8)	−2.0 (−7.2 to 3.3)	2.1 (−6.2 to 10.4)	0.62
Alkaline phosphatase, IU/L				
Intention-to-treat	−2.2 (−5.8 to 1.4)	−2.5 (−6.2 to 1.2)	0.3 (−5.3 to 5.9)	0.91
Intention-to-treat + HEI [4]	−2.3 (−6.0 to 1.5)	−2.4 (−6.2 to 1.4)	0.2 (−5.6 to 5.9)	0.96
Protocol adherence [5,6]	−2.6 (−6.7 to 1.5)	−2.5 (−6.3 to 1.3)	−0.1 (−6.0 to 5.8)	0.98
Protocol adherence + HEI [4–6]	−2.7 (−6.9 to 1.5)	−2.4 (−6.3 to 1.5)	−0.3 (−6.4 to 5.8)	0.92
NAFLD fibrosis score				
Intention-to-treat	0.1 (−0.1 to 0.3)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.3)	0.86
Intention-to-treat + HEI [4]	0.1 (−0.1 to 0.3)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.4)	0.76
Protocol adherence [5,6]	0.1 (−0.1 to 0.3)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.4)	0.88
Protocol adherence + HEI [4–6]	0.1 (−0.1 to 0.4)	0.1 (−0.1 to 0.3)	0.0 (−0.3 to 0.4)	0.78

[1] Defined as change in participant intake from baseline to Month 6. [2] Mean difference is low-high avocado allotment group. [3] From ANCOVA model (adjusted for change in avocado intake and/or Healthy Eating Index 2015 score at Month 6 and/or intervention adherence), where appropriate. [4] Adjusted for Healthy Eating Index 2015 score at Month 6. [5] Adjusted for intervention adherence. [6] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 31/35 at month 6. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

Table 5. Changes in hepatic function outcomes at 6 months after accounting for change in avocado intake [1], stratified by prediabetes status [2], per intention-to-treat and protocol adherence analyses.

Outcome	Within-Group Differences				Mean between-Group Difference [3] (95% CI)			
	Low Avocado Allotment (n = 37)		High Avocado Allotment (n = 35)					
	Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes	p-Value [4]	Mean (95% CI) Prediabetes	p-Value [4]
	Yes (n = 18)	No (n = 19)	Yes (n = 15)	No (n = 20)	Yes		No	
hsCRP, mg/L								
Intention-to-treat	−0.2 (−1.4 to 1.0)	0.0 (−0.6 to 0.6)	−0.3 (−1.6 to 1.1)	−0.3 (−0.9 to 0.3)	0.1 (−1.8 to 1.9)	0.95	0.3 (−0.6 to 1.2)	0.52
Intention-to-treat + HEI [5]	−0.3 (−1.5 to 0.9)	0.1 (−0.5 to 0.7)	−0.2 (−1.5 to 1.1)	−0.4 (−1.0 to 0.2)	−0.1 (−1.9 to 1.7)	0.92	0.5 (−0.5 to 1.5)	0.31
Protocol adherence [6–8]	−0.2 (−1.6 to 1.1)	0.0 (−0.7 to 0.7)	−0.2 (−1.6 to 1.1)	−0.3 (−0.9 to 0.3)	0.0 (−2.0 to 2.0)	0.99	0.3 (−0.7 to 1.3)	0.56
Protocol adherence + HEI [5–8]	−0.3 (−1.7 to 1.0)	0.1 (−0.6 to 0.8)	−0.2 (−1.5 to 1.2)	−0.4 (−1.0 to 0.2)	−0.2 (−2.2 to 1.8)	0.86	0.5 (−0.6 to 1.6)	0.35
GGT, IU/L								
Intention-to-treat	12.2 (−4.3 to 28.6)	−1.9 (−6.5 to 2.6)	−7.5 (−25.7 to 10.6)	2.9 (−1.5 to 7.3)	19.7 (−5.5 to 45.0)	0.12	−4.8 (−11.9 to 2.3)	0.18
Intention-to-treat + HEI [5]	11.5 (−5.1 to 28.0)	−1.4 (−6.2 to 3.5)	−6.7 (−24.9 to 11.5)	2.4 (−2.3 to 7.0)	18.2 (−7.3 to 43.7)	0.16	−3.7 (−11.4 to 4.0)	0.33
Protocol adherence [6–8]	13.8 (−5.3 to 32.7)	−2.1 (−7.3 to 3.0)	−6.6 (−25.6 to 12.4)	2.7 (−1.8 to 7.3)	20.3 (−7.2 to 47.8)	0.14	−4.9 (−12.4 to 2.6)	0.20
Protocol adherence + HEI [5–8]	12.8 (−6.3 to 31.9)	−1.5 (−6.9 to 4.0)	−5.7 (−24.9 to 13.4)	2.2 (−2.5 to 6.9)	18.4 (−9.3 to 46.2)	0.18	−3.7 (−11.8 to 4.4)	0.36
AST, IU/L								
Intention-to-treat	−0.2 (−5.7 to 5.3)	−0.7 (−4.6 to 3.1)	−5.0 (−11.1 to 1.0)	1.2 (−2.6 to 4.9)	4.8 (−3.6 to 13.3)	0.25	−1.9 (−7.8 to 4.0)	0.52
Intention-to-treat + HEI [5]	−0.5 (−5.9 to 5.0)	0.2 (−3.7 to 4.2)	−4.7 (−10.7 to 1.3)	0.2 (−3.6 to 4.0)	4.2 (−4.2 to 12.6)	0.31	0.0 (−6.3 to 6.2)	0.99
Protocol adherence [6–8]	−0.2 (−6.5 to 6.2)	−0.8 (−5.1 to 3.5)	−5.1 (−11.4 to 1.3)	1.1 (−2.7 to 4.9)	4.9 (−4.3 to 14.1)	0.28	−1.9 (−8.2 to 4.3)	0.53
Protocol adherence + HEI [5–8]	−0.6 (−6.9 to 5.7)	0.2 (−4.2 to 4.7)	−4.7 (−11.0 to 1.6)	0.3 (−3.6 to 4.1)	4.1 (−5.1 to 13.3)	0.37	0.0 (−6.6 to 6.6)	0.99
ALT, IU/L								
Intention-to-treat	0.2 (−8.9 to 9.2)	−0.6 (−5.0 to 3.9)	−5.9 (−15.8 to 4.1)	1.5 (−2.8 to 5.8)	6.0 (−7.9 to 19.9)	0.38	−2.1 (−9.0 to 4.8)	0.54
Intention-to-treat + HEI [5]	−0.2 (−9.3 to 8.9)	0.5 (−4.1 to 5.0)	−5.4 (−15.5 to 4.6)	0.6 (−3.9 to 5.0)	5.2 (−8.8 to 19.3)	0.45	−0.1 (−7.4 to 7.2)	0.98
Protocol adherence [6–8]	0.2 (−10.2 to 10.7)	−0.7 (−5.6 to 4.3)	−5.9 (−16.3 to 4.5)	1.5 (−2.8 to 5.9)	6.2 (−8.9 to 21.3)	0.41	−2.2 (−9.4 to 5.1)	0.54
Protocol adherence + HEI [5–8]	−0.2 (−10.7 to 10.3)	0.5 (−4.6 to 5.6)	−5.4 (−16.0 to 5.1)	0.6 (−3.8 to 5.1)	5.2 (−10.1 to 20.5)	0.49	−0.1 (−7.8 to 7.5)	0.97
Alkaline phosphatase, IU/L								
Intention-to-treat	−0.9 (−7.1 to 5.4)	−4.6 (−8.5 to −0.8)	−5.9 (−12.8 to 1.0)	1.2 (−2.5 to 4.9)	5.0 (−4.6 to 14.6)	0.30	−5.8 (−11.8 to 0.1)	0.05
Intention-to-treat + HEI [5]	−0.9 (−7.3 to 5.5)	−5.3 (−9.3 to −1.3)	−5.9 (−12.9 to 1.2)	1.9 (−2.0 to 5.7)	5.0 (−4.9 to 14.9)	0.31	−7.2 (−13.6 to 0.8)	0.03
Protocol adherence [6–8]	−1.2 (−8.4 to 6.0)	−5.1 (−9.4 to −0.9)	−5.7 (−12.9 to 1.5)	0.9 (−2.8 to 4.7)	4.5 (−6.0 to 14.9)	0.39	−6.1 (−12.3 to 0.2)	0.06
Protocol adherence + HEI [5–8]	−1.2 (−8.6 to 6.1)	−5.9 (−10.4 to −1.4)	−5.7 (−13.1 to 1.7)	1.5 (−2.4 to 5.4)	4.4 (−6.3 to 15.2)	0.40	−7.4 (−14.1 to 0.7)	0.03

Table 5. *Cont.*

Outcome	Within-Group Differences				Mean between-Group Difference [3] (95% CI)			
	Low Avocado Allotment (n = 37)		High Avocado Allotment (n = 35)					
	Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes		Mean (95% CI) Prediabetes	p-Value [4]	Mean (95% CI) Prediabetes	p-Value [4]
	Yes (n = 18)	No (n = 19)	Yes (n = 15)	No (n = 20)	Yes		No	
NAFLD fibrosis score								
Intention-to-treat	−0.1 (−0.4 to 0.1)	0.3 (0.0 to 0.6)	0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.6)	0.0 (−0.4 to 0.4)	0.95	0.0 (−0.4 to 0.5)	0.93
Intention-to-treat + HEI [5]	−0.1 (−0.4 to 0.1)	0.3 (0.0 to 0.6)	−0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.5)	0.0 (−0.4 to 0.4)	0.95	0.1 (−0.4 to 0.6)	0.74
Protocol adherence [6–8]	−0.2 (−0.5 to 0.2)	0.3 (0.0 to 0.7)	−0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.6)	0.0 (−0.5 to 0.4)	0.88	0.0 (−0.4 to 0.5)	0.90
Protocol adherence + HEI [5–8]	−0.2 (−0.5 to 0.2)	0.4 (0.0 to 0.7)	−0.1 (−0.4 to 0.2)	0.3 (0.0 to 0.6)	0.0 (−0.5 to 0.4)	0.88	0.1 (−0.4 to 0.6)	0.73

[1] Defined as change in participant intake from baseline to Month 6. [2] Defined as a fasting glucose level ≥100 mg/dL or glycosylated hemoglobin ≥5.7 % and/or reported use of glucose-lowering medication, at baseline. [3] Mean difference is low-high avocado allotment group. [4] From ANCOVA model (adjusted for change in avocado intake and/or Healthy Eating Index 2015 score at Month 6 and/or intervention adherence), where appropriate. [5] Adjusted for Healthy Eating Index 2015 score at Month 6. [6] Adjusted for intervention adherence. [7] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 31/35 at month 6. [8] Sample size by prediabetes/diabetes status (yes/no) for low avocado allotment: 15/16 and for high avocado allotment: 15/20 at month 6. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

Table 6. Between-group differences of multivariable linear regression models assessing 6-month changes in hepatic function markers related to avocado [1] allotment, per intention-to-treat and per protocol adherence analyses [2].

	Mean between-Group Difference [3] (95% CI)							
	Model 1 [4]	R-Squared	Model 2 [5]	R-Squared	Model 3 [6]	R-Squared	Model 4 [7]	R-Squared
hsCRP, mg/L								
Intention-to-treat [8]	−0.1 (−0.9 to 0.8)	0.09	0.0 (−0.9 to 0.8)	0.09	0.0 (−0.9 to 0.8)	0.12	−0.1 (−1.0 to 0.8)	0.12
Protocol adherence [9]	0.0 (−0.9 to 0.9)	0.09	0.0 (−1.0 to 0.9)	0.09	0.0 (−0.9 to 1.0)	0.12	0.0 (−1.0 to 0.9)	0.12
GGT, IU/L								
Intention-to-treat [8]	−2.4 (−11.7 to 7.0)	0.40	−2.9 (−12.5 to 6.6)	0.41	−2.8 (−12.5 to 6.8)	0.41	−3.0 (−12.9 to 7.0)	0.41
Protocol adherence [9]	−1.2 (−11.2 to 8.9)	0.44	−1.8 (−12.0 to 8.4)	0.44	−1.7 (−12.0 to 8.6)	0.45	−1.9 (−12.6 to 8.8)	0.45
AST, IU/L								
Intention-to-treat [8]	0.6 (−3.8 to 4.9)	0.11	1.0 (−3.4 to 5.3)	0.13	1.0 (−3.4 to 5.4)	0.13	1.0 (−3.5 to 5.6)	0.13
Protocol adherence [9]	0.6 (−4.2 to 5.3)	0.11	1.0 (−3.8 to 5.8)	0.14	1.0 (−3.9 to 5.8)	0.14	1.1 (−4.0 to 6.1)	0.14
ALT, IU/L								
Intention-to-treat [8]	0.2 (−6.3 to 6.7)	0.09	0.4 (−6.2 to 7.0)	0.09	0.3 (−6.4 to 6.9)	0.10	0.2 (−6.7 to 7.1)	0.10
Protocol adherence [9]	0.3 (−6.9 to 7.4)	0.09	0.4 (−6.9 to 7.8)	0.09	0.3 (−7.1 to 7.6)	0.10	0.1 (−7.5 to 7.8)	0.10
Alkaline phosphatase, IU/L								
Intention-to-treat [8]	−2.1 (−6.6 to 2.4)	0.23	−2.3 (−6.9 to 2.2)	0.23	−2.3 (−6.9 to 2.3)	0.23	−2.1 (−6.8 to 2.6)	0.24
Protocol adherence [9]	−2.4 (−7.2 to 2.5)	0.24	−2.6 (−7.6 to 2.3)	0.24	−2.6 (−7.6 to 2.4)	0.24	−2.4 (−7.6 to 2.8)	0.25

Table 6. *Cont.*

	Mean between-Group Difference [3] (95% CI)							
	Model 1 [4]	**R-Squared**	**Model 2 [5]**	**R-Squared**	**Model 3 [6]**	**R-Squared**	**Model 4 [7]**	**R-Squared**
NAFLD fibrosis score								
Intention-to-treat [8]	−0.2 (−0.4 to 0.1)	0.13	−0.2 (−0.4 to 0.1)	0.14	−0.2 (−0.4 to 0.1)	0.16	−0.1 (−0.4 to 0.1)	0.17
Protocol adherence [9]	−0.2 (−0.5 to 0.1)	0.13	−0.2 (−0.5 to 0.1)	0.14	−0.2 (−0.5 to 0.1)	0.17	−0.1 (−0.4 to 0.2)	0.18

[1] Allocated avocado group in the trial; thus, intervention status was a covariate in the model. [2] All models had p-value >0.05 unless otherwise stated. [3] Mean difference is low-high avocado allotment group. [4] Model 1 adjusted for: age (years); MVPA (minutes/week); total energy intake (kcals/day); alcohol (grams/day). [5] Model 2 adjusted for: variables in Model 1 and change in fruit and vegetable intake (servings/day). [6] Model 3 adjusted for: variables in Model 2 and change in waist circumference (cm). [7] Model 4 adjusted for: variables in Model 3 and HEI 2015 score at Month 6. [8] n = 72. [9] n = 66. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

Table 7. Between-group differences of multivariable linear regression models assessing 6-month changes in hepatic function markers related to avocado [1] allotment, per intention-to-treat and per protocol adherence analyses [2], stratified by prediabetes status [3].

	Presence of Prediabetes							
	Mean between-Group Difference [4] (95% CI)							
	Model 1 [5]	**R-Squared**	**Model 2 [6]**	**R-Squared**	**Model 3 [7]**	**R-Squared**	**Model 4 [8]**	**R-Squared**
hsCRP, mg/L								
Intention-to-treat [9]	−0.4 (−2.1 to 1.2)	0.26	−0.5 (−2.3 to 1.2)	0.26	−0.3 (−2.1 to 1.5)	0.29	−0.5 (−2.3 to 1.4)	0.31
Protocol adherence [10]	−0.5 (−2.3 to 1.4)	0.26	−0.6 (−2.6 to 1.4)	0.27	−0.4 (−2.4 to 1.7)	0.30	−0.6 (−2.7 to 1.6)	0.32
GGT, IU/L								
Intention-to-treat [9]	5.4 (−6.7 to 17.5)	0.81	5.3 (−7.7 to 18.2)	0.81	4.4 (−9.1 to 17.9)	0.82	4.4 (−9.1 to 17.9)	0.82
Protocol adherence [10]	8.5 (−3.1 to 20.1)	0.87	8.4 (−4.1 to 20.8)	0.87	7.3 (−5.7 to 20.2)	0.88	6.1 (−7.5 to 19.7)	0.88
AST, IU/L								
Intention-to-treat [9]	3.9 (−3.2 to 10.9)	0.37	4.9 (−2.6 to 12.3)	0.39	5.0 (−2.8 to 12.8)	0.39	4.5 (−3.6 to 12.7)	0.40
Protocol adherence [10]	3.8 (−4.3 to 11.9)	0.39	4.9 (−3.6 to 13.4)	0.41	5.1 (−3.9 to 14.0)	0.41	4.3 (−5.1 to 13.7)	0.42
ALT, IU/L								
Intention-to-treat [9]	5.5 (−6.1 to 17.1)	0.36	6.5 (−5.8 to 18.8)	0.37	6.9 (−6.0 to 19.9)	0.37	6.9 (−6.7 to 20.4)	0.37
Protocol adherence [10]	5.9 (−7.3 to 19.1)	0.38	7.1 (−6.9 to 21.1)	0.39	7.6 (−7.2 to 22.4)	0.39	7.2 (−8.5 to 22.9)	0.40
Alkaline phosphatase, IU/L								
Intention-to-treat [9]	1.8 (−6.2 to 9.9)	0.38	2.6 (−5.9 to 11.1)	0.39	2.9 (−6.0 to 11.9)	0.39	3.9 (−5.4 to 13.1)	0.42
Protocol adherence [10]	1.7 (−7.5 to 10.8)	0.40	2.5 (−7.3 to 12.2)	0.41	2.8 (−7.5 to 13.1)	0.41	3.9 (−6.8 to 14.6)	0.43
NAFLD fibrosis score								
Intention-to-treat [9]	0.0 (−0.4 to 0.3)	0.22	−0.1 (−0.4 to 0.3)	0.22	−0.1 (−0.5 to 0.4)	0.22	0.0 (−0.5 to 0.4)	0.22
Protocol adherence [10]	−0.2 (−0.6 to 0.2)	0.28	−0.2 (−0.6 to 0.3)	0.28	−0.2 (−0.6 to 0.3)	0.28	−0.2 (−0.7 to 0.3)	0.28

Table 7. *Cont.*

	Absence of Prediabetes							
	Mean between-Group Difference [4] (95% CI)							
	Model 1 [5]	R-Squared	Model 2 [6]	R-Squared	Model 3 [7]	R-Squared	Model 4 [8]	R-Squared
hsCRP, mg/L								
Intention-to-treat [9]	0.0 (−0.7 to 0.7)	0.28	0.0 (−0.9 to 0.8)	0.09	0.0 (−0.9 to 0.8)	0.12	−0.1 (−1.0 to 0.8)	0.12
Protocol adherence [10]	0.0 (−0.8 to 0.8)	0.28	0.0 (−0.8 to 0.8)	0.30	0.0 (−0.8 to 0.8)	0.30	0.0 (−1.0 to 0.9)	0.30
GGT, IU/L								
Intention-to-treat [9]	−3.8 (−9.8 to 2.3)	0.15	−3.9 (−9.8 to 2.0)	0.22	−4.5 (−10.3 to 1.2)	0.29	−4.5 (−11.0 to 1.9)	0.29
Protocol adherence [10]	−3.9 (−10.4 to 2.7)	0.15	−4.0 (−10.4 to 2.4)	0.22	−4.5 (−10.8 to 1.7)	0.29	−4.4 (−11.5 to 2.6)	0.29
AST, IU/L								
Intention-to-treat [9]	1.6 (−6.9 to 3.7)	0.03	−1.5 (−6.8 to 3.8)	0.07	−1.6 (−7.0 to 3.8)	0.07	0.0 (−5.8 to 5.9)	0.13
Protocol adherence [10]	−1.6 (−7.3 to 4.2)	0.04	−1.5 (−7.2 to 4.2)	0.07	−1.6 (−7.4 to 4.3)	0.08	0.3 (−6.1 to 6.6)	0.14
ALT, IU/L								
Intention-to-treat [9]	−2.2 (−8.2 to 3.9)	0.07	−2.2 (−8.3 to 4.0)	0.07	−2.1 (−8.3 to 4.2)	0.07	−0.4 (−7.3 to 6.5)	0.12
Protocol adherence [10]	−2.3 (−8.8 to 4.2)	0.09	−2.3 (−8.9 to 4.4)	0.09	−2.2 (−8.9 to 4.6)	0.09	−0.2 (−7.6 to 7.2)	0.14
Alkaline phosphatase, IU/L								
Intention-to-treat [9]	−4.0 (−9.2 to 1.2)	0.16	−4.1 (−9.1 to 0.9)	0.24	−4.6 (−9.5 to 0.3) [11]	0.30	−5.0 (−10.5 to 0.4)	0.31
Protocol adherence [10]	−4.5 (−10.0 to 1.1)	0.17	−4.6 (−10.0 to 0.8)	0.25	−5.0 (−10.3 to 0.3) [11]	0.31	−5.6 (−11.5 to 0.4) [11]	0.32
NAFLD fibrosis score								
Intention-to-treat [9]	−0.3 (−0.6 to 0.1)	0.16	−0.2 (−0.6 to 0.1)	0.19	−0.2 (−0.6 to 0.2)	0.20	−0.1 (−0.5 to 0.3)	0.24
Protocol adherence [10]	−0.2 (−0.6 to 0.2)	0.17	−0.2 (−0.6 to 0.2)	0.20	−0.2 (−0.6 to 0.2)	0.20	−0.1 (−0.6 to 0.4)	0.24

[1] Allocated avocado group in the trial; thus, intervention status was a covariate in the model. [2] All models had *p*-value > 0.05 unless otherwise stated. [3] Defined as a fasting glucose level ≥100 mg/dL or glycosylated hemoglobin ≥5.7% and/or reported use of glucose-lowering medication, at baseline. [4] Mean difference is low-high avocado allotment group. [5] Model 1 adjusted for: age (years); MVPA (minutes/week); total energy intake (kcals/day); alcohol (grams/day). [6] Model 2 adjusted for: variables in Model 1 and change in fruit and vegetable intake (servings/day). [7] Model 3 adjusted for: variables in Model 2 and change in waist circumference (cm). [8] Model 4 adjusted for: variables in Model 3 and HEI 2015 score at Month 6. [9] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 37/35 at baseline; where by prediabetes/diabetes status (yes/no) for low avocado allotment: 18/19 and for high avocado allotment: 15/20. [10] Sample size by intervention group allocation (low avocado allotment/high avocado allotment): 31/35 at month 6; where by prediabetes/diabetes status (yes/no) for low avocado allotment: 15/16 and for high avocado allotment: 15/20. [11] *p*-value = 0.06. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, gamma-glutamyl transferase; hsCRP, high-sensitivity c-reactive protein; NAFLD, Non-alcoholic fatty liver disease.

4. Discussion

To our knowledge, this is the first study to test the effects of an avocado supplemented diet intervention on hepatic function and oxidative stress markers in healthy individuals, free of severe chronic disease. We found that Hispanic/Latino adults who were heads of households randomized to a high allotment of avocados (14/week/family) did not significantly reduce their levels of liver enzymes, GGT, hsCRP, or NAFLD fibrosis score, compared to adult heads of households randomized to a low allotment of avocados (3/week/family). Results denote little evidence of avocado effect on hepatic function and oxidative stress. However, there was a suggestive inverse association between high avocado intake and alkaline phosphatase and GGT among those heads of households with existing prediabetes, and evidence of a potential GGT-lowering effect in those with high avocado intake.

It is challenging to compare our findings with those of the existing evidence, since avocados have never been specific studied on biomarkers of NAFLD previously. However, a recent randomized controlled cross-over trial aimed to assess the effects of substituting carbohydrate energy in meals with half or a whole avocado on indices of metabolic and vascular health in overweight and obese middle-aged adults and measured similar biomarkers of inflammation and oxidative stress in the post-prandial period [29]. Although the cross-over trial examined different biomarkers than those used in our study, findings showed an increase in inflammatory marker interleukin−6 (IL−6) post-meal, but were not different between meals with/without avocado. Additionally, plasma concentrations of monocyte chemoattractant protein−1 (MCP−1), an adhesion molecule expressed by the stimulation of endothelial cells by CRP [30], did not differ between meals. Although we did not observe significant between group differences in fasting hsCRP after a longer-term intervention, we did not evaluate post-prandial effects.

Elevated levels of liver enzymes ALT, AST, and GGT, are markers of NAFLD in both individuals with and without type 2 diabetes [31]. Our study population was generally healthy and free of NAFLD, and had normal values of these indicators at baseline. Thus, it is possible we did not observe changes in these hepatic health markers because they were too healthy. However, our results suggest avocado intake might be beneficial in those with existing metabolic abnormalities. In this respect, an in vivo animal study evaluated the effect of avocado oil, compared to olive oil, on hepatic function in sucrose-fed rats and found non-statistically significant differences in liver enzyme values between study groups [32]. While the nutritional profiles of avocado oil vs. whole avocado are not comparable, with the exception of MUFA content, the results are interesting. First, rats were separated into either a control group, which received a basal diet, or a sucrose-fed group, which received the basal diet plus 30% sucrose solution as drinking water. The animals had ad libitum access to food and water for 16 weeks before the sucrose-fed group was divided into one group of four groups: one group maintained a basal diet with sucrose and the three other groups were assigned to a sucrose plus 7.5% of either olive oil, avocado oil extracted via centrifugation, or avocado oil extracted via solvent, as primary source of dietary fat for 4 weeks. At the end of the study, levels of AST in both avocado oil groups (avocado oil extracted by centrifugation and extracted via solvent) were similarly reduced but not significantly different than either the basal diet with sucrose or the control group. However, the olive oil group showed significantly lower levels of AST, when compared with all other study groups. Additionally, the reductions observed in ALT and alkaline phosphatase did not differ when compared to all other study groups [32].

In clinical evaluations of high MUFA dietary patterns, studies that tested either the effects of a MUFA-rich diet in individuals with type 2 diabetes [17] or the impact of a Mediterranean-style diet in participants with NAFLD [18], demonstrated no significant reductions in ALT, AST, or GGT. Bozzeto et al. [17] assessed the 8-week effects of two widely recommended dietary approaches to manage type 2 diabetes (i.e., a high-carbohydrate/high-fiber/low-glycemic index diet and a high-MUFA diet) with and without the association of a structured physical activity program on liver biomarkers and liver fat. Although researchers reported no significant change in liver enzymes and HOMA-

IR between groups, they observed a 29% significant decrease in hepatic fat content after 8-weeks with MUFA diet, independently of physical activity. This was a clinically significant decrease, suggesting that modifications in the intake of MUFA may positively impact fatty liver [17]. Similarly, Ryan et al. [18] examined the effect of the Mediterranean diet on steatosis and insulin sensitivity in twelve subjects free of diabetes (6 Females/6 Males), randomized to a cross-over 6-week dietary intervention study. All subjects followed both a Mediterranean diet and the control diet (low fat-high carbohydrate diet) with a 6-week wash-out period between each diet. While there were no significant reductions in liver enzyme levels, following the Mediterranean diet exhibited a 39 ± 4% significant relative reduction in liver fat content, measured by intrahepatic lipid, at 6-weeks. In contrast, when following the control diet only showed a 7 ± 2% relative decrease in liver fat content. However, there was significant improvement in insulin sensitivity following the MUFA-rich Mediterranean diet [18].

Though the mechanisms associated in the development of NAFLD remain unclear, a "multiple-hit" model has been proposed as an inclusive framework accounting for the interplay of multiple risk factors involved with the pathophysiology of NAFLD, including an individual's dietary pattern, degree of insulin resistance, amount of visceral fat, state of inflammation, oxidative stress, the microbiome's condition, and genetic susceptibility [33]. Oxidative stress, the relative overproduction of reactive oxygen species in excess of available antioxidant defenses, plays a key role as the starting point of the hepatic and extrahepatic damage. Subsequently, oxidative stress leads to lipid peroxidation and protein damage [34,35]. Free radicals react with unsaturated fatty acids (mainly poly-unsaturated fatty acids) producing lipid peroxides, which can damage endothelial tissue and induce impaired nitric oxide production and vasodilatory dysfunction [34].

Our findings were unexpected and contrary to our hypothesis since we would expect a beneficial effect on oxidative stress and hepatic in those participants randomized to the high avocado allotment group. As previously mentioned, a possible explanation for a lack of significant difference between avocado allotment groups could be that, although our population was overweight, they were healthy and without metabolic or severe chronic disease. Furthermore, small reductions were observed in those participants with prediabetes, particularly in the high allotment of avocado group, suggesting a potential floor effect for the participants overall, whereas there was some room for improvement in those with prediabetes. Additionally, it is conceivable that although the individual average weekly intake of avocado was estimated (at 6 months, 1.7 ± 1.7 vs. 5.4 ± 3.3 in low vs. high avocado allotment groups), the heads of household may not have consumed adequate avocados to have had an effect. Unfortunately, due to the design of the parent trial, we could not measure all hepatic function markers in all trial participants, which would have increased our sample size.

