UNIVERSITY OF NAPLES FEDERICO II

DEPARTMENT OF PHARMACY
SCHOOL OF MEDICINE AND SURGERY

PhD course in

NUTRACEUTICALS, FUNCTIONAL FOODS AND HUMAN HEALTH

PhD Thesis

EXPLORING THE POTENTIAL OF COFFEE AND ITS BY-PRODUCTS TO MITIGATE SPECIFIC HEALTH-RELATED CONCERNS

CANDIDATE:

SONIA LOMBARDI

PhD TUTOR:
DOTT.SSA LUANA IZZO

PhD COORDINATOR:
PROF. ANGELO A. IZZO

XXXVI CYCLE
ACADEMIC YEAR 2020-2023

PREFACE

This Ph.D. thesis is submitted as a requirement for obtaining the Ph.D. Degree at the University of Naples Federico II (Italy). It is written based on three-years research conducted by the author, Lombardi Sonia, both at the Department of Pharmacy in Naples, under the supervision of Prof. Alberto Ritieni and Dr. Luana Izzo, and at the Department of Food Chemistry and Toxicology in Valencia (Spain) under the supervision of Prof. Jordi Mañes.

Sonia Lombardi

Naples, April 2024

“Quando io morirò, tu portami il caffè,

e vedrai che io resuscito come Lazzaro”

Peppino De Filippo

ABSTRACT

Coffee is recognized as one of the most popular beverages consumed worldwide due to its appreciated taste and aroma as well as its stimulating properties. The experimental activities of my PhD involved the analysis of alternative ingredients, including coffee brews and their residues, in order to evaluate their chemical, nutritional, and antiproliferative properties. Additionally, I conducted a technological examination of coffee residues, specifically spent coffee grounds, with the aim of developing innovative food products. Thus, the first part of my doctoral project focused on the health-promoting molecules in coffee, specifically polyphenols, and their potential benefits. The anti-inflammatory and antioxidant activity of coffee is higher after *in vitro* simulated gastrointestinal digestion (GiD), because of the release of compounds with higher bioactivity. Digested coffee brews exhibit increased polyphenol levels, higher antioxidant capacity, and reduced cytotoxic effects, suggesting the potential role of coffee in promoting health and well-being. The second section of this study is dedicated to exploring the objective of proposing a specific type of coffee that is suitable for individuals diagnosed with Gastroesophageal Reflux Disease (GERD). To investigate the impact of coffee on GERD, a randomized pilot study involving 40 Italian participants was conducted. The study aimed to evaluate the effects of standard coffee (SC) and dewaxed coffee (DC) on GERD symptoms and quality of life. Results indicated that consumption of DC resulted in a significant reduction in symptom frequency, increased days without heartburn and regurgitation, and

a noteworthy increase in days without the need for antacids. Further analysis using UHPLC-Q-Orbitrap HRMS showed that chlorogenic acids (CGAs) were the primary compounds found in coffee pods. Based on the preliminary data from this pilot study, it can be concluded that consuming DC may be an effective option for individuals with GERD. This finding provides a promising avenue for improving the digestibility and tolerance of coffee for those with this condition. Moreover, the chemical and nutritional characterization of a coffee by-product were investigated. Findings revealed that spent coffee grounds still retained bioactive components that could be utilized to create food items with elevated health-beneficial value. From a technological perspective, it was proposed that the incorporation of spent coffee grounds into traditional product formulations, such as biscuits, could yield food products with desirable nutritional and sensory attributes. Thus, the final part of my doctoral project involved conducting a comprehensive reassessment of coffee processing residues, with a specific focus on Spent Coffee Grounds (SCG). SCG, as one of the prominent waste by-products in the coffee industry, has garnered attention due to its environmental and economic implications in regards to food waste. Consequently, I incorporated Spent Coffee Grounds (SCG) in baked goods, specifically cookies (SCGc), in order to enhance their bioactive properties. I conducted a thorough study on the polyphenolic fraction of both SCG and SCGc, using high-resolution mass spectrometry analysis. Additionally, I evaluated the bioaccessibility of polyphenols and the changes in antioxidant activity (AA) during simulated gastrointestinal digestion (GiD). The data revealed that SCGc contained 780 mg of melanoidins, 16.2 mg of chlorogenic acid (CGA), 6.5 mg of caffeine, and 0.08 mg of phenolic acids per 100 g of samples. Furthermore, the most abundant CGA compound identified in both SCG (116.39 mg/100 g) and SCGc (8.16 mg/100 g) samples was 5-caffeoylquinic acid. The AA was assessed using three spectrophotometric tests, and the total phenolic compounds in SCGc samples showed significantly higher values compared to the control samples. Moreover, during GiD, the highest bioaccessibility of SCGc polyphenols was observed after the colonic stage, suggesting their potential health benefits for humans.

In conclusion, the results of my PhD thesis highlight the potential of coffee and its by-products in promoting health and addressing specific health-related concerns.

LIST OF PUBLICATIONS

The present research project has been documented in six separate articles that have been published in the following academic journals:

Paper I

Castaldo, L., **Lombardi, S.**, Gaspari, A., Rubino, M., Izzo, L., Narváez, A., Ritieni, A. & Grosso, M. (2021). **In vitro bioaccessibility and antioxidant activity of polyphenolic compounds from spent coffee grounds-enriched cookies.** *Foods*, *10*(8), 1837.

Paper II

Castaldo, L., Toriello, M., Sessa, R., Izzo, L., **Lombardi, S.**, Narváez, A., Ritieni, A. & Grosso, M. (2021). **Antioxidant and anti-inflammatory activity of coffee brew evaluated after simulated gastrointestinal digestion**. *Nutrients*, *13*(12), 4368.

Paper III

Castaldo, L., Toriello, M., Izzo, L., Sessa, R., **Lombardi, S.**, Trombetti, S., Ritieni, A. & Grosso, M. (2022). **Effect of Different Coffee Brews on Tryptophan Metabolite-Induced Cytotoxicity in HT-29 Human Colon Cancer Cells.** *Antioxidants*, *11*(12), 2458.

Paper IV

Polese, B., Izzo, L., Mancino, N., Pesce, M., Rurgo, S., Tricarico, M. C., **Lombardi, S.**, De Conno, B., Sarnelli, G., & Ritieni, A. (2022). **Effect of Dewaxed Coffee on Gastroesophageal Symptoms in Patients with GERD: A Randomized Pilot Study.** *Nutrients*, *14*(12), 2510.

PUBLICATION OF DIVULGATIVE SCIENTIFIC ARTICLE

Lombardi, S.* (2022). **Il benessere espresso. "**Salute Mentale e Cognitiva"
NutraHorizons
https://digital.teknoscienze.com/nutra_horizons_3_2022_ita/il_benessere_espresso

Other publications:

1. Castaldo, L., **Lombardi, S.**, Izzo, L., & Ritieni, A. (2023). **Exploring the Chemical Composition of Female Zucchini Flowers for Their Possible Use as Nutraceutical Ingredient.** *Antioxidants*, *12*(12), 2108.

2. Lima da Silva, J., **Lombardi, S.*,** Castaldo, L., Morelli, E., Garda-Buffon, J., Izzo, L., & Ritieni, A. (2023). **Multi-Mycotoxin Analysis in Italian**

Grains Using Ultra-High-Performance Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry. *Toxins*, *15*(9), 562.

3. Narváez, A., Izzo, L., Castaldo, L., **Lombardi, S.**, Rodríguez-Carrasco, Y., & Ritieni, A. (2023). **Multi-Mycotoxin Method Development Using Ultra-High Liquid Chromatography with Orbitrap High-Resolution Mass Spectrometry Detection in Breakfast Cereals from the Campania Region, Italy.** *Toxins*, *15*(2), 148.

4. Izzo, L., Castaldo, L., **Lombardi, S.**, Gaspari, A., Grosso, M., & Ritieni, A. (2022). **Bioaccessibility and antioxidant capacity of bioactive compounds from various typologies of canned tomatoes.** *Frontiers in Nutrition*, *9*, 849163.

5. Izzo, L., Mikušová, P., **Lombardi, S.**, Sulyok, M., & Ritieni, A. (2022). **Analysis of Mycotoxin and Secondary Metabolites in Commercial and Traditional Slovak Cheese Samples.** *Toxins*, *14*(2), 134.

6. Castaldo, L., Izzo, L., **Lombardi, S.**, Gaspari, A., De Pascale, S., Grosso, M., & Ritieni, A. (2022). **Analysis of Polyphenolic Compounds in Water-Based Extracts of Vicia faba L.: A Potential Innovative Source of Nutraceutical Ingredients.** *Antioxidants*, *11*(12), 2453.

7. Castaldo, L., Izzo, L., Gaspari, A., **Lombardi, S.**, Rodríguez-Carrasco, Y., Narváez, A., Grosso M., & Ritieni, A. (2021). **Chemical Composition of green pea (Pisum sativum L.) pods extracts and their potential exploitation as ingredients in nutraceutical formulations.** *Antioxidants*, *11*(1), 105.

LIST OF ABBREVIATIONS

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid assay (ABTS)

2,2-diphenyl-1-picrylhydrazyl assay (DPPH)

AA Antioxidant activity

C-5HT Carboxylic acid-5-hydroxytryptamides

$CaCl_2(H_2O)_2$ Calcium chloride dihydrate

CGA Chlorogenic acid

CGAs Chlorogenic acids

CEN European Committee for Standardization

DC Dewaxed coffee

dSPE Dispersive Solid-Phase Extraction

ENEA Italian institute for new technologies, energy and the environment

ESI Electrospray ionization

ESI-MS Electrospray ionization mass spectrometry

FA Formic acid

FAO Food and Agriculture Organization of the United Nations

$FeCl_3$ Ferric chloride

FRAP Ferric reducing antioxidant power

GC-MS Gas chromatography mass spectrometry

GERD Gastroesophageal reflux disease

GSH Glutathione

H_2O Water

HCOOH Formic acid

HMWM High Molecular Weight Melanoidins

HPLC High performance liquid chromatography

ICO International Coffee Organization

IFN-γ Interferon gamma

ILs Interleukins

$K_2S_2O_8$ Potassium persulfate

KCl Potassium chloride

KH_2PO_4 Potassium dihydrogen phosphate

LC Liquid-chromatography

LC-MS/MS Liquid chromatography tandem mass spectrometry

LDL Low-density lipoprotein

LPC lysophosphatidylcholine

LOD Limit of Detection

LOQ Limit of Quantification

MAO-A Monoamine oxidase A

Magnesium chloride hexahydrate $MgCl_2(H2O)_6$

MeOH Methanol

MCI Mild Cognitive Impairment

MS Mass spectrometry

MS/MS Tandem mass spectrometry

MW Molecular weight

Na_2SO_4 Sodium sulfate

NaCl Sodium chloride

NaH_2PO_4 Monosodium phosphate

$NaH(CO_3)_2$ Ammonium carbonate

$NaHCO_3$ Sodium bicarbonate

NaOH Sodium hydroxide

NMR Nuclear Magnetic Resonance

PAHs Polycyclic aromatic hydrocarbons

QuEChERS quick, easy, cheap, effective, rugged and safe

R_2 Coefficient of determination

ROS Reactive Oxygen Species

RT Retention time

S/N Signal-to-noise ratio

SCG Spent coffee ground

SCGc Spent coffee ground cookies

SGF Simulated Gastric Fluid

SIF Simulated intestinal Fluid

SLE Solid/Liquid extraction

SSF Simulated Salivary Fluid

TPC Total polyphenolics content

TPC Total polyphenol content

TNFα Tumor necrosis factor

TPTZ 2,3,5-triphenyltetrazolio chloride

UHPLC High performance liquid chromatography

WHO World Health Organization

LIST OF CONTENT

Chapter 1
Introduction

Coffee is more than just a morning invigorating tonic beverage; it is a global phenomenon that has transcended cultures and time (Castaldo L., et al. 2020). In this introduction, I explored the world of coffee, covering its origins, varieties, production process, consumption, health benefits, by-products, and economic importance.

1.1. Coffee: an overview

Coffee is a widely consumed and highly esteemed beverage derived from the roasted seeds of the Coffea plant (Belitz, H. D. 2009). Renowned for its delectable flavor and pleasing scent, coffee derives its stimulating properties from caffeine (Ramalakshmi, K., & Raghavan, B. 1999). Coffee holds a significant position in numerous cultures and societies, offering solace and facilitating social connections (Ferreira, J., Ferreira, C., & Bos, E. 2021). Moreover, coffee has played a pivotal role in fostering intellectual discussions, serving as a symbol of social engagement, and contributing to economic growth (Morris, J. 2018). In contemporary times, the coffee culture has expanded to encompass various brewing methods and the surging popularity of specialty coffee captivates enthusiasts worldwide.

The narrative of coffee's origins is replete with legend, culture, and discovery, leaving an indelible impact on the global stage (Teuber, R. 2010). Coffee boasts a captivating history that commences in the highlands of Ethiopia (National Coffee Association). The coffee plant, scientifically known as Coffea and belonging to the *Rubiaceae* family produces cherry-like fruits called coffee cherries, within which the coffee beans are encased. These beans, when skillfully extracted, roasted, and ground, serve as the fundamental components in the creation of the coffee we savor (Belitz, H. D. 2009).

Coffee's cultivation can be traced back to the 9th century in the Arab world, specifically in Yemen. By the 15th century, coffee had reached Persia (modern-day Iran), where coffee houses became an integral part of Persian culture. In the early 17th century, coffee began to make its way to Europe. The first coffeehouse in Venice opened in 1645, followed by coffeehouses in England and France (Myhrvold, N. 2024). In the 18th century, coffee was introduced to the New World. Caribbean, Central, and South American countries embraced coffee cultivation, with Brazil emerging as a dominant coffee-producing nation (Ukers, W. H. 1935). Moreover, the establishment of coffee plantations in the Americas reshaped global coffee production and became a vital part of many Latin American economies in the present days (Harvey, C. A., et al. 2021).

1.1.1. Exploring the world of beans and brews

The genus *Coffea* encompasses numerous plant species. The original taxonomical investigations of the *Coffea* L. genus were conducted by Chevalier in 1947, who classified the species based on the morphology of their flowers. Subsequent studies by Leroy in 1980, Bridson in 1982, and Stoffelen in 1998 contributed to the most recent taxonomical classification, which was adopted by Davis et al. in 2006. This classification includes 103 species, with 41 originating from Africa, 59 from Madagascar, and 3 from the Mascarene Islands—an archipelago located in the Indian Ocean (Davis, A., et al. 2006) Out of all the known species, only three are of significant economic interest: *Coffea arabica*, *Coffea canephora* (or Robusta), and *Coffea liberica* (Davis, A. P., et al. 2022). These species not only differ in terms of plant morphoanatomy but also in the quality of the beverage produced during seed processing. Arabica yields coffee that is considered to be higher in quality, with a less bitter and more persistent flavor, and it accounts for three-quarters of global production. Robusta, on the other hand, constitutes one-quarter of production and produces coffee of lower quality, characterized by a fuller body, weak aroma, very bitter (sometimes astringent) taste, and higher caffeine content. *Liberica* coffee cultivated mostly in Malaysia, exhibits the highest dimensions, with lower bulk density in both berries and beans. Additionally, Coffea arabica, primarily cultivated in Latin America, yields a superior-quality product (Clifford, M. N. 2012; Ismail, I., et al. 2014).

Over fifty countries are engaged in coffee production, and they are situated in the geographical belt spanning from the Tropic of Cancer (23.43695° N) to the Tropic of Capricorn (23.43695° S), encompassing three continents: Africa, Central and Caribbean America, and Asia (Figure 1). The same plantation produces varying fruits from year to year, influenced by seasons, climate, and meteorological conditions (DaMatta, F. M., et al. 2007). The most suitable climate for coffee cultivation is warm and humid, at elevations ranging from 600 to 2000 meters for Arabica coffee, with a required temperature of approximately 18–21°C. On the other hand, Robusta coffee can tolerate temperatures as high as 28°C and can be grown at lower altitudes, between 200 and 900 meters (Pohlan, H. A. J., et al. 2010; Davis, A. et al. 2012).

The growth of coffee as a global commodity was intricately linked to the establishment of coffee plantations. Currently, as mentioned above coffee is cultivated in over fifty countries worldwide, with the majority of production focused in Brazil, Vietnam, and Colombia. These three countries together account for 56.4% of the global coffee supply (International Coffee Organization (ICO), Prices Production; FAOSTAT, 2023).

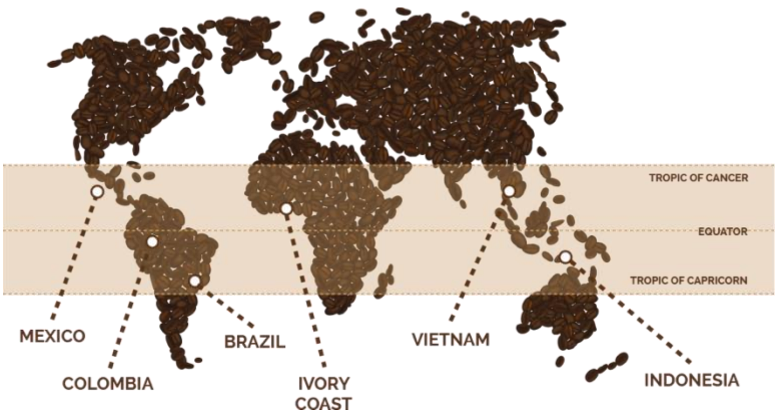


Figure 1. Coffee Belt

1.2. The global coffee industry

In the global context, an astounding 3.1 billion cups of coffee are consumed daily, which is equivalent to approximately 170.8 million 60kg sacks of coffee. The global roasted coffee market was valued at around $120 billion in 2022, and these figures consistently show an upward trend each year. It is projected that coffee consumption will continue to rise and could potentially reach 208 million sacks by 2030, resulting in a staggering 3.8 billion cups consumed per day. Despite the presence of numerous coffee varieties, it is worth noting that only two, namely Arabica and Robusta, hold significant commercial relevance. In 2022, Arabica accounted for 56.2% of global production. However, the Robusta variety has seen a gradual increase in its market share, rising from 39.2% in the 2012/2013 season to 43.8% in the 2021/2022 season. This shift can be attributed to its heightened resistance to climate conditions and pests, as well as its higher yields.