This study had several strengths including internal validity and the fact that this is the first clinical trial to assess risk of NAFLD indicators. Additionally, the study population consisted of Hispanic/Latino adult participants who were overweight/obese and thus at higher risk of developing NAFLD. This study also had limitations. First, we applied a fibrosis score to assess NAFLD status rather than a hepatic ultrasound, CT scan or gold standard biopsy. However, the NAFLD fibrosis score has been validated in 13 studies that included >3000 patients [36], and it is, at present, the most accurate non-invasive test for predicting advanced fibrosis for NAFLD in comparison studies [37,38], and has been endorsed by the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver. Second, we were limited to collecting and reporting lab data on heads of households of families from the parent trial only, of whom the vast majority were women; thus, these results may not generalize to men or other trial participants. Although we would not anticipate sex differences, it would have been interesting to observe effects in both men and women had the sample allowed for sex-specific subgroups. Furthermore, our results cannot be extrapolated to the general population due to the nature of the study design.

In future research, investigations on the effects of avocado on NAFLD should include individuals with insulin resistance and baseline elevated levels of GGT and/or established NAFLD to completely evaluate the effects of this food and its components.

5. Conclusions

After 6 months, adult heads of Hispanic/Latino households of families randomized to a low allotment of avocados (3/week/family) did not have significant changes in biomarkers of NAFLD, compared to adult heads Hispanic/Latino households of families randomized to a high allotment of avocados (14/week/family). These findings suggest increased access and intake of avocados had minimal effect on hepatic function and oxidative stress in healthy, overweight adults. Results should be interpreted with caution and future studies should consider avocado intake effects on individuals with cardiometabolic abnormalities.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University of California San Diego and San Diego State (protocol #160584, approved 5 May 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent was obtained from the participants to publish this paper.

Data Availability Statement: Data described in the manuscript, code book, and analytic code will be made available upon re-quest pending application and approval. Proposals should be directed to malli-son@health.ucsd.edu.

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Modulation of Human Motor Cortical Excitability and Plasticity by Opuntia Ficus Indica Fruit Consumption: Evidence from a Preliminary Study through Non-Invasive Brain Stimulation

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Abstract: Indicaxanthin (IX) from Opuntia Ficus Indica (OFI) has been shown to exert numerous biological effects both in vitro and in vivo, such as antioxidant, anti-inflammatory, neuro-modulatory activity in rodent models. Our goal was to investigate the eventual neuro-active role of orally assumed fruits containing high levels of IX at nutritionally-relevant amounts in healthy subjects, exploring cortical excitability and plasticity in the human motor cortex (M1). To this purpose, we applied paired-pulse transcranial magnetic stimulation and anodal transcranial direct current stimulation (a-tDCS) in basal conditions and followed the consumption of yellow cactus pear fruits containing IX or white cactus pear fruits devoid of IX (placebo). Furthermore, resting state-functional MRI (rs-fMRI) preliminary acquisitions were performed before and after consumption of the same number of yellow fruits. Our data revealed that the consumption of IX-containing fruits could specifically activate intracortical excitatory circuits, differently from the placebo-controlled group. Furthermore, we found that following the ingestion of IX-containing fruits, elevated network activity of glutamatergic intracortical circuits can homeostatically be restored to baseline levels following a-tDCS stimulation. No significant differences were observed through rs-fMRI acquisitions. These outcomes suggest that IX from OFI increases intracortical excitability of M1 and leads to homeostatic cortical plasticity responses.

Keywords: non-invasive brain stimulation; TMS; a-tDCS; indicaxanthin; brain food; cortical excitability; homeostatic plasticity

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1. Introduction

Non-invasive brain stimulation (NIBS) represents a gold-standard neurophysiological approach for modulating brain activity with a view to unveil potential neuronal effects of unknown substances that could implicate repercussions at synaptic, network, and cognitive levels [1,2].

Among the intriguing substances that could play a role in physiological processes, phytochemicals (PhC) have been widely investigated for their bioactive roles [3]. Nevertheless, the putative protective effects of PhC on neuronal functions represent a still debated field of research. On this point, "neuro-nutraceuticals" ascribe to a number of natural, bioactive PhC whose consumption, at nutritionally relevant amounts, is correlated with the ability to modulate neuronal processes and higher functions [4]. Only a few of them can cross the blood-brain barrier (BBB), maintain their molecular integrity, accumulate in some regions of interest, and exert a protective activity on the Central Nervous System

(CNS). Polyphenols, curcumin, and berberine are just some examples of widely studied neuro-nutraceuticals able to cross the BBB [5–8]. Within this framework, specific dietary regimens comprising plant-derived compounds have been suggested to modulate neuronal excitability [9–12]. Neurophysiological measures on cortical excitability by means of TMS have been extensively collected on caffeine or caffeinated energy drinks [13–17], showing contrasting results. De Carvalho [17] found that a high dose of caffeine (~400 mg) caused a reduction of cortical inhibition. Moreover, research that investigated the effects of another PhC, i.e., theophylline on motor cortical excitability, found a reduction in intracortical inhibition, which is thought to depend on $GABA_A$ mediated mechanisms [18].

Opuntia ficus-indica is a plant extensively cultivated worldwide for its fruits and cladodes with high content of phytochemicals and relevant anti-oxidative and anti-inflammatory properties [19,20]. In contrast to cladodes, fruits are hallmarked by a totally distinct PhC fingerprint and are enriched in the betalains pigments. Among the betalains of *Opuntia ficus-indica* fruit, indicaxanthin (IX) has been investigated over the last 20 years by the author's research group for its chemical and nutraceutical properties [21,22]. Thanks to its reducing and amphipathic properties, the PhC modulates specific, redox-dependent signal transduction pathways, thus showing relevant redox-modulating, anti-inflammatory, anti-dysmetabolic, neuroprotective, and neuromodulatory properties [22–24]. In particular, the fruit supplementation was specifically found to exert anti-oxidant and anti-inflammatory effects in healthy subjects [20]. Furthermore, by demonstrating that correlations between circulating levels of inflammatory biomarkers and individual oxidative status can be uncovered in healthy individuals, these previous findings could provide an interesting topic for nutritional research on putative processes affected by these molecular properties [20]. Indeed, since inflammation and oxidative stress contribute to the alterations of physiological processes, assessing their relationships in healthy populations may help to eventually validate the effects of dietary interventions [20,23].

Potential targets underlying IX effects were identified in neuromodulation and inflammatory patterns, but also glutamatergic receptors such as AMPA and Kainate [25]. Interestingly, IX is highly bioavailable, as the ingestion of four cactus pear fruits containing 28 mg indicaxanthin generates a peak plasma concentration of 7 μM of the PhC after 3 h in humans [26]. Moreover, and in contrast with several other PhC, IX is able to cross the BBB and modulate discrete cerebral regions in the rat brain. Indeed, it has been shown that IX, orally administered to rats, modulates the in vivo bioelectric activity of hippocampal neurons, highlighting a predominantly inhibitory effect: this evidence suggested that IX could reduce neuronal excitability in this brain region [27].

Considering the evidence accumulated so far on the effects of IX from *Opuntia Ficus Indica* on both rodent models and healthy subjects, we aimed at investigating putative effects of IX on human cortical excitability and plasticity. To this aim, we applied paired pulse Transcranial Magnetic Stimulation (ppTMS) for assessing intracortical inhibitory and excitatory circuits of the primary motor cortex [28]. Furthermore, in order to evaluate the effect of IX on homeostatic plasticity in terms of neuronal circuit change as a consequence of given stimuli or experience, we employed anodal transcranial Direct Current Stimulation (a-tDCS), widely known to exert an excitatory effect.

In our study, we aimed at investigating the effects induced by the ingestion of a nutritionally-relevant serving of *Opuntia Ficus Indica* yellow fruits by comparing with those obtained from the consumption of the white ones, known to be devoid of IX [29] and thus examined as placebo control. Yellow fruits were chosen according to previous data on the same cultivar, showing the highest levels of IX when compared to other cultivars [29]. To do so, we applied ppTMS and tDCS to provide insight on cortical processes that could be ascribed to underlying mechanisms of intracortical facilitation (ICF) subserved by glutamatergic circuits, short intracortical inhibition (SICI) related to GABAAergic networks [28], and neuronal excitability and plasticity, such as Long Term Potentiation (LTP) and Long Term Depression (LTD) [30]. LTP and LTD represent the paradigmatic binomial phenomena implicated in the cellular substrate of learning and

memory, but also in physiopathology of different brain diseases, especially excitability-based ones [31,32].

Following in the footsteps of the human brain as a complex network, we also conducted preliminary neuroimaging evaluations to explore whether functional connectivity during the resting state (rs-fMRI) could be influenced by the consumption of the PhC present in the yellow fruits.

All considered, our scientific investigation will attempt to add knowledge on the effects of brain stimulation on the consumption of IX-containing *Opuntia* pear fruits, which could be suggested as a source of neuro-active nutraceuticals in diet regimens in healthy subjects.

2. Materials and Methods

Two NIBS and non-invasive neuromodulation (NIN) sessions (baseline, post-fruit intake) per volunteer took place, separated by at least one week (as in Figure 1). Session order was randomized and balanced across subjects.

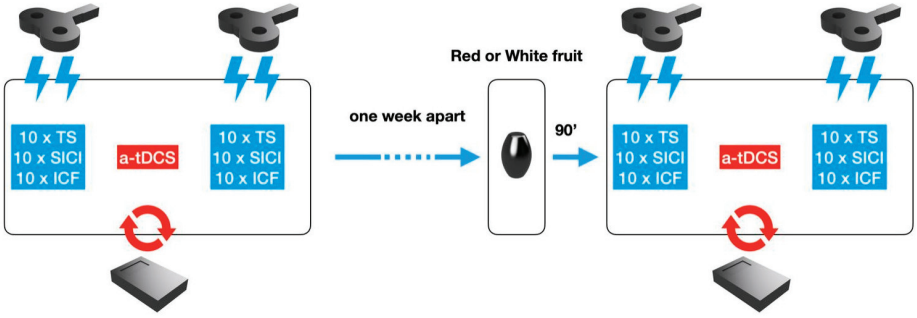

Figure 1. Schematic procedure of the NIBS and NIN sessions. TMS stimulation in baseline conditions was performed before and after a-tDCS to record values of Test stimulus (TS), Short Intracortical Inhibition (SICI), and Intracortical Facilitation (ICF) in random order. Next, one week apart, TMS and a-tDCS stimulations were performed 90 min after red or green fruits ingestion.

2.1. Subjects

Twenty healthy male subjects, with a mean age of 20–45 years, were enrolled for the study. The volunteers were all right-handed (as measured by Edinburgh Inventory [33]: LQ > 40). They all fulfilled the requirements for NIBS and NIN, as assessed by a clinical neurologist, rigidly applying safety guidelines. According to the clinical evaluation by two experienced physicians, nobody had ongoing psychiatric disorders based on the DSM-5 criteria [34] or showed neurologic symptoms. Each volunteer underwent anamnesis questions in order to evaluate the TMS eligibility [35].

All subjects gave their written consent to the study. The experimental procedure was conducted in accordance with ethical standards of the Declaration of Helsinki (Ethical approval code: 'Comitato Etico Palermo1_03.2022').

2.2. Experimental Procedure

Our study was conceived as a single blinded and placebo-controlled design. Each subject underwent two experimental sessions. Before each session a structured interview on dietary habits was recorded and subjects were instructed to keep nutritional intake constant for all the duration of the study. In the first session (T0), 10 Test Stimulus (TS), 10 SICI, and 10 ICF were collected before and after the at-DCS session (20 min). In the second session (T1), at least one week apart, the same TMS parameters were collected 90 min after having orally assumed 400 g. of yellow cactus pear fruits (IX Group, $N = 12$) or 90 min after the consumption of white cactus pear fruits (devoid of indicaxanthin) at the same quantity (Placebo Group, $N = 8$). Four subjects had to be at last excluded from the placebo group since they did not respect the required nutritional intake during the study.

2.2.1. ppTMS

Subjects were seated on a comfortable armchair, asked to be as relaxed as possible, and asked to wear a tight plastic swimmer's cap that was later used to mark the optimal stimulation site. Electromyographic (EMG) activity was recorded by means of 0.9 cm diameter Ag-AgCl surface electrodes placed 3 cm apart with a belly and tendon montage over the right abductor pollicis brevis muscle (APB). Signals were stored with a bandpass between 10 and 1000 Hz and display gain ranging from 50 to 200 V/cm. EMG allowed a TMS simultaneous neuronal responses control, and its accurate temporary resolution was useful to verify the inhibitory or the facilitatory paired pulse provided. Nonetheless, the whole track was analyzed offline and each MEP measurement was collected per subject for subsequent statistical analyses.

The stimulating figure-of-eight coil was connected to two Magstim 200 stimulators through a Bistim module (Magstim Co., Whitland, UK). The coil was placed over the ABP- hotspot on the left primary motor cortex (Brodmann area 4) to elicit responses in the contralateral target muscle. The orientation and position of the coil were kept constant for each subject.

The resting motor threshold (rMT) for eliciting responses in the relaxed APB muscle was defined as the intensity of stimulation needed to obtain a motor evoked potential (MEP) of at least 50 μV in at least 3 out of 6 trials [36]. The subject received audio-visual feedback of EMG activity to keep a completely relaxed state. In case of MT higher than 80% of the output, aMT (active motor threshold) was found as the minimum stimulation intensity necessary to elicit a MEP of at least 50 μV during voluntary contraction.

Once the resting MT was found, the testing stimulus and the conditioning were calculated, the former was set to 120% of the MT, the latter at an intensity of 80% of the motor threshold. ICF and SICI of primary motor cortex were assessed by means of a paired-pulse paradigm. The interstimulus interval (ISI) was 2 ms between CS and TS to record SICI, 10 ms to record ICF. The Test Stimulus (TS) was evoked alone, turning off the CS machine. The interval between each paired pulse stimulus and TS was at least 10 s. The 30 stimuli TMS set was composed by 10 SICI, 10 ICF, and 10 TS stimuli intermixed in random order [36].

2.2.2. a-TDCS

In each experimental session, after the first TMS 30 stimuli set, the subject underwent anodal transcranial Direct Current Stimulation (a-tDCS) (Magstim Company Ltd., Whitland, Wales, UK). The two electrodes (35 cm^2 (7 × 5) size), covered with saline-soaked sponges, were applied to the scalp. Anodal tDCS was delivered at 1.5 mA, with a fade-in and fade-out time of 8 s, for 20 min over the motor hotspot for the ABP muscle [37].

2.2.3. Rs-fMRI Acquisition

A preliminary investigation was conducted on a dedicated sub-group of healthy subjects that also underwent evaluation of functional connectivity, following acute ingestion of yellow cactus pear fruits. Six participants were analyzed via brain MRI scan using a 1.5 T MRI unit (SignaHDxt; GE Medical System, Milwaukee, WI, USA) before and 90 min after consumption of 400 g. of yellow fruits. Acquisitions were performed through an eight-channel brain phased array coil. Structural images were obtained via a T1-weighted sagittal three-dimensional (3D) 1.2 mm thick Fast Spoiled GRadient-echo (FSPGR) prepped inversion recovery pulse sequence (acquisition matrix 256 × 256; slice thickness 1.2 mm; TR 12.4 ms; TE 5 ms; IT 450 ms; FA 20; parallel imaging method: Array coil Spatial Sensitivity Encoding, ASSET). RS-fMRI data were acquired with a two-dimensional (2D) axial T2*-weighted gradient-echo Echo-Planar (EP) pulse sequence parallel to the anterior commissure–posterior commissure (AC–PC) line over the entire brain (acquisition matrix 64 × 64; 33 slices; slice thickness 3 mm; gap 1 mm; TR 3000 ms; TE 60 ms; FA 90); the first 10 scans were discarded to allow T1 saturation to reach equilibrium; a 10-minute (200 volumes) fMRI scan was performed on each participant. The instructions provided

to the subjects were the following: avoid moving during the MRI scan and quietly rest in the scanner with the open eyes and avoid thinking of anything specific. An expert neuroradiologist evaluated the images to exclude the presence of any imaging finding and artifacts that could affect the results of the rs-fMRI analyses.

Preprocessing was performed following the pipeline adopted in literature [38]. Various modules of the FMRIB's Software Library (FSL version 5.0.9 www.fmrib.ox.ac.uk/fsl, access date 25 June 2022) were used. The analysis pipeline was composed of the following steps: motion correction, slice timing correction using Fourier-space time-series phase-shifting, spatial smoothing with a Gaussian kernel of FWHM 6.0 mm, high pass temporal filtering with sigma = 100.0 s, pre-whitening, and global spatial smoothing using a Gaussian kernel with a full width at a half maximum of 8 mm. After preprocessing, the registration of the functional images to the 2 mm isotropic MNI-152 standard space image used non-linear registration with 12 degrees of freedom. A visual quality check was guaranteed by two qualified raters to ensure correct registration.

2.3. Statistical Analysis

Data were analyzed by means of Repeated Measures ANOVA (rmANOVA) to compare SICI, ICF, and TEST in baseline (T0), post-yellow fruit or white fruit consumption (T1) in pre vs. post tDCS condition, by using Stat Soft Statistica (version 8.0; Dell Software, Tulsa, OK, USA). SICI and ICF were normalized and expressed as percentage of TS value. Two separate rmANOVA, with two times (T0 vs. T1) and two conditions (pre- vs. post-tDCS) as intrasubject variables in both IX and Placebo groups were performed. The post-hoc Bonferroni test was adopted to highlight potential significant differences. We considered values as statistically significant when $p < 0.05$. Data are reported as mean ± vertical bars, denoting 0.95 confidence intervals.

As for rs-fMRI acquisition, probabilistic independent component analysis (PICA) was performed through the FSL's MELODIC toolbox. The multi-session temporal concatenated ICA (Concat-ICA) approach was chosen. The groups of scans before and after consumption of yellow cactus pear fruits were compared to identify significantly different regions. The subjects of each couple of groups considered were concatenated for the ICA analysis. A total of 30 independent components (IC) maps were extracted. The inference on estimated maps was accomplished through a mixture model performing variance normalization, thresholding IC maps, and checking the local false-discovery rate at $p < 0.5$. The different component maps are tested voxel-wise for statistically significant differences between the groups using FSL dual regression. In particular, FSL randomized non-parametric permutation testing, with 10,000 permutations, was performed using a threshold free cluster enhanced (TFCE) technique. Correction for multiple comparisons across space was applied assuming an overall significance of $p < 0.05$ using permutation testing and TFCE.

Examples of the Independent Components obtained are reported in the figure below (Figure 2):

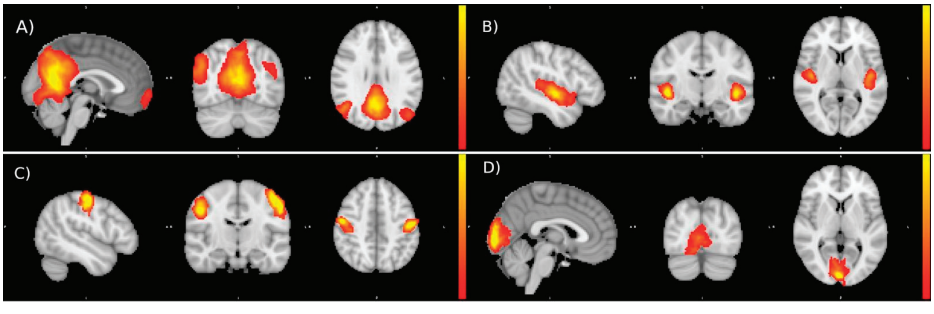

Figure 2. Examples of resting state networks identified by ICA: default mode network (**A**), auditory network (**B**), sensory-motor network (**C**), and visual network (**D**).

3. Results

No significant harmful effects of TMS and tDCS were reported. Sixteen participants (80%) reported a slight itching sensation during the stimulation sessions, which completely subsided after a few minutes of stimulation.

3.1. Effects of Cactus Pear Fruit on Corticospinal Excitability (TS), Intracortical Facilitation (ICF), Intracortical Inhibition (SICI), and Cortical Plasticity (a-tDCS) in IX Group

Statistical analyses were conducted on healthy subjects following the consumption of 400 g yellow cactus pear fruits (T1) containing indicaxanthin. Our data show that the fruit ingestion is associated with the increase in intracortical excitatory circuits. Indeed, the evaluation of MEP values recorded in TEST stimulus and of normalized SICI values did not show any significant effect, respectively ($F_{(1117)}$= 0.28; p = 0.59; Figure 3A) and ($F_{(1119)}$= 1.35; p = 0.24; Figure 3B). Whereas, rmANOVA on normalized ICF % values recorded from subjects that assumed red fruits containing IX revealed a significant main effect of the time T0 versus T1 ($F_{(1119)}$ = 13.75; p = 0.00032; Figure 3C).

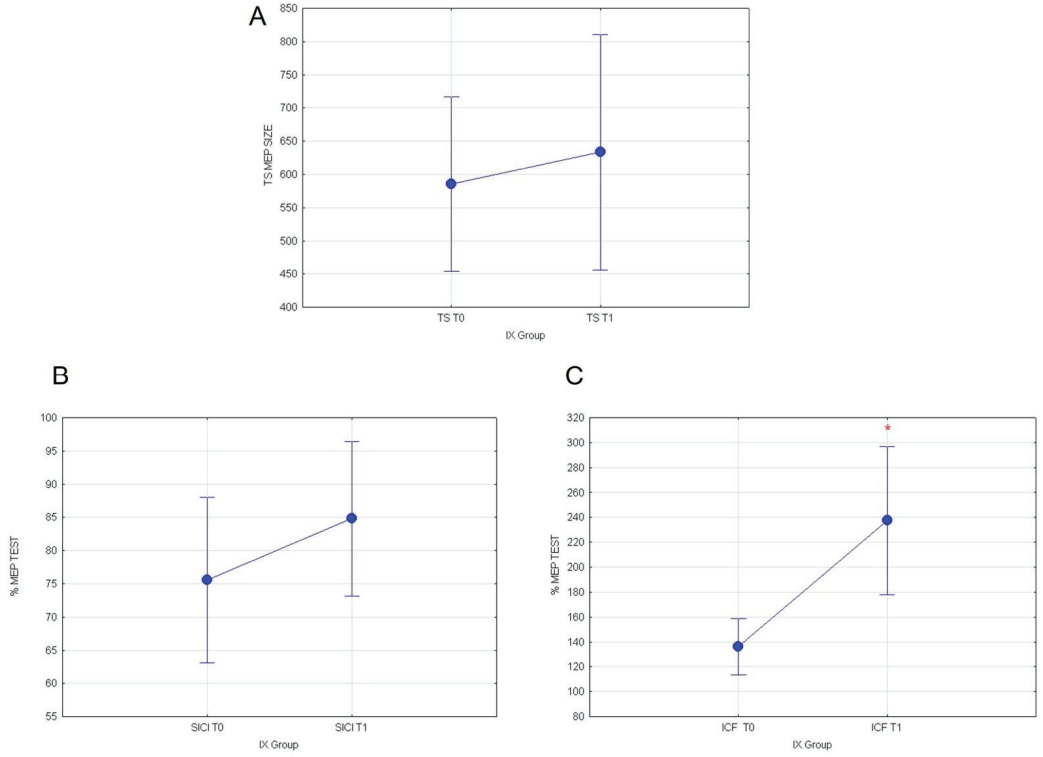

Figure 3. (**A**) MEP values for TEST stimulus, (**B**) normalized % MEP for SICI, and (**C**) normalized % MEP for ICF, at T0 vs. T1 for IX group are shown. Vertical bars denote 0.95 confidence intervals. (*) for $p < 0.05$.

Our results reveal that in the IX group, in the post-tDCS condition, the consumption of yellow fruits exerts a significant main effect on intracortical excitatory circuits, elevated by a-tDCS stimulation. In particular, a rmANOVA was conducted to compare the mean response values in T1 with two conditions (pre- vs. post-tDCS) as intra-subject variables. Neither the evaluation of TEST values outline any differences ($F_{(1116)}$ = 2.06; p = 0.153; Figure 4A), nor of normalized % SICI ($F_{(1119)}$ = 0.22; p = 0.63; Figure 4B) at T1 in pre- vs. post-tDCS conditions. Remarkably, statistical comparisons on normalized ICF % responses at T1 revealed

a significant decreased in recorded MEP values ($F_{(1119)} = 14.567$; $p = 0.00022$; Figure 4C) in post-tDCS vs. pre-tDCS, following the ingestion of red fruits containing indicaxanthin. No other significant results were found.

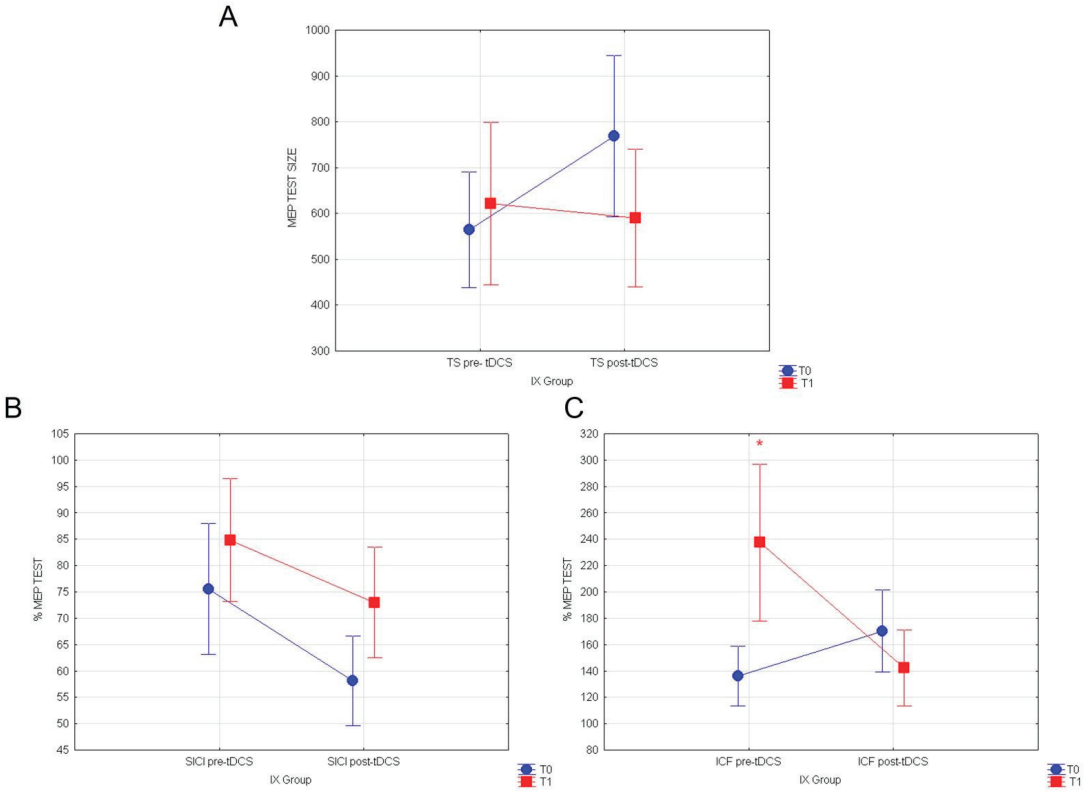

Figure 4. (**A**) MEP values for TEST stimulus, (**B**) normalized % MEP for SICI, and (**C**) normalized % MEP for ICF, at T0 vs. T1 for the IX group, are shown in pre vs. post-TDCS. Vertical bars denote 0.95 confidence intervals. (*) for $p < 0.05$.

3.2. Effects of Cactus Pear Fruit on Corticospinal Excitability (TS), Intracortical Facilitation (ICF), Intracortical Inhibition (SICI), and Cortical Plasticity (a-tDCS) in the Placebo Group

Statistical assessment was also carried out on healthy subjects following the consumption (T1) of 400 g white cactus pear fruits (Placebo Group) devoid of indicaxanthin. Our results show that the fruit ingestion does not modulate cortical excitability. Indeed, the rmANOVA performed on mean response values of MEP revealed no differences in T0 vs. T1, respectively, for TEST stimuli: ($F_{(1.79)} = 0.85$; $p = 0.35$); normalized SICI ($F_{(1.89)} = 2.72$; $p = 0.10$) and ICF ($F_{(1.89)} = 0.76192$; $p = 0.38$).

The rm ANOVA conducted on Placebo group at T1 comparing pre- vs. post-TDCS conditions outlined a significant main effect for TEST stimulus post-tDCS following white pear fruits ingestion ($F_{(1.79)} = 6.17$; $p = 0.015$). No significant effects were found for SICI or ICF values.

3.3. Effects of a-tDCS on Corticospinal Excitability (TS), Intracortical Facilitation (ICF), and Inhibition (SICI) before Assuming Cactus Pear Fruit in IX and Placebo Groups

Statistical analyses performed on healthy subjects stimulated with a-tDCS at T0, before the ingestion of yellow cactus pear fruits (IX group), showed that a-tDCS can increase corticospinal excitability and intracortical GLU activity, while it inhibits intracortical GABA circuits

in healthy subjects. Indeed, repeated measures ANOVA comparing the mean response values of MEP at T0 revealed a significant main effect of the condition pre vs. post-TDCS for TEST stimulus ($F_{(1118)} = 3.96$; $p = 0.0487$; Figure 5A). Additionally, evaluation of normalized SICI and ICF respectively showed significant reductions of intracortical inhibitory ($F_{(1119)} = 5.28$; $p = 0.023$; Figure 5B) and increase in excitatory circuits ($F_{(1119)} = 4.00$; $p = 0.047$, Figure 5C), following a-tDCS.

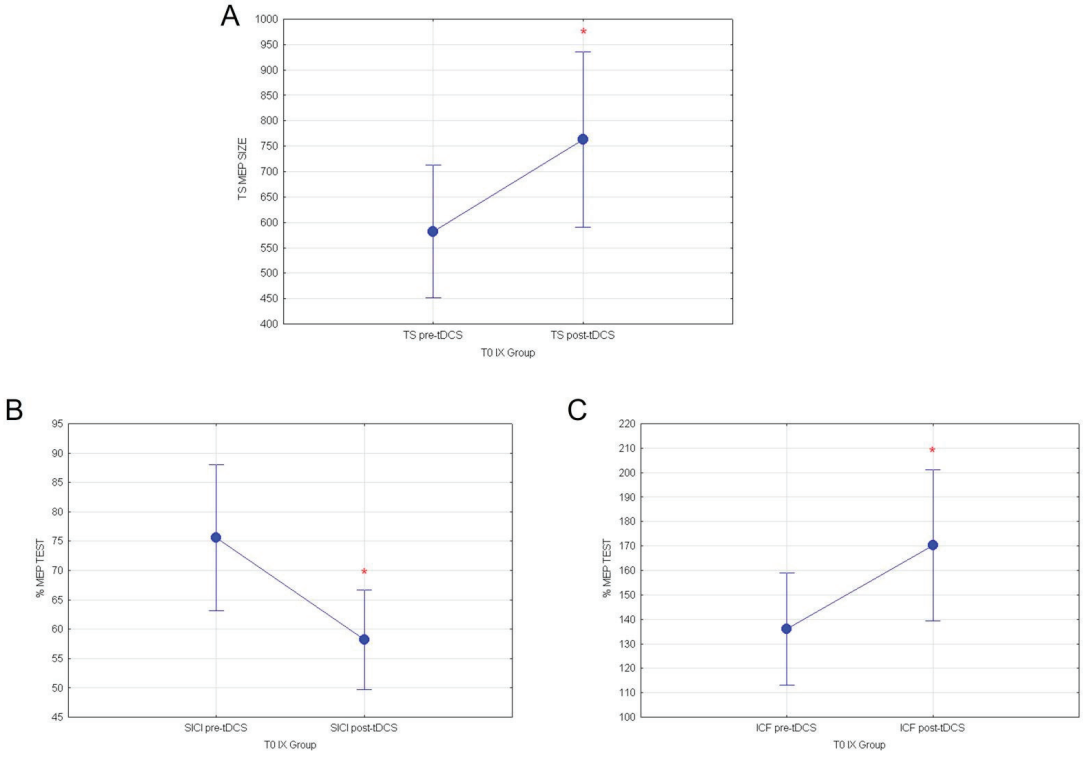

Figure 5. (**A**) MEP values for TEST stimulus, (**B**) normalized % MEP for SICI and (**C**) normalized % MEP for ICF, at T0 for IX group are shown in pre vs. post-TDCS. Vertical bars denote 0.95 confidence intervals. (*) for $p < 0.05$.

Furthermore, analyses on healthy subjects stimulated with a-tDCS at T0, before the consumption of white cactus pear fruits (placebo Group), similarly showed that a-tDCS induces an increase in cortico-spinal excitability and intracortical GLU activity, concomitantly inhibiting intracortical GABA circuits. The mean response values of MEP revealed a tendency to increase in TEST stimulus following a-tDCS, which is almost significant ($F_{(1.79)} = 3.85$; $p = 0.053$; Figure 6A). On the other hand, rmANOVA comparing normalized SICI before white fruit ingestion pre vs. post-tDCS showed a significant inhibitory effect ($F_{(1.89)} = 6.55$; $p = 0.012$; Figure 6B). Finally, normalized ICF values were remarkably potentiated by a-tDCS stimulation at T0 ($F_{(1.89)} = 5.13$; $p = 0.0259$; Figure 6C). Significance of a-TDCS in both group are summarized in Table 1.

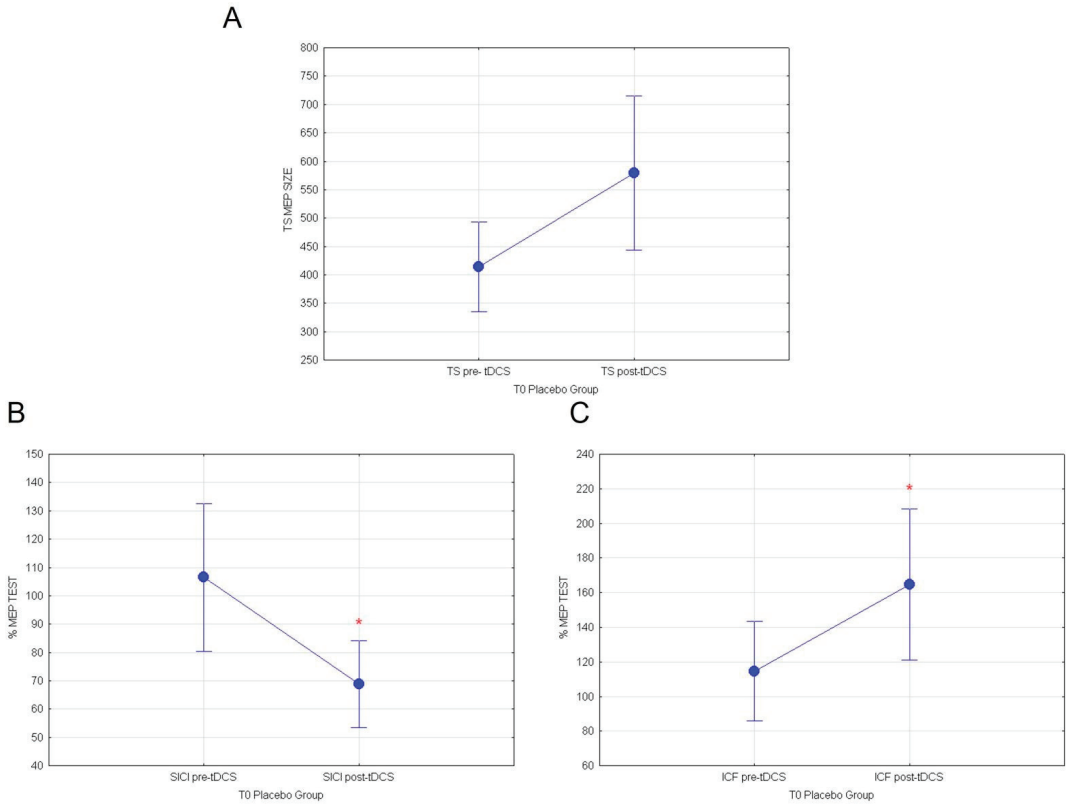

Figure 6. (**A**) MEP values for TEST stimulus, (**B**) normalized % MEP for SICI, and (**C**) normalized % MEP for ICF at T0 for placebo group are shown in pre vs. post-TDCS. Vertical bars denote 0.95 confidence intervals. (*) for $p < 0.05$.

Table 1. Summary of pre-tDCS vs. post-tDCS in baseline conditions (T0) for IX and Placebo groups.

	Pre-tdcs vs. Post-tdcs
IX Group (t0)	
TS	$p = 0.0487$
SICI	$p = 0.023$
ICF	$p = 0.047$
Placebo Group (t0)	
TS	$p = 0.053$
SICI	$p = 0.012$
ICF	$p = 0.0259$

3.4. Preliminary Functional Connectivity Investigations after Cactus Pear Fruit Consumption

Dual regression analysis on rs-fMRI datasets showed no increased resting state-functional connectivity in the six subjects that underwent MRI acquisitions before and after oral consumption of 400 g. of yellow cactus pear fruits.

Indeed, there are no brain regions with significantly larger resting state functional connectivity after the consumption of IX-.containing fruits, considering an overall significance threshold of $p < 0.05$.

4. Discussion

The present work falls within the area of research on brain stimulation and neuromodulatory effects of PhC at dietary consistent amounts in healthy subjects. Here, we aim to explore the acute impact of Opuntia Ficus Indica fruit consumption on human motor cortical circuits [26]. It was indeed elucidated that IX contained in the yellow fruits is able to cross the rat BBB and exert dose-dependent effects [24,27]. In detail, brain distribution of IX was evidenced, namely in rat cortex, hippocampus, globus pallidus, thalamus and subthalamic nucleus, and neuronal modulation, which was revealed with excitatory and inhibitory effects depending on the targeted brain area. Noticeably, these findings were obtained administering an amount of the yellow betalain comparable to those measured in human plasma in volunteers that have assumed Opuntia ficus indica fruits at a dietary consumption.

Considering the outcomes from the rodent model and from human bioavailability studies, we hypothesized that orally administered IX present in the yellow fruits could also play a role on neuronal processes in the healthy human brain. With the purpose of assessing the putative effect of IX from Opuntia Ficus Indica on basic functions of the CNS, such as cortical excitability and plasticity, we applied a neurostimulation and neuromodulation approach on the left motor cortex via TMS and a-tDCS on healthy subjects.

We examined the difference within baseline (T0) and IX or "placebo" (T1) groups, both in pre- and in post-a-tDCS. Firstly, our results confirmed the excitatory effect induced by a-tDCS stimulation, in line with literature. Indeed, a-tDCS effects on MEP, SICI, and ICF immediately after stimulation were clearly described [39]. Additionally, evidence supports that the a-tDCS application enhances corticospinal excitability (CSE) up to 90 min, depending on the duration of a single a-tDCS session [40]. The biochemical key of a-tDCS activity could be found in the modulation of N-methyl-D-aspartate (NMDA) receptors, the main component of the glutamatergic system, and of GABA receptors, by promoting long-term potentiation LTP [41]. Symmetrically, cathodal tDCS promotes long-term depression mechanisms [42]. These LTP and LTD mechanisms can induce synaptic facilitation or depression, thus modulating synaptic efficacy. Furthermore, it was agreed that a-tDCS provides a disinhibitory after-effect corresponding to an increase in CSE, along with the reduction in SICI [43,44]. Indeed, available data on pharmacology of intracortical circuits suggest that SICI reflects the short-term postsynaptic inhibition mediated by the GABAAR, though it is still to be unveiled the exact mechanism mediated by a-tDCS. [28]. In agreement with this, we have shown specific a-tDCS effects increasing ICF and reducing SICI in basal conditions.

Following the consumption of yellow fruits containing IX, our results revealed that IX exerted a specific role on intracortical excitability upon TMS stimulation in pre-tDCS condition. In particular, we have provided evidence that there is a major increase only in ICF values, leaving SICI unaffected. Therefore, IX contained in yellow fruits seems to achieve an excitatory drive on motor cortical excitability, corresponding to a significant raise of the intracortical facilitation, specifically subserved by glutamatergic circuits.

Furthermore, healthy subjects were stimulated by a-tDCS after the fruit consumption and our data revealed a paradoxical effect exerted by IX-containing fruits on ICF that was reduced after anodal tDCS. This may support the idea that IX from yellow fruits induces an excitatory impact leading to homeostatic responses on motor cortical plasticity. This could be interpreted as a safety mechanism, thanks to which activity-dependent synaptic plastic changes can occur only within a physiological range. This homeostatic plasticity mechanism could play a role in neuroprotection from excitotoxicity in the context of hyperexcitability-driven diseases, known to be linked to oxidative, inflammatory, and synaptic insults [45,46].

These outcomes were further confirmed by assessing the placebo experimental group, unveiling the differences between IX-rich yellow and IX-devoid white fruits. The data obtained revealed that white fruit does not influence intracortical excitability even if it seems to increase corticospinal excitability. Although it could appear counterintuitive, a similar increase on motor cortical excitability has been reported as due to the placebo

effect [47]. It seems to be specific for white pear fruit, as the yellow fruit did not affect the same parameter. A possible molecular explanation for the evidence provided could be ascribed to several receptors involved in IX-mediated effects. Its potential implication in the glutamatergic system has already been hinted at thanks to pharmacological and molecular docking approaches, showing a putative affinity with the N2A subunit of the NMDR. This kind of macromolecular complex in mammalian species is the most represented of the glutamate receptor family in hippocampus, the region where LTP was described at first [48].

Effects of other PhC on cortical excitability have been studied in the last decade. For instance, valerianic acid was found to reduce ICF, whereas no effects were reported on SICI [49]. It was also demonstrated that Hypericum perforatum modulated LTD by converting it into facilitation, via inhibition of serotonin, norepinephrine, and dopamine uptake [50,51]. Noteworthy, to the best of our knowledge, no other PhC have been proven to exert an homeostatic effect on intracortical facilitation. This could in principle play a beneficial role in disexcitability-related diseases, such migraines [31,45], or even in complex modulation of synaptic efficacy by endogenous molecular targets [52,53]. A PhC able to modulate cortical excitability could represent a safe and powerful tool to induce homeostatic responses when needed. On this point, it is worth noting that a growing body of evidence points to maladaptive or dysfunctional homeostatic plasticity as a key mechanism in many neurological and psychiatric diseases, such as Alzheimer's Disease, Autism Spectrum Disorder [54], and depression [55]. Furthermore, diseases characterized by fluctuating cortical excitability, such as migraines [31], could take advantage of an acute treatment with IX-containing fruits. Further case-control experiments also recording behavioral data, such as motion analysis or cognitive tests, could unveil other putative effects in diseased people.

Our study also provided a preliminary evaluation of functional connectivity during resting state in healthy subjects that assumed yellow cactus pear fruits. The neuroimaging data did not identify any alterations in network connectivity. This could be due to the limited number of subjects included in the evaluation or could fit well with a specific action of IX-containing fruits that modulate intracortical circuits without modifying the physiological balance of network connectivity in acute conditions. Undoubtedly, further experiments could in future unveil eventual effects after chronic consumption, as for other PhC [56].

In the light of all this, our study paves the way to the idea that the yellow fruits containing IX could exert a neuromodulatory role on healthy subjects, and the resulting putative multi-receptorial interactions suggest conclusively an excitatory input to cortical processes. This outcome unveiling a role for IX-containing fruits in the modulation of intracortical excitation fit well with the already-evidenced neuronal effects obtained upon in vivo administration in rats. Although it should be said that IX-mediated bioelectrical effects in rats were measured in single neurons in discrete brain regions, by means of a microiontophoretic approach, here, we applied NIBS and NIN tools to investigate the overall bioelectrical activity deriving from the networks in the whole motor cortex. Notwithstanding eventual common traits, translating the preclinical study to humans allow to explore network effects due to different neuronal density, neurotransmitters, and receptorial pools circuits.

5. Conclusions

Possible Limitations and Future Directions

The main limitation of our study can be found in the small sample size, even if it is to point out that the study design is based on within-subject comparisons and thus more reliable than a between-subjects design with a similar sample size. Given these pitfalls, our results should be considered as preliminary, thus further experiments with a larger series of subjects are worth being performed in order to explore alternative explanation or control for unspecific effects. Nevertheless, the lack of effect on intracortical excitability of white pear fruit seems to rule out an unspecific effect of treatment and suggest a specific modulatory

role of IX present in yellow fruits that is known to exert powerful antioxidant and anti-inflammatory effects at a cellular level [20]. However, future experiments, including a different stimulation site as control condition, could strengthen conclusions on placebo effect. Indeed, also applying cathodal tDCS could add important pieces to the puzzle of red-orange cactus pear fruit effects on human motor cortical excitability, giving insight also on the putative role of Long-Term Depression (LTD) -like mechanisms.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data can be requested by mail to giuditta.gambino@unipa.it.

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Physicochemical Properties and Effects of Fruit Pulps from the Amazon Biome on Physiological Parameters in Rats

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Abstract: This study aimed to analyze the physicochemical characteristics and the effects of Amazonian pulp fruits consumption, such as araçá-boi (*Eugenia stipitata*), abiu grande (*Pouteria caimito*), araticum (*Annona crassiflora*), biri-biri (*Averrhoa bilimbi* L.), and yellow mangosteen (*Garcinia xanthochymus*), on hematologic, metabolic, renal, and hepatic function parameters in Wistar rats (n = 10 rats/group). The pulp of abiu had the highest levels of soluble solids, sugars, and pH. Biri-biri pulp had the highest levels of ascorbic acid and total titratable acidity, and a low pH. The araticum pulp had higher ($p \leq 0.05$) ash content, total phenolic compounds, and antioxidant activity than the pulp of other analyzed fruits. No significant increase in hematocrit, nor reduction of blood glucose, plasma cholesterol, and serum levels of glutamic-pyruvic transaminase (TGP), creatinine, and urea was observed in experimental groups relative to the control group of rats after the consumption of fruits pulp. The intake of abiu and araticum pulps promoted a significant reduction ($p \leq 0.05$) in total leukocytes of the experimental groups as compared to the control group and only the intake of araticum significantly increased ($p \leq 0.05$) triglyceride blood levels in rats (99.50 mg/dL). The regular consumption of biri-biri pulp for 30 days significantly ($p \leq 0.05$) increased serum glutamic-oxaloacetic transaminase (TGO) levels in rats (116.83 U/L) compared to the control group (98.00 U/L). More researches are needed to generate knowledge about these promising Amazonian fruits, supporting the native fruit production, in addition to promoting health in the population and sustainability in the Amazon region.

Keywords: Amazonian fruits; composition; metabolic effects

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1. Introduction

Brazil is the third largest producer of fruits in the world, and Amazonian fruits are known to contain bioactive compounds. However, despite their high nutritional value and potential for promoting health benefits, these fruits are still relatively unexplored [1–3]. The production and commercialization of açai is currently notable in the Amazon biome, due to its bioactive and antioxidant potential [4]. However, there are other fruits in this biome with similar economic and nutritional importance for the local population, and that have good marketing potential. These include araçá-boi (*Eugenia stipitata*), abiu grande (*Pouteria caimito*), araticum (*Annona crassiflora*), biri-biri (*Averrhoa bilimbi* L.), and yellow mangosteen (*Garcinia xanthochymus*), which can be consumed fresh or in the form of juices, liqueurs, ice creams, and jellies [5].

Scarce scientific studies have characterized araçá-boi, abiu grande, araticum, biri-biri and yellow mangosteen fruits. The bioactive compounds and total antioxidant activity have

been determined in abiu [6,7], cowberry [3,7,8], biri-biri [9], araticum [10,11] and yellow mangosteen [3]. Araçá-boi is characterized by its high content of phenolic compounds, flavonoids and antioxidant activity [1], as well as carotenoids [3] and antimutagenic and antigenotoxic activity [12]. Araticum, besides having a high carotenoid content, is an excellent source of vitamins C and A [13], and a study by Virgolin et al. [3] showed a high content of yellow flavonoids (flavonones and flavonols) in yellow mangosteen.

Numerous studies have shown that fruit consumption is associated with a chronic non-communicable diseases (NCDs) risk reduction [14–16]. NCDs, including cardiovascular, neoplastic, ischemic cerebrovascular, and chronic respiratory diseases, as well as diabetes, represent a major public health challenge for the next decade [17]. According to Chaabane et al. (2012) [18], metabolic dysfunctions and oxidative states favor the development of these diseases. Therefore, a diet rich in bioactive compounds which exhibit antioxidant activity, such as ascorbic acid, phenolic compounds, and carotenoids, present in fruits, is capable of inhibiting oxidation processes in the body, therefore representing a promising alternative in NCDs prevention and treatment [19–21].

Bioactive compounds from fruits have been shown to regulate prostaglandin synthesis and cholesterol synthesis and absorption, to reduce platelet aggregation, as well as lowering blood pressure [22]. High fruit consumption is also associated with changes in specific antioxidant markers or early indicators associated with the risk of pathologies, including cholesterol oxidation products, plasma antioxidant capacity, total circulating glucose, and body weight [23].

It is believed that the increase in fruit consumption derives from the awareness of its benefits, thus additional experimental and clinical studies that scientifically demonstrate, through reliable biomarkers, the proposed benefits should be conducted [24]. It is noteworthy that araçá-boi, abiu grande, araticum, biri-biri, and yellow mangosteen are relatively unknown fruits. Thus, it is also important to evaluate their safety for consumption.

There are scarce studies in the literature that correlate the consumption of Amazonian fruits with biological parameters. Thus, Amazonian fruits offer new perspectives for functional foods characterization which, in addition to basic nutritional functions, produce metabolic and/or physiological beneficial effects to health when consumed as part of the regular diet [25].

Given the above, considering the effort in the field of public health to discover alternative sources to reduce the burden of NCDs without presenting toxicity, and that there are few studies conducted in Brazil assessing beneficial potential of Amazonian fruits, the objective of this study was to analyze the effects of consuming the pulp of Amazonian fruits (araçá-boi, abiu grande, araticum, biri-biri, and yellow mangosteen) on hematological, metabolic, liver, and renal function parameters in rats. We also aimed to analyze the chemical composition of these pulps to promote health and quality of life in the population and sustainability in the Amazonian region.

2. Material and Methods

2.1. Raw Material

The pulps of araçá-boi, abiu grande, araticum, biri-biri, and yellow mangosteen were acquired in the frozen form as 100 g packages from an agro-industry company (latitude: 11°26′19″ S and longitude: 61°26′50″ W) in the city of Cacoal, RO, in February 2016. The pulps were stored in a freezer at −18 °C until analysis.

2.2. Physicochemical Analysis

Total soluble solids, total titratable acidity, reducing sugars, non-reducing sugars, total sugars, and pH of the fruit pulps were determined using methods described by the Association of Official Analytical Chemists (AOAC), 2005 [26]. The results were expressed as follows: total soluble solids as degrees brix (°Bx), total titratable acidity as g citric acid/100 g pulp, and reducing sugars, non-reducing sugars, and total sugars as g glucose/100 g pulp. Determination of the ascorbic acid content of fruit pulps was based

on the oxidation of ascorbic acid by 2,6-dichlorophenolindophenol reagent [26,27] and the result was expressed as mg ascorbic acid/100 g pulp. The moisture content, ash, lipids, and proteins (total nitrogen by the Kjeldahl method and conversion factor of 6.25) in the fruit pulps were evaluated using previously established methods [26].

Extraction of total phenolic compounds was performed using a method described by Macoris et al. (2012) [28] and their content was determined using the Folin-Ciocalteu colorimetric method [29]. Absorbance readings were performed at 720 nm on a Beckman DU-640 spectrophotometer (Fullerton, CA, USA). Quantification was performed using a calibration curve obtained using standard gallic acid (Sigma Aldrich, St. Louis, MO, USA) solutions (72 to 200 μg/mL) and the results were expressed as mg of gallic acid equivalent (GAE)/100 g pulp.

Total antioxidant activity of fruit pulp was determined using DPPH (Sigma Aldrich, St. Louis, MO, USA) free radical scavenging [4]. The absorbance reading was performed at 515 nm and methanol was used as a blank to calibrate the Beckman DU-640 spectrophotometer (Fullerton, CA, USA). Quantification was performed using a calibration curve obtained using standard Trolox (Sigma Aldrich, St. Louis, MO, USA) solutions (200 to 800 μM). The results were expressed as μmol of Trolox/mg of pulp. Among the antioxidant activity methods (ABTS, DPPH, FRAP, and oxygen radical absorbance capacity (ORAC)), the DPPH method proved to be more efficient for the fruits analyzed in this study according to a study previously carried out by Virgolin et al. [3].

All analyses were performed in triplicates at the food analysis laboratory of the Food Engineering Department, IBILCE, São José do Rio Preto.

2.3. Biological Assays

After obtaining the approval for the study from the Ethics Committee in the Use of Animals (CEUA) of the Federal University of Rondônia Foundation—UNIR (Protocol number: PP 020/2014) the entire animal experimentation protocol was carried out according to the rules established by the Brazilian College of Experimentation on Animals, Federal University of São Paulo, World Health Organization and Society for Neuroscience [30].

The biological assays were performed using 60 Wistar rats (*Rattus norvegicus*) born on the same day acquired from the Federal University of Mato Grosso (UFMT). Newly weaned male rats with masses between 90 g and 100 g were separated and placed in polypropylene cages with a stainless steel lid (maximum of two rats per cage) in a controlled environment. The rats were kept in 12-h dark/12-h light cycles at 23 ± 1 °C and 55% relative humidity. The animals received standard diet and drinking water ad libitum for seven days. After this period, they were divided into groups of ten animals to form the six treatment groups (T): T1—Control, T2—Araçá-Boi, T3—Abiu-Grande, T4—Araticum, T5—Biri-biri, and T6—Yellow Mangosteen.

The experiment was conducted from March 16 to April 15 of 2016, lasting 30 days (sub-chronic effect). In this period, the animals had free access to water and pelleted feed (Presence®) with a balanced nutritional composition (Code No.: 05.7883.40.20; Lot No.: 41EX150844109) for rodents and mortality was monitored. The fruit pulp was administered once a day to the animals through a syringe attached to a curved needle with a rounded tip that was introduced into the animal's stomach by gastric intubation (gavage), so that an exact amount was administered [31]. The amount administered was 0.3214 mL of pulp/100 g of body mass, which is equivalent to the intake of 500 mL of juice (prepared with 45% pulp) by an adult weighing 70 kg. The animals were weighed daily and the values obtained were recorded to calculate the pulp dose to be administered. After the experimental period, intraperitoneal anesthesia was performed using pentobarbital sodium (50 mg/kg) (Abbott, IL, USA) [32], followed by laparotomy to collect 1–2 mL of blood sample from the abdominal aorta and euthanasia by severing the abdominal aorta [33–35].

2.3.1. Hematological Analysis

The hematocrit percentage was determined using the microhematocrit technique (Quimis® microhematocrit centrifuge, São Paulo, Brazil).

Platelet count was performed via a blood smear stained with hematoxylin and eosin (Renylab, Minas Gerais, Brazil) and analyzed using light microscopy (monochrome microscope Primo Star ZEISS, Oberkochen, Germany) with an immersion objective (100× magnification). The counting was performed in 10 random fields. The mean platelet number per field was then multiplied by 20,000 (correction factor for monochrome microscopes).

Total leukocyte count was performed on 0.4 mL of Turk's solution (Renylab, Minas Gerais, Brasil) added to 20 uL of whole blood (1:21 dilution) in a test tube. The count was then performed using a hemocytometer Olen Neubauer Chamber (Forlabexpress, Rio de Janeiro, Brazil) in the four quadrants of the chamber, and the calculation was performed according to the following equation:

$$\text{Leukocytes } (\text{mm}^3) \;=\; \frac{\text{Number of leukocytes } \times\ 21 \times 10}{4}$$

where 10 is the conversion factor for μL, 21 is the dilution conversion factor, and 4 is the denominator [33].

2.3.2. Biochemical Analysis

The protocols and reference values adopted in biochemical analyzes followed those of similar studies previously carried out with experimental animals [34–36]. The collected blood was centrifuged for 10 min at 3500 *g* to obtain the plasma. The analyses were performed by spectrophotometry using an automatic analyzer for biochemical and immunochemical tests Labmax 240® (Labtest, Minas Gerais, Brazil). The liver enzymes, glutamic-oxaloacetic transaminase (TGO) and glutamic-pyruvic transaminase (TGP), were measured by spectrophotometry at 340 nm. Total cholesterol, triacylglycerols, and glucose levels were determined by spectrophotometry at 500 nm, and creatinine and urea were measured by spectrophotometry at 504 nm [37,38]. The results were expressed as mg/dl.

2.3.3. Statistical Analysis

The results were tested for normal distribution and homogeneity of variance. When the conditions for applying parametric statistical tests to compare the means were met, the comparison between the results was performed for independent samples using analysis of variance (ANOVA) followed by Tukey's test at a significance level of 5%. In addition, marginally significant results at the 10% significance level ($p < 0.10$), were also indicated. The Kruskal–Wallis nonparametric statistical test was used in the set of results for which normal distribution and, especially, homogeneity of variance was not observed. The results were expressed as mean ± standard deviation. Statistical analyses were performed using the Statistica 12.0 software (TIBCO, PALO ALTO, CA, USA).

3. Results and Discussion

The results of the physicochemical analyses are presented in Table 1. The pulps of abiu and araçá-boi had the highest ($p \leq 0.05$) and lowest levels of soluble solids, respectively. Among the various fruit components, total soluble solids (°Bx) play a key role in determining fruit quality due to their influence on the thermophysical, chemical, and biological properties of the fruit [39].

The pulp of araçá-boi had the highest level of total titratable acidity ($p \leq 0.05$), indicating the presence of citric acid, the chief organic acid found in some fruits. Biri-biri pulp had the highest level of ascorbic acid ($p \leq 0.05$). The levels of ascorbic acid in fruit pulps can be lower than that in fresh fruits because this compound is degraded during food preparation and storage, due to the action of light, temperature, high pH, metal ions (Cu^{+2} and Fe^{+3}), reactive oxygen species, and humidity [40]. Smirnoff and Wheeler (2000) [41]

suggest that the immediate precursors of ascorbic acid biosynthesis are D-glucosone and L-sorbosone, explaining the low levels of reducing, non-reducing, and total sugars in the araçá-boi and biri-biri pulps, and high levels in the abiu and yellow mangosteen pulps. Moisture content was higher than 80% in all pulps. However, it was relatively lower in the abiu pulp ($p \leq 0.05$), which is explained by the high level of soluble solids in it (Table 1).

Table 1. Physicochemical composition of fruit pulps from the Brazilian Amazon biome.