Although coffee cultivation is geographically limited, the trade of coffee knows no such bounds. As of October 2023, the global export volume of green coffee beans reached 8.57 million bags. Additionally, it is expected that global coffee consumption will experience a 2.2% increase, reaching 178.0 million bags during the 2023/24 year (International Coffee Organization, ICO 2023). In terms of consumption, Europe stands as the largest consumer, accounting for 31.7% of the total, followed by North America at 18.7%. Other regions serve as both production and consumption areas, with Asia/Oceania at 23.9%, Central and South America at 18.8%, and Africa at 6.9%. Northern European countries such as Finland, Sweden, and Norway exhibit the highest coffee consumption rates, with Finland averaging 4.4 cups per day, Sweden at 3.2, and Norway at 2.6. Italy ranks seventh globally, consuming roughly 5.2 million sacks annually, which translates to approximately 95 million cups of coffee per day. Ground coffee dominates the market, accounting for 73.6% of total sales. Moreover, the sale of coffee pods and capsules experienced a significant increase of 18.8% between 2020 and 2021. However, formats such as whole beans and instant coffee are less favored.

Italy is particularly renowned for its excellence in coffee roasting and stands as the sixth-largest global exporter of coffee, boasting the highest quantity of roasted coffee. Within the European Union, Italy ranks as the second-largest exporter, second only to Germany. It is also worth noting that the majority of Italian coffee roasters are located in the southern regions (ICO; EUROSTAT; FAO; Area Studi Mediobanca). Italy boasts a flourishing coffee culture that places paramount emphasis on the cultivation of high-quality espresso. Typically served in small cups, the iconic espresso shot is firmly associated with Italian coffee, particularly in cities like Naples. These cities are praised for their coffee customs, as coffee has become deeply ingrained in the fabric of Italian daily life, with significant focus on the convivial aspects of enjoying caffeinated beverages at cafes.

As we carefully analyze the complex patterns and data related to the dynamics of the coffee industry and consumer consumption behaviors, it becomes clear that our examination goes beyond simple numerical representations. This thorough examination serves as a gateway to a more comprehensive reflection on the trajectory of the coffee sector, as it enters a new era characterized by significant challenges and a pressing commitment to sustainability.

In conclusion, the interdependent relationship between current industry trends and discerning consumer preferences has propelled the coffee sector within the European Union, particularly in Italy, into a transformative phase. Beyond the realm of quantitative measures, there are qualitative shifts that indicate an era in which challenges not only act as obstacles to overcome, but also as drivers of innovation and resilience. The combination of increasing demands, growing environmental awareness, and prevailing market dynamics places the industry at a crucial point, demanding a strategic realignment towards sustainable practices. This transition represents a profound evolution, where navigating through obstacles becomes synonymous with steering the industry towards a more sustainable and ethically grounded future.

1.2.1 The coffee processing stages: from seed to cup

Figura 2. Kahwei Yoong - November 16, 2021

The journey from coffee cherry to roasted bean is a meticulous process that greatly impacts the final flavor profile. This process involves various layers and methods, such as silver skin, parchment, pectin, pulp, and outer skin, which protect the coffee bean nestled within the cherry. The extraction process of the bean may vary slightly depending on coffee type and local tradition. However, in general, it can be divided into several steps including harvesting, separation, cleaning, peeling (for natural coffees only), bean extraction, fermentation, washing, drying, degreasing (for washed coffee only), separation, classification, and packaging. The initial stage of coffee production is the harvesting of ripe coffee fruits from the plants. This can be done manually or with the assistance of machinery. During the harvesting process, care is taken to separate overripe or unripe fruits from those at the appropriate level of ripeness to ensure the coffee's flavor is not negatively affected. Following the harvest, there are two primary methods for processing the coffee beans: wet processing and dry processing (Vincent, J. C. 1987).

Wet processing, also referred to as washed processing, involves a meticulous and controlled approach to extracting the coffee beans. The outer skin and pulp are mechanically removed, exposing the parchment layer. Subsequently, the beans undergo fermentation, which aids in the breakdown of the mucilage surrounding the parchment. After thorough washing and drying, the coffee beans are extracted. This method is renowned for producing beans with clean, vibrant flavors that showcase the unique characteristics of the coffee. In contrast, the natural or dry-process is a more traditional method commonly employed in regions of South America. After harvesting, the coffee cherries are laid out to dry, still encompassed by the parchment and mucilage layers. As the cherries dry, the pulp disintegrates, allowing the beans to absorb fruity flavors from the mucilage. Once fully dried, the beans are hulled to reveal the coveted coffee seed. This process imparts a robust and fruity flavor profile to the beans, characterized by a full body and pronounced sweetness. After the fermentation process, the coffee beans undergo a comprehensive washing procedure to eliminate any residual mucilage or impurities. Following this, they are placed on tables or racks to be dried, which reduces their moisture content and prepares them for storage. The "washed" processing method may include an additional step of degreasing to eliminate any remaining fat that could potentially impact the flavor of the coffee. A third processing method, known as semi-washed, employs a combination of both dry and wet techniques. Initially, coffee cherries are washed and sorted in flotation tanks, followed by depulping without fermentation. Subsequently, the depulped coffee, still encased in its mucilage, undergoes the drying process directly. Notably, this approach has been adopted in coffee-producing regions such as Central Africa and Brazil, resulting in the production of a coffee type known as natural depulped coffee (Poltronieri, P., & Rossi, F. 2016).

Traditional processing methods, such as natural, washed, and semi-washed, have been enhanced by the integration of innovative techniques (Várady, M. et al. 2022) that impart unique characteristics upon the coffee beans. Some of these novel processing approaches include carbonic maceration, which involves the fermentation of beans in an environment rich in carbon dioxide; honey processing, a method that influences the duration of fermentation, the type of mucilage removed, and other factors; and fermentation processing, which encompasses the controlled fermentation of coffee cherries to influence flavor. Fermentation processing can be conducted with or without water, resulting in intricate flavor profiles. Another method is soaking processing, where coffee beans are immersed in water to remove the pulp prior to the drying phase. Therefore, each processing method impacts the aromatic complexity, acidity, body, and other sensory attributes of coffee, yielding a wide range of flavor profiles (Elhalis, H., et al. 2021).

Following the drying phase, the coffee beans are sorted based on size and quality to ensure uniformity within each coffee batch regarding size and appearance. Once all processing steps are finalized, the coffee beans are packaged in suitable bags or containers for transportation and sale (Clifford, M. N., et al. 2012).

Therefore, the primary commodity being exchanged is the green coffee bean, which is produced during the initial processing phase. As previously mentioned, the green coffee bean undergoes the roasting process upon importation by different countries, depending on their desired coffee qualities. The color, flavor, and aroma of the coffee are determined based on the preferences of the consuming country. The process of obtaining the desired coffee involves three stages: (i) in the first phase, the moisture content in the bean is partially reduced at a temperature of 180°C; (ii) by increasing the temperature (200-300°C), chemical reactions, including the Maillard reaction, occur, resulting in the formation of compounds responsible for the aroma and flavor; (iii) the final phase involves cooling the beans with streams of cold air or water (Pereira, et al. 2019).

1.2.1.1. Impact of roasting methods

The roasting process of coffee beans induces considerable alterations in their chemical composition. The process entails various chemical reactions that bring about the creation and transformation of diverse compounds. Throughout the roasting phase, several crucial modifications transpire in the chemical composition of coffee, including a reduction in chlorogenic acids and trigonelline, as well as the formation of desirable and undesirable compounds. The Maillard reaction and caramelization that occur during roasting give rise to compounds that contribute to the appealing flavor, aroma, and color of coffee. Nevertheless, the production of potentially carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs) and acrylamide can also transpire (Esquivel and Jiménez, 2012). Moreover, compounds that have an impact on the flavor and aroma of coffee can be influenced, as over 1000 chemical compounds, including acids, alkaloids, phenols, and antioxidants, undergo modifications during the roasting process (Tarigan, E. B., et al, 2019; Diviš, P., et al, 2019).

Following the roasting process, the Coffee Silverskin, which is the innermost coating of the bean, is removed (Castaldo, L., et al. 2020). However, it is not only the Silverskin that necessitates a virtuous recycling loop within the coffee production cycle. Coffee Silverskin and Spent Coffee Grounds (SCG) significantly contribute to environmental pollution due to their high concentration of organic compounds. These compounds require a considerable amount of oxygen for degradation. Additionally, substances like caffeine, polyphenols, and tannins increase the risk of acute toxicity. Therefore, if Coffee Silverskin and Spent Coffee Grounds are disposed of in the environment without treatment, they could pose a potential pollution threat (Arya S. S., et al. 2022). Furthermore, the quality of drinking water and the survival of various aquatic species are compromised. Wet processing, indeed, not only consumes excessive amounts of water but also contaminates wastewater with these compounds, endangering aquatic life (Woldesenbet et al. 2014). Hence, it is crucial to integrate the coffee production cycle into a circular economy context and adopt an approach that addresses the needs of environmental, social, and economic sustainability.

1.2.1.2. The dewaxing process

Green coffee beans are composed of a thin waxy layer known as coffee wax. The wax typically constitutes approximately 0.2-0.3% of the total weight of the coffee bean (Speer, K., & Kölling-Speer, I. 2006). The process used to serve the primary purpose of eliminating the waxy coating that envelops the coffee bean is named dewaxing (Van der Stegen, G. H. D. 1979).

The primary constituents of coffee wax are carboxylic acid-5-hydroxytryptamides (C-5HT). This group encompasses amides of serotonin (5-hydroxytryptamine, 5HT) and fatty acids with varying chain lengths. Initial investigations by Wurziger and colleagues identified three 5HT compounds containing arachidic, behenic, and lignoceric acid. Subsequent research revealed additional acid-5HT compounds, including stearic acid-5HT, 20-hydroxy-arachidic acid, and 22-hydroxy-behenic acid-5HT.

Technological procedures such as polishing, dewaxing, steaming, or decaffeinating coffee beans not only reduce the presence of carboxylic acid-5-hydroxytryptamides (C-5HT) in coffee wax but also enhance the digestibility of the coffee. This discovery, supported by multiple studies, prompted the development of steaming methods in 1933 to minimize potential digestive discomfort for certain coffee consumers (Lendrich, P. 1933).

The method employed for dewaxing involves the utilization of solvent-based techniques, specifically the use of a chlorinated organic solvent known as dichloromethane, in order to selectively extract coffee wax from the beans. Nevertheless, it should be noted that this process also leads to the concurrent removal of caffeine from the beans (European patent application, 1985). Thus, the dewaxing process requires meticulous calibration to ensure that the treated coffee maintains a caffeine content higher than 0.10%. Should the caffeine level fall below this threshold, the coffee must be classified as decaffeinated. The production and trade of dewaxed coffee are regulated by the Ministerial Decree of 22 June 1983. This decree outlines the toasted coffee product must not contain more than 250 ppm (parts per million) of waxy residues, equivalent to 250 mg per kilogram. The residual traces of dichloromethane used in the dewaxing process should not exceed 2 mg per kilogram in the toasted coffee product. The raw coffee must not have a moisture content exceeding 11%. The product must be clearly labeled as "dewaxed coffee" upon being introduced to the market. Furthermore, the product label must prominently display the humidity level and specify that the wax content does not surpass 30% of the original quantity (Italian Ministerial Decree of 22 June 1983). In conclusion, the elimination of waxes during the coffee production process yields several health-related advantages. These include enhanced digestibility and decreased acidity levels. Additionally, the coffee retains substantial amounts of polyphenols and antioxidants, despite the removal of waxes. Finally, it is finely ground and possesses a delicate quality, making it a suitable choice for individuals afflicted with heartburn and stomach acidity. Consequently, opting for this specific coffee variety enables individuals to indulge in moments of relaxation without triggering any discomfort to their digestive system.

1.3. Coffee's impact extends far beyond its flavor

"A strong association between coffee consumption and pancreatic cancer was evident in both sexes", asserted Brian MacMahon and his colleagues in 1981, as documented in their publication in the New England Journal of Medicine (MacMahon, B., et al. 1981). However, it was later discovered that this particular study had flaws. Many individuals were cautious about discussing the apparent risks associated with the consumption of one of the world's most widely enjoyed beverages, deeply rooted in the cultural traditions of every nation (Coffee, Tea or Paranoia, The Washington Post, 1981). Subsequently, numerous studies have convincingly demonstrated the beneficial health effects that can be derived from the habit of consuming coffee, a practice that remains deeply entrenched and virtually irreplaceable.

The most well-known effect associated with coffee is arguably its stimulating property. Due to its caffeine content, the consumption of coffee leads to increased alertness throughout the day, providing a boost of energy that is beneficial in managing the demands of modern times. Caffeine, by virtue of its chemical composition, has the ability to interact with adenosine receptors, acting as an antagonist. Through competitive antagonism on these receptors, caffeine increases dopamine levels, which are responsible for the stimulating and addictive effects (reference is made to 1.4.2.1.). Additionally, caffeine works in collaboration with adrenaline and noradrenaline to activate the sympathetic nervous system, thus synergistically functioning with these neurotransmitters (Rodak, K., et al. 2021). Improved perception, reduced fatigue, and decreased drowsiness are some of the stimulating effects attributed to caffeine, while its long-term effects include implications for memory consolidation (Borota, D., et al. 2014). Furthermore, caffeine has been associated with a protective role in Alzheimer's disease, as demonstrated by research conducted by Lindsay, et al. in 2002.

1.3.1. A short focus on mental health benefit

Coffee is a complex mixture of bioactive compounds, which presents a challenge in isolating the specific effects of individual components within the beverage. The beneficial properties of this plant-based drink are often attributed to polyphenols, a type of antioxidant molecule. Research has shown that certain polyphenols have anti-inflammatory properties and can protect against certain diseases (reference is made to 1.4.1.). Notably, studies indicate that decaffeinated coffee also exhibits some of the same risk-reducing effects for diabetes and heart disease, suggesting that caffeine alone is not solely responsible for the beneficial effects associated with coffee consumption (Ding, M., et al. 2014).

Numerous epidemiological studies and meta-analyses have been conducted on coffee, consistently reporting its positive effects and highlighting a reduced risk of developing various chronic diseases (Tajik, N., et al. 2021). Moreover, it plays a fundamental role in protecting against degenerative diseases (Castaldo, L., et al. 2021), as well as exhibiting neuroprotective properties and positive effects on mood and/or anxiety disorders (Carneiro, S.M. et al. 2021; Wasim, S., et al. 2020; Yenisetti, S.C., et al. 2016; Chu, Y. F., et al. 2009).

1.3.1.1. In search of mental well-being

Nowadays, individuals are faced with fast-paced and demanding lifestyles that contribute to elevated levels of anxiety and stress. According to the World Health Organization (WHO), mental health is a state of well-being that enables individuals to realize their potential, effectively cope with everyday pressures, achieve productive and fulfilling work, and make valuable contributions to their communities (WHO/Europe, Fact sheet - Mental health, 2019). Consequently, an inadequate social environment, socioeconomic disparities, or sudden traumatic experiences can disrupt an individual's mental well-being. Mental and neurological disorders, such as depression, anxiety, and dementia, constitute 13% of the global disease burden, surpassing cardiovascular diseases and cancer. The number of people suffering from depressive disorders has exceeded 300 million, with an increase of more than 18% between 2005 and 2015 (Owen, L. et al. 2017). Furthermore, recent data published in Lancet indicate that the COVID-19 pandemic has resulted in an additional 53 million cases of depression (+28%) and 76 million cases of anxiety disorders (+26%) worldwide in 2020 (Santomauro D. F., et al. 2021).

1.3.1.2. A complex blend: Anxiety, Depression and Cognitive decline

A systematic literature review of the role of coffee and its constituents, particularly caffeine, indicates that moderate consumption of caffeine has a positive impact on mood, cognitive functions, and physical abilities. Furthermore, it is linked to a decrease in the likelihood of experiencing depressive symptoms (McLellan, T. M., et al. 2016). Caffeine is widely recognized within the scientific community as a cognitive enhancer; consuming doses ranging from 32 to 300 mg of caffeine results in improved attention, alertness, and reaction time. Conversely, consuming high doses (equivalent to 5 or more cups of coffee per day) may trigger panic attacks in individuals with heightened sensitivity and induce anxiety in both healthy adults and those who are more susceptible (Klevebrant, L., et al. 2022). Additional investigations revealed that for caffeine at higher doses to produce anxiogenic effects, a predisposition to specific anxiety disorders must be present. However, it is improbable that complete abstinence from caffeine would lead to a significant improvement in mood and/or anxiety disorders (Lara, D. R., et al. 2010).