Parameters [1]	Araçá-Boi (*Eugenia stipitata*)	Abiu Grande (*Pouteria caimito*)	Araticum (*Annona crassiflora*)	Biri-Biri (*Averrhoa bilimbi* L.)	Yellow Mangosteen (*Garcinia xanthochymus*)
Total soluble solids (°Brix)	3.63 ^{e} ± 0.12	17.50 ^{a} ± 0.00	8.37 ^{b} ± 0.23	4.83 ^{d} ± 0.29	7.00 ^{c} ± 0.00
Total titratable acidity (g citric acid/100 g)	2.34 ^{a} ± 0.05	0.10 ^{e} ± 0.01	0.66 ^{d} ± 0.00	1.02 ^{c} ± 0.01	1.41 ^{b} ± 0.01
Ascorbic acid (mg ascorbic acid/100 g)	13.71 ^{b} ± 0.02	3.11 ^{d} ± 0.31	3.47 ^{d} ± 0.84	20.23 ^{a} ± 0.22	5.60 ^{c} ± 0.62
Reducing sugars (g glucose/100 g)	0.27 ^{d} ± 0.03	5.78 ^{a} ± 0.31	5.18 ^{b} ± 0.04	2.75 ^{c} ± 0.09	5.86 ^{a} ± 0.30
Non-reducing sugars (g glucose/100 g)	0.57 ^{b} ± 0.07	3.40 ^{a} ± 0.24	0.43 ^{b} ± 0.16	0.43 ^{b} ± 0.22	0.65 ^{b} ± 0.020
Total sugars (g glucose/100 g)	0.84 ^{e} ± 0.02	9.19 ^{a} ± 0.32	5.60 ^{c} ± 0.14	3.14 ^{d} ± 0.12	6.51 ^{b} ± 0.50
pH	2.81 ^{d} ± 0.01	5.84 ^{a} ± 0.01	4.06 ^{b} ± 0.01	2.15 ^{e} ± 0.01	2.97 ^{c} ± 0.00
Moisture (%)	95.35 ^{b} ± 0.02	81.65 ^{e} ± 0.03	89.25 ^{d} ± 0.14	96.23 ^{a} ± 0.03	91.65 ^{c} ± 0.05
Ashes (%)	0.19 ^{b} ± 0.01	0.32 ^{b} ± 0.02	2.32 ^{b} ± 0.30	0.20 ^{a} ± 0.07	0.31 ^{b} ± 0.00
Lipids (%)	0.07 ^{c} ± 0.02	0.13 ^{b} ± 0.01	0.09 ^{c} ± 0.00	0.06 ^{c} ± 0.02	0.28 ^{a} ± 0.01
Protein (%)	2.30 ^{b} ± 0.01	4.60 ^{a} ± 0.31	2.52 ^{b} ± 0.11	1.27 ^{c} ± 0.12	1.19 ^{c} ± 0.00
Total phenolic compounds (mg EAG/100 g fruit)	144.0 ^{c} ± 0.06	60.0 ^{d} ± 0.015	258.04 ^{a} ± 0.036	30.0 ^{e} ± 0.005	204.0 ^{b} ± 0.03
Antioxidant activity (µmol Trolox/100 g)	6.09 ^{a} ± 0.29	2.89 ^{c} ± 0.00	6.27 ^{a} ± 0.06	0.62 ^{d} ± 0.00	4.29 ^{b} ± 0.26

[1] Mean ± standard deviation. Means followed by the same letters in a line do not differ significantly ($p \leq 0.05$) by the one-way ANOVA test followed by post-hoc Tukey test.

Araticum pulp had the highest ash content ($p \leq 0.05$) of all pulps. According to Damiani et al. (2011) [5], the predominant mineral in araticum pulp is magnesium (350 mg/kg), followed by phosphorus (220 mg/kg). There are several factors that interfere with the mineral content of fruits, as confirmed in this study, such as variety, stage of maturation, soil type and conditions, fertilization, irrigation, and temperature [42]. Additionally, all fruits had low levels of lipids (≤0.28%) and proteins (≤4.60%).

The araticum, yellow mangosteen, and araçá-boi pulps had the highest contents of total phenolic compounds (258.04, 204.0, and 144.0 g EAG/100 g, respectively; $p \leq 0.05$), and were thus classified as having medium polyphenol content (100–500 mg EAG/100 g) whereas the other pulps were classified as having low polyphenol content (<100 mg EAG/100 g) according to classification proposed by Vasco, Ruales and Kamal-Eldin (2008) [43].

The pulp of araticum and araçá-boi had the highest total antioxidant activity values (6.27 and 6.09 µmol of Trolox/g, respectively; $p \leq 0.05$), higher values than those found by Garzón et al. (2012) [8] and Vinholes et al. (2017) [44] that reported total antioxidant activity for araçá pulp of 0.8 µmol and 3.34 µmol of Trolox/g, respectively, using the DPPH method. Souza et al. (2012) [10] reported higher values for antioxidant activity (131.58 µmol of Trolox/g) and total phenolic compounds (739.37 mg EAG/100 g) in araticum from Cerrado; additionally, their values for ascorbic acid and β-carotene levels were 59.05 mg/100 g and 0.57 mg/100 g, respectively. Antioxidant content in fruits is influenced by genetic factors, edaphoclimatic conditions, stage of development, and cultivation system [28].

The results obtained in the physical-chemical characterization of the pulps have driven us to deepen our research on the physiological and metabolic effects beneficial to the health of these Amazonian fruit pulps (Table S1). This is because, in addition to the functional potential, these fruit pulps could help in prevention and treatment of some diseases [45]. Figure 1 depicts the results of the experimental tests of pulp consumption and its physiological effects on the Wistar rats.

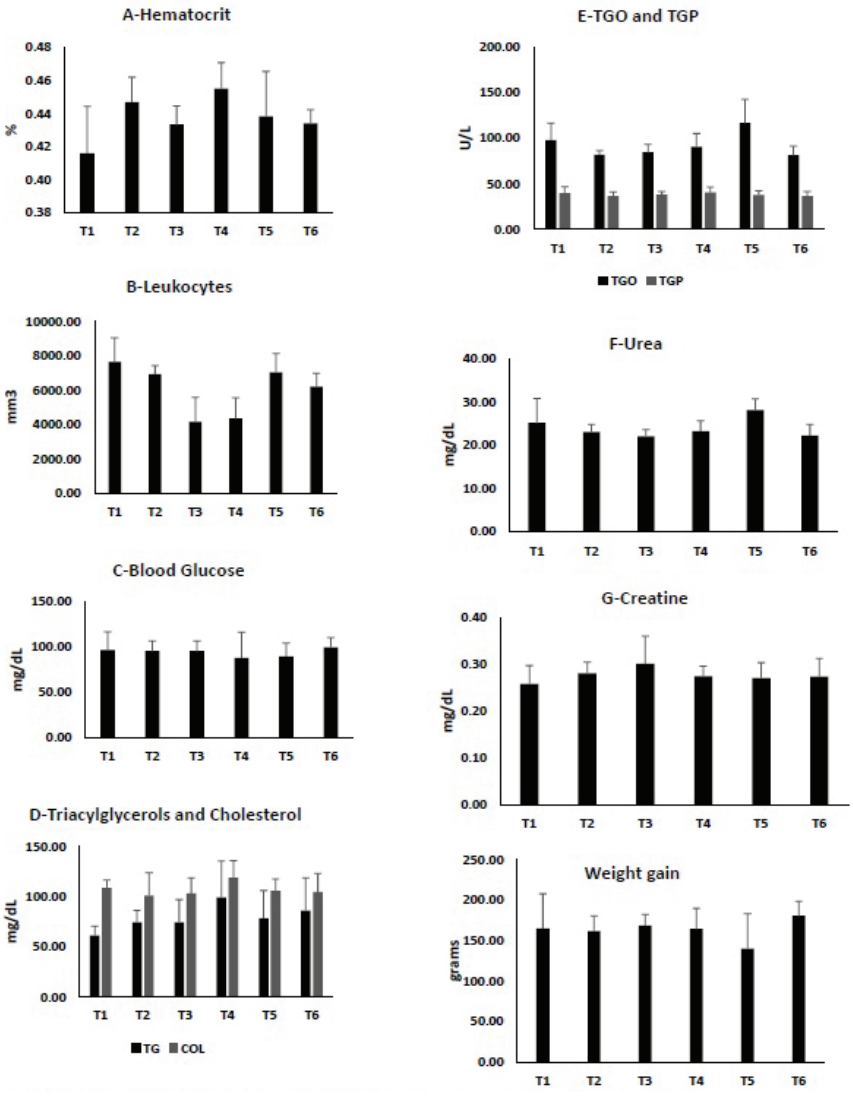

Figure 1. Biochemical results after Amazonian pulp fruits consumption in Wistar rats ($n = 10$ rats/group), T1—Control; T2—Araçá-Boi; T3—Abiu-grande; T4—Araticum; T5—Biri-biri; T6—Yellow mangosteen; TGO—glutamic-oxaloacetic transaminase; TGP—glutamic-pyruvic transaminase; TG—triacylglycerol and Col—Cholesterol. Mean values and standard deviation are presented.

Figure 1A shows that the consumption of araticum pulp (T4) marginally increased ($p \leq 0.10$) the hematocrit percentage (0.46%) in experimental groups relative to the control group (0.42%) of rats. It may be possible that the increase in hematocrit is due to the antioxidant activity of phenolic compounds found in fruits [46]. According to a study by Lage (2014) [47] the araticum pulp contain mainly flavonoids, such as O-glycosides quercetin and peltatoside, aporphine alkaloid, norstephalagine, and epicatechin, that are the compounds that protect normal cells from the toxic effect of highly reactive oxygen concentration generated during cellular metabolism [48,49]. Although a study conducted by Xu et al. (2017) [50] demonstrated that the ethanolic extract of *Garcinia xanthochymus* increased the activities of antioxidant enzymes (superoxide dismutase, catalase, and heme

oxygenase-1) and decreased reactive oxygen species in Pheochromacytoma (PC12) cells in rats, this result differs from that found in our study, where no significant increase in hematocrit was observed after the consumption of yellow mangosteen pulp.

With regard to total leukocytes (Figure 1B), which are cells that participate in the inflammatory process, the results suggest that the consumption of abiu and araticum pulps promoted a significant reduction relative to the control group (4166.67 mm^3, 4365.00 mm^3 and 7670.00 mm^3 respectively, $p \leq 0.05$). A previous study conducted by Rocha et al. (2016) [51] with ethanolic extract of araticum demonstrated that oral treatment with 300 mg/kg of extract significantly inhibited the formation of carrageenan-induced edema ($p \leq 0.05$) and decreased the number of leukocytes ($p \leq 0.05$) in the tested animals, indicating that the extract has chemopreventive and anti-inflammatory potential due to the presence of kaempferol. Similar results were reported by Meira et al. (2014) [52], where the administration of ethanolic extract of abiu leaves in rats had an effect against inflammatory pain and anti-hypersensitive action.

Fruit pulps generally contain compounds with functional potential, such as vitamins, carotenoids, flavonoids, steroids, and fibers (mainly soluble fiber and pectin) [15], that improve glycemic control and have been shown to be effective as adjuvants in diabetes treatment [53]. As shown in Figure 1C, the intake of fruit pulp did not cause changes in blood glucose in the tested rats as all animals had normal fasting blood glucose. However, the consumption of araticum (88.10 mg/dL) and biri-biri (89.83 mg/dL) pulps marginally reduced their blood glucose ($p \leq 0.10$).

Previous studies conducted by Anitha, Geetha, and Lakshmi (2011) [54] and Pushparaj, Tanb, and Tana (2001) [55] demonstrated that an ethanolic extract of biri-biri leaves had hypoglycemic activity in rats by decreasing glucose-6-phosphatase activity without affecting cytochrome P_{450}. The aqueous extracts of yellow mangosteen and abiu fruits, that was previously studied, contain bioflavonoids, including GB2a glucoside, GB2a, and fukugetinde [56]; additionally, according to Souza et al. (2012) [10], they strongly inhibit the activity of alpha-amylase and alpha-glucosidase. According to previous studies, the antihyperglycemic effect via the inhibition of alpha-glucosidase and carbohydrate metabolism enzymes of glycosylated quercetin derivatives, are found at high levels in araçá-boi pulp [57,58]. However, these results are not in accordance with our study, that observed no significant reduction in blood glucose ($p > 0.05$) after the consumption of abiu, yellow mangosteen, and araçá-boi pulps by rats.

The consumption of fruits and their phytochemical components, especially polyphenols and fibers is related to the reduction or control of blood cholesterol and triglycerides levels [59]. This statement is partially in agreement with our results (Figure 1D) that demonstrate that, with the exception of araticum pulp (T4), the consumption of Amazonian fruit pulps causes a marginal reduction in plasma cholesterol and a marginal increase in triglycerides in rats, although not significantly ($p \leq 0.10$). The increase in triglycerides may be associated with the fact that most fruits have high concentrations of simple sugars, in particular fructose [60], a fact that may have modulated the metabolism of rats that consume the pulps of Amazonian fruits. As already reported, a significant difference ($p \leq 0.05$) in triglycerides was observed between the control group (61.44 mg/dL) and the araticum pulp group (99.50 mg/dL). This could be due to the low ascorbic acid content in the fruit pulp (3.47 mg of ascorbic acid/100 g), in addition to the content of total sugars (5.6 g of glucose/100 g), parameters analyzed in our study, and mineral iron (0.43 g/100 g), a parameter analyzed in another study [61]. Despite the beneficial effects of minerals, high iron content can cause lipid peroxidation via the formation of free radicals and is thus a risk factor for atherosclerosis [62].

One way to assess liver function is to perform routine biochemical testing of the serum liver enzymes, such as cytoplasmic TGO and TGP, as well as serum levels of creatinine and urea to assess renal function [63,64]. As shown in Figure 1E, serum levels of TGP in the different experimental groups were not significantly different ($p > 0.05$) when compared to the control group of rats. Although previous study show low in vitro cytotoxicity of biri-biri methanolic extract [65], in our study the regular consumption of its pulp for 30 days

significantly ($p \leq 0.05$) increased serum TGO levels in rats (116.83 U/L), compared to the control group (98.00 U/L). It is noteworthy that three Wistar rats from the group that consumed biri-biri pulp died within the 30-day experiment. According to the literature, elevated levels of TGO and TGP induce an increase in bilirubins, in particular direct bilirubin, alkaline phosphatase, and gamma-glutamyl transferase, which indicate liver toxicity [66].

Creatinine is a catabolite of muscle metabolism of creatine-phosphate; it is primarily eliminated via glomerular filtration, with a small amount being excreted via tubular secretion. It has been used as a biomarker of chronic kidney disease and acute kidney injury [67]. No significant difference ($p > 0.05$) was observed in creatinine after the consumption of the fruit pulps in the experimental groups relative to the control group (Figure 1F).

Urea is a waste product excreted in the urine. However, it also plays an important role in human physiology by assisting the function of nephrons, which are microscopic structures capable of eliminating metabolic waste from the blood, maintaining the hydroelectrolytic and acid–base balance, controlling the amount of fluids, regulating blood pressure, secreting hormones, and producing urine [68]. Herein, the results of urea test (Figure 1G) demonstrated that only the experimental group that consumed biri-biri pulp had a marginal increase ($p \leq 0.10$) in urea levels (28.17 mg/dL). The result obtained in this study is similar to the findings of Bakul et al. (2013) [69]. Biri-biri has a high oxalic acid content and its consumption is linked to a high risk of developing acute renal failure due to deposition of calcium oxalate crystals in the renal tubules. Paschoalin et al., (2014) [70] reported in a case study, acute kidney injury associated with lumbar pain, hiccups, and diarrhea in a 50-year-old hypertensive patient with normal kidney function who consumed a large amount of biri-biri juice after fasting to treat hypertension. Thus, between the evaluated Amazonian fruit pulps by us, only the intake of biri-biri pulp showed risks of liver and kidney dysfunction in rats.

4. Conclusions

Among the Amazonian fruits studied, Araticum pulp had higher ash content, total phenolic compounds, and antioxidant activity than the other analyzed pulps. Moreover, araticum pulp consumption by rats marginally increased the hematocrit percentage ($p \leq 0.10$), promoted a significant reduction in total leukocytes ($p \leq 0.05$), and a marginal reduction on blood glucose ($p \leq 0.10$).

Abiu pulp had the highest levels of soluble solids, total and reducing sugars, and pH, and the lowest level of moisture. Furthermore, abiu consumption promoted a significant reduction ($p \leq 0.05$) in total leukocytes of rats.

Biri-biri pulp had the highest levels of ascorbic acid and total titratable acidity and a low pH and its consumption promoted a marginal reduction on blood glucose ($p \leq 0.10$). However, attention should be paid to its consumption due the risks of liver and kidney dysfunction observed.

Additionally, as all fruit pulps had low lipid and protein contents, the consumption of Amazonian fruit pulps caused a marginal reduction in plasma cholesterol, but a marginal increase in triglycerides in rats, although not significantly ($p \leq 0.10$). Exception should be made to Araticum consumption, where the increase in triglycerides was significant ($p \leq 0.05$).

These results generate knowledge mainly with regard to the chemical and nutritional composition and effects of the intake of native/cultivated fruits in the Amazon biome. However, more researches are needed to generate more knowledge about these promising Amazonian fruits so as to promote the native fruit production, in addition to promoting health in the population and sustainability in the Amazon region.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13051484/s1, Table S1. Biochemical results after Amazonian pulp fruits consumption in Wistar rats (n = 10 rats/group).

Author Contributions: Physicochemical analysis, F.R.F.S. and L.B.V.; biological assays, F.R.F.S. and B.K.B.; statistical analysis, B.K.B. and L.C.G.; supervision, N.S.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and were approved by the Ethics Committee in the Use of Animals (CEUA) of the Federal University of Rondônia Foundation—UNIR (Protocol number: PP 020/2014).

Informed Consent Statement: Not applicable.

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Acetylcholine and Royal Jelly Fatty Acid Combinations as Potential Dry Eye Treatment Components in Mice

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Abstract: Dry eye is a multifactorial disease characterized by ocular discomfort and visual impairment. Our previous studies have shown that royal jelly (RJ) has restored the capacity for tear secretion by modulating muscarinic calcium signaling. RJ contains acetylcholine, which is a major cholinergic neurotransmitter, and a unique set of fatty acids with C 8 to 12 chains, which are expected to be associated with health benefits. The purpose of the present study was to investigate the active components involved in tear secretion capacity, focusing on acetylcholine and fatty acids in RJ. Using the stress-induced dry-eye model mice, it was confirmed that acetylcholine with three fatty acids (10-hydroxydecanoic acid, 8-hydroxyoctanoic acid, and (*R*)-3,10-dihydroxydecanoic acid) was essential for tear secretion. In ex vivo Ca^{2+} imaging, these three fatty acids suppressed the decrease in intracellular modulation of Ca^{2+} in the lacrimal gland by acetylcholine when treated with acetylcholinesterase, indicating that the specific type of RJ fatty acids contributed to the stability of acetylcholine. To our knowledge, this study is the first to confirm that a specific compound combination is important for the pharmacological activities of RJ. Our results elucidate the active molecules and efficacy mechanisms of RJ.

Keywords: royal jelly; acetylcholine; fatty acid; ophthalmology; dry eye

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1. Introduction

Royal jelly (RJ) is a yellowish white substance, a cream secreted by the cephalic glands of honey bees (*Apis mellifera* L.) and used as nutrition for young larvae and queen bees. RJ is known to induce epigenetic development in the queen bee and is considered to be involved in many advantages for the queen bee, including enabling a large size (double that of worker bees), fertility, and a long lifespan (about 5–6 years, compared to worker bees with a lifespan of 35–40 days) [1]. RJ has a multitude of pharmacological activities reported in clinical trials, including antihypertensive effects [2]; the alleviation of chills [3], neck muscle strain [4], and female menopausal symptoms [5]; reduction of blood sugar in patients with type 2 diabetes [6]; prevention of age-associated muscle strength decline [7]; and maintenance of skin moisture [8]. RJ consists of water (60–70%), proteins (9–18%), sugar (7.5%), lipids (3–8%), and other trace compounds. (*E*)-10-Hydroxy-2-decenoic acid (10H2DA) and 10-hydroxydecanoic acid (10HDAA) are known to be major fatty acids in RJ and represent 60–80% of RJ lipids [9]. These fatty acids have been reported to activate TRPA1 channels expressed in HEK293 cells [10], induce estrogen receptor β recruitment to the promoter in MCF-7 cells [11], express GLUT4 in skeletal muscle by activating AMPK signaling in vivo [12], and enhance filaggrin production in a human three-dimensional epidermis model [13]. Moreover, the composition of RJ fatty acids has been characterized

as a set of C8, C10, and C12 fatty acids [14]. In the case of minor fatty acids, several pharmacological activities have been reported, such as anticancer [15], antimicrobial [16], and anti-inflammatory [17] activities. Therefore, RJ fatty acids are expected to play an important role in the pharmacological activities of RJ reported in clinical trials.

Dry eye is a multifactorial disease characterized by an unstable tear film that leads to a variety of symptoms and potentially even visual impairment and ocular surface damage [18]. The incidence of dry eye has increased because of the use of air conditioning and digital devices, and the prevalence of dry-eye disease has been reported to range from 5 to 50% [19]. Current treatments are generally the instillation of artificial tears that can provide temporary relief, but the effect of topical treatments is not persistent because it does not solve the underlying causes [20].

Previously, our research group conducted a double-blind, placebo-controlled trial to investigate the effects of RJ on dry eye signs and symptoms in human patients. In this study, we found that supplementation with oral RJ for 8 weeks can improve tear secretion, as assessed by the Schirmer score, in patients with dry-eye symptoms [21]. In addition, we investigated the underlying lacrimal mechanisms in relation to the results of this study using a rodent model of dry eye. A rat blink-suppressed dry-eye model, simulating the effect on dry eye in patients whose etiology is associated with excessive staring at a computer display, which corresponds to a human trial, has been investigated. It has been assumed that these effects occur alongside an increase in ATP, mitochondrial function, and phosphorylation of AMPK by modulating the muscarinic calcium-signaling pathway stimulated by RJ, reflecting the restoration of the energy state of the lacrimal gland (LG) [22]. Given that acetylcholine (ACh), a major cholinergic neurotransmitter involved in tear secretion, has been shown to be 1 mg/g in RJ [23], ACh may be a potential RJ component for restoring tear secretion. In the present study, in order to investigate a potential RJ component for the treatment of dry eye, we evaluated the effect of RJ components on tear secretion capacity using a rodent dry-eye model focused on RJ constitutive fatty acids and ACh.

2. Materials and Methods

2.1. Materials and Chemicals

Enzyme-treated RJ powder (ETRJ), which degrades RJ proteins to peptides and amino acids by proteases to reduce the immunoreactivity and allergenicity [24], was obtained from Yamada Bee Company, Inc. (Okayama, Japan), and standardized to contain a minimum of 3.5% 10H2DA and 0.6% 10HDAA. Table 1 shows the content of ETRJ compounds and the source or reference of each compound used in this study. The synthetic method and analytical data are provided in Supporting Information (Figures S1 and S2).

Table 1. The composition of the enzyme-treated royal jelly (ETRJ) and source or reference of the compounds used in this study.

Compounds	(%) [1]	Oral Administration Dosage (mg/kg) [2]	Source or Reference
acetylcholine (ACh)	0.023	0.069	Tokyo Chemical (Tokyo, Japan)
8-hydroxyoctanoic acid (8HOA)	0.312	0.936	Sigma-Aldrich (St. Louis, MO, USA)
(*R*)-3,10-dihydroxydecanoic acid (3,10DDA)	0.363	1.089	Isolation from RJ according to the previous report (Noda et al. [25]).
10-hydroxydecanoic acid (10HDAA)	1.347	4.041	Combi-Blocks (San Diego, CA, USA)
(*E*)-9,10-dihydroxy-2-decenoic acid (9,10D2DA)	0.001	0.003	Prepared by the mixing of synthetic (*R*) and (*S*)-acids in a ratio of $R/S = 3.5/1$ (Tani et al. [26])

Table 1. *Cont.*

Compounds	(%) [1]	Oral Administration Dosage (mg/kg) [2]	Source or Reference
(*E*)-10-hydroxy-2-decenoic acid (10H2DA)	4.267	12.801	Hangzhou Eastbiopharm (Hangzhou, China)
(*E*)-2-decenedioic acid (2DA)	0.435	1.305	Sundia MediTech (Shanghai, China)
sebacic acid (SA)	0.279	0.837	Sigma-Aldrich (St. Louis, MO, USA)
(*E*,*R*)-11,12-dihydroxy-2-dodecenoic acid (11,12D2DA)	0.001	0.003	Prepared according to the procedures described in the patent [27] and Supporting Information (Scheme S1). Analytical data are provided in the Supporting Information (Figures S1 and S2).
12-hydroxydodecanoic acid (12HDA)	0.049	0.147	MP Biomedicals (Santa Ana, CA, USA)

[1] ACh, 9,10D2DA, and 11,12D2DA content in ETRJ were analyzed by LC/MS/MS, and the other fatty acid content in ETRJ was based on data from a previous report [28]. [2] Equivalent to the content in 300 mg/kg ETRJ.

2.2. Animals

All animal experiments in this study were approved by the Ethics Committee for Animal Research at the Keio University School of Medicine (approval no. 11008). All mice were treated according to the Association of Research and Vision in Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic Vision Research.

Female C57BL/6 mice (Charles River, Yokohama, Japan) and yellow cameleon-Nano15 (YCNano15) transgenic mice were used in this study (8 weeks old, 20–23 g). YCNano15 is a mouse line expressing the YCNano15 Ca^{2+} sensor probe under the control of the CAG promoter [29]. They were quarantined and acclimatized for 1 week prior to the experiments under the following general conditions: room temperature of 23 ± 2 °C, relative humidity of 60 ± 10%, alternating 12 h light-dark cycle (8 a.m.–8 p.m.), water, and food ad libitum.

2.3. Stress-Induced Dry-Eye Model in Mice

We used a mouse stress-induced dry-eye model that simulates evaporative dry eye in patients whose etiology is associated with excessive staring at a computer display [30]. In brief, the mice were restrained in a 50 mL plastic conical tube and treated with a flow of air directed toward their heads at a rate of 0.5–1.0 m/s for 4 h. They were placed individually in cages, with water and food available ad libitum for the remaining time. ETRJ was suspended in distilled water, and a dose of 300 mg/kg was administered orally using a feeding needle (20 gauge) before stress exposure. To determine the active component of RJ, oral administration of ACh and each fatty acid corresponding to the content of 300 mg/kg ETRJ (Table 1) was performed individually or in combination with distilled water. Distilled water was used as a vehicle control. Six mice were used in each experiment.

2.4. Post-Ganglionic Denervation of the Lacrimal Gland (PGD)

PGD was performed as described previously [31]. In brief, the mice were placed in a prone position, and the skin on the temporal side of the head was incised under deep anesthesia using a combination of medetomidine (0.75 mg/kg), butorphanol (5 mg/kg), and midazolam (4 mg/kg). The post-ganglionic nerve bundle was detached from the blood vessels at the caudal root site of the ventral surface of the LG and denervated under a stereomicroscope. PGD was performed unilaterally. They were placed individually in cages, with water and food available ad libitum during the experimental period. Three hundred mg/kg ETRJ, ACh, three selected fatty acids (10HDAA, 8HOA, and 3,10DDA) mixture, and a combination of ACh with a mixture of three fatty acids were dissolved in distilled water and administered orally using a feeding needle (20 gauge). Distilled water was used as a vehicle control. The dose of ACh and each of the three selected fatty acids corresponded to the content of 300 mg/kg ETRJ. Each solution was administered orally

twice daily after PGD surgery. In the sham group, distilled water was administered orally twice daily. Five mice were used in each experiment.

2.5. Measurement of Tear Secretion in Mice

Tear secretion was measured using a modified phenol red thread test with a phenol red thread (Zone-Quick; Showa Yakuhin Kako, Tokyo, Japan) [30]. It was placed on the temporal side of the conjunctiva between the limbus and outer canthus for 15 s. The length of the moistened area at the edge was measured to be within 0.5 mm. For the stress-induced dry-eye model in mice, tear secretion was measured before and 1 d after stress exposure. The average values of both the left and right eyes were used for the analysis. The percentage of tear secretion value at 1 d after stress exposure relative to that before stress exposure was calculated. For PGD mice, tear secretion was measured before and 1, 2, 3, 5, and 7 days after PGD in the PDG-treated unilateral eye. The values of the PGD-treated eyes were used in the analysis.

2.6. Histopathological Analysis

PGD mice were euthanized with an overdose of sodium pentobarbital 7 days after PGD surgery and their LGs were dissected. The entire LG was fixed in 10% formalin solution and embedded in paraffin. Sections (5 mm thick) were obtained from the middle of the horizontal dissection of the LG. The sections were then stained with hematoxylin and eosin. The images were captured using a BIOREVO BZ-9000 (Keyence, Osaka, Japan) optical microscope. To quantify acinar cell size, the size of 20 acinar cells was measured in three randomly selected areas in each section using the BZ-Analyzer 2.1 software (Keyence, Osaka, Japan).

2.7. Acetylcholinesterase Treatment

ETRJ (500 mg), the corresponding amounts of acetylcholine, and three selected fatty acids (10HDAA, 8HOA, and 3,10DDA) were suspended in 10 mL of saline solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM dextrose [pH7.4]). These solutions were incubated with 0.08 U/mL of acetylcholinesterase (AChE) at 37 °C for 30 min. After incubation, rivastigmine, an AChE inhibitor, was added at a final concentration of 100 μM to stop the reaction. These mixtures, before and after AChE treatment, were evaluated by intracellular Ca^{2+} imaging, and the acetylcholine in these samples was quantified by LC/MS/MS.

2.8. Ex Vivo Ca^{2+} Imaging in the Lacrimal Gland

YCNano15 transgenic mice were euthanized with an overdose of sodium pentobarbital. The LGs dissected from YCNano15 mice were transferred to round coverslips that were mounted on the bath region of a perfusion chamber and continuously perfused with a saline solution through polyethylene tubes connected to a Masterflex peristaltic pump (Cole-Parmer, Chicago, IL, USA) at a flow rate of 0.8 mL per minutes. The mixture of ETRJ (500 μg/mL), ACh (0.115 μg/mL), and the three fatty acids (10HDAA: 6.735 μg/mL, 8HOA: 1.560 μg/mL, and 3,10DDA: 1.815 μg/mL) was diluted to the desired concentration with a saline solution before use. Each stimulant that reacted with and without AChE was applied to the LG for 1 min at 5 min intervals.

Ca^{2+} imaging was performed in accordance with previous reports [29]. Briefly, Ca^{2+} mobilization was observed with a two-photon microscope equipped with a 25× water-immersion objective lens by measuring the change in the fluorescence resonance energy transfer (FRET) ratio, which was calculated as the ratio of YFP to CFP fluorescence intensity. An excitation wavelength of 830 nm was used for FRET imaging. Two-photon excited fluorescence images of CFP and YFP were acquired in separate channels through dichroic mirrors and emission filters, namely BP460-500 nm and BP520-560 nm, respectively. The fluorescence images were acquired at approximately 1 frame/s for 13 min at 2 μm depth-intervals from the LG surface at a maximum depth of 10 μm. Fluorescence images were

reconstructed using Imaris software and analyzed using MATLAB software (MathWorks, Natick, MA, USA).

2.9. Analysis of Acetylcholine and RJ Fatty Acids by LC/MS/MS

LC/MS/MS was performed on a UPLC system (Ultimate 3000, Thermo Scientific, Waltham, MA, USA) with an MS orbitrap system (Q-Exactive Focus, Thermo Scientific).

ACh contained in ETRJ was extracted with 20% methanol and diluted to 100 μg/mL. Acetylcholine was analyzed using a normal phase column (Atlantis Hilic Silica, 2.1 × 50 mm, 3 μm i.d., Waters, Milford, MA, USA) at 40 °C. The mobile phase consisting of water with 0.1% formic acid (A) and acetonitrile (B) was pumped at a flow rate of 0.3 mL/min. The gradient system was as follows: 95% B (0–2 min), 95–20% B (2–8 min), 20% B (8–12 min), and 95% B (12–20 min). The typical injection volume was 3 μL. RJ fatty acid analysis was performed according to a previous report [28]. Briefly, RJ fatty acids contained in ETRJ were extracted with 100% methanol and diluted to 10 or 100 μg/mL. The sample was injected onto a reversed-phase column (Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm i.d., Waters) and eluted using a gradient with solvent A, 0.01% acetic acid and solvent B, acetonitrile at a flow rate of 0.3 mL/min at 40 °C. The gradient system was as follows: 5% B (0–2 min), 5–100% B (2–17 min), 100% B (17–20 min), and 5% B (20–25 min). MS/MS was performed in positive and negative modes with full mass monitoring (range m/z 70–1000). MS conditions were the same as those described in our previous report [28]. The validation data of the analysis methods are provided in the Supporting Information (Table S1).

2.10. Statistical Analysis

All results are presented as mean ± SEM, and statistical analyses were performed using JMP12 software (version 12.2; SAS Institute, Cary, NC, USA). Comparisons between the two groups were performed using an F-test, followed by a *t*-test for parametric variables and a U-test for nonparametric variables. Multiple comparisons were performed using a one-way analysis of variance, followed by the Tukey-Kramer or Dunnett test. Differences between the measurement variables were considered significant if the resulting *p*-value was ≤ 0.05.

3. Results

3.1. ACh and RJ Fatty Acids Are Necessary Components of Dry-Eye Suppression in Royal Jelly

Initially, we orally administered ACh or a mixture of nine RJ fatty acids (nine RJ FAs) in a stress-induced dry-eye mouse model. The effects of ETRJ, ACh, and nine RJ FAs are shown in Figure 1A. ETRJ and significantly preserved tear secretion ($p < 0.001$ vs. vehicle), and ACh and nine RJ FAs did not affect the decreased tear secretion compared to the vehicle.

We then evaluated the combined effects of ACh and nine RJ FAs or each fatty acid. The ameliorative effect of tear secretion was not observed in ACh treated with RJ fatty acids. Interestingly, significant restoration of tear secretion was observed in ACh with nine RJ FAs compared to the vehicle (Figure 1B). Typical changes in the tear secretory patterns are shown in Figure 1C. These results show that ACh with two or more RJ fatty acids is essential to restore decreased tear secretion. To select a suitable combination of RJ fatty acids, nine RJ FAs were grouped into saturated C8-10 chain fatty acids (SC8-10 FAs; 10HDAA, 8HOA, 3,10DDA, and SA), and the other fatty acids (10H2DA, 2DA, 9,10D2DA, 11,12D2DA, and 12HDA). The mixture of ACh with SC8-10 FAs restored the reduction in tear secretion to the same level as that of 9 RJ FAs. In contrast, the mixture of other FAs did not restore reduced tear secretion (Figure 2A).

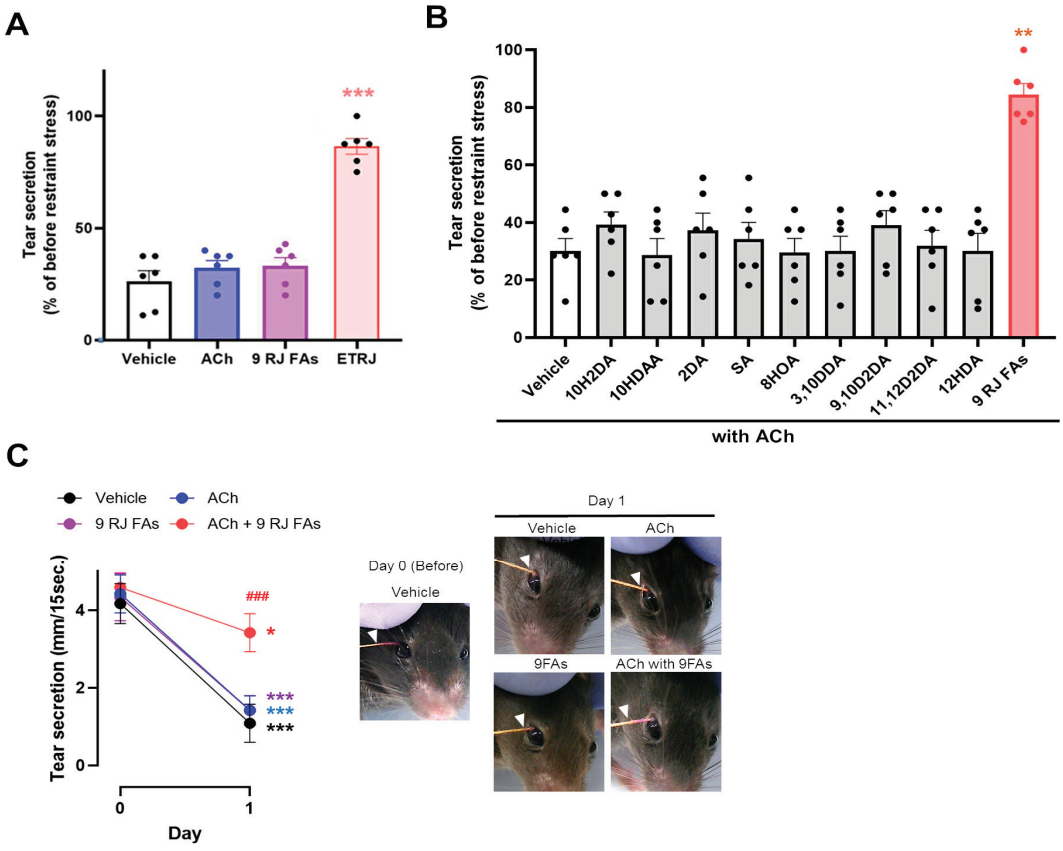
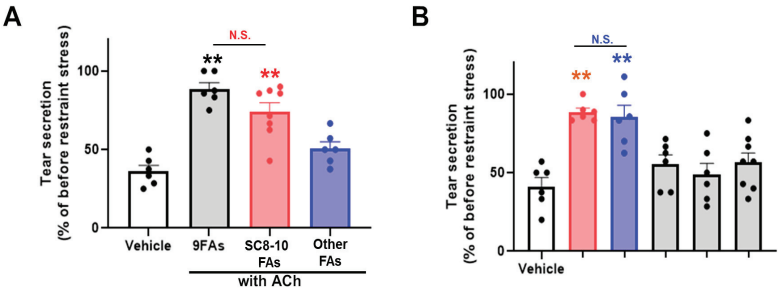


Figure 1. ACh with two or more RJ fatty acids were essential to restore decreased tear secretion. (**A**) Effect of ACh and 9 RJ fatty acids (9 RJ FAs). (**B**) Effect of a combination of ACh with RJ fatty acids on tear secretion. (**C**) Change in measured value in tear secretion (**left**) and representative photographs measured with a cotton thread (**right**). The value was calculated as a percentage of tear secretion at 1 day after restraint stress exposure compared to before restraint stress. (**A**,**B**) All data represent the mean ± SEM, $n = 6$ mice eyes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle (**A**,**B**) or day 0, ### $p < 0.001$ versus vehicle (**C**).

Next, to identify potent combinations of fatty acids, four SC8-10 FAs were grouped into five mixtures composed of two or three SC8-10 FAs (Figure 2B). The ameliorating effects of ACh with these SC8-10 FA mixtures on the reduction of tear secretion were compared. For ACh either with 10HDAA/8HOA, 8HOA/3,10DDA, or 10HDAA/3,10DDA, the reduction in tear secretion was at the same level as that of the vehicle. For ACh with a mixture of 10HDAA, 8HOA, and 3,10DDA (10HDAA/8HOA/3,10DDA), tear secretion was restored to the same level as ACh with a mixture of four SC8-10 FAs (Figure 2B). These results suggest that ACh with 10HDAA/8HOA/3,10DDA is an essential component for the anti-dry-eye effect of RJ.



Ach		-	+	+	+
SC8-10 FAs	10HDAA	-	+	+	-
	SA	-	+	+	-
	8HOA	-	+	+	-
	3,10DDA	-	+	+	-
Other FAs	10H2DA	-	+	-	+
	2DA	-	+	-	+
	9,10D2DA	-	+	-	+
	11,12D2DA	-	+	-	+
	12HDA	-	+	-	+

Ach		-	+	+	+	+	+
SC8-10 FAs	10HDAA	-	+	+	-	+	+
	SA	-	+	-	-	-	-
	8HOA	-	+	+	+	-	+
	3,10DDA	-	+	+	+	+	-

Figure 2. ACh with 10HDAA/8HOA/3,10DDA were the essential components for the anti-dry-eye effect of RJ. (**A**) Effect of saturated C8-10 chains fatty acids (SC8-10 FAs) and the other fatty acids (other FAs) on tear secretion (upper). (**B**) Effect of SC8-10 FAs on tear secretion. All data represent the mean ± SEM, $n = 6$ mice eyes. ** $p < 0.01$ versus vehicle. N.S. indicates no significant difference.

3.2. ACh with 10HDAA/8HOA/3,10DDA Preserved Tear Secretion Capacity and LG Morphology in the LG Post-Ganglionic Denervation Dry-Eye Model

Our previous study demonstrated that the oral administration of RJ restores tear secretion and LG structure when neuronal LG stimuli are interrupted [21]. These findings indicate that direct effects on LGs are a potential mechanism of RJ supplementation in patients with dry eye. To further confirm the underlying mechanism of ACh with 10HDAA/8HOA/3,10DDA on the restoration of tear secretion similar to that of RJ, we compared changes in body weight, tear secretion, and LG pathology between six groups: (1) sham, (2) vehicle, (3) ETRJ, (4) ACh, (5) 10HDAA/8HOA/3,10DDA, and (6) ACh with 10HDAA/8HOA/3,10DDA. The evaluation was performed when neuronal stimuli from the autonomic central nervous system to the LG were interrupted in the PGD dry-eye model.

There was no change in body weight between the groups (Figure 3A). In the vehicle group, tear secretion significantly decreased immediately after denervation, and this reduction was sustained until day 7. In the ETRJ group, significant preservation of tear secretion was observed compared to that in the vehicle group during the experimental period. The values were approximately 80% before PGD. In the ACh and 10HDAA/8HOA/3,10DDA group, tear secretion was at the same level as that in the vehicle group. For the ACh with 10HDAA/8HOA/3,10DDA group, significant preservation of tear secretion was observed compared to the vehicle group. The values and patterns were similar to those in the ETRJ group. The sham eye did not affect tear secretion (Figure 3B). Since decreased tear secretion and LG atrophy were characteristic changes in this dry eye model, gross pathological and histological changes were evaluated. Typical changes among these groups are shown in Figure 3C on the left. PGD-induced LG atrophy, reduced organ size (Figure 3C, upper panels), and acinar cell area (Figure 3C, lower panels), were significantly suppressed in the ETRJ and ACh with 10HDAA/8HOA/3,10DDA groups (Figure 3C, right).

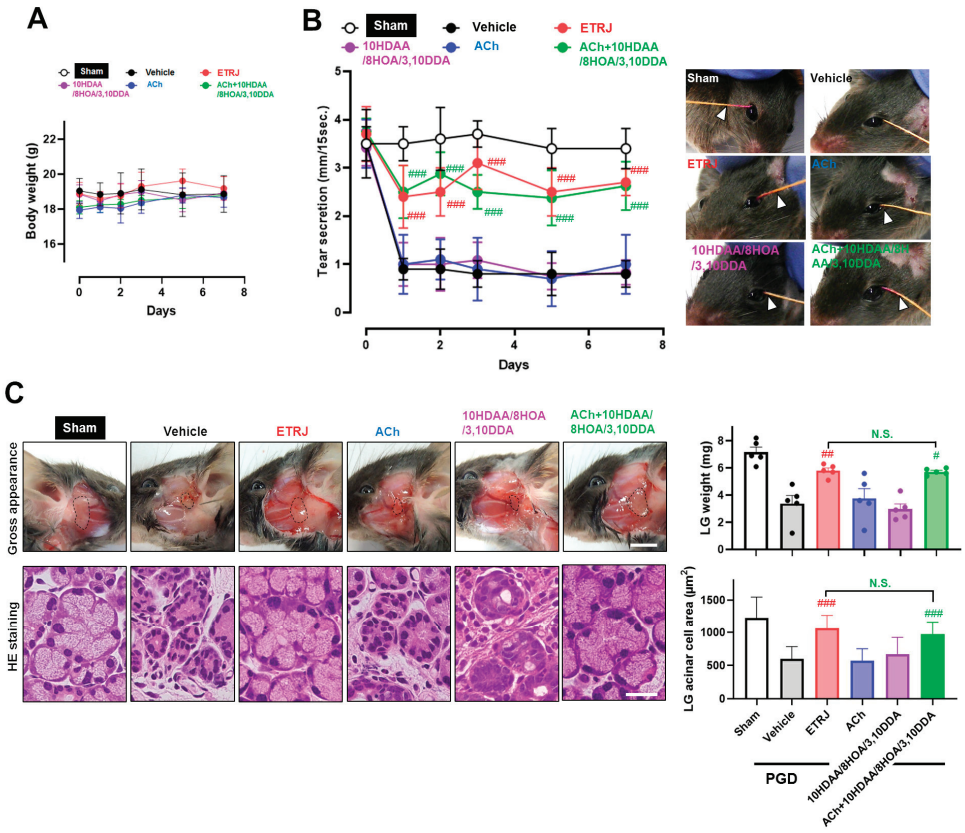


Figure 3. The mechanism of tear secretion restoration by ACh with 10HDAA/8HOA/3,10DDA is similar to that of RJ. Changes in (**A**) body weight and (**B**) tear secretion. The photographs on the right side show representative tear secretion patterns measured with a cotton thread 7 days after post-ganglionic denervation (PGD) surgery. (**B**) The arrow indicates the wetted length due to tear secretion. (**C**) Gross (upper) and histological (lower) changes in the LG. The upper and lower right bar chart show the LG weight and LG acinar cell area, respectively. The scale bar is 5 mm (upper) and 20 μm (lower). All data represent the mean ± SEM, $n = 5$ mice. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus vehicle. N.S. indicates the no significant difference between ETRJ and ACh + 10HDAA/8HOA/3,10DDA.

3.3. 10HDAA/8HOA/3,10DDA Suppressed the Decrease of ACh-Modulated [Ca^{2+}]i in the LG by Acetylcholinesterase Treatment

ACh is rapidly hydrolyzed and diminishes the physiological activity of acetylcholinesterase (AChE) at brain cholinergic synapses and neuromuscular junctions [32]. To investigate whether 10HDAA/8HOA/3,10DDA preserves the activity of ACh from AChE decomposition, we evaluated the effect of 10HDAA/8HOA/3,10DDA on Ach-modulated LG [Ca^{2+}]i changes. Modulation of LG [Ca^{2+}]i by ACh was diminished after treatment with AChE (Figure 4A). Modulation of LG [Ca^{2+}]i by ETRJ (Figure 4B) and ACh with 10HDAA/8HOA/3,10DDA were preserved after AChE treatment (Figure 4C). Significant preservation of LG [Ca^{2+}]i changes was observed in ACh with 10HDAA/8HOA/3,10DDA compared to ACh. The LG [Ca^{2+}]i changes were preserved at the same level as those induced by ETRJ (Figure 4D). Corresponding to the response to LG [Ca^{2+}]i changes, the analytical quantification of ACh by LCMS showed that 10HDAA/8HOA/3,10DDA preserved the amount of ACh from AChE (Figure 4E).

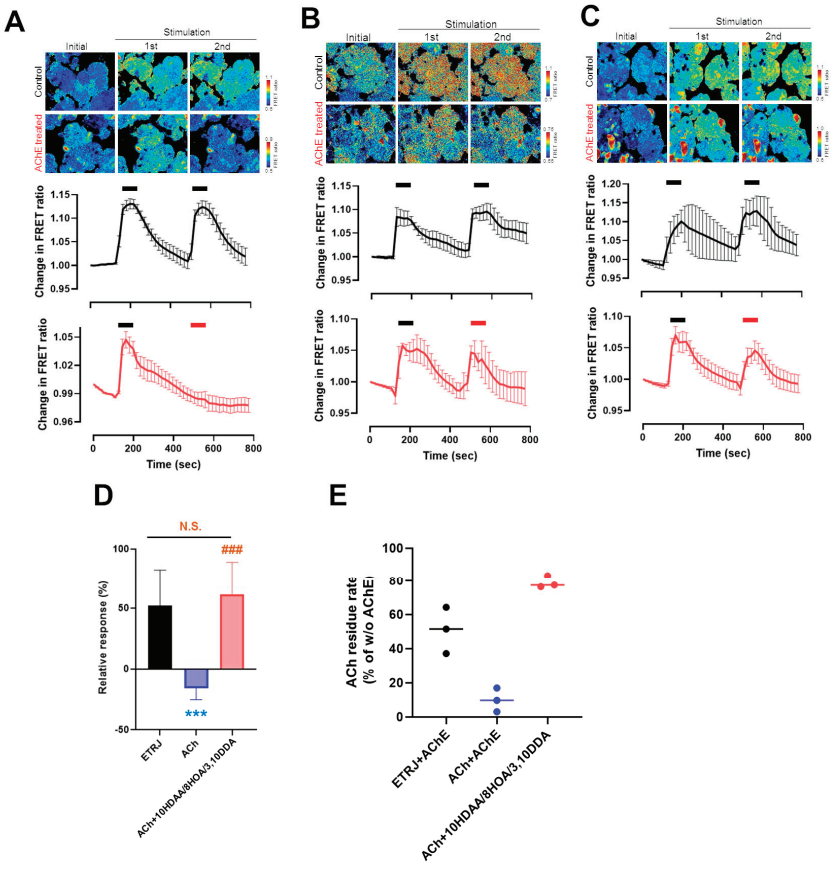


Figure 4. 10HDAA/8HOA/3,10DDA suppressed the decrease of Ach-modulated LG $[Ca^{2+}]$i by ACh esterase. $[Ca^{2+}]$i increase in LG acinar cells exposed to (**A**) ACh, (**B**) ETRJ, and (**C**) ACh + 10HDAA/8HOA/3,10DDA treated with or without ACh esterase (AChE). The upper panels show pseudo-colored images of $[Ca^{2+}]$i. The lower trace shows the $[Ca^{2+}]$i response to each stimulus. The black and red bar over the trace indicates when each stimulus reacted without and with AChE, respectively. (**D**) Summarized data on amplitudes of $[Ca^{2+}]$i responses. Relative responses were calculated as a percentage of stimulus reacted with the AChE induced $[Ca^{2+}]$i response relative to the stimulation induced without AChE. (**E**) Analytical quantification of ACh. All data represent the mean ± SEM, n = 4–10 LGs (**A**–**D**) and n = 3 (**E**). *** $p < 0.001$ versus ETRJ, ### $p < 0.001$ versus ACh. N.S. indicates no significant difference between ETRJ and ACh with 10HDAA/8HOA/3,10DDA (**D**).

4. Discussion

In the present study, we found that three RJ fatty acids consisting of C8-10 chains, 10HDAA/8HOA/3,10DDA, in combination with ACh, are the RJ components that may have potential application in the treatment of dry eye.

ACh is an important neurotransmitter that is synthesized in preganglionic neurons in both sympathetic and parasympathetic autonomic ganglia. ACh is released at cholinergic synaptic sites in response to neural excitability. The released ACh is rapidly hydrolyzed by AChE and decreases physiological activity at brain cholinergic synapses and neuromuscular junctions. Furthermore, it has been reported that AChE and butyrylcholinesterase were expressed in the blood and intestine epithelial cells [33,34]. Therefore, it is assumed that orally administered ACh is mostly degenerated in the process of absorption and blood transport before it reaches the ACh receptor expressed in brain cholinergic synapses and autonomously innervated organs, such as smooth muscles of blood vessels and intestines, pancreas, and LGs. Nevertheless, it has been reported that orally administered RJ has

ACh-like effects, such as improvement of Alzheimer's disease [35], vasodilation induced by nitric oxide production [36], and induction of insulin secretion [37]. Moreover, our previous report showed that RJ had a tear secretion capability via muscarinic acetylcholine receptor (mAChR) signaling induced by intracellular Ca^{2+} increase in LGs, which was suppressed by the mAChR antagonist atropine and by inhibitors of phospholipase C and ER-Ca^{2+}-ATPase essential enzyme pathways [23]. These reports and our results (Figures 3 and 4) indicate that ACh in RJ retains its activity even after oral administration of RJ, possibly due to the effect of RJ fatty acids, preventing ACh degradation by AChE and transporting ACh to targeted sites efficiently. Additionally, decanoic acid (capric acid) has been reported to interact with mAChR agonists in the ileum and jejunum [38]. Thus, 10HDAA, 8HOA, and 3,10DDA, which are saturated medium-chain fatty acids similar to capric acid, could interact with mAChR. In the nicotinic ACh receptor (nAChR), lipids have been reported to be potent modulators that influence receptor function both by conformational selection and by kinetic mechanisms via membrane stabilization [39]. In addition, the binding affinity of mAChR and its substrates has been reported to change depending on the membrane lipid composition [40]. 10HDAA, 8HOA, and 3,10DDA could play the role of mAChR modulators to stabilize lipid membranes and/or binding to allosteric sites. Further studies are needed to calculate the molecular modeling of the mAChR-fatty acid interactions to clarify the mechanism of RJ tear secretion capacity.

5. Conclusions

For the first time, we identified RJ components for the treatment of dry eye. Our results suggest that the combination of ACh with three saturated fatty acids, namely 10HDAA, 8HOA, and 3,10DDA, which have a terminal hydroxyl group, was critical for the therapeutic effect of RJ in dry eye. The restoration of Ach content using these three fatty acids may function by inhibition of AChE activity as a possible mechanism.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13082536/s1. Scheme S1: Synthesis of (*E*, *R*)-11, 12-dihydroxy-2-dodecenoic acid. Figure S1: ^{1}H NMR spectrum of (*E*, *R*)-11, 12-dihydroxy-2-dodecenoic acid. Figure S2: ^{13}C NMR spectrum of (*E*, *R*)-11, 12-dihydroxy-2-dodecenoic acid. Table S1: Validation data of the analytical methods for acetylcholine and RJ fatty acids.

Author Contributions: Conceptualization, M.Y. and T.I.; methodology, M.Y. and T.I.; validation, T.I. and S.N.; formal analysis, T.I.; investigation, M.Y. and T.I.; data curation, T.I.; writing—original draft preparation, M.Y. and T.I.; writing—review and editing, H.T., A.Y., and S.N.; visualization, T.I.; supervision, S.N. and K.T.; project administration, S.N. and K.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Ethics Committee for Animal Research at the Keio University School of Medicine (Approval No. 11008). All mice were treated according to the Association of Research and Vision in Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic Vision Research.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: Kazuo Tsubota is the CEO of Tsubota Laboratory, Inc., Tokyo, Japan, a company developing treatment, prevention, and medical devices for dry eyes. The remaining authors declare

that there are no conflicts of interest that could affect the impartiality of the research reported. M.Y., H.T., and A.Y. are employees of the Yamada Bee Company, Inc.

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Anti-Fatigue and Exercise Performance Improvement Effect of *Glossogyne tenuifolia* Extract in Mice

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Abstract: *Glossogyne tenuifolia* (GT) is a native perennial plant growing across the coastline areas in Taiwan. The current study aimed to examine the efficacy of GT extract in ameliorating physical fatigue during exercise and increasing exercise performance. Fifty male Institute of Cancer Research (ICR) mice were randomly segregated into five groups (n = 10) to GT extract orally for 4 weeks, at different concentrations (50, 100, 250, and 500 mg/kg BW/day): LGT 1X, MGT 2X, HGT 5X, and HGT 10X groups. Forelimb grip strength, endurance swimming time, serum biochemical marker levels, blood lipid profile and histological analysis of various organs were performed to assess the anti-fatigue effect and exercise performance of GT extract. The forelimb-grips strength and endurance-swimming time of GT-administered mice were increased significantly in a dose-dependent manner when compared to the control. Serum glucose, creatine kinase, and lactate levels were increased significantly in the HGT 10X group. Liver marker serum glutamic-oxaloacetic transaminase (GOT) was increased in the HGT 5X and HGT 10X groups, whereas Serum Glutamic Pyruvic Transaminase (GPT) was not altered. Renal markers, creatinine and uric acid levels, were not altered. Muscle and hepatic glycogen levels, which are essential for energy sources during exercise, were also significantly increased in a dose-dependent manner in all GT extract groups. No visible histological aberrations were observed in the vital organs after GT extract administration. The supplementation with GT extract could have beneficial effects on exercise performance and anti-fatigue function without toxicity at a higher dose.

Keywords: *Glossogyne tenuifolia*; exercise; forelimb grip strength; lactate; ammonia; creatine kinase

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1. Introduction

Fatigue is defined as the inability to maintain power output and strength, impairing physical performance. Redundant physical load, insufficient rest, and mental stress/pressure are the causative factors of physiological fatigue, which is further divided into central and peripheral fatigue [1]. Factors affecting the brain, spinal cord, and motor neurons in the central nervous system could result in central fatigue, whereas peripheral fatigue results from muscle weakness induced by changes at the neuromuscular junction [2]. A decline in functional performance could also be associated with physiological fatigue [3]. According to the exhaustion theory, a decrease in energy sources, such as glucose, and accumulation of metabolites due to physical performance are causative factors of fatigue [4].

Fatigue is associated with high-intensity exercise-induced exhaustion, suggesting that the functioning muscle capacity has been substantially impaired [5]. During high-intensity exercise, the body utilizes key energy sources, such as glucose, hepatic, and

muscle glycogen; on the other hand, the aggregation of metabolites, such as lactic acid, ammonia, blood urea nitrogen (BUN), and inorganic phosphorus, results in intracellular acidosis, which contributes to muscle fatigue [6]. A decrease in energy sources, such as glucose and liver and muscle glycogen, and accumulation of metabolites, such as lactic acid and ammonia, are the key factors in peripheral fatigue. Peripheral fatigue can be reversed by sustaining the availability of energy sources and clearing the metabolic products [7]. Chronic low-grade inflammation has also been linked to a wide spectrum of chronic illnesses that are characterized by fatigue. Lower stamina and fatigue may occur from an energy deficit caused by mitochondrial dysfunction. Inflammation has been linked to mitochondrial dysfunction and oxidative stress [8]. Recovery from exercise-induced fatigue needs the repair of muscular injury by the metabolites, as well as the removal of metabolites produced during exercise. As a result, frequent exercise coupled with a well-balanced diet can help to prevent muscle fatigue during exercise [9]. In particular, when the intake of dietary protein and energy fail to meet individual demands, body fat and muscle are catabolized to provide energy, leading to symptoms such as fatigue [10]. Previous studies have shown that natural products boost athletic performance and decrease or prevent fatigue without causing adverse effects [11].

The medicinal properties of many Chinese herbal teas or medicines are known and include being rich in antioxidants, notably phenolics, alkaloids and antioxidant vitamins [12,13]. The *G. tenuifolia* perennial herb belongs to the Asteraceae family growing in South Asia and Australia. *Glossogyne tenuifolia* (GT) originated from coastal areas of Penghu Islands, Taiwan. In Taiwan, the GT plant was used for preparing herbal tea and in folk medicines. Aqueous extract of dried GT powder is used as an antipyretic, hepatoprotective, and anti-inflammatory medicine in traditional Chinese medicine [13–16]. Herbal teas/drinks prepared using GT have been used to prevent sunstroke [17]. In our previous studies, we showed that GT extract has antioxidant and hepatoprotective activities against acetaminophen-induced hepatotoxicity and high-fat-diet-induced diabetes [14,18]. Furthermore, we have also shown the hypoglycemic and hypolipidemic effects of GT extracts in animal models [13,19]. We have also shown the presence of major phenolic compounds, such as luteolin, luteolin-7-glucoside, and oleanolic acid, in GT extract by HPLC analysis Figure 1 [14,18,20].