An important enzyme responsible for the deamination of neurotransmitters, such as serotonin (5-HT), in the central nervous system is monoamine oxidase A (MAO-A). The decrease in 5-HT levels can lead to affective disorders, resulting in depression and imbalances in the sense of satiety. A recent study assessed the extent of MAO-A inhibition by chlorogenic acids found in green coffee beans and roasted coffee extracts. The analysis demonstrated that coffee, particularly in its green form, has the potential to function as an antidepressant by inhibiting MAO-A, thereby increasing the concentration of 5-HT and enhancing its bioavailability (Grzelczyk, J., et al. 2021). Additionally, according to Asil et al. 2021, the relationship between caffeine and depression varies depending on the quantity of daily caffeine intake. Several studies have indicated that individuals who consume four or more cups of coffee per day have a significantly lower risk of depression compared to those who consume one cup of coffee or less. Consistent with these findings, a study found that consuming 450-600 mg of caffeine resulted in a

61% reduction in the risk of depression compared to consuming 0-300 mg of caffeine (Rucci, S., et al. 2011).

Thanks to a recent scientific study, the association between caffeine and the risk of dementia and/or cognitive decline has been examined. The study analyzed 61 clinical studies conducted between 1990 and 2020, involving a total of 153,070 subjects. By assessing both short and long-term effects, the investigation revealed positive associations that were influenced by the amount and type of caffeine consumed, as well as the gender and age of the patients. Specifically, a positive short-term effect was observed, particularly when moderate quantities of caffeine (100 - 400 mg/day) were consumed consistently. Additionally, the positive effects were found to be more pronounced in women compared to men. However, it is worth noting that a few studies reported conflicting results based on other factors such as age, dietary habits, or previous illnesses (Chen, J. Q., et al. 2020). In fact, a separate study monitored the plasma concentration of caffeine in patients with Mild Cognitive Impairment (MCI) over a period of 2 to 4 years and observed a reduction in disease progression when caffeine plasma levels exceeded 1200 ng/mL (Cao, C. et al. 2012).

Thus, the importance of proper nutrition and diet for maintaining good health is increasingly recognized (Manippa, V. 2021). Coffee has been identified as a valuable asset in this regard, prompting the question: how much coffee should be consumed to enhance mental health? The impact of caffeine on mental well-being can be influenced by factors such as gender, body weight, and individual variations in caffeine sensitivity. Consequently, providing a definitive answer is a formidable task. Nonetheless, the scientific community concurs that in order to experience beneficial and neuroprotective effects, it is advisable to consume coffee in moderation and avoid excessive intake (EFSA; Nehlig, A. et al. 2016).

1.3.2. A brief examination of the beneficial effects of coffee consumption

Multiple scientific studies have demonstrated the positive impact of coffee consumption on human health. One significant advantage is its ability to decrease the risk of metabolic syndromes and diabetes mellitus (Shang, F., et al. 2016). This can be attributed to the antioxidant properties of coffee and its capacity to enhance insulin sensitivity (Shahinfar, H., et al. 2021). Additionally, coffee consumption has the potential to act as a protective factor against colorectal cancer (Vitaglione, P., et al. 2012). Certain components present in coffee have been identified as having chemopreventive properties (Galeone, C., et al. 2010 - Higdon, J., Frei, B. 2006 - George, S. E., et al. 2008). Furthermore, coffee has been associated with cardiovascular health benefits, including a reduced likelihood of developing coronary heart disease and an improvement in antioxidative status (Butt, M. S., & Sultan, M. T. 2011). This can be attributed to ingredients such as chlorogenic acid and caffeic acid, which can inhibit inflammation and safeguard against endothelial damage

(O'Keefe, J., et al. 2013 - O'Keefe, J., et al. 2018). Moreover, animal and in vitro studies have indicated that coffee consumption stimulates autophagy, a cellular process crucial for muscle turnover, maintenance, and regeneration (Dirks-Naylor, A. J. 2015). Through the stimulation of autophagy in various tissues, including skeletal muscle, regular coffee consumption can enhance insulin sensitivity and help prevent sarcopenia, the age-related loss of muscle mass and strength (Guo, Y., et al. 2014). In fact, elderly mice that consumed caffeinated coffee exhibited greater muscle weight and strength, along with lower levels of serum inflammatory mediators. These findings suggest that coffee may play a role in preserving muscle function and preventing age-related decline (Dirks-Naylor, A. J. 2015) (for a more comprehensive understanding of the specific benefits provided by the distinct major components of coffee, please consult section 1.4.).

1.4. The chemistry of coffee

Coffee is a beverage that possesses a remarkably intricate composition, consisting of over 1000 phytochemicals from various chemical classes. Among these are phenolic compounds, namely chlorogenic acids and their derivatives, diterpenes such as cafezol and kahweol, methylxanthines like caffeine, theobromine, and theophylline, as well as nicotinic acid (vitamin B3) and trigonelline (de Melo Pereira, G. V. 2020). The purpose of this section is to explore the molecular profile of coffee, with a specific emphasis on its fundamental constituents that play a significant role in shaping its unique characteristics and widespread appeal as a popular beverage.

The constitution of green coffee beans has been extensively studied. Carbohydrates constitute approximately 50% of the dry weight and encompass both soluble and insoluble polysaccharides such as cellulose, galactomannans, and arabinogalactans, as well as oligosaccharides, disaccharides, and monosaccharides like glucose, galactose, arabinose, fructose, mannose, mannitol, xylose, and ribose (Farah, A. 2012). Lipids make up about 18% of the composition and consist of triglycerides, sterols, and fatty acids, primarily of the unsaturated variety, present in both free and esterified forms with diterpene alcohols. Proteins, peptides, and free amino acids collectively contribute to 16% of the dry weight, with notable amino acids including asparagine, lysine, alanine, aspartic acid, and glutamic acid. The mineral content is observed to be around 4%. Following the roasting process, the chemical composition of coffee undergoes minor alterations. The main bioactive compounds in green coffee decrease slightly, while roasted coffee contains approximately 29% of small molecules called melanoidins, which are formed as a result of the Maillard reaction. Nonetheless, the primary beneficial effects associated with coffee consumption are attributed to various bioactive compounds, including alkaloids, polyphenols, and melanoidins (Hu, W., et al. 2019; Barbosa, M. D. S. G., et al. 2019).

1.4.1. Polyphenols

Polyphenols are a large group of secondary metabolites synthesized by plants as a defense mechanism against ultraviolet radiation and pathogenic attacks (Manach, C., et al. 2004). Recently, there has been increasing interest in these compounds due to their well-documented antioxidant activity in laboratory and animal studies. One of the primary functions of polyphenols is their ability to convert reactive oxygen species (ROS) into non-radical species (Figure 3). They work in collaboration with endogenous antioxidants, such as glutathione (GSH), glutathione peroxidase, superoxide dismutase, catalase, and dietary vitamins, to counteract oxidative stress in cells (Quideau, S., et al. 2011). Polyphenols can be classified into two main classes based on their chemical structure: flavonoids and non-flavonoids. The flavonoid class includes flavanols, flavones, anthocyanidins, isoflavonoids, and neoflavonoids. On the other hand, the non-flavonoid class consists of phenolic acids, lignans, tannins, and stilbenes. Among the phenolic acids, hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic) are the most abundant polyphenolic constituents found in coffee and coffee by-products. The esterification of hydroxycinnamic acids with quinic acid leads to the formation of chlorogenic acids (CGAs), which are considered to be one of the most important bioactive compounds present in coffee.

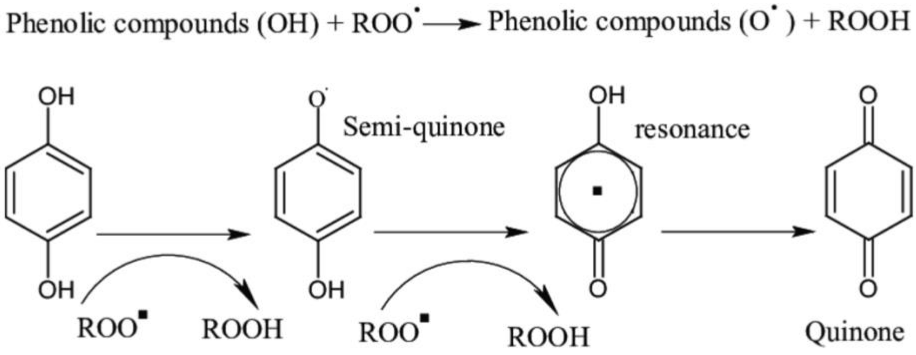


Figure 3. Antioxidant action mechanism of polyphenols – from Ref. Smetanska, I. (2018)

Chlorogenic acids (CGAs) are the primary polyphenolic compounds identify in coffee. Numerous studies have shown that CGAs possess significant physiological properties, including strong free radical scavenging activity and anti-inflammatory and antioxidant effects. These properties may help explain the preventive health benefits associated with coffee consumption. Furthermore, the antioxidant and anti-inflammatory activities of chlorogenic acids contribute to the regulation of disorders related to metabolic syndrome, which involve various inflammatory processes. For example, CGAs protect the endothelium by inhibiting the action of proinflammatory molecules such as interleukins, TNFα, lysophosphatidylcholine (LPC) - a lipid found in oxidized LDL with atherogenic properties - and endothelial adhesion molecules (Tajik, N., et al. 2017).

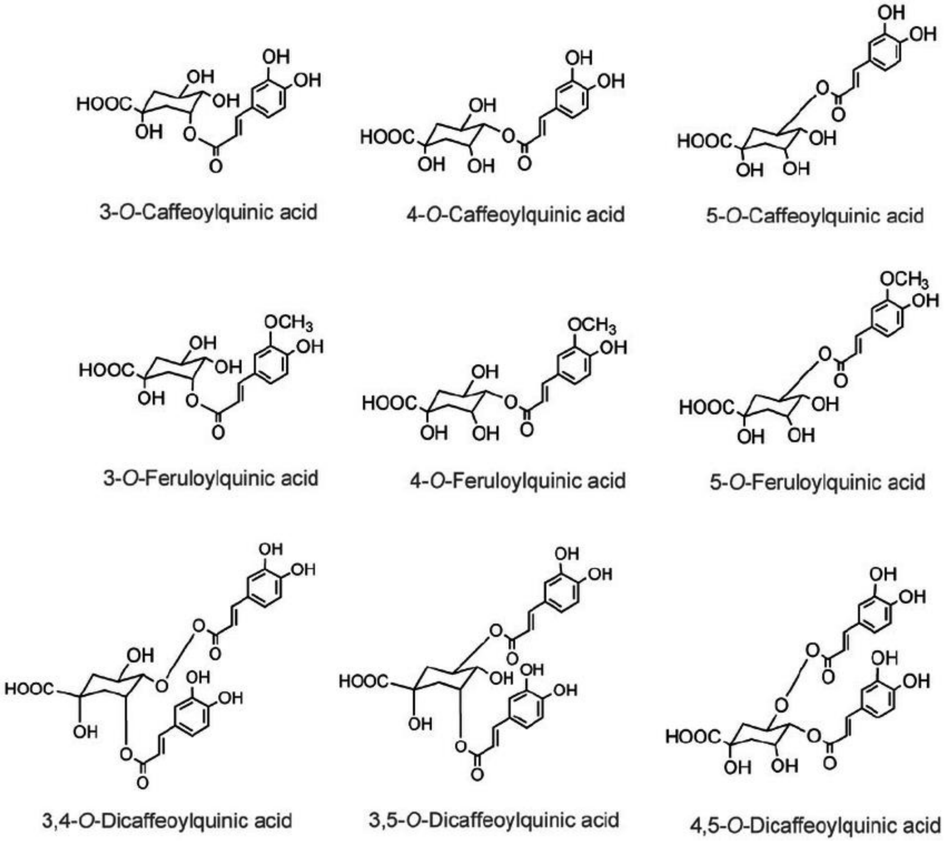


Figure 4. Major chlorogenic acids found in coffee - from ref. Stalmach, A. (2006)

1.4.2. Alkaloids

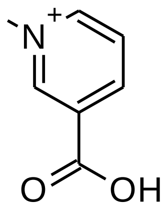


Figure 5. Trigonelline structure

Trigonelline, caffeine, theobromine, and theophylline are the main alkaloids present in coffee. Trigonelline (Figure 3), a pyridine alkaloid derived from nicotinic acid, undergoes thermal degradation during the coffee roasting process. This degradation results in the formation of volatile compounds that contribute to the aroma of coffee. Extensive research has been conducted on the bioactivity of trigonelline, exploring its potential in preventing cardiovascular disorders, providing neuroprotection, and inhibiting carcinogenesis. Trigonelline has been found to regulate key enzymes involved in glucose and lipid metabolism, including glucokinase, glucose-6-phosphatase, fatty acid synthase, and carnitine palmitoyl transferase. As a result of this regulation, diabetic mice exhibited reduced levels of blood sugar, lipids, and cholesterol. (Zhou, J. 2012). Furthermore, the anti-carcinogenic properties of trigonelline can be attributed to two primary mechanisms: inhibition of cellular invasion, crucial for tumor cell proliferation (Hirakawa, N., et al. 2005), and regulation of Nrf2 transcription factor expression. Nrf2, activated under normal conditions to protect cells from damage, is overexpressed in tumor cells, leading to chemotherapy resistance. However, trigonelline enhances sensitivity to chemotherapeutic agents by inhibiting Nrf2 expression (Arlt, A., et al. 2013).

1.4.2.1. Caffeine

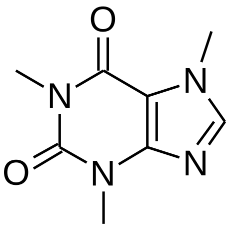


Figure 6. Caffeine Structure

Caffeine is a natural alkaloid that can be found in various substances, including coffee, cocoa beans, tea leaves, Guarana berries, Cola nuts, and is also produced synthetically as a food additive (Figure 4). However, the majority of daily caffeine intake comes from coffee, with the amount varying depending on the beverage type. Caffeine belongs to the xanthine class, which are purine bases formed through the deamination of the guanidine nucleus and the oxidation of hypoxanthine catalyzed by xanthine oxidase. Positions 1, 3, and 7 of caffeine undergo methylation by a group of methyltransferases known as SABATH, resulting in the formation of theobromine (3,7-dimethylxanthine) and then caffeine (Huang, R., et al. 2016). Caffeine is known to have 100% oral bioaccessibility and is metabolized by cytochrome CYP1A2 in the liver, resulting in the formation of paraxanthine, theophylline, and theobromine (Schwarzschild, M., et al. 2003). The elimination half-life of caffeine ranges from 4 to 9 hours, with various factors such as gender, liver function, pregnancy, contraceptive use, and age influencing its duration in the body. It is worth noting that the effects of caffeine tend to last longer in children and young individuals (Fredholm, B. B., et al. 1999).

Caffeine acts as an antagonist on several subtypes of adenosine receptors including A1, A2a, A2b, and A3 due to its chemical structure. A1 receptors are predominantly found in the central nervous system, including regions like the hippocampus, cerebellum, and cortex, along with peripheral organs such as the lungs, kidneys, bladder, and heart. Studies have demonstrated that blocking these receptors can potentially yield beneficial outcomes such as hypertension reduction, improved cognitive function, amelioration of Alzheimer's disease symptoms, anxiety mitigation, and enhanced renal function. A majority of A2a receptors are located in brain tissues associated with dopamine production, while the A2b subtype is present in the gastrointestinal system. The A3 subtype, on the other hand, is distributed in various organs including the liver, kidneys, lungs, heart, and inflammatory cells on their surface (Hu, G. L., et al. 2019). Through competitive antagonism of adenosine receptors, caffeine elevates dopamine levels, resulting in its stimulant properties and addictive potential. Furthermore, caffeine synergizes with adrenaline and noradrenaline to activate the sympathetic nervous system (Lean, M.E.J. et al. 2012). These stimulant effects include heightened alertness, reduced fatigue, and decreased sleepiness. Long-term investigations indicate that caffeine may also influence memory consolidation and potentially play a protective role in the progression of Alzheimer's disease (Borota, D., et al., 2014; Lindsay, J., et al. 2002). Additionally, by increasing dopamine release through A2 receptor blockade, caffeine may alleviate symptoms associated with Parkinson's disease, such as tremors, muscle rigidity, and "freezing gait". This is significant in the context of Parkinson's, a neurodegenerative disorder characterized by the gradual loss of dopaminergic neurons in the extrapyramidal system's substantia nigra

(Trevitt, J., et al. 2009; Kitagawa, M., Houzen, H., and Tashiro, K. 2007). Research conducted on healthy individuals suggests an inverse relationship between caffeine consumption and the onset of Parkinson's symptoms (Ascherio, A., et al. 2001), while patients may experience slower disease progression (Hong, C. T., et al. 2020). Another extensively recognized effect of caffeine is its antinociceptive property, rendering it a valuable adjunct to analgesic medications, particularly in the treatment of headaches. The analgesic impact of caffeine is attributed to its vasoconstrictive action, which arises from inhibiting the vasodilatory activity of adenosine. This aligns with the "purinergic" theory of migraines put forth in 1989, positing that migraines are triggered by vasodilation resulting from the activation of P2y receptors in the cerebral region (Burnstock, G. 1989). Furthermore, the analgesic activity of caffeine seems to be connected to the restraint of leukotriene and prostaglandin synthesis, both of which play a role in migraine pathophysiology (Antonova, M., et al. 2013).

1.4.3. Melanoidins

Melanoidins, also known as High Molecular Weight Melanoidins (HMWM), are heterogeneous high-molecular-weight compounds present in coffee beans after the roasting process, which contribute to their brown coloration. The formation of melanoidins occurs during the Maillard reaction, a non-enzymatic chemical reaction that is initiated by high temperatures. This reaction involves the interaction between a reducing sugar and an amino acid

(Nooshkam, M., Varidi, M., & Bashash, M. 2019). The Maillard reaction can be divided into three stages: (i) in the first stage, the carbonyl group of the reducing sugar reacts with the amino group of the amino acid, leading to the formation of a Schiff base. This Schiff base then undergoes cyclization to form a substituted N-glycosylamine (Figure 7); (ii) in the second stage, Amadori rearrangement products are degraded into ketones, furfural, and other intermediate compounds that can further react with amino acids. Simultaneously, the open form of ketosamine from the first stage undergoes dehydration and deamination, yielding dicarbonyls; (iii) in the final stage, these dicarbonyls, through Strecker degradation and a series of condensation reactions with various compounds, result in the formation of melanoidins (Echavarría, A. P., Pagán, J., & Ibarz, A. 2012).

Scientific studies have revealed that melanoidins exhibit a protective effect against diseases induced by free radicals, such as hepatitis, atherosclerosis, and colon carcinoma. This protective effect is attributed to the intrinsic antioxidant activity of these compounds (Yu, M. et al. 2018; Daglia, M. et al. 2008). Both the soluble and insoluble fractions of melanoidins function as dietary fiber, remaining intact until they reach the intestine. There, they are subjected to fermentation by the gut microbiota, displaying properties commonly associated with soluble fiber (Gniechwitz, D., et al. 2008). In vitro studies have also demonstrated that melanoidins are capable of reducing the risk of colon cancer by inhibiting the activity of metalloproteinases (MMPs), a family of endopeptidases that play a pivotal role in tumor growth and metastasis (Rufián-Henares, J. A., & Morales, F. J. 2007). Based on these findings and future research, melanoidins are believed to play a crucial role in the defense and prevention of various health conditions.

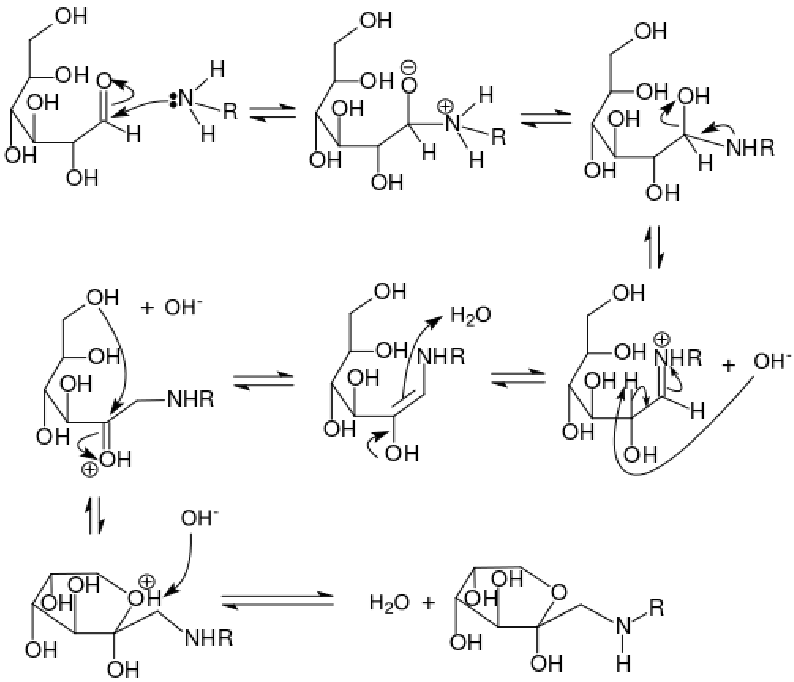


Figure 7. First stage of the Maillard reaction: attachment of the amino function to the carbonyl group of the reducing sugar, formation of the Schiff base and intramolecular rearrangement, with formation of N-substituted glycosylamine

1.4.4. Waxes

Oils and waxes are essential components, comprising 8 to 18% of the total dry mass (Esquival P., Jimenez V. M. 2011). One of the molecules found in coffee beans that has received relatively little attention in scientific research is -Nβ-

Alkanoyl-5-hydroxytryptamine (C-5-HT), which falls under the category of waxy substances. These waxes are situated in the cortical region of the coffee bean and are challenging for the human body to digest and absorb due to their solubility properties (they become soluble at approximately 65 °C) (Van der Stegen, G.H.D. 1979; Rentz, J.A., et al. 2004). In certain individuals, they may even cause mild irritation to the gastric mucosa. To address these concerns, an innovative and delicate extraction process known as dewaxing is employed to eliminate the waxy layer from unroasted coffee, along with a small quantity of caffeine, using an organic solvent (reference is made to paragraph 1.2.1.2.).

1.5. Coffee in a Circular Economy

The concept of the circular economy has emerged as a crucial notion in Europe, signifying a transition towards sustainability, resource efficiency, and environmental accountability (Lacy, P., et al. 2016). The main challenge faced by member states of the European Union revolves around achieving sustainability through the effective and sustainable reuse of the Earth's resources, while minimizing environmental impact. The circular economy embodies this idea by establishing a system in which all activities, from extraction to production, are organized in a manner that ensures waste becomes a resource for another process. This economic model not only safeguards the environment but also enables cost savings in production and management (Gunasekaran, A., & Spalanzani, A. 2012). At the core of the circular economy lies the principle of decoupling economic growth from resource depletion. This is achieved through the promotion of a system that emphasizes the reuse, refurbishment, remanufacturing, and recycling of products and materials, in contrast to the linear economy model of "take, make, dispose" (Camacho-Otero, J., et al. 2018) The circular economy prioritizes waste reduction, prolonging the lifespan of products, and adopting a regenerative approach to resource utilization (Ngan, S. L., et al. 2019). Europe has taken a leading role in driving the circular economy forward through robust policy initiatives and frameworks. The European Green Deal, adopted in 2019, places sustainability at the forefront and includes specific commitments to the circular economy. Within the European Community, Italy stands out as a frontrunner in this field. The country boasts the highest waste recycling rate in Europe, reaching approximately 75% for total municipal and special waste, which is double the European average. Moreover, Italy ranks first in circularity among the five largest European economies (Italy, Spain, France, Poland, Germany), as indicated by the recent "Report on the Circular Economy in Italy" by CEN and ENEA.

Europe's dedication to the circular economy exemplifies a visionary approach to addressing the interconnected challenges of resource scarcity, environmental degradation, and climate change (Yang, M., et al. 2023). By embracing circularity, Europe not only strives for economic resilience but also positions itself as a global leader in sustainable development.

1.5.1. Coffee and Sustainability: brewing a greener future

The initial phase of the coffee production chain involves producers from economically disadvantaged nations, with limited safeguards for workers and the environment. Several studies indicate that farmers contribute only a small percentage, not exceeding 3%, to the overall value of a cup of coffee (Coffeebarometer, 2023). This situation has prompted major roasters to prioritize sustainability issues. Large coffee companies have acknowledged the environmental and social impacts of coffee production and have taken steps to enhance sustainability in the industry (Bager, S. L., et al. 2020). By implementing specific requirements for sustainable coffee production and quality, these companies aim to mitigate the adverse effects on the environment (Lingnau, V., et al. 2019).

Deforestation poses a significant environmental challenge in coffee production. The rising demand for coffee has led to intensified cultivation, which contributes to environmental impacts, such as the replacement of tropical forests with coffee plantations, leading to soil erosion (Rattan, S. et al. 2015). Moreover, the excessive use of nitrogen-intensive fertilizers contributes to climate change and exacerbates environmental problems. These challenges underscore the need for more sustainable farming practices that prioritize the conservation of natural resources and reduce the carbon footprint of coffee production (Beverfood, 2020).

In terms of water consumption, the Water Footprint Network states that approximately 130 liters of water are required to produce a 125 ml cup of coffee, a ratio deemed unsustainable and requiring urgent reduction. Considering the vast number of coffee consumers worldwide, this issue becomes particularly critical. It is also important to note that the coffee production chain encompasses over 120 million individuals who depend on the cultivation and processing of coffee in more than 70 countries worldwide (Troncone, A., 2020). Given the large number of stakeholders in this sector, it is inevitable to assert that the coffee industry carries numerous responsibilities, both environmentally and socially.

Finally, the growing awareness of this issue emphasizes the urgent need for more sustainable production practices and an effective waste management system, including alternative utilization of by-products by the food industry.

1.5.1.1. Kimbo Spa: from local roots to global success

Kimbo is an Italian coffee company that started as a small family-run bar-pastry shop in Naples after World War II. The Rubino brothers, Francesco, Gerardo, and Elio, transformed their business into a small roastery and later into an industrial operation. In 1955, they established Café do Brasil, which later changed its name to Kimbo Caffè in 1963. The company's success grew as they capitalized on new coffee packaging techniques and introduced a revolutionary vacuum-sealed can. This allowed them to export their coffee beyond Naples and eventually throughout Italy and even internationally.

The success of Kimbo can be attributed to its association with the traditional Neapolitan coffee culture and its commitment to quality and excellence. The company has invested in infrastructure, such as its Melito factory near Naples, which undergoes continuous updates to ensure high production standards and social and environmental sustainability. In the 2000s, Kimbo embraced a managerial approach while maintaining its family ownership. The company focused on international growth, particularly in the United States and Asia. Kimbo's mission is to promote Italian coffee worldwide, offering a product inspired by Neapolitan tradition and made with advanced technologies while respecting people and the environment. The company emphasizes quality control throughout the coffee production process, from selecting the best beans to roasting and blending. The packaging is also carefully done to preserve the aroma and taste.

Committed to social responsibility and environmental protection, the Kimbo Melito factory has received certifications for environmental policies and quality control. With a turnover of approximately 200 million euros and over 600 employees, Kimbo is a major player in the Italian coffee market. The company has also expanded internationally, with a presence in over 80 countries and around 22% of its total turnover coming from abroad.

Specifically, as its sustainability initiative, Kimbo has developed and implemented the "Integrity" project. This program has made the Latin American plantations, which produce the new Kimbo-branded blend, sustainable and certified by the Rainforest Alliance. This organization aims to protect the ecosystem, conserve biodiversity, and provide real opportunities for growth to the local populations, rather than impoverishing the territory through the purchase of raw materials from developing countries. Hence, the Kimbo company has consistently demonstrated its commitment to promoting sustainable agriculture, protecting the ecosystem, and addressing social issues within the supply chain (Kimbo, 2022). Regardless, there is unfortunately limited information about the project available on the website or other sources. Thus, some may argue that the existing insufficiency of information is insufficient for a company that holds the second-largest retail position in the Italian market, particularly when considering the imperative to prioritize sustainability initiatives. Nevertheless, over the past three years, the company has made substantial investments in research to address the global issue of product reuse more effectively. The aim is to extend the lifespan of their products and mitigate the environmental impacts of waste generation.

Moreover, in order to thrive in an increasingly competitive and intricate market, the company must take into account other significant factors, such as the growing consumer emphasis on health and well-being (MarkUp, 2022). To address these emerging needs, the company has developed enhancer products that align with the realm of coffee. For instance, they have formulated new blends like Kimbo "Amico," which involves reducing the wax coating on raw coffee beans. This results in a less acidic coffee with a rich flavor, preserving all its properties and aromas.

In conclusion, the company's continual dedication to this context is apparent in its proactive commitment to sustainability and innovation. While upholding its distinct quality, the company demonstrates its awareness of global challenges and its aspiration to adopt more environmentally-friendly business practices.

1.5.1.2. Valorization of Agri-food waste

In the pursuit of sustainable practices, there has been a shift in the focus of scientific inquiry towards the valorization of vegetable waste. In this paragraph, I will delve into the utilization of food-grade extraction methods for extracting valuable compounds from vegetative by-products.
With the global population expanding and environmental concerns mounting, it is imperative to address waste management and tap into previously unexplored resources. The incorporation of food-grade solvents in the extraction process represents a conscientious endeavor to align scientific innovation with principles of sustainability (Chemat, F., et al. 2012). By exploring the potential of vegetable waste as a reservoir for bioactive compounds and employing extraction techniques that adhere to food-grade standards, this research aims to make a contribution to the ongoing discourse

on eco-friendly strategies in waste management and value addition in the agri-food sector.

1.5.1.2.1. The importance of sustainable extraction techniques

The utilization of the "food grade" extraction method presents several advantages in comparison to conventional extraction techniques employing organic solvents.

Water emerges as an environmentally friendly and cost-effective solvent. It possesses characteristics such as non-toxicity, non-flammability, and the potential for clean processing and pollution prevention. The solubility of substances in water extraction fluctuates with temperature, with low-temperature extraction favoring water-soluble substances and high-temperature extraction targeting less soluble compounds. Procedures that necessitate simple water-based extraction are generally safe for human consumption (Dominguez, H., & Muñoz, M. J. G. 2017). This solvent can be completely eliminated from the final product, reducing the risk of contamination from organic solvent residues and enhancing food safety. From an environmental sustainability standpoint, the utilization of "food grade" solvents diminishes the environmental impact in comparison to traditional organic solvents (Mahato, N., et al. 2017). Furthermore, the implementation of "food grade" solvents aids in maintaining the quality of the extracted ingredients (Norshazila, S. 2017). They assist in preserving the flavor, aroma, and nutritional value, resulting in higher-quality final products. Lastly, the industry can reap benefits from the adoption of "food grade" extraction techniques. Products obtained through these procedures comply with food regulations and regulatory requirements, ensuring production that adheres to safety standards.

To summarize, the adoption of "food grade" extraction techniques not only enhances food safety, but also presents environmental and operational advantages to the industry. This contributes to sustainable development and the production of high-quality products.

1.6. Analytical strategies for bioactive compounds: methods and approaches

Analyzing bioactive compounds entails the utilization of sophisticated analytical methodologies to identify and quantify these substances within complex matrices. Present-day scientific research strongly emphasizes the application of green chemistry. In order to guide the design of processes and materials in research laboratories, Anastas and Warner's 12 principles of Green Chemistry have been introduced (Anastas P. T., et al. 2000). These principles center around reducing the consumption of reagents and solvents, minimizing emissions and waste, eliminating toxic reagents, and decreasing labor and energy consumption. To align with these principles, various strategies are implemented, including the use of alternative extraction methods (such as Supercritical Fluid Extraction and Pressurized Liquid

Extraction) for sample preparation without the use of solvents, real-time sensor-based direct analysis for on-site measurement without hazardous substances, and the implementation of "greener" reaction conditions such as photochemical, microwave-assisted, and ultrasound-assisted techniques. The aim of these techniques is to enhance product yield, conserve energy, and minimize waste production (Duarte K., et al. 2014).

1.6.1. Sampling and Extraction techniques

Current methods for polyphenol determination typically involve the preparation of the sample using compatible solvents to extract these bioactive compounds (Gil-Martín, E., et al 2022). Subsequently, a purification step is usually performed to obtain a more refined extract prior to quantification (Wollgast, J., & Anklam, E 2000).

1.6.1.1. Solid Liquid Extraction

The structural diversity of polyphenols and their presence in various plant matrices present a challenge when attempting to establish a standardized extraction protocol for these compounds. Solid/liquid extraction (SLE) is a commonly utilized technique for this purpose (Teixeira, D. M., et al. 2006). The process begins by preserving the sample through lyophilization or freezing. Once preserved, the sample is dissolved in solvents such as water, methanol, ethanol, acetone, or a combination thereof. The pH of the solvent used is an important factor to considerduring the extraction process. Polyphenols exhibit greater stability in acidic environments as they primarily exist in their neutral form under such conditions (Deng, J., et al. 2018). Consequently, a small amount of strong acid is added to acidify the mixture. However, excessive acidification can lead to hydrolysis of the compounds, particularly since polyphenols are predominantly present in glycosylated or esterified forms. This can potentially result in changes to chromatographic profiles during separation. Acidification is particularly advantageous when dealing with complex glycosides as it simplifies the separation process (Tsao, R. 2010). Solid-liquid extraction often incorporates the use of an ultrasonic bath, which employs mechanically amplified vibrations to penetrate the cells of the matrix and enhance the transfer of cellular content, including polyphenols, into the solvent (Horžic, et al. 2012).

1.6.1.2. Novel extraction techniques

Newer extraction techniques include Pressurized Liquid Extraction, which utilizes elevated pressures and temperatures to enhance extraction efficiency, and Microwave-Assisted Extraction (MAE). MAE is an innovative extraction technique where a predominantly polar solvent, selected based on its dielectric constant, dipole moment, and energy dissipation factor, is employed. The solvent, along with polyphenols, which are permanent dipoles, absorbs electromagnetic radiation and converts it into heat energy through dielectric heating (Eskilsson, C. S. 2000).

1.6.2. Instrumental techniques for bioactive compounds characterization

Within the realm of scientific research, a range of instrumental techniques are employed to identify and quantify bioactive compounds derived from natural sources (Donno, D., et al. 2020). One widely utilized method is high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), which enables the separation and detection of diverse compounds with high sensitivity. Nuclear magnetic resonance (NMR) spectroscopy provides valuable structural information about bioactive molecules, facilitating their characterization. Additionally, gas chromatography-mass spectrometry (GC-MS) is utilized for the analysis of volatile compounds. Gas Chromatography-Mass Spectrometry (GC-MS), indeed, has limited applicability in the investigation of polyphenols due to the requirement of converting the sample into the vapor phase, which can compromise the chemical stability of thermolabile polyphenols. In GC-MS analysis, the sample is vaporized before being injected into the system. However, this process can lead to the decomposition or loss of polyphenols that are sensitive to heat. Therefore, alternative methodologies such as Liquid Chromatography-Mass Spectrometry (LC-MS) are often preferred for polyphenol analysis. LC-MS enables a gentler separation process and better preservation of the chemical stability of these thermolabile compounds (Câmara, J. S., et al. 2020).