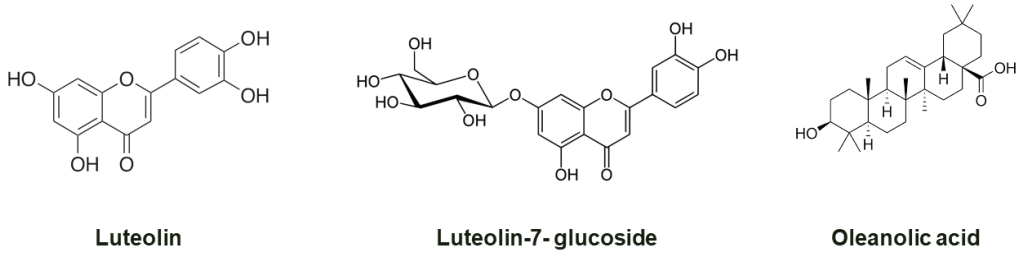

Figure 1. Major compounds present in GT extract. Structure of luteolin, luteolin-7-glucoside, and oleanolic acid.

However, the anti-fatigue effect and exercise performance of GT extract were not reported previously. The purpose of the present study was to assess the potential beneficial effects of GT extract on anti-fatigue and ergogenic functions following physiological challenges.

2. Materials and Methods

2.1. GT Extract Preparation and HPLC Analysis

GT plant was procured from Kaohsiung District Agricultural Improvement Station in Penghu, Taiwan, Republic of China (ROC). The plant was identified by a taxonomist at Tunghai University, Taichung City, Taiwan, ROC, and a specimen of the plant was

deposited in the Tunghai University Taichung City, Taiwan, ROC. GT extract was prepared based on our previous study [18]. Safety reports from the Department of Agricultural Pharmaceutical Drug Test, Executive Yuan, Kaohsiung, have shown that Lethal dose 50 (LD_{50}) of GT extract is higher than 10 g/kg BW.

2.2. Animal Experiments

Male Institute of Cancer Research (ICR) mice were used in the present study. Eight-week-old male ICR mice were procured from BioLASCO, Taiwan. Animals were maintained in the university animal house with a constant temperature of 25 ± 1 °C under the 12:12 h light–dark cycle. Animals were freely allowed to access food and water. Standard commercial laboratory mice feed (PMI Feeds, Inc., Brentwood, MO, USA) and water ad libitum were used. After 1 week of acclimatization, mice were randomly divided into 5 groups (n = 10). Control group received vehicle treatment, LGT 1X—low-dose GT extract (50 mg/kg BW/day), MGT 2X—medium-dose GT extract (100 mg/kg BW/day), HGT 5X—high-dose GT extract (250 mg/kg BW/day), and HGT 10X—high-dose GT extract (500 mg/kg BW/day). GT extract was administered for 28 days. All animal treatment procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication NO 85-23, raised 1996). Animal-experiment procedures in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of Tunghai University.

At the end of treatment, mice were euthanized by 95% CO_2, blood was collected, and serum was separated by using centrifugation and stored at −80 °C. Organs, such as the liver, lung, heart, kidney, adipose tissue, and skeletal muscles, were collected, weighed, and stored at −80 °C. A small portion of tissue sections was stored in formalin for histological analysis. A small portion of liver and muscle tissue used for estimating glycogen content was washed in saline and stored separately.

2.3. Forelimb Grip Strength Test

For estimating the forelimb grip strength of the mice in the different treatment groups, low-force testing system (Model-RX-5, Aikoh Engineering, Nagoya, Japan) was used. Only the front paws of the mice were permitted to grab the pull bar on the grip wire, which was gradually drawn back until they lost their grip on the metal bar. A detailed experiment procedure was described in the previous study [21]. The grip strength of each mouse has measured 10 times, and the longest duration in each trial was recorded.

2.4. Weight-Loaded Swimming Test

A weight-loaded swimming test in the mice was performed to evaluate the exercise endurance time based on the previous report [22]. After GT-extract administration, a weight (5% of the mice body weight) was attached to the tail and allowed to swim in a small plastic water tank with a depth of 30 cm of warm water (28 °C). Mice were considered to be exhausted when they failed to rise above the water surface in 10 s.

2.5. Biochemical Parameters Associated with Fatigue

After 1 h of GT extract administration, animals were allowed for a swimming test for 15 min without weight. The blood sample was collected after swimming exercise from the submandibular duct of mice, serum was separated by centrifugation and used to analyze fatigue-associated biochemical parameters, such as serum glucose, BUN (blood urea nitrogen), lactic acid, ammonia, CK(creatine kinase) and LDH(lactate dehydrogenase), using an autoanalyzer (Hitachi 7060, Hitachi, Tokyo, Japan).

2.6. Serum Marker Analysis

At the end of the experimental period, all mice were euthanized by using 95% CO_2. Then the blood was collected, and the serum was separated by centrifugation. Biochemical markers, such as GOT, GPT, creatinine, CK, uric acid, LDH, CPK(creatine phosphokinase),

total cholesterol (TC), TG (triglycerides), HDL (high-density lipoprotein), LDL (Low-density lipoprotein), BUN, ammonia, and glucose, were measured by using an autoanalyzer (Hitachi 7060, Hitachi, Tokyo, Japan).

2.7. Tissue Glycogen Analysis

Liver and skeletal muscles are the two primary sites for glycogen storage; we tested the effect of GT extract administration on the liver and skeletal muscle tissues. Glycogen content in the liver and muscle tissues was quantified based on the previous report [23].

2.8. Histological Analysis

Liver, skeletal muscle, heart, lung, kidney, pancreas, and adipose were collected from all treatment groups. A small portion of tissue was fixed in 10% neutral buffered formalin and covered with wax. Then 0.2 μm–size sections were cut from paraffin-embedded tissue blocks, using a microtome. Slides were prepared by deparaffinization, stained with hematoxylin and eosin (H&E), dehydrated through a series of graded alcohols (100%, 95%, and 75%), and rinsed twice in xylene. Photomicrographs were obtained by using a Zeiss Axiophot microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.9. Statistical Analysis

Data analyses were performed by using SPSS 17 software (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA). The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way analysis of variants. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of GT Extract on Body Weight and Organ Weight

After 28 days of treatment with low, medium, and high dosages (LGT 1X, MGT 2X, HGT 5X, and HGT 10X) of GT extract, the final-day bodyweight of mice was measured. The bodyweight of GT-administered mice was not significantly different when compared to control. Food and water intake were also significantly changed in the GT-treated group and control (Table 1).

Table 1. Bodyweight and organ weight of the treatment group.

Parameters	Control	LGT 1X	MGT 2X	HGT 5X	HGT 10X
Final BW (g)	40.92 ± 0.68	41.88 ± 0.63	41.86 ± 0.63	42.22 ± 0.39	39.87 ± 0.23
Muscle (g)	0.419 ± 0.010 ^{b}	0.402 ± 0.013 ^{b}	0.420 ± 0.016 ^{b}	0.400 ± 0.009 ^{ab}	0.368 ± 0.009 ^{a}
Liver (g)	1.585 ± 0.050 ^{ab}	1.558 ± 0.100 ^{ab}	1.575 ± 0.056 ^{ab}	1.845 ± 0.298 ^{b}	1.358 ± 0.037 ^{a}
Heart (g)	0.187 ± 0.006 ^{a}	0.207 ± 0.012 ^{ab}	0.219 ± 0.014 ^{b}	0.208 ± 0.008 ^{ab}	0.180 ± 0.008 ^{a}
Pancreas (g)	0.226 ± 0.007 ^{c}	0.189 ± 0.013 ^{b}	0.186 ± 0.009 ^{b}	0.152 ± 0.015 ^{a}	0.175 ± 0.007 ^{ab}
Lung (g)	0.220 ± 0.005 ^{a}	0.211 ± 0.003 ^{a}	0.212 ± 0.005 ^{a}	0.208 ± 0.004 ^{a}	0.216 ± 0.003 ^{a}
Kidney (g)	0.580 ± 0.013 ^{bc}	0.533 ± 0.016 ^{ab}	0.592 ± 0.033 ^{c}	0.527 ± 0.017 ^{a}	0.484 ± 0.014 ^{a}
Spleen (g)	0.1081 ± 0.0035 ^{b}	0.1093 ± 0.0048 ^{b}	0.0974 ± 0.0049 ^{ab}	0.0898 ± 0.0050 ^{a}	0.0902 ± 0.0029 ^{a}
Testis (g)	0.262 ± 0.016 ^{a}	0.269 ± 0.008 ^{a}	0.245 ± 0.010 ^{a}	0.237 ± 0.010 ^{a}	0.246 ± 0.009 ^{a}
Epididymis (g)	0.116 ± 0.020	0.087 ± 0.009	0.094 ± 0.015	0.068 ± 0.010	0.059 ± 0.002
Epididymis fat (g)	0.97 ± 0.1 ^{b}	0.77 ± 0.0 ^{a}	0.74 ± 0.1 ^{a}	0.76 ± 0.0 ^{a}	0.82 ± 0.1 ^{ab}

Data are the mean ± SEM for $n = 10$ mice in each group. Values in the same row with different superscript letters (^{a}, ^{b}, and ^{c}) differ significantly, $p < 0.05$.

3.2. GT Extract Increases Forelimb Grip Strength and Endurance-Swimming Time in a Dose-Dependent Manner

The forelimb-grip-strength test in animal models measures the changes in neuromuscular coordination, the intensity of muscle, and total functional capability. The forelimb-grip strength of the control group was around 95.8, which was found to be increased after GT treatment. Grip strength of the animals increased significantly in a dose-dependent

manner, where maximum forelimb grip was achieved in the HGT 5X and HGT 10X groups (Figure 2A). Another parameter used for assessing exercise endurance is the duration of the workout/exercise. It is used for testing natural compound ability in alleviating fatigue in high-intensity exercise performance. Endurance-swimming time of the different treatment groups was shown in Figure 2B. Endurance-swimming time of the mice was increased in all GT-extract-administered groups. Low and medium GT doses (LGT 1X and MGT 2X) significantly increased the endurance time. However, high doses of GT (HGT 5X and HGT 10X) showed significantly increased endurance-swimming time than the control and low and medium GT doses.

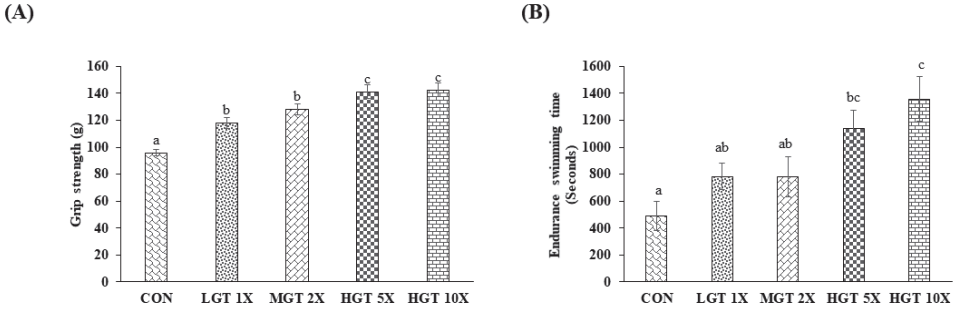

Figure 2. GT extract increases forelimb grip strength and endurance swimming time in a dose-dependent manner: (**A**) forelimb grip strength and (**B**) endurance swimming time. Data are the mean ± SEM for $n = 10$ mice in each group. Values in the same row with different superscript letters (a, b, and c) differ significantly, $p < 0.05$.

3.3. Effect of GT Extract on Biochemical Markers Associated with Fatigue

Figure 3 shows the effect of GT extract treatment on fatigue-associated biochemical parameters, such as serum glucose, BUN, lactate, ammonia, CK, and LDH, after swimming exercise. Levels of serum glucose, BUN, lactic acid, ammonia, CK, and LDH in the blood were quantified to evaluate the anti-fatigue effect of GT extract. Medium and high doses of GT extract significantly increased the glucose level in the blood when compared to control. However, low-dose GT extract has no significant changes in glucose level compared to the control. GT extract has no effect on the BUN level. Low dose and medium doses (LGT 1X and MGT 2X) do not significantly reduce the lactate and ammonia levels, whereas high doses of GT (HGT 5X and HGT 10X) significantly lowered the levels of lactic acid and ammonia after exercise. Creatinine kinase and LDH levels in the blood indicate tissue/muscular damage after exercise. In our present study, HGT 5X and HGT 10X groups significantly lowered the enzyme activity of CK and LDH more than the control and low- and medium-GT doses (LGT 1X and MGT 2X).

3.4. Effect of GT Extract on Liver and Muscle Glycogen

Glycogen is a key energy source utilized during exercise, and higher tissue glycogen levels in the liver and muscle promote physical performance. Estimating the tissue glycogen level helps in identifying the severity of fatigue. GT extract significantly increased the liver and muscle glycogen level. However, MGT 2X, HGT 5X, and HGT 10X groups show significantly higher tissue glycogen levels than the low-dose GT (LGT 1X) group (Figure 4).

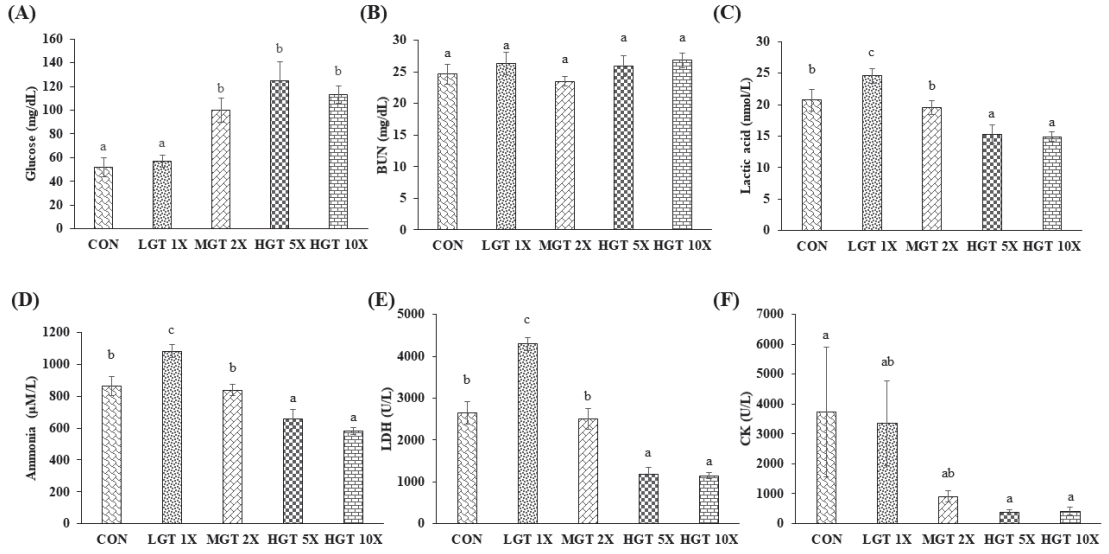

Figure 3. Effect of GT extract on biochemical markers associated with fatigue: (**A**) glucose, (**B**) BUN, (**C**) lactic acid, (**D**) ammonia, (**E**) LDH, and (**F**) CK. Data are the mean ± SEM for $n = 10$ mice in each group. Values in the same row with different superscript letters (a, b, and c) differ significantly, $p < 0.05$.

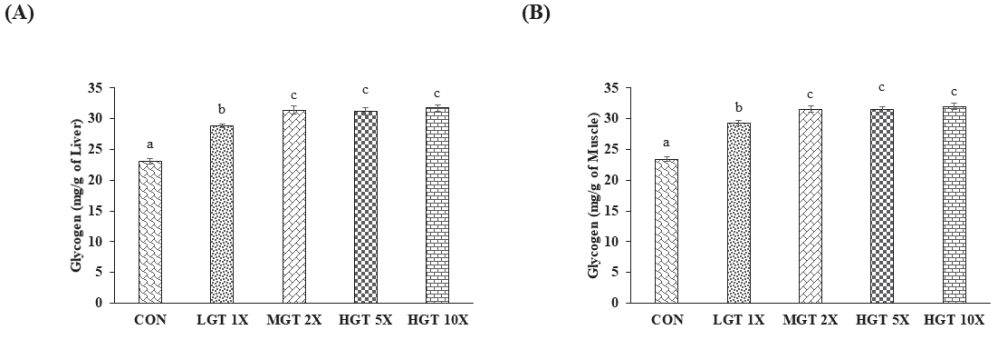

Figure 4. Effect of GT extract on liver and muscle glycogen: (**A**) liver and (**B**) muscle. Data are the mean ± SEM for $n = 10$ mice in each group. Values in the same row with different superscript letters (a, b, and c) differ significantly, $p < 0.05$.

3.5. Effect of GT Extract on Biochemical Parameters at the End of the Treatment Period

At the end of the treatment period, biochemical parameters for liver, kidney, heart, and muscle injury markers, such as GOT, GPT, creatinine, uric acid, LDH, and CPK; lipid profile; glucose; BUN; and ammonia levels, were quantified to evaluate the anti-fatigue effect GT extract (Table 2). Liver markers GOT and GPT, kidney markers creatinine and uric acid, heart, and muscle injury markers LDH and CPK do not significantly change after GT treatment. In lipid profile total cholesterol, triglyceride and HDL levels were not significantly altered in GT treated group; however, GT extract (LGT 1X, MGT 2X, HGT 5X, and HGT 10X) decreased the LDL level significantly in a dose-dependent manner. No significant changes were observed in glucose, BUN, and ammonia levels in all the groups.

Table 2. Biochemical analysis at the end of the treatment period in treatment groups.

Parameters	Control	LGT 1X	MGT 2X	HGT 5X	HGT 10X
GOT (U/L)	117.9 ± 18.8 ^{a}	101.5 ± 6.8 ^{a}	116.2 ± 15.1 ^{a}	140.8 ± 24.5 ^{ab}	176.3 ± 29.5 ^{b}
GST (U/L)	27.8 ± 2.8 ^{a}	23.0 ± 1.0 ^{a}	26.6 ± 3.6 ^{a}	44.9 ± 16.6 ^{a}	28.0 ± 2.1 ^{a}
Triglyceride (mg/dL)	66.8 ± 6.1 ^{a}	90.1 ± 15.5 ^{a}	84.8 ± 7.9 ^{a}	75.2 ± 6.8 ^{a}	80.1 ± 7.2 ^{a}
Total cholesterol (mg/dL)	153.7 ± 6.4 ^{a}	154.6 ± 5.9 ^{a}	142.7 ± 6.5 ^{a}	151.6 ± 5.4 ^{a}	153.7 ± 8.0 ^{a}
HDL (mg/dL)	117.7 ± 4.29 ^{a}	120.8 ± 5.63 ^{a}	114.0 ± 5.58 ^{a}	122.5 ± 4.09 ^{a}	123.9 ± 7.24 ^{a}
LDL (mg/dL)	25.0 ± 2.09 ^{b}	22.1 ± 1.72 ^{ab}	19.8 ± 1.28 ^{a}	20.9 ± 0.98 ^{ab}	19.5 ± 1.42 ^{a}
BUN (mg/dL)	24.9 ± 0.70 ^{a}	27.3 ± 1.32 ^{ab}	28.6 ± 1.74 ^{bc}	31.0 ± 0.77 ^{c}	25.9 ± 1.55 ^{ab}
Creatinine (mg/dL)	0.228 ± 0.014 ^{a}	0.225 ± 0.017 ^{a}	0.235 ± 0.022 ^{ab}	0.214 ± 0.014 ^{a}	0.277 ± 0.014 ^{b}
Uric acid (mg/dL)	2.44 ± 0.20 ^{ab}	3.01 ± 0.22 ^{b}	2.13 ± 0.18 ^{a}	2.00 ± 0.24 ^{a}	2.35 ± 0.24 ^{a}
Glucose (mg/dL)	131.7 ± 10.8 ^{a}	160.5 ± 5.3 ^{bc}	152.3 ± 6.7 ^{ab}	166.9 ± 11.1 ^{c}	138.9 ± 6.6 ^{ab}
CPK (U/L)	5789 ± 1495 ^{ab}	3420 ± 490 ^{a}	4762 ± 921 ^{a}	6019 ± 1632 ^{ab}	9168 ± 1841 ^{b}
LDH (U/L)	1214 ± 193 ^{a}	721 ± 134 ^{a}	810 ± 190 ^{a}	924 ± 259 ^{a}	1012 ± 111 ^{a}
Ammonia (μM/L)	414 ± 87 ^{a}	485 ± 106 ^{a}	632 ± 132 ^{a}	528 ± 124 ^{a}	645 ± 103 ^{a}

Data are the mean ± SEM for n = 10 mice in each group. Values in the same row with different superscript letters (^{a}, ^{b}, and ^{c}) differ significantly, $p < 0.05$.

3.6. Effect of GT Extract on Histology in Various Organs

A histological analysis of various organs, such as the liver, kidney, heart, muscle, lungs, adipose tissue, and pancreas, was performed to understand the morphological damage in the tissues. Even high doses of GT extract (HGT 5X and HGT 10X) do not induce any morphological changes in the liver, kidney, lungs, and muscle. Heart and muscle sections also display normal cellular architecture in GT-treated groups. The morphology of pancreas and adipose tissue do not alter after GT treatment (Figure 5).

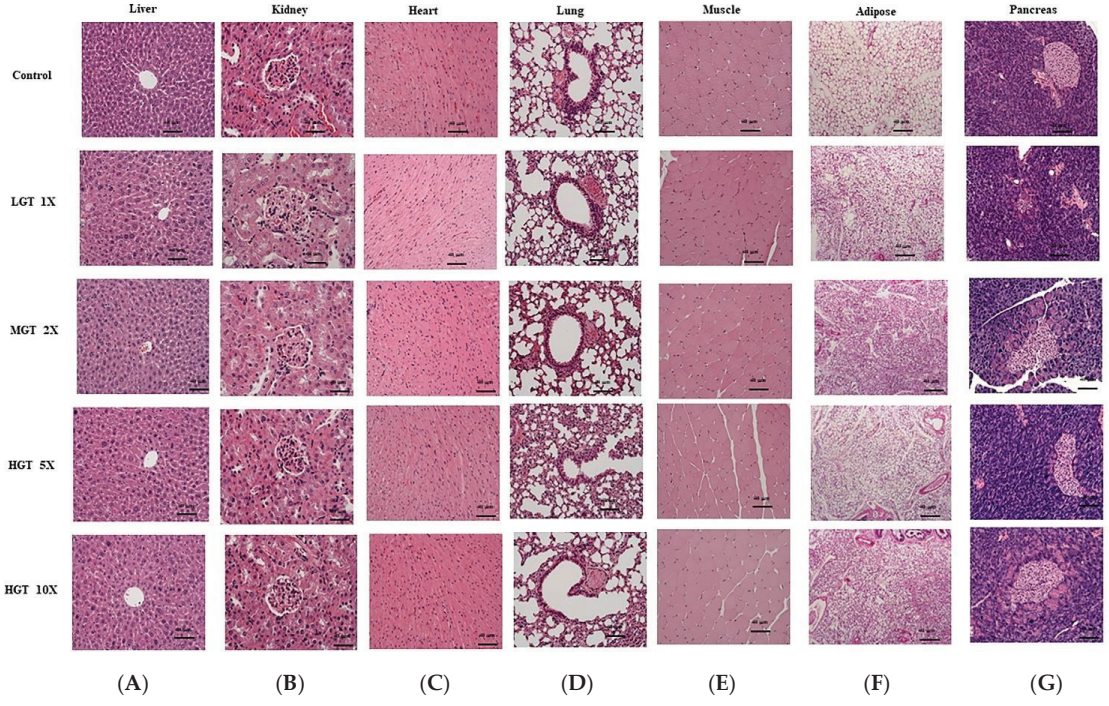

Figure 5. Effect of GT extract on histology in various organs: (**A**) liver, (**B**) kidney, (**C**) heart, (**D**) lung, (**E**) muscle, (**F**) adipose, and (**G**) pancreas. H&E stain (100×).

4. Discussion

Endurance-swimming time, forelimb-grip strength, and changes in biochemical marker levels were studied and a histological analysis of muscle and other tissues was performed to assess the fatigue in animal models [24,25]. Endurance-swimming time in a forced-swimming test, forelimb-grip test, and rotary rod test have been widely used in animal models for evaluating the anti-fatigue efficacy of drugs or natural compounds [26,27]. The enhancement of exercise endurance time is the key manifestation of the anti-fatigue effect of the drug or natural compounds. In our present study, GT-extract administration increased the forelimb-grip strength significantly compared to the control. A higher GT dose, HGT 5X, and HGT 10X have significantly higher forelimb-grip strength than LGT 1X and MGT 2X. This suggests that the potential benefits of GT administration have a beneficial effect on increasing grip strength without training. Further, GT extract significantly improved the endurance-swimming time in a time-dependent manner compared to the control. The administration of phytochemicals, such as resveratrol, capsaicin, and curcumin, has been shown to improve the forelimb-grip strength and endurance-swimming time in animal models without training [22,28,29]. Supplementation of Chinese herbal extract from Cornu cervi pantotrichum increased the swimming time and forelimb-grip strength [30].

Apart from swimming tests, blood biochemical parameters are also used as a marker for fatigue. Glucose is produced from tissue glycogen in the liver and muscle and released in blood for the energy source [31]. High-intensive exercises have a high energy demand and consume glucose from tissue glycogen and increase their concentration in the blood [32]. Low-dose GT extract (LGT 1X) does not alter the serum glucose level significantly after the exercise. However, in MGT 2X, HGT 5X, and HGT 10X, the glucose levels were significantly increased after exercise when compared to the control. Thus, GT extract increases exercise performance by supplying/maintaining high glucose levels in the blood. Blood lactate is the metabolic end-product during glycolysis of carbohydrates under anaerobic conditions, and anaerobic glycolysis serves as a key energy source during intensive exercise [33]. Accumulated lactic acid during intensive exercise in the blood decreases the pH of muscles, and blood can cause acidosis. Acidosis is known to damage muscle and other vital organs and cause fatigue [34]. BUN is a metabolic product of protein metabolism. In amino acid metabolism, ammonia is produced as a metabolic end-product. BUN and ammonia accumulation increased during high-intensity exercise, reducing the performance and causing fatigue [35]. Preventing the accumulation and removal of lactic acid, BUN, and ammonia from the blood could increase exercise performance and prevent exercise-induce fatigue. In rats, *Moringa oleifera* extracts reduced the accumulation of LA, BUN, and ammonia and increased the endurance swimming time [36]. In our study, we found that GT extract does not alter BUN levels significantly in all groups. However, lactic acid and ammonia levels increased in LGT 1X, but higher GT doses, namely MGT 2X, HGT 5X, and HGT 10X, significantly decreased the lactic acid and ammonia levels. This suggests the potential anti-fatigue effect of GT.

Tissue glycogen from the muscle and liver is the key energy source during glycolysis and oxidative phosphorylation and increases the glucose level in blood during physical exercise [27]. High levels of hepatic and muscle glycogen could increase endurance time and performance during exercise with high intensity and reduce fatigue [37]. Compounds increasing the tissue glycogen level could have an anti-fatigue effect. *Antrodia camphorata*–extract treatment in mice increased the hepatic and muscular glycogen level and increased the endurance swimming time [38]. GT treatment in the mice increased the muscle and liver glycogen levels.

Liver function marker alanine aminotransferase (ALT); aspartate aminotransferase (AST); kidney function marker creatinine and uric acid; and other organ markers, such as creatine kinase (CK), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH), are key markers for tissue damage during high-intensity exercise [39]. *Antrodia camphorata* extract, an edible fungus extract with high triterpenoids content has an anti-fatigue effect by increasing glucose level reducing the accumulation of BUN and lactic acid in blood and

decreasing CK activity [38]. In our study, GT-extract treatment does not change the activity of these enzymes, suggesting no organ damage during exercise performance. The presence of phenolic compounds, such as luteolin and luteolin-7-glucoside, in the GT extract has been shown to have a hepatoprotective effect [14,18].

Fats are the second macronutrient that gives energy after glucose. During exercise, lipolysis is a major contributor to elevated triglyceride levels during rest [40]. Some natural phytochemicals induce fat/lipid metabolism, which delays the glycogen as an energy source. Aqueous extract of *Millettiae speciosae* Champ. supplementation increased fat utilization and reduced triglyceride and muscle glycogen usage as an energy source; thus, it possesses an anti-fatigue effect [41]. However, in our present study, GT extract did not change the lipid profile. Apart from forelimb-grip strength, endurance-swimming time and biochemical parameters related to exercise-induced fatigue, histological analysis of liver, kidney, heart, muscle, lung, and testis tissue were used to evaluate the impairment induced during exercise [42]. GT administration does not cause any morphological abnormalities in the liver, kidney, heart, muscle, lung, and pancreas.

5. Conclusions

G. tenuifolia is a traditional antipyretic and hepatoprotective herb used in Penghu Island and may soon become an important economic healthy food. However, the information on effective ingredients was still rare and unclear until now. In our previous study, some active ingredients, including luteolin and luteolin-7-glucoside, were reported in the GT extract [14,18]. Safety reports of the GT extract have also shown no toxicity of up to 10 g/b.wt. The experimental results show that this plant extract could be used for formulating food supplementation/health drinks exhibiting anti-fatigue effect and improving exercise performance.

In conclusion, GT extract treatment in mice could enhance physical performance, including forelimb-grip strength and endurance-swimming time. Furthermore, fatigue blood biochemical parameters indicate that GT extract improves glucose level and prevents the accumulation of lactic acid, BUN, and ammonia from the blood. GT possesses an anti-fatigue effect by increasing tissue glycogen levels in the liver and muscle. Taken together, herbal extract GT can be used to mitigate fatigue during exercise and increase exercise performance.

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Institutional Review Board Statement: All animal-treatment procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication NO 85-23, raised 1996). This study was conducted following the IACUC-105-25 protocol and approved by the institutional animal care and use committee (IACUC) of Tunghai University, Taiwan.