HPLC-MS, which stands for High-performance Liquid Chromatography-Mass Spectrometry, is an advanced chemistry technique that combines the separation capabilities of liquid chromatography, with the precise mass analysis capabilities of mass spectrometry. This analytical method is widely utilized for various applications due to its exceptional sensitivity and selectivity. An HPLC-MS system enables swift and mass-directed purification of natural-product extracts and novel molecular entities. These applications are particularly relevant to industries such as food, pharmaceuticals, and agrochemicals. Typically, HPLC-MS is employed for the detection and potential identification and quantification of individual components of mixtures, particularly when dealing with organic compounds spanning low to high polarity in liquid or solid matrices. It is renowned for its exceptional performance and rapid execution, primarily attributed to the high-pressure pump system that propels the eluent into the column, as well as the reduced particle size (3-10 μm) of the stationary phase. Regarding the analysis of polyphenols, it has been observed that the most effective columns are those featuring a C18 reverse-phase (silica derivatized with 18-carbon alkyl chains) (Bajkacz, S., et al. 2018). In recent years, HPLC has been superseded by its successor, Ultra High-Performance Liquid Chromatography (UHPLC), in both qualitative and quantitative analyses of diverse compounds. UHPLC presents significant advantages over conventional HPLC, as it employs a packed stationary phase with a smaller particle diameter (< 2 μm), allowing it to surpass the conventional pressure limits of 400 bar. This facilitates increased chromatographic speed without compromising efficiency (Chesnut, S. M., and Salisbury, J. J. 2007). The instrumental evolution of UHPLC translates into higher chromatogram efficiency and resolution, resulting in narrower and well-separated peaks.

Mass spectrometry (MS) is the widely utilized technique for compound characterization, as it provides information on analyte masses and molecular formulas in the form of molecular ions. The basic principle involves chemically ionizing compounds using an energy beam from various sources. The mass-to-charge ratio (m/z) is then employed to separate ions in the analyzer, thereby determining the sensitivity and resolution of the analysis. In recent years, High-Resolution Mass Spectrometry (HRMS) has emerged, offering enhanced precision and accuracy in mass measurement. This enables more reliable results and higher resolution, capable of distinguishing ions with very similar m/z values (Wood, M. 2019). Among the most advanced analyzers within HRMS, the Q-Orbitrap stands out. The Q-Orbitrap technology commences by subjecting ions to filtration via a quadrupole prior to their entry into the Orbitrap compartment. This compartment is equipped with electrodes positioned at both ends, in addition to a central electrode. Once within the compartment, the ions initiate oscillation around the central electrode at their individual frequencies, subsequently translated into m/z (mass-to-charge ratio) data. In the case of fragmentation events, the precursor ions are directed towards a dissociation chamber. The resulting product ions subsequently return to the Orbitrap for the determination of their respective m/z values. This technique provides precision and accuracy in detecting molecular ions within the ppb (μg/kg) range (Makarov, et al. 2006). Moreover, it offers enhanced selectivity for analyzing matrices such as food, which contain a diverse array of compounds. Additionally, it possesses the capability to conduct full scan analysis and retrospective data analysis without the need for re-runs, thereby facilitating untargeted compound identification (Van Wijk, X. M., et al. 2019).

1.6.3. Antioxidant Activity and Total Polyphenol Content

Taking on a pivotal role within biological systems, antioxidants are recognized as powerful guardians against oxidative stress, diligently working to maintain cellular balance. This esteemed group of defenders includes well-known entities such as vitamins C and E, flavonoids, polyphenols, as well as key enzymes like superoxide dismutase and catalase. Their primary objective involves counteracting the potentially detrimental effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS), natural byproducts of cellular metabolism that, if left uncontrolled, can lead to damage (Halliwell, B., & Gutteridge, J. M. 2015). Antioxidants employ a strategic approach, generously donating electrons or hydrogen atoms to neutralize and eliminate free radicals. Additionally, they demonstrate proficiency as metal chelators, hindering the formation of free radicals (Gutteridge, J. M., & Halliwell, B. 2010). The assessment of antioxidant capabilities is carried out through various assays, including the dynamic 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the insightful ferric reducing antioxidant power (FRAP) assay. Spectrophotometric methods play a central role in unveiling the overall polyphenol content, offering a detailed glimpse into the phenolic composition that defines the antioxidant profile of each sample.

Polyphenols are commonly evaluated for their antioxidant activity through the employment of spectrophotometric assays. The most frequently employed assays are the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the ferric reducing antioxidant power (FRAP) assay. Within the ABTS and DPPH assays, the sample reacts with a chromogenic radical ($ABTS^{\cdot+}$ and $DPPH^{\cdot}$), and the antioxidant compound neutralizes it, leading to a modification in color. This alteration in color is then quantified using a spectrophotometer. As for the FRAP assay, there is no presence of a radical; instead, the reagent supplies the Fe^{3+} cation. This cation undergoes a redox reaction with the antioxidant, leading to its reduction to Fe^{2+}. The antioxidant capacity is determined in Trolox equivalents, which serves as a reference standard possessing a high antioxidant potential (Shahidi, F., and Zhong, Y. 2015).
Finally, it is important to emphasize the Folin-Ciocalteau assay, a method employed for quantifying the overall polyphenol content. This particular technique evaluates the capacity of polyphenols to reduce the Folin-Ciocalteau reagent, which consists of phosphomolybdic and phosphotungstic acid within an alkaline medium. Subsequently, a blue chromophore is

generated through a resulting reaction, and this chromophore possesses an ability to absorb light at 760 nm. To determine the total polyphenol content (TPC) in a given sample, the absorbance value derived from this reaction is compared to that of gallic acid, which is employed as a standard for constructing a calibration curve. This curve is then utilized to convert absorbance values into concentration units, thereby facilitating the quantification of the total polyphenol content within the sample (Singleton, V. L., & Rossi, J. A. 1965).

1.6.4. Assessment of bioavaibility

The process of substance transportation throughout the human body comprises several stages: absorption, distribution, metabolism, and excretion. These stages give rise to three fundamental concepts: bioavailability, bioaccessibility, and bioactivity. Bioavailability quantifies the amount of a substance that enters the bloodstream upon introduction to the body, offering valuable insights into its effectiveness. It includes both bioaccessibility and bioactivity. On the other hand, bioaccessibility refers to the percentage of a nutrient or bioactive compound that is released from a food matrix and becomes accessible for absorption in the gastrointestinal tract during digestion. Additionally, bioaccessibility plays a crucial role in determining the bioactivity of phenolic compounds in various forms, such as pharmaceuticals and functional foods. It is important to note that the bioactivity of phenolic compounds observed in vitro may differ significantly from that observed in vivo. This discrepancy primarily stems from the lower bioaccessibility of certain phenolic compounds within the digestive system (Grgić, J., et al. 2020). However, by exploring the realms of bioavailability and bioaccessibility, we can develop a comprehensive understanding of the complex dynamics governing the assimilation and impact of substances within the human body (Figure 8).

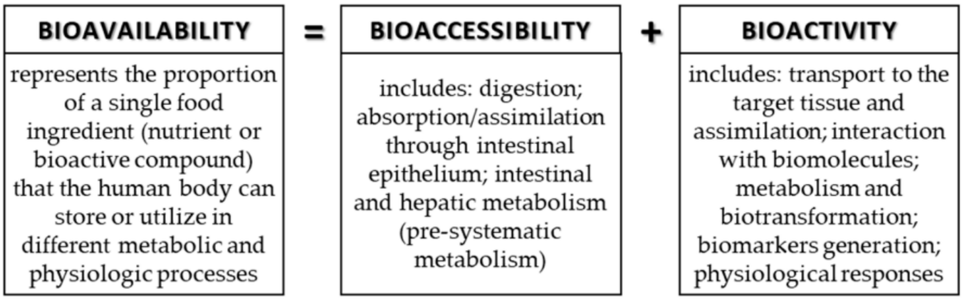


Figure 8. Connection of terms: bioavailability, bioaccesibility and bioactivity, from Ref. Grgić, J., et al. 2020.

Therefore, a comprehensive comprehension of the intricate food digestion process is paramount for comprehending the journey of nutrients throughout the human body and their subsequent effects on human health. Significant resources have been devoted by scientists to replicate this process under controlled laboratory conditions. In this regard, static in vitro simulation of the gastrointestinal system has emerged as a valuable technique, enabling researchers to mimic the physiological conditions of the human digestive system. By employing this method, researchers can effectively investigate the mechanisms underlying the breakdown, absorption, and release of nutrients during the initial stages (Brodkorb, A., et al. 2019). Figure 9 schematized in vitro gastrointestinal digestion (GiD).

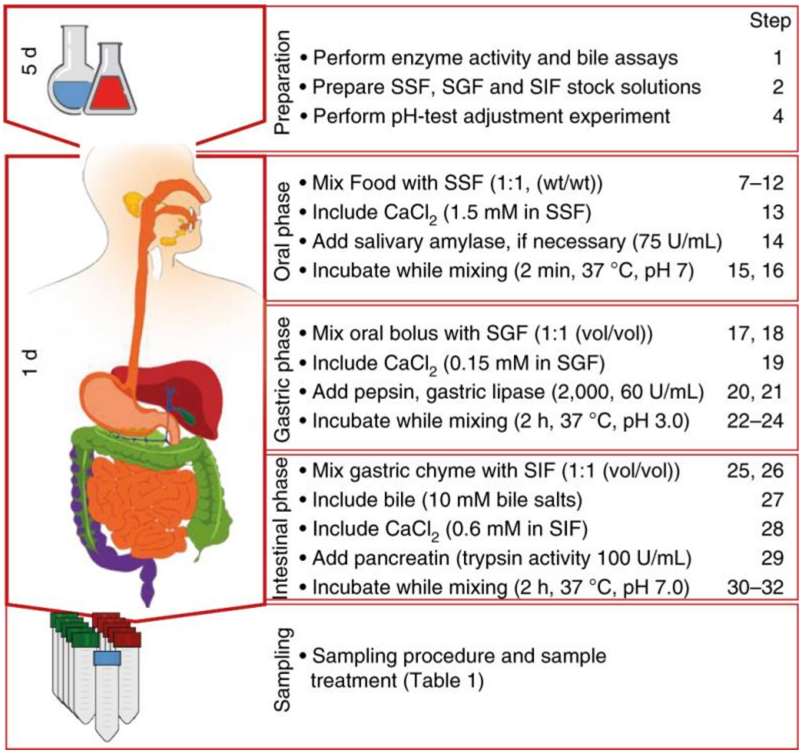


Figure 9. Flow diagram of the INFOGEST 2.0 digestion method, from ref. Brodkorb A. 2019.

1.6.5. Assessment of Anti-inflammatory and Antiproliferative Functions

It is well established that natural antioxidants have a protective effect against several lifestyle-related diseases, including colon cancer, by efficiently reducing reactive oxygen species (ROS) formation and preventing colonic inflammation (Martín, M. A., et al. 2019; Sarnelli, G., et al. 2017). Inflammation is recognized as an immune response that is triggered by microbial infection or tissue damage in humans (Muruve, D. A., et al. 2008). As a result of this response, several pro-inflammatory cytokines are released, including interleukin-12 (IL-12), IL-6, IL-1β, chemokine IL-8, tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) (Opree, A., & Kress, M. 2000). Moreover, a variety of antiinflammatory cytokines are generated, such as IL-10 and IL-4 (Van der Meeren, A., et al. 1999). Furthermore, the increased formation of ROS may prompt tissue damage and inflammation of intestinal mucosa, resulting in a higher risk of cancer development (Yahfoufi, N., et al. 2018; Ullman, T. A., & Itzkowitz, S. H. 2011).

Nowadays, the human colon cancer cell line HT-29 is extensively utilized in scientific investigations, particularly in the realm of colorectal cancer studies. Originating from a primary tumor of the human colon, these cells are cultured in laboratory settings for experimental purposes. HT-29 cells exhibit typical features of adenocarcinomas, a prevalent form of cancer arising from glandular tissues. In vitro experiments involving HT-29 cells are commonly conducted to explore the impact of bioactive compounds on colon cancer cell growth at a cellular level (Martínez-Maqueda, D. 2015). Initially, HT-29 cells are cultured and stimulated in a controlled environment, typically within a culture plate. These cells are supplied with a nutrient-rich medium containing vital nutrients, growth factors, and other necessary components for their sustenance and propagation (Castaldo, L., et al. 2021).

Subsequent evaluations of cell viability and proliferation often incorporate standard assessments such as MTT assays, which gauge mitochondrial activity, and trypan blue exclusion tests, aimed at evaluating cell membrane integrity (Supino, R. 1995; Van Meerloo, J., et al. 2011). Furthermore, molecular and biochemical analyses are performed to deepen our comprehension of the molecular and biochemical alterations triggered by the applied treatments. This investigative process may encompass scrutinizing the expression of specific genes, proteins, or signaling pathways linked to cell proliferation, apoptosis, or other cellular mechanisms. Typically, this analysis involves protein extraction and subsequent assessment through Western blot analysis. Western blotting is a method enabling the identification of specific proteins within a sample based on their size and charge, proving instrumental in examining protein expression, quantifying protein levels, and exploring post-translational modifications (Pillai-Kastoori, L., et al. 2020).

References

- Anastas, P. T., & Warner, J. C. (2000). *Green chemistry: theory and practice*. Oxford university press.
- Area Studi Mediobanca - Il settore del caffè in Italia e nel mondo (ed. 2023)
- Antonova, M., Wienecke, T., Olesen, J., & Ashina, M. (2013). Prostaglandins in migraine: update. *Current opinion in neurology*, *26*(3), 269-275.
- Arlt, A., Sebens, S., Krebs, S., Geismann, C., Grossmann, M., Kruse, M. L., ... & Schäfer, H. (2013). Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. *Oncogene*, *32*(40), 4825-4835.
- Arya, S. S., Venkatram, R., More, P. R., & Vijayan, P. (2022). The wastes of coffee bean processing for utilization in food: A review. *Journal of Food Science and Technology*, 1-16.
- Ascherio, A., Zhang, S. M., Hernán, M. A., Kawachi, I., Colditz, G. A., Speizer, F. E., & Willett, W. C. (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *50*(1), 56-63.

- Asil, E., Yılmaz, M. V., & Yardimci, H. (2021). Effects of black tea consumption and caffeine intake on depression risk in black tea consumers. *African Health Sciences*, *21*(2), 858-865.
- Bager, S. L., & Lambin, E. F. (2020). Sustainability strategies by companies in the global coffee sector. *Business Strategy and the Environment*, *29*(8), 3555-3570.
- Bajkacz, S., Baranowska, I., Buszewski, B., Kowalski, B., & Ligor, M. (2018). Determination of flavonoids and phenolic acids in plant materials using SLE-SPE-UHPLC-MS/MS method. *Food Analytical Methods*, *11*, 3563-3575.
- Barbosa, M. D. S. G., dos Santos Scholz, M. B., Kitzberger, C. S. G., & de Toledo Benassi, M. (2019). Correlation between the composition of green Arabica coffee beans and the sensory quality of coffee brews. *Food Chemistry*, *292*, 275-280.
- Belitz, H. D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. *Food chemistry*, 938-970.
- Beverfood 2020 - https://www.beverfood.com/istituto-espresso-italiano-sostenibilita-evoluzione-settore-caffe-wd/
- Bolka, M., & Emire, S. (2020). Effects of coffee roasting technologies on cup quality and bioactive compounds of specialty coffee beans. *Food science & nutrition*, *8*(11), 6120-6130.

- Borota, D., Murray, E., Keceli, G., Chang, A., Watabe, J. M., Ly, M., ... & Yassa, M. A. (2014). Post-study caffeine administration enhances memory consolidation in humans. *Nature neuroscience*, *17*(2), 201-203.
- Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... & Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature protocols*, *14*(4), 991-1014.
- Burnstock, G. (1989). The role of adenosine triphosphate in migraine. *Biomedicine & pharmacotherapy*, *43*(10), 727-736.
- Butt, M. S., & Sultan, M. T. (2011). Coffee and its consumption: benefits and risks. *Critical reviews in food science and nutrition*, *51*(4), 363-373.
- Camacho-Otero, J., Boks, C., & Pettersen, I. N. (2018). Consumption in the circular economy: A literature review. *Sustainability*, *10*(8), 2758.
- Câmara, J. S., Albuquerque, B. R., Aguiar, J., Corrêa, R. C., Gonçalves, J. L., Granato, D., & Ferreira, I. C. (2020). Food bioactive compounds and emerging techniques for their extraction: Polyphenols as a case study. *Foods*, *10*(1), 37.
- Cao, C., Loewenstein, D. A., Lin, X., Zhang, C., Wang, L., Duara, R., ... & Arendash, G. W. (2012). High blood caffeine levels in MCI linked to lack of progression to dementia. *Journal of Alzheimer's Disease*, *30*(3), 559-572.
- Carneiro, S. M., Oliveira, M. B. P., & Alves, R. C. (2021). Neuroprotective properties of coffee: An update. *Trends in Food Science & Technology*.

- Castaldo, L., Narváez, A., Izzo, L., Graziani, G., & Ritieni, A. (2020). In vitro bioaccessibility and antioxidant activity of coffee silverskin polyphenolic extract and characterization of bioactive compounds using UHPLC-Q-Orbitrap HRMS. *Molecules*, *25*(9), 2132.
- Castaldo, L., Toriello, M., Sessa, R., Izzo, L., Lombardi, S., Narváez, A., Ritieni, A., & Grosso, M. (2021). Antioxidant and Anti-Inflammatory Activity of Coffee Brew Evaluated after Simulated Gastrointestinal Digestion. *Nutrients*, *13*(12), 4368.
- Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: Concept and principles. *International journal of molecular sciences*, *13*(7), 8615-8627.
- Chen, J. Q., Scheltens, P., Groot, C., & Ossenkoppele, R. (2020). Associations between caffeine consumption, cognitive decline, and dementia: a systematic review. *Journal of Alzheimer's Disease*, *78*(4), 1519-1546.
- Chesnut, S. M., & Salisbury, J. J. (2007). The role of UHPLC in pharmaceutical development. *Journal of separation science*, *30*(8), 1183-1190.
- Chevalier, A. (1947). Coffees of the World. Classification of coffee and pseudo-coffee trees. Diseases and pests. *Coffees of the World. Classification of coffee and pseudo-coffee trees. Diseases and pests.*
- Chu, Y. F., Brown, P. H., Lyle, B. J., Chen, Y., Black, R. M., Williams, C. E., Lin, Y., Hsu, C. & Cheng, I. H. (2009). Roasted coffees high in

lipophilic antioxidants and chlorogenic acid lactones are more neuroprotective than green coffees. *Journal of Agricultural and Food Chemistry*, *57*(20), 9801-9808.

- Clifford, M. N. (Ed.). (2012). *Coffee: botany, biochemistry and production of beans and beverage*. Springer Science & Business Media
- Coffeebarometer, 2023 - https://coffeebarometer.org/documents_resources/coffee_barometer_2023.pdf
- Daglia, M., Papetti, A., Aceti, C., Sordelli, B., Gregotti, C., & Gazzani, G. (2008). Isolation of high molecular weight components and contribution to the protective activity of coffee against lipid peroxidation in a rat liver microsome system. *Journal of Agricultural and Food Chemistry*, *56*(24), 11653-11660.
- DaMatta, F. M., Ronchi, C. P., Maestri, M., & Barros, R. S. (2007). Ecophysiology of coffee growth and production. *Brazilian journal of plant physiology*, *19*, 485-510.
- Davis, A. P., Gole, T. W., Baena, S., & Moat, J. (2012). The impact of climate change on indigenous arabica coffee (Coffea arabica): predicting future trends and identifying priorities. *PloS one*, *7*(11), e47981.
- Davis, A. P., Govaerts, R., Bridson, D. M., & Stoffelen, P. (2006). An annotated taxonomic conspectus of the genus Coffea (Rubiaceae). *Botanical Journal of the Linnean Society*, *152*(4), 465-512

- Davis, A. P., Kiwuka, C., Faruk, A., Walubiri, M. J., & Kalema, J. (2022). The re-emergence of Liberica coffee as a major crop plant. *Nature Plants*, *8*(12), 1322-1328
- de Melo Pereira, G. V., de Carvalho Neto, D. P., Júnior, A. I. M., do Prado, F. G., Pagnoncelli, M. G. B., Karp, S. G., & Soccol, C. R. (2020). Chemical composition and health properties of coffee and coffee by-products. *Advances in food and nutrition research*, *91*, 65-96.
- Deng, J., Yang, H., Capanoglu, E., Cao, H., & Xiao, J. (2018). Technological aspects and stability of polyphenols. In *Polyphenols: Properties, recovery, and applications* (pp. 295-323). Woodhead Publishing.
- Ding, M., Bhupathiraju, S. N., Chen, M., Van Dam, R. M., & Hu, F. B. (2014). Caffeinated and decaffeinated coffee consumption and risk of type 2 diabetes: a systematic review and a dose-response meta-analysis. *Diabetes care*, *37*(2), 569-586.
- Dirks-Naylor, A. J. (2015). The benefits of coffee on skeletal muscle. *Life sciences*, *143*, 182-186.
- Distribuzione Moderna, 2011 https://distribuzionemoderna.info/sostenibilita/kimbo-presenta-una-nuova-referenza-allinsegna-della-sostenibilit
- Diviš, P., Pořízka, J., & Křikala, J. (2019). The effect of coffee beans roasting on its chemical composition. Potravinarstvo, 13(1)

- Dominguez, H., & Muñoz, M. J. G. (Eds.). (2017). *Water extraction of bioactive compounds: from plants to drug development*. Elsevier.
- Donno, D., Mellano, M. G., Gamba, G., Riondato, I., & Beccaro, G. L. (2020). Analytical strategies for fingerprinting of antioxidants, nutritional substances, and bioactive compounds in foodstuffs based on high performance liquid chromatography–mass spectrometry: An overview. *Foods*, *9*(12), 1734.
- Duarte, K., Justino, C. I. L., Gomes, A. M., Rocha-Santos, T., & Duarte, A. C. (2014). Green analytical methodologies for preparation of extracts and analysis of bioactive compounds. In *Comprehensive analytical chemistry* (Vol. 65, pp. 59-78). Elsevier.
- Echavarría, A. P., Pagán, J., & Ibarz, A. (2012). Melanoidins formed by Maillard reaction in food and their biological activity. *Food Engineering Reviews*, *4*, 203-223.
- Elhalis, H., Cox, J., Frank, D., & Zhao, J. (2021). The role of wet fermentation in enhancing coffee flavor, aroma and sensory quality. *European Food Research and Technology*, *247*, 485-498.
- Eskilsson, C. S., & Björklund, E. (2000). Analytical-scale microwave-assisted extraction. *Journal of chromatography A*, *902*(1), 227-250.
- Esquivel, P., & Jimenez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food research international*, *46*(2), 488-495.
- European Food Safety Authority. EFSA. Caffeina. https://www.efsa.europa.eu/it/topics/topic/caffeine

- EUROPEAN PATENT APPLICATION- https://patentimages.storage.googleapis.com/27/aa/e5/1c38dc3f126dfd/EP0158381A1.pdf
- EUROSTAT - https://ec.europa.eu/eurostat
- FAOSTAT - https://www.fao.org/faostat/en/#data/TCL
- Farah, A. (2012). Coffee constituents. *Coffee: Emerging health effects and disease prevention*, *1*, 22-58.
- Ferreira, J., Ferreira, C., & Bos, E. (2021). Spaces of consumption, connection, and community: Exploring the role of the coffee shop in urban lives. *Geoforum*, *119*, 21-29.
- Food and Agriculture Organization. (2019). Food Balance Sheet. https://www.fao.org/faostat/en/#data/FBS.
- Fredholm, B. B., Bättig, K., Holmén, J., Nehlig, A., & Zvartau, E. E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacological reviews*, *51*(1), 83-133.
- Galeone, C., Turati, F., La Vecchia, C., & Tavani, A. (2010). Coffee consumption and risk of colorectal cancer: a meta-analysis of case–control studies. *Cancer Causes & Control*, *21*, 1949-1959.
- George, S. E., Ramalakshmi, K., & Mohan Rao, L. J. (2008). A perception on health benefits of coffee. *Critical reviews in food science and nutrition*, *48*(5), 464-486.

- Gil-Martín, E., Forbes-Hernández, T., Romero, A., Cianciosi, D., Giampieri, F., & Battino, M. (2022). Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products. *Food Chemistry*, *378*, 131918.
- Gniechwitz, D., Reichardt, N., Meiss, E., Ralph, J., Steinhart, H., Blaut, M., & Bunzel, M. (2008). Characterization and fermentability of an ethanol soluble high molecular weight coffee fraction. *Journal of agricultural and food chemistry*, *56*(14), 5960-5969.
- Grgić, J., Šelo, G., Planinić, M., Tišma, M., & Bucić-Kojić, A. (2020). Role of the encapsulation in bioavailability of phenolic compounds. *Antioxidants*, *9*(10), 923.
- Grzelczyk, J., Budryn, G., Peña-García, J., Szwajgier, D., Gałązka-Czarnecka, I., Oracz, J., & Pérez-Sánchez, H. (2021). Evaluation of the inhibition of monoamine oxidase A by bioactive coffee compounds protecting serotonin degradation. *Food Chemistry*, *348*, 129108.
- Gunasekaran, A., & Spalanzani, A. (2012). Sustainability of manufacturing and services: Investigations for research and applications. *International journal of production economics*, *140*(1), 35-47.
- Guo, Y., Niu, K., Okazaki, T., Wu, H., Yoshikawa, T., Ohrui, T., ... & Nagatomi, R. (2014). Coffee treatment prevents the progression of sarcopenia in aged mice in vivo and in vitro. *Experimental gerontology*, *50*, 1-8.

- Gutteridge, J. M., & Halliwell, B. (2010). Antioxidants: Molecules, medicines, and myths. *Biochemical and biophysical research communications*, *393*(4), 561-564.
- Halliwell, B., & Gutteridge, J. M. (2015). *Free radicals in biology and medicine*. Oxford university press, USA.
- Harvey, C. A., Pritts, A. A., Zwetsloot, M. J., Jansen, K., Pulleman, M. M., Armbrecht, I., Valencia, V. (2021). Transformation of coffee-growing landscapes across Latin America. A review. *Agronomy for sustainable development*, *41*(5), 62.
- Higdon, J. V., & Frei, B. (2006). Coffee and health: a review of recent human research. *Critical reviews in food science and nutrition*, *46*(2), 101-123.
- Hirakawa, N., Okauchi, R., Miura, Y., & Yagasaki, K. (2005). Anti-invasive activity of niacin and trigonelline against cancer cells. *Bioscience, biotechnology, and biochemistry*, *69*(3), 653-658.
- Hong, C. T., Chan, L., & Bai, C. H. (2020). The effect of caffeine on the risk and progression of Parkinson's disease: A meta-analysis. *Nutrients*, *12*(6), 1860.
- Horžic, D., A. R. Jambrak, A. Belšcak-Cvitanovic, D. Komes, e V. Lelas. « Comparison of Conventional and Ultrasound Assisted Extraction Techniques of Yellow Tea and Bioactive Composition of Obtained Extracts.» *Food and Bioprocess Technology*, 2012: 2858–2870.

- Hu, G. L., Wang, X., Zhang, L., & Qiu, M. H. (2019). The sources and mechanisms of bioactive ingredients in coffee. *Food & Function*, *10*(6), 3113-3126.
- Huang, R., O'Donnell, A. J., Barboline, J. J., & Barkman, T. J. (2016). Convergent evolution of caffeine in plants by co-option of exapted ancestral enzymes. *Proceedings of the National Academy of Sciences*, *113*(38), 10613-10618.
- ICO International Coffee Organization https://www.icocoffee.org/documents/cy2023-24/cmr-1123-e.pdf; https://www.ico.org/documents/cy2022-23/coffee-development-report-2021.pdf;
- International Coffee Organization (2019). Coffee Development Report 2019. http://www.ico.org/documents/cy2018-19/ed-2318e-overview-flagship-report.pdf
- International Coffee Organization (ICO), Prices Production - https://www.ico.org/prices/po-production.pdf
- Ismail, I., Anuar, M. S., & Shamsudin, R. (2014). Physical properties of Liberica coffee (Coffea liberica) berries and beans. *Pertanika Journal of Science and Technology*, *22*(1), 65-79.
- Kitagawa, M., Houzen, H., & Tashiro, K. (2007). Effects of caffeine on the freezing of gait in Parkinson's disease. *Movement disorders*, *22*(5), 710-712.

- Klevebrant, L., & Frick, A. (2022). Effects of caffeine on anxiety and panic attacks in patients with panic disorder: A systematic review and meta-analysis. *General hospital psychiatry*, *74*, 22-31.
- Klevebrant, L., & Frick, A. (2022). Effects of caffeine on anxiety and panic attacks in patients with panic disorder: A systematic review and meta-analysis. *General hospital psychiatry*, *74*, 22-31.
- Lacy, P., Rutqvist, J., & Lamonica, B. (2016). *Circular economy: Dallo spreco al valore*. EGEA spa.
- Lara, D. R. (2010). Caffeine, mental health, and psychiatric disorders. *Journal of Alzheimer's disease*, *20*(s1), S239-S248.
- Lean, M. E., Ashihara, H., Clifford, M. N., & Crozier, A. (2011). Purine alkaloids: a focus on caffeine and related compounds in beverages. *Teas, cocoa and coffee: Plant secondary metabolites and health*, 25-44.
- Lindsay, J., Laurin, D., Verreault, R., Hébert, R., Helliwell, B., Hill, G. B., & McDowell, I. (2002). Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. *American journal of epidemiology*, *156*(5), 445-453
- Lingnau, V., Fuchs, F., & Beham, F. (2019). The impact of sustainability in coffee production on consumers' willingness to pay–new evidence from the field of ethical consumption. *Journal of Management Control*, *30*, 65-93.

- MacMahon, B., Yen, S., Trichopoulos, D., Warren, K., & Nardi, G. (1981). Coffee and cancer of the pancreas. *New England Journal of Medicine*, *304*(11), 630-633
- Mahato, N., Sinha, M., Sharma, K., Koteswararao, R., & Cho, M. H. (2019). Modern extraction and purification techniques for obtaining high purity food-grade bioactive compounds and value-added co-products from citrus wastes. *Foods*, *8*(11), 523.
- Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American journal of clinical nutrition*, *79*(5), 727-747.
- Manippa, V., Lupo, R., Tommasi, L., & Brancucci, A. (2021). Italian breakfast in mind: The effect of caffeine, carbohydrate and protein on physiological state, mood and cognitive performance. *Physiology & Behavior*, *234*, 113371.
- Martín, M. A., Goya, L., & Ramos, S. (2016). Preventive effects of cocoa and cocoa antioxidants in colon cancer. *Diseases*, *4*(1), 6.
- Martínez-Maqueda, D., Miralles, B., & Recio, I. (2015). HT29 cell line. *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, 113-124.
- McLellan, T. M., Caldwell, J. A., & Lieberman, H. R. (2016). A review of caffeine's effects on cognitive, physical and occupational performance. *Neuroscience & Biobehavioral Reviews*, *71*, 294-312.

- McLellan, T. M., Caldwell, J. A., & Lieberman, H. R. (2016). A review of caffeine's effects on cognitive, physical and occupational performance. *Neuroscience & Biobehavioral Reviews*, *71*, 294-312.
- Morris, J. (2018). *Coffee: A global history*. Reaktion Books.
- Muruve, D. A., Pétrilli, V., Zaiss, A. K., White, L. R., Clark, S. A., Ross, P. J., ... & Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature*, *452*(7183), 103-107.
- Myhrvold, N. (2024, March 6). coffee. *Encyclopedia Britannica*. https://www.britannica.com/topic/coffee
- National Coffee Association - https://www.ncausa.org/about-coffee/history-of-coffee
- Nehlig, A. (2016). Effects of coffee/caffeine on brain health and disease: What should I tell my patients? Practical neurology, 16(2), 89-95
- Ngan, S. L., How, B. S., Teng, S. Y., Promentilla, M. A. B., Yatim, P., Er, A. C., & Lam, H. L. (2019). Prioritization of sustainability indicators for promoting the circular economy: The case of developing countries. *Renewable and Sustainable Energy Reviews*, *111*, 314-331.
- Niseteo, T., Komes, D., Belščak-Cvitanović, A., Horžić, D., & Budeč, M. (2012). Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. *Food chemistry*, *134*(4), 1870-1877

- Nooshkam, M., Varidi, M., & Bashash, M. (2019). The Maillard reaction products as food-born antioxidant and antibrowning agents in model and real food systems. *Food chemistry*, *275*, 644-660.
- Norshazila, S., Koy, C. N., Rashidi, O., Ho, L. H., Azrina, I., Zaizuliana, N. R., & Zarinah, Z. (2017). The effect of time, temperature and solid to solvent ratio on pumpkin carotenoids extracted using food grade solvents. *Sains Malaysiana*, *46*(2), 231-237.
- O'Keefe, J. H., Bhatti, S. K., Patil, H. R., DiNicolantonio, J. J., Lucan, S. C., & Lavie, C. J. (2013). Effects of habitual coffee consumption on cardiometabolic disease, cardiovascular health, and all-cause mortality. *Journal of the American College of Cardiology*, *62*(12), 1043-1051.
- O'Keefe, J. H., DiNicolantonio, J. J., & Lavie, C. J. (2018). Coffee for cardioprotection and longevity. *Progress in cardiovascular diseases*, *61*(1), 38-42.
- Opree, A., & Kress, M. (2000). Involvement of the proinflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. *Journal of Neuroscience*, *20*(16), 6289-6293.
- Owen, L., & Corfe, B. (2017). The role of diet and nutrition on mental health and wellbeing. *Proceedings of the Nutrition Society*, *76*(4), 425-426.