Data Availability Statement: The data that support the findings of this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Potential Effect of Glutamine in the Improvement of Intestinal Stem Cell Proliferation and the Alleviation of Burn-Induced Intestinal Injury via Activating YAP: A Preliminary Study

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Abstract: Burn injury is a common form of traumatic injury that leads to high mortality worldwide. A severe burn injury usually induces gut barrier dysfunction, partially resulting from the impairment in the proliferation and self-renewal of intestinal stem cells (ISCs) post burns. As a main energy substance of small intestinal enterocytes, glutamine (Gln) is important for intestinal cell viability and growth, while its roles in ISCs-induced regeneration after burns are still unclear. To demonstrate the potential effects of Gln in improving ISCs proliferation and alleviating burn-induced intestinal injury, in this study, we verified that Gln significantly alleviated small intestine injury in burned mice model. It showed that Gln could significantly decrease the ferroptosis of crypt cells in the ileum, promote the proliferation of ISCs, and repair the crypt. These effects of Gln were also confirmed in the mouse small intestine organoids model. Further research found that Yes-associated protein (YAP) is suppressed after burn injury, and Gln could improve cell proliferation and accelerate the renewal of the damaged intestinal mucosal barrier after burns by activating YAP. YAP is closely associated with the changes in intestinal stem cell proliferation after burn injury and could be served as a potential target for severe burns.

Keywords: glutamine; intestinal stem cells; crypt; proliferation; burns

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1. Introduction

Intestinal injury is a common complication in severe burn patients. The damaged intestine not only affects the absorption of nutrients, leading to malnutrition in patients, but more importantly, it is the pathophysiological basis of gut-derived infection and enterogenic hypermetabolism, which is an important factor leading to poor prognosis [1,2]. Various studies have shown that the repair and functional re-establishment of the damaged intestinal mucosal barrier relies on a reserve stem cell population; its proliferation and self-renewal are key factors in intestinal tract injury repair [3,4]. When the intestine is irritated by external damage, the repair function of the stem cells is initiated through relevant mechanisms at the molecular level, which stimulates the intestinal stem cell (ISC) proliferation and generates progeny cells to substitute the damaged intestinal epithelial cells and regenerate the intestinal barrier [5]. During the process of stem cell division and proliferation, their demand for nucleic acid and protein increases due to DNA replication and protein synthesis [6,7]. Therefore, the proliferation of ISCs is highly dependent on

anabolism. In this process, in addition to the essential growth stimulation hormones and growth factors, some special nutrients, including glutamine (Gln), glucose, ascorbic acid, and lipids can promote cells anabolism, improve the proliferation activity of stem cells, and accelerate the repair of damaged intestinal mucosa [8]. Among these nutrients, the role of Gln is particularly noteworthy.

Gln is the most plentiful amino acid in mammals, accounting for more than half of the free amino acids [9]. In recent years, Gln has aroused extensive concern in view of its significant metabolic changes in diseases; several studies have shown that Gln is an important precursor for the synthesis of pyrimidine, purine nucleotide, and amino sugar as a conditionally required amino acid [10]. Gln is a precursor for the synthesis of reduced glutathione (GSH), an important antioxidant in vivo. It can improve the antioxidant capacity of the body by maintaining and increasing the GSH reserve in tissue cells [11]. Gln has been found to have trophic effects on ISCs and crypt cells. Supplementation of Gln decreased crypt depth and increased the villus/crypt ratio in three-week-old weaned mice [12,13]. These results support that Gln can promote the proliferation of ISCs, increase the formation of crypt organoids, and maintain their stability. Collectively, these studies demonstrate that Gln can promote the proliferation of ISCs, but its mechanism, especially the signaling mechanism, is not fully understood so far.

Stem cell proliferation is a complex physiological process regulated by multiple signals, in which the Hippo pathway is gradually gaining attention [14]. Recent studies demonstrated that the Hippo pathway was related to the physiological processes of stem cell proliferation, differentiation, and self-renewal [15]. The Hippo pathway is composed of several conserved kinases, such as mammalian sterile 20-like kinase 1 (MST1) and Yes-associated protein (YAP); as transcriptional coactivators, they are the main effectors of the Hippo pathway, which can promote the proliferation of ISCs and accelerate the repair of damaged intestinal epithelium [16]. Studies have reported that MST1 transcription and YAP activity are closely related to Hippo signaling pathway activation [16]. This signaling pathway is involved in the regulation of ISCs and plays an indispensable role in cell proliferation, apoptosis, differentiation, and development [17]. In a previous study, YAP could lead to the accumulation of intermediate products in glycolysis by promoting the expression of glucose transporter [18]. Studies have reported that Gln not only has glucose replacement effects but also could regulate glycolysis [19]. Therefore, we hypothesized that Gln might regulate YAP activity through the glycolytic pathway, thereby promoting the proliferation of ISCs. In this study, the expected research objectives are to find the cause of YAP changes and functional degeneration of ISCs after burns and how Gln regulates the activation of YAP to promote the proliferation of ISCs and improve the repair of the damaged intestinal mucosa.

2. Results

2.1. Gln Improves Crypt Survival and Mitigates Pathological Damage of Small Intestine after Burns in Mice

Gln is a crucial metabolic source for small intestinal cells and is important for the growth of intestinal cells. In view of the importance of Gln in intestinal homeostasis, we added Gln to observe whether it could reduce the damage of ISCs after burns. We first constructed a burned mouse model, as shown in Figure S1A; the scabby area on the back of mice was increased time-dependently. Subsequently, we found that the body weight of mice decreased significantly over time (Figure S1B). We observed the effects of Gln treatment on the small intestine under a light microscope after burns. As presented in Figure 1A–C, the intestinal tract was obviously atrophic, the length of the small intestine was significantly shortened, the intestine was perforated, and the blood vessels on the surface of the intestinal tract were increased in the Burn group. Histopathological scores for small intestine injury were used to evaluate the extent of intestinal injury (Figure 1C). Compared with the Burns group, the injury scores of small intestinal in the Burn+Gln group had significantly decreased. Next, the results of hematoxylin–eosin (H&E) staining showed that burns stress

induced remarkable morphological alterations in the small intestine (Figure 1D,E). In both jejunum and ileum, the villi were disordered, the depth of crypts became shallow, and obvious vacuoles appeared in the crypts, accompanied by the thickening of intestinal basal tissues (Figure 1D–G). However, the addition of Gln could rescue the pathological damage of the crypts of small intestinal by burns. For instance, as shown in Figure 1D–F, it was found that the length of crypts in the jejunum after burns was significantly reduced compared with the control group. After Gln treatment, the length of crypts in the jejunum increased compared with the Burn group. As well, in Figure 1G, the ileum basal tissue increased significantly compared with the control group, while the basal tissue thickness decreased significantly after Gln treatment.

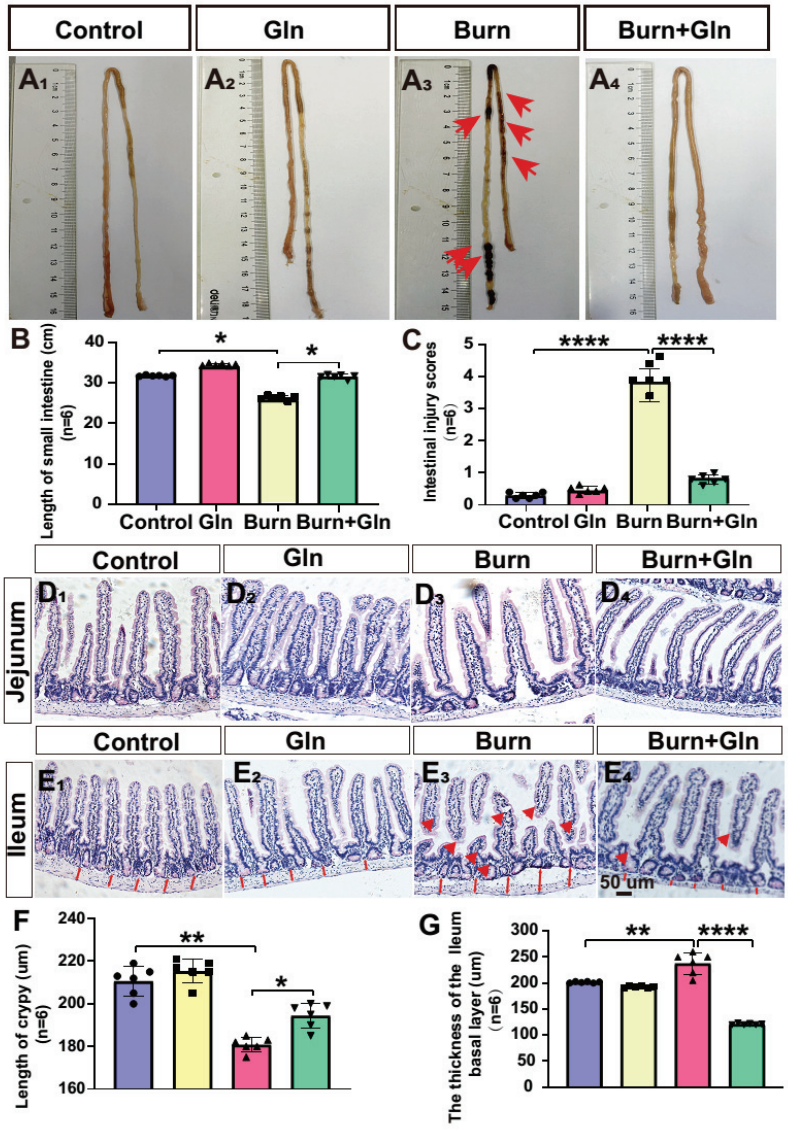

Figure 1. Effect of burn injury on the pathological structure of small intestinal crypts. The villus length and crypt depth were measured as indicated in the image (×100; $n = 6$). Yellow arrows indicate

goblet cell infiltration in the small intestine. The red bidirectional arrows indicate the thickness of the basal tissue of the small intestinal crypt. (**A_1**–**A_4**) Representative images of the small intestine of each group under a light microscope: (**A_1**) Control group; (**A_2**) Burn group; (**A_3**) Gln group; and (**A_4**) Burn+Gln group. (**B**) Quantitative analysis of the length of the small intestine under different treatments. (**C**) Quantitative analysis of the number of preservation injury scores of the small intestine of different groups. (**D_1**–**D_4**) Representative images of the hematoxylin and eosin (H&E) staining of the small intestine sections in jejunum from control\Gln\Burn and Burn+Gln group at day 3. (**E_1**–**E_4**) Representative images of H&E staining of the small intestine sections in ileum from control\Gln\Burn and Burn+Gln group at day 3. (**F**) The statistical analysis of the length of crypts from images shown in (**E_1**–**E_4**). (**G**) The statistical analysis of the thickness of the lleum basal layer in ileum from images shown at (**E_1**–**E_4**). The data are Mean ± SEM with an $n = 6$. * $p < 0.1$, ** $p < 0.01$, **** $p < 0.0001$ using one-way ANOVA and post hoc Tukey's test.

In addition, the results of Periodic acid Schiff (PAS) staining showed obviously visible goblet cell hyperplasia in the Burn group compared to the control group. Goblet cell expression in the Burn group was significantly higher than that in the control group (Figure S2A,B). However, we found that goblet cell proliferation was reduced after Gln treatment (Figure S2A,B). These results added further evidence for the pathological changes in the small intestine, and the damage to crypts was serious after burns. On the other hand, Gln treatment mitigated the pathological injury and improved the survival of the crypts of the small intestine, indicating its protective effects on burns-induced intestinal damage.

2.2. Gln Promotes Budding in Three-Dimensional (3D) Cultured Intestinal Organoids after Burns

Subsequently, we further utilized an intestinal organoids model to determine the effects of Gln on the survival and proliferation of intestinal stem cells. It showed that the intestinal crypt organs of the control group were spherical, the hidden nest organs began to sprout in 24 h after burns, and the germination of 48 h saw a protruding germination phenotype, which is called budding in biology (Figure 2A–C). The spheroid area of organoids increased significantly in an incubation time-dependent manner. It was found that the spheres of the intestinal 3D organoid were significantly contracted and accompanied by large numbers of dead cell fragments after burns, and the buddings were reduced compared with the control group, then accompanied by large numbers of dead cell fragments. However, Gln treatment increased the number of spheres of the intestinal organs and the area of spheres (Figure 2C,D). It indicated that ISCs were seriously damaged by burn injury from the perspective of 3D organoids. In contrast, Gln treatment can relieve the damage of intestinal organoids and improve the budding rate, thereby accelerating the proliferation of intestinal organoids.

2.3. Effects of Gln on Endogenous Apoptosis and Ferroptosis of ISCs after Burns

To investigate the salvage effects of Gln treatment on ISCs, we first detected the endogenous apoptosis of ISCs after burns. We verified the expression of two apoptotic proteins (BAX and Caspase9), the endogenous pro-apoptotic effectors. Due to the severe intestinal injury observed by previous H&E staining on the third day after burns, we tested the protein levels of BAX and Caspase9 in ISCs on the third day after burns. As indicated in Figure 3A,B, there were no significant differences in BAX protein levels at different time points after burns, and Gln supplementation and the repeats of each group maintained consistent homogeneity. Similarly, the protein level of Caspase9 had no significant difference between each group on the third day after burns in the expression level in the control group, the Gln group, the Burn group, and the post-burn treated with the Gln group (Figure 3C). Subsequently, we verified the changes of ferroptosis marker GPX4 in ISCs by immunofluorescence on the third day after burn injury. A large number of $GPX4^+$ cells were found in the Burn group compared with the control one, and we speculated that the increase in GPX4 in the Burn group may be due to the stress states of the body after burns. However, the fluorescence of GPX4+ cells decreased after Gln treatment on ISCs after burns, which indicated that the addition of Gln could decrease the ferroptosis of crypt

cells in the ileum. This may be related to the antioxidant properties of Gln itself (Figure 3D). These results suggested that the damages of ISCs after burns were unrelated to endogenous apoptosis. However, the addition of Gln alleviated ferroptosis of ISCs after burn injury.

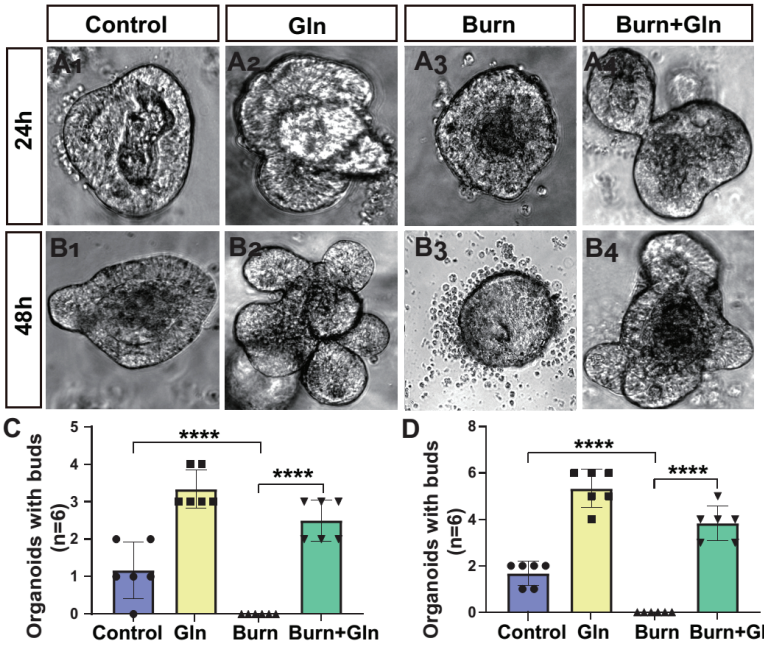

Figure 2. Regeneration of the organoids of the mouse small intestine crypts after Gln treatment. (**A$_1$**–**A$_4$**) are representative images of the organoids of the mouse small intestine crypt in control group, Gln group, Burn group and Burn+Gln group 24 h after burns injury. (**B$_1$**–**B$_4$**) are representative images of the organoids of the mouse small intestine crypt in control group, Gln group, Burn group, and Burn+Gln group 48 h after burns injury. (**C**) Comparison of the number of budding crypts in a single organoid of the mouse small intestine crypt 24 h after burns injury. (**D**) Comparison of the number of budding crypts in a single organoid of mice small intestine crypt post burns 48 h. Data were expressed as mean ± SEM from 6 independent experiments (n = 6). **** $p < 0.0001$ using 1-way ANOVA and post hoc Tukey's test.

2.4. Gln Treatment Promotes Self-Renewal of ISCs by Speeding Up the Cell Cycle and Promoting Proliferation

Apart from the endogenous apoptosis and ferroptosis effect, we next measured the influence of Gln on the cell proliferation and cell stemness of ISCs. Firstly, we assessed the proliferation of each group after burn injury. Crypts were marked by the white boxes on each image (Figure 4A–D), and the corresponding magnified images were displayed in Figure 4(A$_5$–D$_5$). The number of proliferation cell nuclear antigen (PCNA)-positive cells was greatly decreased after burns, and Gln supplementation reversed the burn-induced inhibitory effects on ISCs (Figure 4A–E), indicating that Gln can maintain cell proliferation and inhibit crypt injury after burns. Olfm4 is a stem cell marker of ISCs. Similarly, the number of olfactomedin 4$^+$ (Olfm4$^+$) positive cells was significantly decreased after burns, while Gln treatment significantly aggrandized the number of Olfm4$^+$ positive cells compared with the Burn group, especially at crypt bottoms, which was the location of ISCs (Figure 4A–D,F). Furthermore, we used 5-bromodeoxyuridine (BrdU) staining to identify and quantify cells undergoing mitosis. We found that the BrdU-positive cells in the crypts were significantly reduced after burns, indicated that the cell cycle delay in ISCs. However, the expression of BrdU-positive cells was significantly elevated in the

Burn+Gln group (Figure 5A–E). In addition, the trends of ATP binding cassette subfamily G member 2 ($ABCG_2$), another stemness marker, were consistent with the trends of Olfm4 results (Figure 5A–D,F). To further determine the regulation of the cell cycle in ISCs by Gln supplementation after burn injury, we examined the cell cycle by flow cytometry. The results showed that the proportion of the G0/G1 phase was significantly increased, and the proportion of the S phase was decreased after burns. However, Gln supplementation reversed the cell cycle changes after burns and promoted the transition of the G1-S phase in ISCs (Figure 6A–C). These results indicated that complementary Gln treatment accelerates the cell cycle and promotes ISCs proliferation after burns in vitro, which was consistent with the results of PCNA staining.

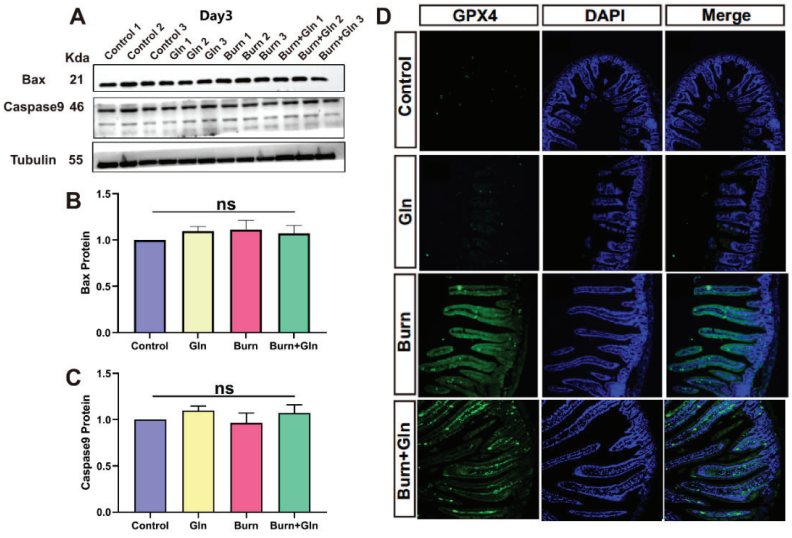

Figure 3. Effects of Gln treatment on apoptosis and ferroptosis in the small intestine after severe burns. (**A**) Gln supplementation after severe burns for 3 days. The expressions of Bax and Caspase9 were detected by immunoblotting (n = 6). (**B**) The relative intensities of Bax on the 3rd day (Day 3) after burn of bands were quantified using ImageJ. (**C**) The relative intensities of Caspase9 on the 3rd day (Day 3) after burn of bands were quantified using ImageJ. (**D**) Representative images of immunofluorescence for Glutathione Peroxidase 4 (GPX4) (green) and diamidino-phenyl-indole (DAPI) (blue): Control group; Burn group; Gln group; and Burn+Gln group. Data were expressed as mean ± SEM from 6 independent experiments (n = 6).

To further clarify the regulatory effects of Gln on the proliferation and stemness of ISCs after burns, the mRNA levels of the targets that related to proliferation and stemness were validated by Real-Time Quantitativeq Polymerase Chain Reaction (RT-qPCR). The results showed that the mRNA levels of Sulfur Oxides 9 (SOX9), PCNA, Cyclin-Dependent Kinase 2 (CDK2), and Cyclin-Dependent Kinase 4 (CDK4) were down-regulated after burn injury but elevated by Gln supplementation. Although the levels of CDK4 were of no significance between the Burns and Control group, it up-regulated by Gln compared with the Burns group (Figure 7A–F). Hippo-Yes-associated Protein (Hippo-YAP) signaling notoriously plays a vital role in stem cell proliferation, cell cycle, and tissue regeneration in mammalians. Coincidentally, these targets related to proliferation and stemness were the signature of YAP. Additionally, the mRNA levels of YAP and MST1, which was the pivot of Hippo-YAP signaling, were remarkably decreased after burn injury, while aggrandized by Gln (Figure 7G,H). These results revealed that the proliferation and stemness of ISCs were eroded after burns, and Gln supplementation could antagonize the damnification, thereby

promoting the endogenous proliferation of ISCs to maintain the renewal of intestinal epithelial cells.

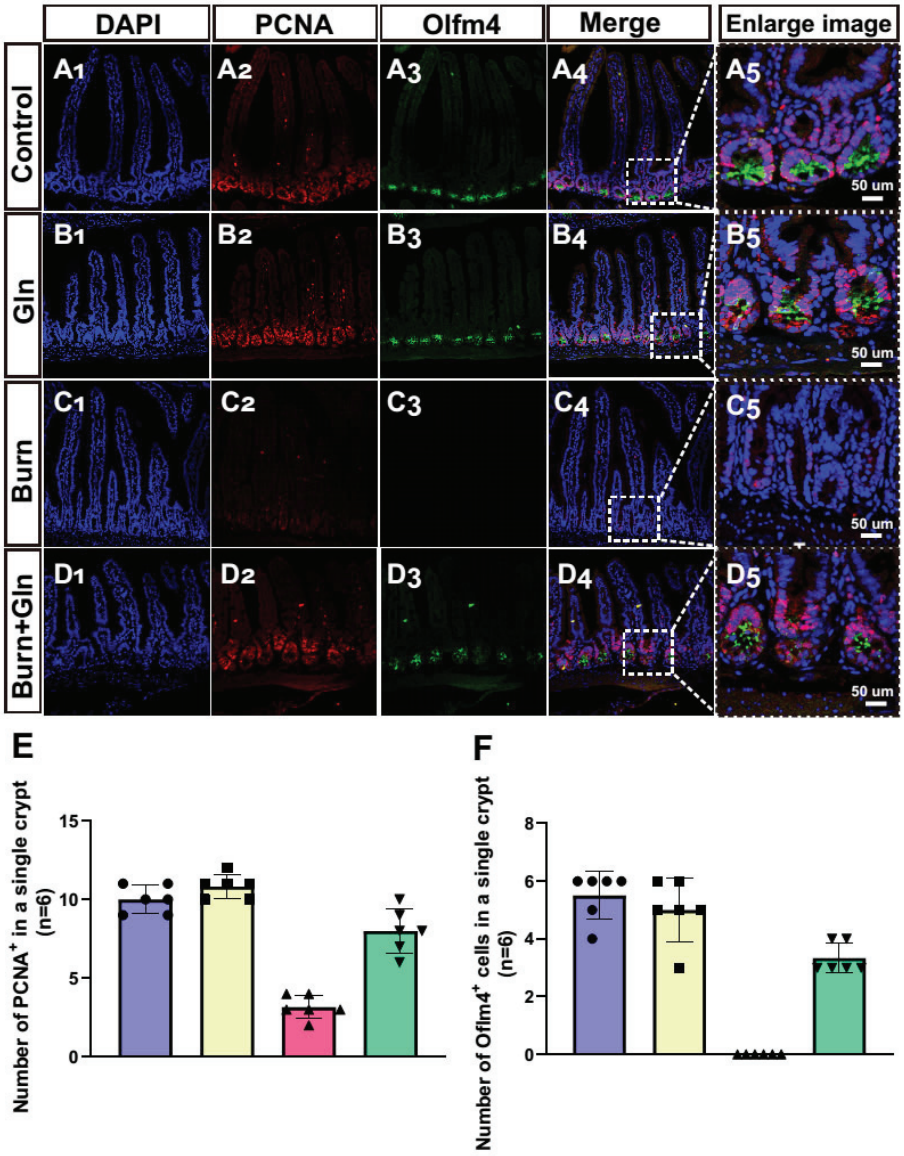

Figure 4. Influences of Gln treatment on proliferation and stemness of small intestinal crypts and ISCs of C57 mice after burn injury. (**A**–**C**) Representative images of immunofluorescence for proliferation cell nuclear antigen (PCNA) (red), olfactomedin 4 (Olfm4) (green), and DAPI (blue) in different groups: (**A$_1$**–**A$_5$**) Control group. (**B$_1$**–**B$_5$**) Gln group. (**C$_1$**–**C$_5$**) Burn group. (**D$_1$**–**D$_5$**) Burn+Gln group. (**E**) Comparison of the number of PCNA-positive cells in a single crypt in different groups. (**F**) Comparison of the number of Olfm4-positive cells in a single crypt in different groups. Data were expressed as mean ± SEM from 6 independent experiments (n = 6).

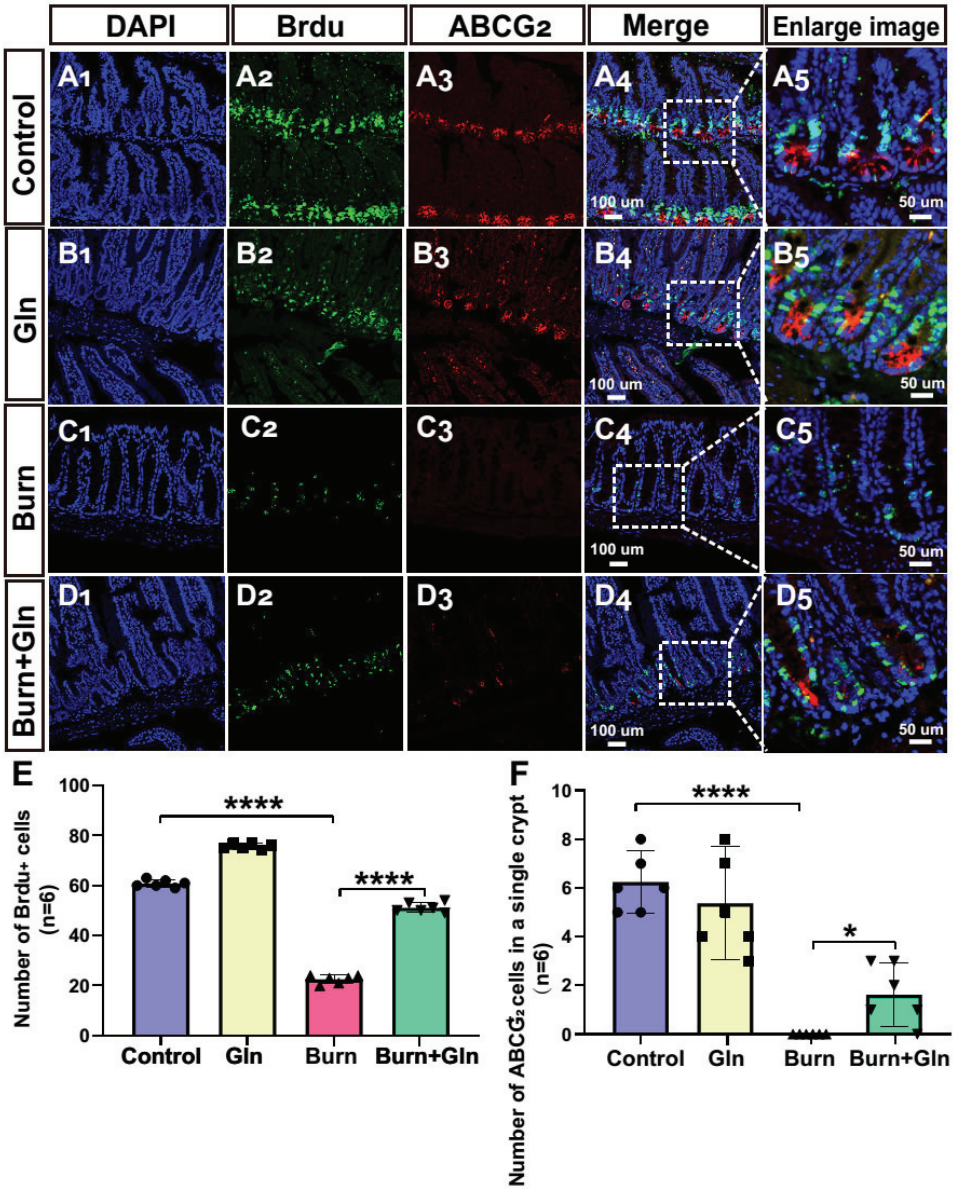

Figure 5. Gln treatment accelerates the small intestinal stem cell cycle and promotes the dry expression of ISCs C57 mice after burn injury. (**A**–**D**) Representative images of immunofluorescence for BrdU (**green**), ATP binding cassette subfamily G member 2 ($ABCG_2$) (red), and DAPI (blue) in different groups: ($\mathbf{A_1}$–$\mathbf{A_5}$) Control group. ($\mathbf{B_1}$–$\mathbf{B_5}$) Gln group. ($\mathbf{C_1}$–$\mathbf{C_5}$) burn group. ($\mathbf{D_1}$–$\mathbf{D_5}$) Burn+Gln group. (**E**) Comparison of the number of BrdU-positive cells in a single crypt in different groups (n = 6). (**F**) Comparison of the number of $ABCG_2$-positive cells in a single crypt in different groups. Data were expressed as mean ± SEM from 6 independent experiments (n = 6). * $p < 0.1$, **** $p < 0.0001$ using 1-way ANOVA and post hoc Tukey's test.