- Pereira de Melo, G. V., de Carvalho Neto, D. P., Júnior, A. I. M., Vásquez, Z. S., Medeiros, A. B., Vandenberghe, L. P., & Soccol, C. R. (2019). Exploring the impacts of postharvest processing on the aroma formation of coffee beans–A review. *Food chemistry*, *272*, 441-452.
- Pillai-Kastoori, L., Schutz-Geschwender, A. R., & Harford, J. A. (2020). A systematic approach to quantitative Western blot analysis. *Analytical biochemistry*, *593*, 113608.
- Pohlan, H. A. J., & Janssens, M. J. (2010). Growth and production of coffee. *Soils, plant growth and crop production*, *3*, 101.
- Poltronieri, P., & Rossi, F. (2016). Challenges in specialty coffee processing and quality assurance. *Challenges*, *7*(2), 19.
- Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouységu, L. (2011). Plant polyphenols: chemical properties, biological activities, and synthesis. *Angewandte Chemie International Edition*, *50*(3), 586-621.
- Ramalakshmi, K., & Raghavan, B. (1999). Caffeine in coffee: its removal. Why and how?. *Critical reviews in food science and nutrition*, *39*(5), 441-456.
- Rattan, S., Parande, A. K., Nagaraju, V. D., & Ghiwari, G. K. (2015). A comprehensive review on utilization of wastewater from coffee processing. *Environmental Science and Pollution Research*, *22*, 6461-6472.
- Rodak, K., Kokot, I., & Kratz, E. M. (2021). Caffeine as a factor influencing the functioning of the human body—Friend or foe. *Nutrients*, *13*(9), 3088.

- Rucci, S., Bonuccelli, A., Angelini, F., Negro, M., & Marzatico, F. (2011). Caffeina: alleata o nemica. *Rivista della Società Italiana di Medicina Generale [revista en internet], 5.*
- Rufián-Henares, J. A., & Morales, F. J. (2007). Effect of in vitro enzymatic digestion on antioxidant activity of coffee melanoidins and fractions. *Journal of Agricultural and Food Chemistry*, *55*(24), 10016-10021.
- Santomauro, D. F., Herrera, A. M. M., Shadid, J., Zheng, P., Ashbaugh, C., Pigott, D. M., ... & Ferrari, A. J. (2021). Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. *The Lancet*, *398*(10312), 1700-1712.
- Sarnelli, G., Grosso, M., Palumbo, I., Pesce, M., D'Alessandro, A., Zaninotto, G., ... & Cuomo, R. (2017). Allele-specific transcriptional activity of the variable number of tandem repeats of the inducible nitric oxide synthase gene is associated with idiopathic achalasia. *United European Gastroenterology Journal*, *5*(2), 200-207.
- Schwarzschild, M. A., Xu, K., Oztas, E., Petzer, J. P., Castagnoli, K., Castagnoli Jr, N., & Chen, J. F. (2003). Neuroprotection by caffeine and more specific A2A receptor antagonists in animal models of Parkinson's disease. *Neurology*, *61*(11_suppl_6), S55-S61.
- Shahidi, F., & Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of functional foods*, *18*, 757-781.

- Shahinfar, H., Jayedi, A., Khan, T. A., & Shab-Bidar, S. (2021). Coffee consumption and cardiovascular diseases and mortality in patients with type 2 diabetes: a systematic review and dose–response meta-analysis of cohort studies. *Nutrition, Metabolism and Cardiovascular Diseases*, *31*(9), 2526-2538.
- Shang, F., Li, X., & Jiang, X. (2016). Coffee consumption and risk of the metabolic syndrome: A meta-analysis. *Diabetes & metabolism*, *42*(2), 80-87.
- Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*(3), 144-158.
- Smetanska, I. (2018). Sustainable production of polyphenols and antioxidants by plant in vitro cultures. *Bioprocessing of plant in vitro systems*, 225-269.
- Stalmach, A., Mullen, W., Nagai, C., & Crozier, A. (2006). On-line HPLC analysis of the antioxidant activity of phenolic compounds in brewed, paper-filtered coffee. *Brazilian Journal of Plant Physiology*, *18*, 253-262.
- Supino, R. (1995). MTT assays. *In vitro toxicity testing protocols*, 137-149.
- Tajik, N., Tajik, M., Mack, I., & Enck, P. (2017). The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. *European journal of nutrition*, *56*(7), 2215-2244.

- Tarigan, E. B., Wardiana, E., Hilmi, Y. S., & Komarudin, N. A. (2022). The changes in chemical properties of coffee during roasting: A review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 974, No. 1, p. 012115). IOP Publishing.
- Teixeira, D. M., Patão, R. F., Coelho, A. V., & da Costa, C. T. (2006). Comparison between sample disruption methods and solid–liquid extraction (SLE) to extract phenolic compounds from Ficus carica leaves. *Journal of Chromatography A*, *1103*(1), 22-28.
- Teuber, R. (2010). Geographical indications of origin as a tool of product differentiation: The case of coffee. *Journal of International Food & Agribusiness Marketing*, *22*(3-4), 277-298.
- The Washington Post 1981 - Coffee, Tea or Paranoia, The Washington Post 1981.
- Trevitt, J., Kawa, K., Jalali, A., & Larsen, C. (2009). Differential effects of adenosine antagonists in two models of parkinsonian tremor. *Pharmacology Biochemistry and Behavior*, *94*(1), 24-29.
- Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231-1246.
- Ukers, W. H. (1935). All about coffee (Vol. 1). *Library of Alexandria*
- Ullman, T. A., & Itzkowitz, S. H. (2011). Intestinal inflammation and cancer. *Gastroenterology*, *140*(6), 1807-1816.
- Van der Meeren, A., Squiban, C., Gourmelon, P., Lafont, H., & Gaugler, M. H. (1999). Differential regulation by IL-4 and IL-10 of

radiation-induced IL-6 and IL-8 production and ICAM-1 expression by human endothelial cells. *Cytokine*, *11*(11), 831-838.

- Van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Cancer cell culture: methods and protocols*, 237-245.
- Van Wijk, X. M., Goodnough, R., & Colby, J. M. (2019). Mass spectrometry in emergency toxicology: Current state and future applications. *Critical reviews in clinical laboratory sciences*, *56*(4), 225-238.
- Várady, M., Tauchen, J., Fraňková, A., Klouček, P., & Popelka, P. (2022). Effect of method of processing specialty coffee beans (natural, washed, honey, fermentation, maceration) on bioactive and volatile compounds. LWT, 172, 114245.
- Vincent, J. C. (1987). Green coffee processing. In *Coffee: Volume 2: Technology* (pp. 1-33). Dordrecht: Springer Netherlands.
- Vitaglione, P., Fogliano, V., & Pellegrini, N. (2012). Coffee, colon function and colorectal cancer. *Food & function*, *3*(9), 916-922.
- Wasim, S., Kukkar, V., Awad, V. M., Sakhamuru, S., & Malik, B. H. (2020). Neuroprotective and neurodegenerative aspects of coffee and its active ingredients in view of scientific literature. *Cureus*, *12*(8).
- Wasim, S., Kukkar, V., Awad, V. M., Sakhamuru, S., & Malik, B. H. (2020). Neuroprotective and neurodegenerative aspects of coffee and its active ingredients in view of scientific literature. *Cureus*, *12*(8).
- Water Footprint Network https://www.waterfootprint.org/

- WHO/Europe. (2019). Fact sheet, Mental health. https://www.euro.who.int/__data/assets/pdf_file/0004/404851/MNH_FactSheet_ENG.pdf.
- Woldesenbet, A. G., Woldeyes, B., & Chandravanshi, B. S. (2014). Characteristics of wet coffee processing waste and its environmental impact in Ethiopia. *International Journal of Research in Engineering and Science*, *2*(4), 1-5
- Wollgast, J., & Anklam, E. (2000). Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International*, *33*(6), 423-447.
- Wood, M. (2019). High-resolution mass spectrometry: an emerging analytical method for drug testing. In *Critical Issues in Alcohol and Drugs of Abuse Testing* (pp. 173-188). Academic Press.
- Yahfoufi, N., Alsadi, N., Jambi, M., & Matar, C. (2018). The immunomodulatory and anti-inflammatory role of polyphenols. *Nutrients*, *10*(11), 1618.
- Yang, M., Chen, L., Wang, J., Msigwa, G., Osman, A. I., Fawzy, S., ... & Yap, P. S. (2023). Circular economy strategies for combating climate change and other environmental issues. *Environmental Chemistry Letters*, *21*(1), 55-80.

- Yenisetti, S. C. (2016). Beneficial role of coffee and caffeine in neurodegenerative diseases: A minireview. *AIMS public health*, *3*(2), 407.
- Yu, M., He, S., Tang, M., Zhang, Z., Zhu, Y., & Sun, H. (2018). Antioxidant activity and sensory characteristics of Maillard reaction products derived from different peptide fractions of soybean meal hydrolysate. *Food Chemistry*, *243*, 249-257.
- Zhou, J., Chan, L., & Zhou, S. (2012). Trigonelline: a plant alkaloid with therapeutic potential for diabetes and central nervous system disease. *Current medicinal chemistry*, *19*(21), 3523-3531.

Chapter 2
Research objectives

2.1. General objectives

The objective of my thesis was an over-expanding investigation on coffee brewing and its byproducts. By focusing on the chemical composition, nutritional content, anti-inflammatory, antioxidant and antiproliferative characteristics, this study aims to provide a comprehensive understanding of how coffee consumption can positively impact on human health. The investigation of by-products deriving from the industrial process in depth uncovers new insights that can help industry practices and consumer choices. The coffee industry, as a significant player in the global market, is currently experiencing a shift in perspective towards environmental consciousness and social responsibility. Kimbo Spa, being a forward-thinking company, recognizes the importance of integrating sustainable practices into its operations. This collaboration signifies a pivotal moment, signifying both a dedication and a call to action to align with a more sustainable world through innovative approaches and progressive methodologies.

Through interdisciplinary inquiry that explored the intricate interactions of compounds in coffee and their influence on human health, the significance of coffee transcends its mere status as a beverage as it underscores its potential as a functional resource for the enhancement of overall well-being.

2.2. Specific objectives

1. Assess of the anti-inflammatory and antioxidant effects of different coffee brew samples on HT-29 cells exposed to varying concentrations of coffee brews and skatole, a tryptophan metabolite released from intestinal putrefactive fermentation in the presence of excessive dietary animal protein intake. This evaluation aims to comprehend the influence of different coffee varieties and their extracts on cellular responses associated with inflammation and oxidative stress. Specifically, the focus is on understanding their capacity to regulate the molecular pathways involved in the development and progression of colon cancer.
2. Evaluation of the impact of simulated gastrointestinal digestion (GiD) on the polyphenol composition of coffee brews and assess the changes in antioxidant and anti-inflammatory properties using the HT-29 human colon cancer cell model. The primary purpose of this investigation is to gain insight into the modifications that occur in the bioactive compounds of coffee during the digestion process, as well as their potential health benefits, particularly in relation to reducing inflammation and oxidative stress associated with colon cancer.
3. Study of the correlation between the consumption of standard coffee and dewaxed coffee and the symptoms of Gastroesophageal Reflux Disease (GERD) by employing a GERD questionnaire in a pilot study. The main objective of this study is to determine if the process of

dewaxing coffee has an impact on its association with GERD. Additionally, the polyphenolic profile of coffee pods' extracts were analyzed using ultra-high-performance liquid chromatography coupled with high-resolution Orbitrap mass spectrometry (UHPLC Q-Orbitrap HRMS) in order to identify the specific polyphenolic compounds present. Through the identification and measurement of polyphenols in coffee pods, this research aims to comprehend their role in mediating the correlation between coffee consumption and GERD.

4. Investigation of the chemical composition of byproduct that emerges at the end of the coffee production: the spent coffee grounds (SCG). The valorization of this waste into a valuable asset could lead to minimize waste and increasing the overall sustainability of the coffee production process. SCG possesses an unexplored source of bioactive compounds useful to implement SCG in a baked food, such as cookies, to increase its antioxidant activity and polyphenolic compound content to create innovative, healthy food. Moreover, a complete simulated GiD was used to assess the SCGc polyphenol bioaccessibility as well as the variations in the antioxidant activity in order to evaluate the application of SCG in the development of healthy bakery products.

Chapter 3
Results and Discussion

3.1. Effect of Different Coffee Brews on Tryptophan Metabolite-Induced Cytotoxicity in HT-29 Human Colon Cancer Cells

Materials and Methods

Sampling

Three different kinds of coffee brews were investigated in the present article: instant coffee (n = 10), espresso (n = 10), and Americano coffee brews (n = 10). Instant coffee powder/granule samples and medium-roasted coffee beans (*Coffea arabica* L.) were obtained from local Italian markets. The coffee brews were prepared as described in the previous work (Castaldo, L.; et al. 2021).

In Vitro Gastrointestinal Digestion

In vitro GI digestion was performed on the three coffee brew samples under investigation to simulate the effects of human digestion using the protocol proposed by the INFOGEST network, as used in the previous article (Castaldo, L.; et al. 2021).

Cell Culture

The human colorectal cancer cell line HT-29 from American Type Culture Collection (ATCC, Manassas, VA, USA) was maintained in high glucose

RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum plus 4 mM glutamine, at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged by trypsinization when reaching 70–80% confluence (all reagents were from Sigma-Aldrich, Saint Louis, MO, USA). To avoid mycoplasma contamination, cells were routinely checked with a PCR Mycoplasma Test Kit (AppliChem A3744, Darmstadt, Germany).

Cell Treatment

HT-29 cells were treated either with coffee samples (espresso, Americano, and instant coffee brew) prepared in the cell culture medium at 0.250 and 0.500 mg/mL as previously reported (Castaldo, L.; et al. 2021), or with different concentrations of skatole (#M51458-5G/Sigma-Aldrich, Saint Louis, MO, USA) prepared from a 1 M DMSO stock solution in accordance with previous studies (Kurata, K.; et al. 2019). Furthermore, co-treatment experiments were performed in HT-29 cells treated with the assayed coffee samples and different concentrations of skatole for 24 h. Appropriate control cell cultures treated with the same amount of DMSO were included in each experiment and maintained at a final concentration of DMSO (*v*/*v*) of under 0.5% in the mock control. Cells were subsequently seeded to perform a cell viability assay, evaluate the intracellular ROS level, and to extract total mRNA for real-time PCR analysis.

Analysis of Cell Viability

Cell viability was measured using a thiazolyl blue tetrazolium bromide (MTT) colorimetric method (Roche, Mannheim, Germany) following the procedure described by Riccio et al. (Riccio, P.; et al. 2019). In brief, HT-29 cells were seeded onto 96-well plates at a density of 5.5×10^4 cells/mL in 100 µL cell suspension per well. After 24 h, cells were treated with different skatole concentrations (250, 500, 750, 1000 µM) or with coffee samples (0.250 and 0.500 mg/mL) for 24 h. Then, 10 µL of MTT labeling reagent (Cell Proliferation Kit I; Roche, Mannheim, Germany) was added to the cell culture. After 4 h of incubation at 37 °C to dissolve the MTT insoluble formazan crystals, 100 µL of detergent solubilization buffer 1× (10% SDS in 0.01M HCl) was added to each well, according to the manufacturer's instructions. The absorbance was read at 570/690 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The cell viability was calculated as a percentage as follows: (absorbance of the experimental group/absorbance of the control group) × 100.

Assessment of Intracellular ROS Production

The generation of intracellular ROS was measured using a spectrofluorometric test using an H_2DCF-DA (2′7′-dichlorodihydrofluorescein diacetate) fluorescent probe (Voloboueva, L.A.; et al. 2005). HT-29 cells were plated onto 96-well black plates at a density of 5.5×10^4 cells/mL in 100 µL cell suspension per well. Cells were treated with assayed coffee samples (0.250 and 0.500 mg/mL) or skatole (250 and 500 μM) for 24 h. In the positive control, ROS were generated by incubating HT-29 cells with 100 μM of hydrogen peroxide (H_2O_2), followed by H_2DCF-DA incubation as previously described (Castaldo, L.; et al. 2021). To evaluate the intracellular ROS levels under challenging conditions, cells were pretreated with 250 or 500 μM of skatole for 6 h and then treated with 0.250 or 0.500 mg/mL of coffee samples and skatole (250 or 500 μM) for an additional 18 h. After treatment, Dulbecco's phosphate buffered saline (DPBS) was used to wash the cells twice. The cells were then exposed to 10 μM of H_2DCF-DA diluted in Hank's Balanced Salt Solution (HBSS) for 20 min in the dark at 37 °C. The extracellular dye was then removed from the cells by two washes with 1 × DPBS. The fluorescence intensity was detected using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at excitation/emission wavelengths of 485/538 nm. The percentage of intracellular ROS was calculated as follows: (fluorescence intensity of the experimental group/fluorescence intensity of the control group) × 100.

RNA Extraction

HT-29 cells were plated onto 6-well plates at a density of 1.5×10^5 cells/mL. Cells were treated with skatole at a concentration of 250 μM for 24 h. In the positive control, inflammation response was stimulated by incubating the HT-29 cells with 10 ng/mL of LPS (Sigma Aldrich) (Wang, T.; et al. 2004 - Hsu, H.; Wen, M. 2002). In order to test inflammation in challenging conditions, cells were pretreated with skatole (250 μM) for 6 h, and then treated in combination with the assayed coffee samples (0.250 mg/mL) plus skatole (250 μM) for an additional 18 h. After treatment, cells were trypsinized and harvested to perform total RNA extraction. Total RNAs were extracted with QIAzol reagent (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was checked for purity and stability using gel electrophoresis and UV spectrometry. Absorption at 260 and 280 nm was measured, and RNA quantity was calculated.

Real-Time PCR Analysis

Real-time PCR analysis was performed with 1 μg of RNA reverse-transcribed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Berkeley, CA, USA) in a final volume of 20 μL, according to the manufacturer's instructions. This mixture was incubated at 42 °C for 3 min, and then at 95 °C for 3 min, and subsequently used for real-time RT-PCR procedures on a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA).