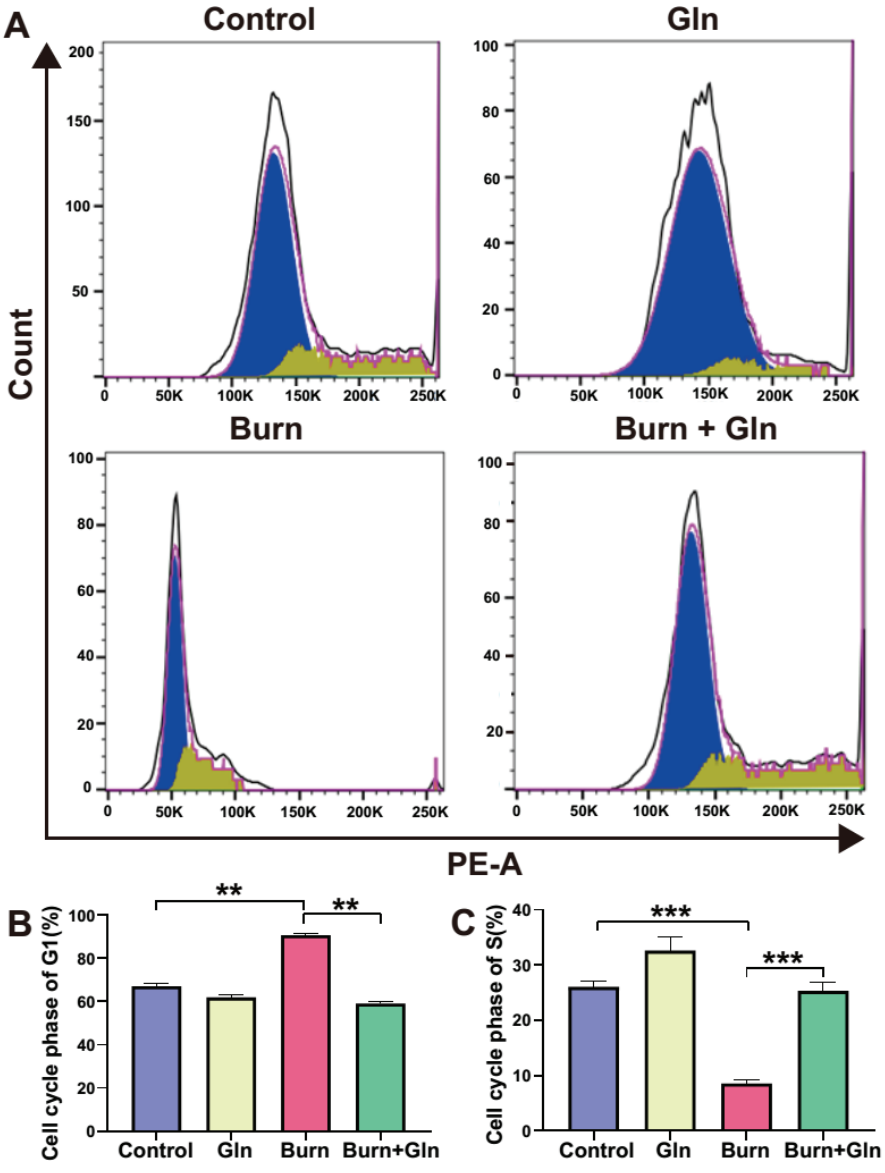

Figure 6. Gln could promote proliferation and accelerate cell cycle. (**A**) Flow cytometry was used to analyze the typical cell cycle of small intestinal stem cells treated in different groups. (**B**) The proportion of G1 phase of cell cycle in different groups. (**C**) The proportion of S phase of cell cycle in different groups. Data were expressed as mean ± SEM from 6 independent experiments ($n = 6$). PE-A represents the curve area of fluorescence intensity. The blue part represents the cell cycle phase of G1 and the yellow part represents the cell cycle phase of S. ** $p < 0.01$, *** $p < 0.001$ using 1-way ANOVA and post hoc Tukey's test.

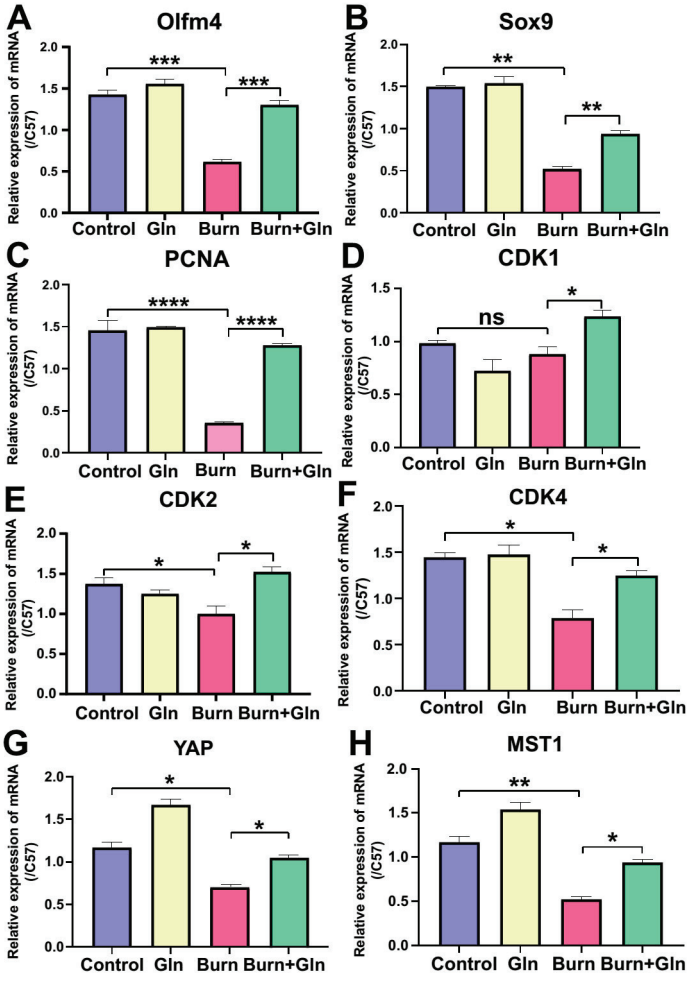

Figure 7. Gln accelerates the small intestine stem cell cycle process and promotes self-renewal. (**A**–**C**) The expression of the mRNA with stem cell markers in the four different groups according to the quantitative by Real-Time Quantitativeq Polymerase Chain Reaction (RT-qPCR). (**A**) Olfactomedin 4 (Olfm4) mRNA expression. (**B**) Sulfur Oxides 9 (SOX9) mRNA expression. (**C**) Proliferation Cell Nuclear Antigen (PCNA) mRNA expression. (**D**–**F**) The expression of the mRNA related to cell cycle of mouse small intestine crypts was measured by RT-PCR from the 4 different groups on day 3 after burn: (**D**) Cyclin-Dependent Kinase12 (CDK1) mRNA expression. (**E**) Cyclin-Dependent Kinase 2 (CDK2) mRNA expression. (**F**) Cyclin-Dependent Kinase 4 (CDK4) mRNA expression. (**G**) Yes-associated Protein (YAP) mRNA expression. (**H**) Mammalian sterile 20-like kinase 1 (MST1) mRNA expression. Data were expressed as mean ± SEM from 6 independent experiments ($n = 6$), ns no significance, * $p < 0.1$, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$ using 1-way ANOVA and post hoc Tukey's test.

2.5. Gln Promotes Proliferation, Stemness and Modulates Cell Cycle Progression of ISCs via an Activation of YAP

To determine whether Gln promotes proliferation and stemness and modulates the cell cycle of ISCs after burn injury through activating YAP. We detected the protein levels of YAP and p-YAP by Western blot. As shown in Figure 8A, the levels of Olfm4, PCNA, Cyclin

D1, and YAP were significantly decreased in the Burn group compared with control one on the first and third day, whereas they were remarkably elevated in the Burn+Gln group. Together, the level of p-YAP was ascending by burn injury on the first and third day while declining in the Burn+Gln group. In addition, the changes of these proteins on the fifth and seventh days were slight compared to the first and third days in the Burn group, but there was still a facilitative effect of Gln on these proteins (Figure 8A–F). These results suggested that YAP was restrained after burn injury, and Gln supplementation could increase the activation of YAP.

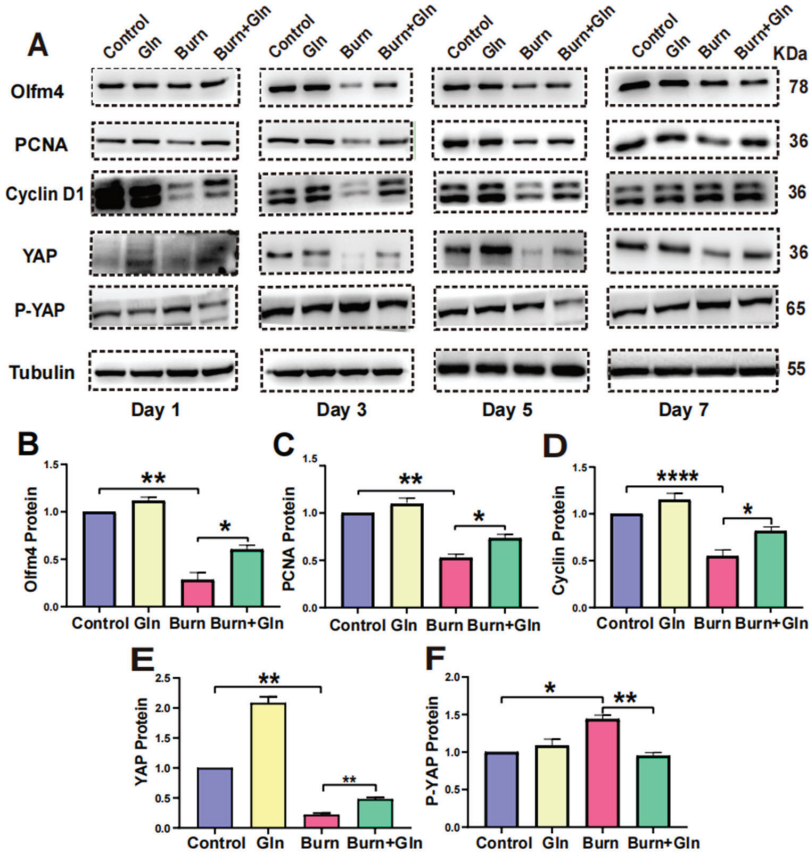

Figure 8. Gln promotes proliferation by activating the YAP-Hippo signaling pathway. (**A**) Protein abundances in crypts from C57 mice of Olfm4, PCNA, Cyclin D1, YAP, and p-Yes-associated Protein (p-YAP) were measured by immunoblotting using antibodies in small intestinal stem cell tissue treated with Gln after burn ($n = 6$). (**B**) Quantification of Olfm4 protein expression in crypts from C57 mice on Day 3. (**C**) Representative immunoblot analysis of PCNA in treated Gln. (**D**) Quantification of Cyclin D1 protein expression in crypts from C57 mice on Day 3. (**E**) Quantification of YAP expression in crypts from C57 mice on Day 3. (**F**) Quantification of p-YAP expression in crypts from C57 mice on Day3. Data were expressed as Mean ± SEM from 6 independent experiments ($n = 6$). * $p < 0.1$, ** $p < 0.01$, **** $p < 0.0001$ using 1-way ANOVA and post hoc Tukey's test.

3. Discussion

Severe burns can be led to intestinal barrier damage and inhibit its repair, which in turn triggers a series of pathophysiological changes. Determining methods to mitigate burn-induced intestinal injury and promote repair is one of the core issues in burn surgery and

intensive care medicine. In this research, we demonstrated that the intestinal pathological tissues and crypts were seriously damaged after burns. Gln treatment alleviated the damage to the pathological structure and crypts of the small intestine. Then, the experimental results certified that Gln supplementation facilitated the proliferation repair and self-renewal of ISCs by accelerating the cell cycle, thereby alleviating the injury of ISCs. Finally, this study found that the mechanism of Gln promoted the proliferation and the repair of damaged intestinal mucosa of ISCs after burn injury by activating YAP.

A previous study showed that Gln has beneficial effects on the repair of intestinal barrier damage post-burn by providing fuel for metabolism and alleviating intestinal injury, accelerating intestinal repair [9]. Whereas the impact of Gln treatment on ISCs has been reported only in very few studies [20]. Gln has an antioxidant function, which plays an important role in maintaining the redox balance of the body [8,21]. This study verified that Gln treatment decreased small intestinal injury scores, mitigated pathological injury, increased the length of the crypt, relieved crypt atrophy, and improved the survival of the crypts of small intestinal. In addition, supplementation with Gln had no significant changes in the expression levels of Caspase9 and BAX proteins of ISCs after burns (Figure 3A–C), indicating that Gln had no effects on endogenous apoptosis of ISCs. Studies have reported that ferroptosis is a new type of programmed cell death [22]. It is iron-dependent and with characteristics mainly of oxidative stress and different from apoptosis, cell necrosis, and autophagy [23]. However, Gln supplementation could decrease the ferroptosis of crypt cells in the ileum. Therefore, we were convinced that the mechanism by which Gln could alleviate the damages of ISCs and repair intestinal mucosa was independent of endogenous apoptosis while dependent on ferroptosis.

This study focused on the effects of Gln treatment on the proliferation and self-renewal of ISCs for experiments. It is reported that regulating ISC proliferation can accelerate nutrient metabolism and promote small intestinal maturation [12]. Several studies have shown that the promotion of Gln on intestinal cell proliferation is also observed in rats [24], chicks [25], and mice [26]. Our experiments also revealed that Gln treatment increased the ISCs proliferation markers (PCNA and BrdU) and stemness markers (Olfm4 and $ABCG_2$) in the crypts after burns. Interestingly, we found that the proliferating cells overlapped with ISCs in the crypts, speculating that these cells are likely to be progenitor cells [27]. The regeneration of the intestinal epithelium is driven by active ISCs at the bases of the crypts. The multipotent ISCs proliferate continuously to replace the old epithelial cells to maintain the integrity of the epithelial cells [28]. The higher quantities and percentages of multipotent cells mean an increase in the stem cells niche. A series of studies have demonstrated that cyclins play a role in enhancing pluripotency and promoting proliferation [29,30]. It was found that Gln treatment increased the ratio of cell cycle G1 of ISCs and promoted the transcription levels of cyclin CDK1/CDK2/CDK4 in our research. Cyclin D1 is a key factor in the proliferation of ISCs and is a target of the Hippo-YAP signal [31]. This study found that the supplementation of Gln promoted the expression of protein Cyclin D1 and improved ISCs proliferation after burns. Therefore, we believed that the reason why Gln supplementation promotes the proliferation, repairment, and self-renewal of stem cells after burns are due to the acceleration of the process of the stem cell cycle.

Some literature has confirmed that the alteration of YAP activity plays an important role in regulating cell injury response and post-injury repair [32]. The Hippo pathway governs organ size and tissue homeostasis in higher-order vertebrates by regulating cell proliferation and cell death [33]. YAP is a key effector in the Hippo pathway, which can promote stem cell proliferation and tissue regeneration [34]. The point of view of improving stem cell proliferation and self-renewal and promoting the phosphorylation of YAP could cause epithelial regeneration in ulcerative colitis post-inflammatory [35,36]. In order to deepen the mechanism, we clarified the regulatory relationship between MST1 in regulating the proliferation and development of ISCs through the Hippo-YAP signaling pathway. In mammals, the core of the Hippo pathway is a kinase cascade, and MST1 and YAP families are considered the key components [37,38]. In this study, it was found that Gln administra-

tion could promote the activation of YAP significantly, thereby promoting the proliferation of ISCs. Previous studies have demonstrated that the activation of YAP can promote the repair of cardiac damage [39]. Therefore, this study evidenced that the activation of YAP after Gln supplementation may explain the enhancement in the proliferation and stemness of ISCs after burns. This study demonstrated the supplementation of Gln could decrease the MST1 expression and promote the activation of YAP after burns. It was suggested that MST1 is the upstream regulatory transcription factor of the YAP protein.

In addition, the results of this paper are also verified in the literature. For instance, the activation of YAP is altered when MST1 is inactivated in response to external signals in mammals, such as low cell density, to regulate the YAP downstream domain family of transcription factors TEAD [40]. This can regulate cell proliferation and death. MST1 is homology with YAP and could act as a transcription coactivator, but there is a lack of a linear relationship between MST1 and YAP. Therefore, we speculated that the change in the activation of YAP might be regulated by other YAP regulatory factors besides MST1, such as TEAD. To further explore these issues, future studies will need to observe changes in ISCs in MST1-deficient mice. MST1 has multiple targets and may regulate YAP through multiple targets rather than one. These will become the next research focus of our future study.

4. Conclusions

This study demonstrated for the first time in the burned model and 3D organoid model that Gln supplementation alleviated proliferation inhibition and promoted self-renewal of ISCs to mitigate burn-induced intestine damage. Gln supplementation could promote the activation of YAP, expedite the cell cycle of ISCs, and alleviate intestinal mucosa damage post-burn. These findings deepen the understanding of the mechanism of Gln promoting the repair of the damaged intestinal mucosa and helping to find new regulatory targets to maintain the intestinal mucosal barrier after burns.

5. Material and Methods

5.1. Experimental Animals

The mice used in the experiment were all male C57BL/6, which were fed in the specific pathogen-free (SPF) grade animal facilities of the Clinical Medical Research Center, Southwest Hospital of Third Military Medical University. The mice were kept in the animal feeding room for one week to adapt to the environment, and then the burned mouse model was constructed. All animal experiments were conducted in strict accordance with the national and Third Military Medical University guidelines for the use of experimental animals and were approved by the Medical Ethics Committee of Third Military Medical University (ethical approval code: AMUWEC20224200).

5.2. Murine Model of Burn Injury

Based on the previous study, we constructed a burned mouse model [41]. Briefly, the 1% pentobarbital sodium (40 mg/kg) was used to anesthetize mice; the hair on the back of mice was removed with a razor and coated with hair removal cream for 20 s. Additionally, the cream was gently wiped away with medical gauze soaked with PBS so that the exposed area was about 30% of the total surface of the body. According to the experimental requirements, the mice were randomly divided into 4 groups, named Control group, Gln group, Burn group, and Burn+Gln group. The hair-free backs of mice were fixed with a special hollowed-out container so that the backs leaked out at the bottom. The backs of mice were placed in a 95 °C water bath for 12 s, then the water on the backs of mice was dried, and 1 mL of normal saline was injected into each mouse for rehydration. Gln treatment was used with intraperitoneal injection after the mice woke up, and the same volume of saline was injected in Control group. The burned mice were kept at 37 °C for 72 h and divided into 1 cage per mouse to ensure that the burned area would not be scratched by other mice.

5.3. H&E Staining and Determination of Villi Length and Crypt Depth

As described in previous study [21], we made tissue sections using small intestine tissue and performed H&E staining. Small intestine tissues of mice at different time points were taken, and the ileum was fixed with 4% paraformaldehyde. The small intestine was paraffin-embedded and sliced into 5 μm sections for long-term preservation and follow-up experiments. The paraffin sections were dewaxed and stained with hematoxylin and eosin (H&E). The staining effect was observed under microscope, then taken photos to evaluate the morphological changes of intestinal villi and crypt in each group. The Fiji ImageJ 1.8.0.345 software was used to measure the villi length and crypt depth.

5.4. PAS Staining

Goblet cells were stained using the periodic acid Schiff staining kit (Beyotime, Shanghai, China) [42]. Briefly, the paraffin sections of each group were first dewaxed with xylene and gradient ethanol, cleaned with distilled water, purified with iodate for 10 min, rinsed with tap water for 10 min and Schiff solution for 10 min, rinsed with PBS for 5 min, and nucleated with harisoxylin or Meyer hematoxylin for 3 min (too deep nuclear staining can be differentiated by hydrochloric acid alcohol). They were rinsed with running water for 5 min. Finally, routine dehydrated by gradient ethanol and xylene, sealed with neutral resin, microscopic observation, and photography records. A total of 30 experimental animals C57BL/6 mice were used.

5.5. Crypt Isolation and Culture

As described in previous study [43], crypt isolation: Fresh intestine tissues were taken from mice and rinsed with normal saline to remove contaminants, the intestine was cut lengthwise, and the inner wall of the intestine was gently rinsed. Additionally, they were then cut into small sections of 0.5 cm, impregnated in a centrifuge tube containing 40 mL sterile chelating agent (1X dissociation buffer, Ethylene Diamine Tetra-acetic Acid (EDTA) and Ethylene Glycol Tetra-acetic Acid (EGTA) 400 μL each). The tube was put in a shaker at 4 °C for 30 min, the chelating agent was discarded, and the small intestinal fragment was washed with sterile Phosphate Buffered Saline (PBS). The centrifuge tube was driven up and down with arm to separate the crypts, and the 70 μm filter was used to separate the villi and crypt. The filtrate was centrifuged at 700 rpm for 10 min, and the white precipitate on the bottom of tube was intestinal crypts. The obtained crypts were divided into three parts for culture, one part was used for culture, one part was used for extraction of total protein, and the other part was used for extraction of total RNA.

Crypt organoid culture: The obtained crypts were added to 40 mL sterile PBS containing BSA for re-suspension and centrifuged at 700 rpm at 4 °C for 5 min, and supernatant was removed. 0.5 mL washing medium was added (10 mL was prepared according to the ratio of 9.7 mL F12 base medium (Gibco, San Diego, CA, USA), 100 μL Penicillin Streptomycin (Beyotime, Shanghai, China), 100 μL Glutamax (Gibco, San Diego, CA, USA), and 100 μL NAC (Sigma, Reeds Spring, MO, USA)) to resuspend the crypts. The crypts were settled naturally for 5 min (this step can be omitted if a small number of crypts are collected), the total volume of crypts can be calculated directly according to the number of crypts required by each hole (10–30 crypts/10 μL matrix glue/96 holes). The corresponding volume of recess suspension was taken and centrifuged at 700 rpm at 4 °C for 5 min. The supernatant was discarded, and the corresponding volume of matrigel was added to the ice to re-suspend the crypts. The matrigel was dispensed at 10 μL per well to the center of the 96-well plate and then promptly placed in the incubator at 37 °C for 17 min. The 10 mL ISC culture medium consisted of 9.5 mL DMEM (Gibco, San Diego, CA, USA), 200 μL B27 (Gibco, San Diego, CA, USA), 100 μL N2 (Gibco, San Diego, CA, USA), 100 μL Glutamax (Gibco, San Diego, CA, USA), 100 μL Penicillin Streptomycin (Beyotime, Shanghai, China), and 10 μL NAC (Sigma, Reeds Spring, MO, USA). 100 μL complete medium (7 mL IntestiCult™ OGM (Stemcell, Vancouver, BC, Canada) and 100 μL Y27632 (Sigma, Reeds Spring,

MO, USA), 3 mL ISC culture medium) were added to each well, and the medium was changed every 2 days. A total of 30 experimental animals C57BL/6 mice were used.

5.6. Protein Extraction and Western Blot Analysis

Based on the previous study [21], 200 μL Radioimmunoprecipitation (RIPA) solution (Beyotime, Shanghai, China), which contains 1x protease inhibitor, was added to crack the crypts and shaken at 4 °C for 20 min to fully crack the crypts. The protein concentration was determined by Bicinchoninic Acid (BCA) kit (Beyotime, Shanghai, China). After adding the protein loading buffer, the protein was denatured at 100 °C for 10 min and stored at −80 °C refrigerator. The protein samples with the calculated concentration were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel electrophoresis. Then, they were gummed, transferred, sealed, and incubated primary antibody overnight at 4 °C. Protein primary antibody information and dilution ratio: BAX (1:1000, Proteintech, Wuhan, China), Caspase9 (1:1000, CST, Boston, MA, USA), PCNA (1:1000, Abcam, Cambridge, UK), Olfm4 (1:1000, CST, Boston, MA, USA), P-YAP (1:1000, Abclonal, Wuhan, China), YAP (1:1000, Abcam, Cambridge, UK), Cyclin D1 (1:1000, CST, Boston, MA, USA). Finally, we incubated secondary antibodies, and the ECL solution was used to reveal the bands. A total of 30 experimental animals C57BL/6 mice were used.

5.7. Immunofluorescence

As described in previous study [21], paraffin sections of mouse small intestine were used for immunofluorescence analysis. The paraffin sections were first baked at 60 °C for 2 h, then dewaxed and cleaned with PBS. The tissue sections were placed in 0.01 M sodium citrate buffer for antigen repair by microwave method, cleaned with PBS, and then coated with 3% hydrogen peroxide solution. Incubated in a wet box for 15 min to inactivate endogenous peroxidase. Goat serum was added and incubated at room temperature for 20 min to block the nonspecific antigen. The serum was discarded, and diluted primary antibody working solution was added to incubate overnight at 4 °C. Protein primary antibody information and dilution ratio: Olfm4 (1:100, CST, Boston, MA, USA), PCNA (1:100, Abcam, Cambridge, UK), Cyclin D1 (1:100, CST, Boston, MA, USA), GPX4 (1:100, CST, Boston, MA, USA), BrdU (1:100, CST, Boston, MA, USA), Olfm4 (1:100, CST, Boston, MA, USA), Cyclin D1 (1:100, CST, Boston, MA, USA), followed by drops of Alexa Fluor 488 or 594 fluorophores (Invitrogen, CA, USA), incubated at room temperature for 30 min against light, and sealed with anti-fluorescence quenching tablets, and confocal laser imaging was used. A total of 30 experimental animals C57BL/6 mice were used.

5.8. Cell Cycle Detection

As described in previous study [44], the isolated crypts were transferred to a new 15 mL centrifuge tube, resuspended with 2 mL TrypLE Express (Gibco, San Diego, CA, USA) digestive solution, and incubated at 37 °C for 5–10 min. After being blown and re-suspended several times with a 1 mL pipette, 10 μL cell suspension was taken for microscopic examination. A final concentration of 10% FBS was added, and the cell suspension was filtered through 40 μm cell sieve. The suspensions of the filtered crypts were centrifuged at 4 °C for 5 min at 500× *g*. Cells were cleaned with Basal medium twice and centrifuged at 4 °C for 5 min. Precooled 70% ethanol was added, gently blown and mixed, fixed at 4 °C overnight, and centrifuged at 1000× *g* for 5 min; the supernatant was discarded; the cells were cleaned with precooled PBS; propyl iodide staining solution was added; the cells were stained at room temperature for 30 min; the flow cytometry was used to detect the changes of cell cycle. A total of 30 experimental animals C57BL/6 mice were used.

5.9. RNA Extraction and Quantitative Real-Time PCR Analysis

As described in previous study [45], the isolated crypts were cracked by TRizol (Takara, Osaka, Japan) on ice for 10 min, the chloroform was added into an upside-down tube and

mixed, centrifuged at 4 °C at 12,000 rpm for 15 min. The upper water phase was transferred to a new tube, and the same volume of isopropyl alcohol was added and mixed, precipitated for 2 h at −20 °C, and centrifuged for 10 min at 12,000 rpm at 4 °C. The white precipitate at the bottom of the tube was RNA. The RNA was washed twice with 75% ice ethanol and dissolved in enzyme-free water after air drying in the ventilation cabinet. Nanodrop 2000 was used to measure the concentration and purity of RNA, the performed reverse transcription (Takara, Osaka, Japan), and qRT-PCR (Takara, Osaka, Japan) according to the kit instructions. A total of 30 experimental animals C57BL/6 mice were used.

5.10. Statistical Analysis

All the experimental data in this study were carried out in three independent experiments. Results were tabulated with mean ± standard deviation (SD). The GraphPad Prism 8.0 software was used to conduct single-sample *T*-test for the significant difference between the two groups, the difference between multiple samples was tested by Analysis of Variance (ANOVA). The *p*-value: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ defined a statistically significant difference between groups.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15071766/s1. Figure S1. Establishment of burn mouse model. (A) Renderings of the scab area on the back of mice in the Burn group and the Control group. (B) Body weight statistics of Burn group and Control group for 7 consecutive days after burns. Figure S2. Effects of Gln treatment on pathological damage of the small intestine after burns injury. The black arrow represents the site of injury in the small intestine. (A_1–A_4) Representative full-transverse sections of the proximal jejunum from control and Gln-treated mice stained with PAS staining: (A_1) Control group; (A_2) Burn group; (A_3) Gln group; (A_4) Burn+Gln group. (B_1–B_4) Enlarged image at the black box in (B_1–B_4) ($n = 6$). Table S1. List of primer.

Author Contributions: X.P. and F.W. provided the study idea and supervised the experiment process; X.C., P.Z., Z.Y. and Y.Z. conducted the animal experiments and collected the intestine tissues; X.C., P.Z., Y.W. and S.F. obtained test data; X.C. and P.Z. processed the data and wrote the article. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (AMUMEC20224200) on 28 February 2022.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data from this study are included in the article and Supplementary Materials. Other data could be uploaded as required.

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Conflicts of Interest: The authors declare no competing interest.

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