Primers for quantitative real-time PCR analysis are reported in Table 1 (Duary, R.K.; et al. 2014). β-actin mRNA was used as an endogenous control. Each real-time PCR experiment was performed in triplicate in a 20 μL reaction mix containing 10 μL of 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.38 μL of a 20 μM primer mix, and 6.6 μL of cDNA (1/2 volume of RT-PCR product). The cycling conditions were set up as follows: initial denaturation step at 98 °C for 30 s, followed by 40 cycles (95 °C for 15 s, 60 °C for 30 s). A calibration curve was calculated to assess the efficiency of the PCR, as previously reported (Trombetti, S.; et al. 2021). Real-time PCR reactions were performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories) and CT values were obtained from automated threshold analysis. Data were analyzed using CFX Manager 3.0 software (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer's specifications, and a relative quantification of gene expression was determined using the ΔΔCT method.

Table 1. Primer sequences used for quantitative Real-time PCR analysis.

Transcript	Primer	Sequence 5′-3′	Amplicon Size (bp)
TNF-α	For Rev	AGCCCATGTTGTAGCAAACC TGAGGTACAGGCCCTCTGAT	134
IL-1β	For Rev	CATGGGATAACGAGGCTTATG CCACTTGTTGCTCCATATCC	149
IL-8	For Rev	TGGCTCTCTTGGCAGCCTTC TGCACCCAGTTTTCCTTGGG	238
IL-12	For Rev	TTCACCACTCCCAAAACCTGC GAGGCCAGGCAACTCCCATTA	225
β-actin	For Rev	CGACAGGATGCAGAAGGAGA CGTCATACTCCTGCTTGCTG	160

Statistical Analysis

The experiments were carried out in triplicate and the results are provided as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) or two-way ANOVA test was used to determine the statistical differences between the control and treated cell groups. Where appropriate, Dunnett's test and/or Student's *t*-tests were performed. The *p*-values of 0.05, 0.01, and 0.001 (*, **, and ***, respectively) were used to determine the level of significance.

Results

Cell Viability in HT-29 Cells

The HT-29 cell viability after the exposure to the assayed coffee samples was investigated. Figure 1 shows the cell viability evaluated with an MTT assay after treatment with different coffee extracts (0.250 and 0.500 mg/mL). The results highlighted that the cells treated with the instant and Americano coffee samples at both assayed concentrations (0.250 and 0.500 mg/mL), showed a significant dose-dependent increase in cell viability (10% and 20%, respectively) compared to the mock control, whereas no significant differences were observed between those treated with espresso coffee and the mock control. To exclude effects mediated by the digestion fluid, the blank control resulting from in vitro digestion was analyzed by MTT assay and compared to untreated cells. The results (Figure 10A–C) indicated no significant differences between the blank control and untreated cells at 24 h of treatment. In addition, the cytotoxic effects of skatole treatment at different concentrations (from 250 to 1000 μM) on HT-29 cells was investigated by MTT assay after 24 h exposure (Figure 10D). As shown in Figure 10D, the colorimetric assay indicated no cytotoxicity effects in cells exposed for 24 h to skatole concentrations lower than 500 μM. However, at higher concentrations (750 μM and 1000 μM) a dose-dependent decrease was observed in cell viability, equal to 87% and 78% of that in untreated cells, respectively. Since exposure to the lower doses of coffee extracts (0.250 mg/mL) or skatole (250 and 500 μM) did not affect cell viability, these conditions were used for subsequent experiments.

Intracellular ROS Levels in HT-29 Cells

The effect of skatole and the three types of assayed coffee samples on intracellular ROS generation in HT-29 cells was examined after 24 h using a fluorometric test employing the specific dye H_2DCF-DA. Cells treated only with H_2O_2 were used as a positive control. The intracellular ROS level results are shown in Figure 11. The fluorescence intensity (Figure 11A) decreased remarkably following coffee treatment at both 0.250 and 0.500 mg/mL concentrations, compared with the control group that received no coffee treatment. Specifically, in cells treated with coffee extracts at 0.250 and 0.500 mg/mL, the reduction in fluorescence intensity was 19% and 36.5% for instant coffee, 17.5% and 36% for Americano coffee, and 21.5% and 31.2% for espresso coffee, respectively. A blank control resulting from in vitro digestion was also examined and compared to the mock control to exclude side effects mediated by the digestion fluid, and it showed no significant differences compared to the control cells (Figure 11A). In addition, the possible pro-oxidant activity of skatole at non-toxic concentrations (250 and 500 μM) after 24 h of treatment was investigated (Figure 11B), showing significantly increased intracellular ROS levels in cells treated with skatole at 250 μM (13%) and 500 μM (8%) compared to the mock control (cells treated only with DMSO). In order to examine the antioxidant capacity of the assayed coffee samples, we used an

H_2DCF-DA assay to evaluate the intracellular ROS levels in HT-29 cells after 24 h treatment with skatole (250 and 500 µM) and the respective coffee treatments at 0.250 mg/mL. The data obtained are shown in Figure 12. After stimulation with 250 µM of skatole (Figure 12A), fluorescence intensity significantly decreased for all assayed coffee samples, at a rate of 28% for instant coffee, 39% for Americano coffee, and 45% for espresso coffee. After stimulation with 500 µM of skatole (Figure 12B), the fluorescence intensity decreased for all assayed samples at the following rates: 11% for instant coffee, 30% for Americano coffee, and 39% for espresso coffee.

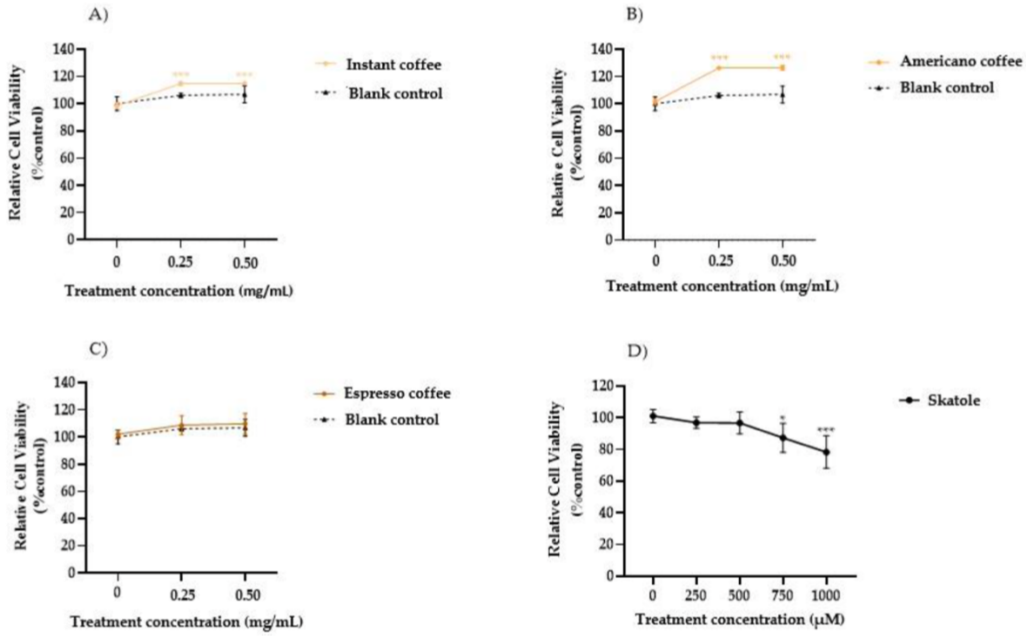


Figure 10. Evaluation of cell viability in HT-29 cells. The effect of treatment with different types of coffee extract: instant (**A**), Americano (**B**), and espresso

(**C**), at 0.250 and 0.500 mg/mL on cell viability was evaluated using the MTT assay after 24 h with respect to the mock control. The MTT test was used to determine the impact of skatole treatment (**D**) on cell viability after 24 h at the concentrations of 250, 500, 750, and 1000 μM compared with control cells (treated only with DMSO). The graph represented the mean and SD of three separate experiments. * p-value ≤ 0.05 and *** p-value ≤ 0.001 compared to the control group (calculated as fold-change relative to control cells, arbitrarily set at 100%).

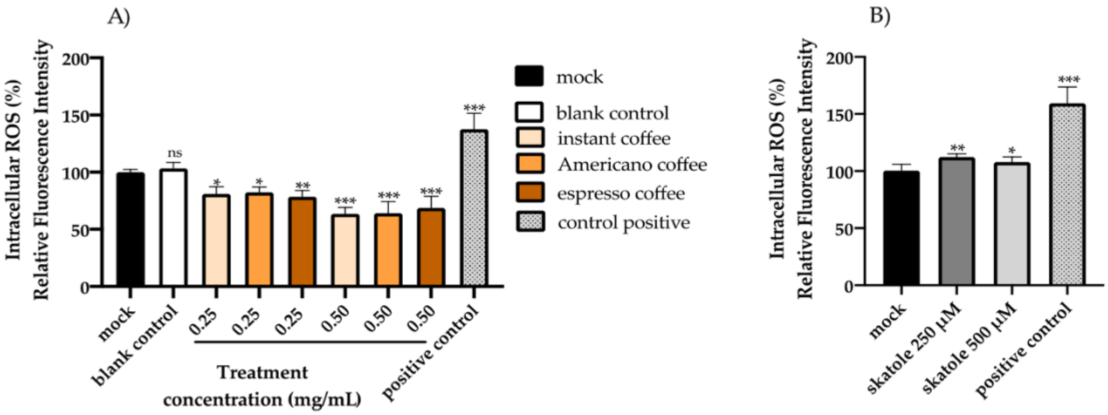


Figure 11. Evaluation of intracellular ROS level in HT-29 cells. The effect on the generation of intracellular ROS levels after treatment with a blank control (resulting from in vitro digestion) and the different types of coffee extract (instant, Americano, and espresso) at 0.250 and 0.500 mg/mL was evaluated using the H_2DCF-DA assay after 24 h of treatment, and compared with the mock control (untreated cells) (**A**). The effect of skatole treatment (**B**) on the

production of intracellular ROS was estimated by fluorometric assay after 24 h at the concentrations of 250 and 500 µM and compared to the control cells (treated with DMSO only). Cells treated only with H_2O_2 were used as a positive control. The graphs represent the mean and SD of three separate experiments. * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01 and *** *p*-value ≤ 0.001 compared to the control group (calculated as fold-change relative to control cells, arbitrarily set at 100%).

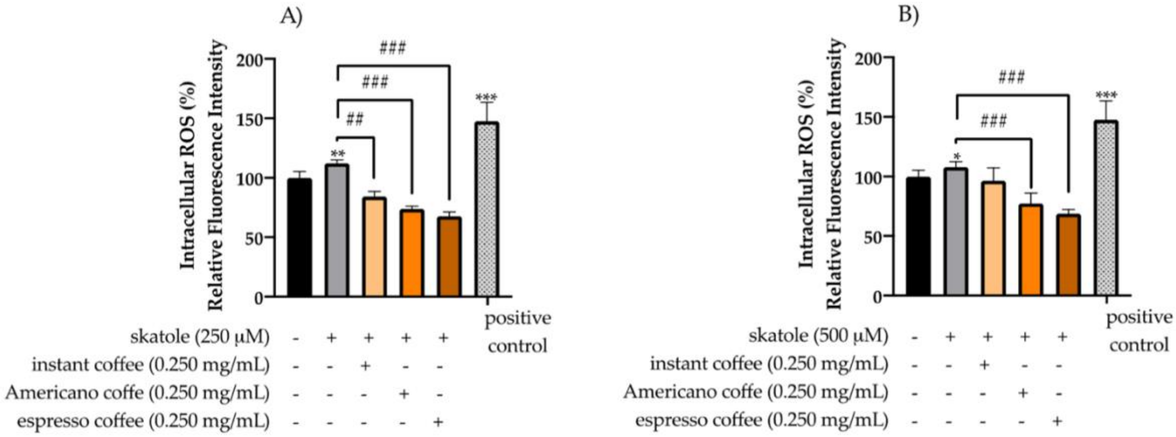


Figure 12. Evaluation of intracellular ROS levels under challenging conditions in HT-29 cells. The effect of treatment with different types of coffee extract (instant, Americano, and espresso) at 0.250 mg/mL after skatole treatment of 250 µM (**A**) and 500 µM (**B**) on the production of intracellular ROS levels was determined by H_2DCF-DA assay after 24 h of treatment and compared to the mock control. Cells treated only with H_2O_2 were used as a positive control. The graphs represent the mean and SD of three separate experiments. * *p*-value ≤ 0.05, ** *p*-value ≤ 0.01 and *** *p*-value ≤ 0.001 compared to untreated

control (calculated as fold-change relative to control cells, arbitrarily set at 100%). ## p-value ≤ 0.01 and ### p-value ≤ 0.001 skatole versus coffee treatment.

Anti-Inflammatory Effects of Coffee Extracts on Cytokine mRNA Expression Levels in HT-29 Cells

Real-time PCR analysis was performed to investigate the expression levels of mRNAs encoding the pro-inflammatory cytokines and chemokines TNF-α, IL-1β, IL-8, and IL12 in HT-29 cells under challenging conditions. In accordance with the literature (Perrone, D.; et al. 2012- Sarnelli, G.; et al. 2017), cells treated only with LPS were used as a positive control. Our data showed that cells treated with 250 μM of skatole for 24 h led to a significant up-regulation of TNF-α, IL-1β, IL-8, and IL-12 (1.61-, 3.07-, 1.92-, and 2.17-fold changes with respect to the mock control, respectively) (Figure 13). On the other hand, co-treatments with coffee extracts counterbalanced the pro-inflammatory effects mediated by skatole by down-modulating the expression of the analyzed cytokines to values almost comparable with the mock control. In particular, the mRNA expression levels of TNF-α (Figure 13A), IL-1β (Figure 13B), IL-8 (Figure 13C), and IL-12 (Figure 13D) were found to be significantly decreased when the cells were co-treated with espresso coffee (0.84-, 1.08-, 0.64-, and 0.55-fold changes with respect to the mock control, respectively). However, the levels of IL-8 (Figure 13C) and IL-12 (Figure 13D) were found to be markedly decreased in cells co-treated with instant coffee (1.13- and 1.19-fold changes with respect to the mock control, respectively), and IL-12 levels (Figure 13D) also decreased considerably upon co-treatment with Americano coffee (a 1.31-fold change with respect to the mock control).

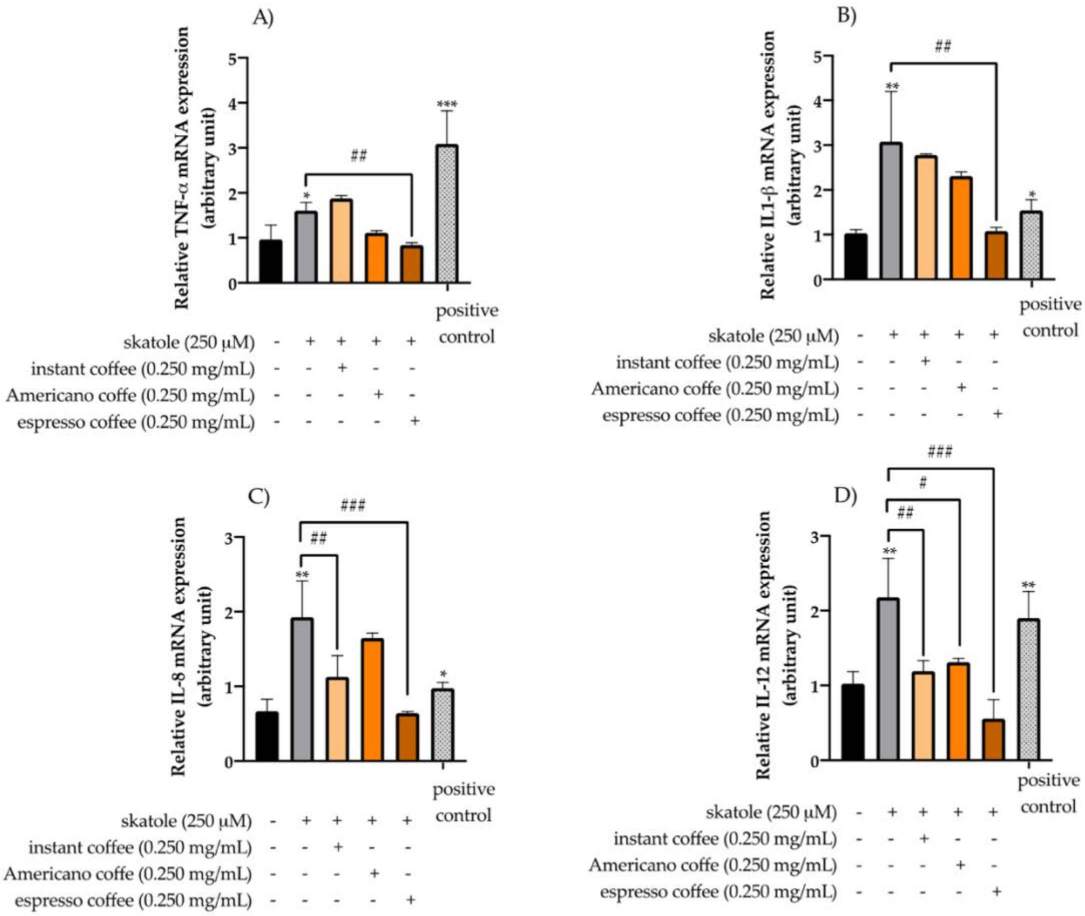


Figure 13. Evaluation of mRNA expression levels in HT-29 cells. The effect of treatment with the different types of coffee extract (instant, Americano and espresso) at 0.250 mg/mL after stimulation with 250 μM skatole on the expression level of TNF-α (**A**), IL-1β (**B**), IL-8 (**C**), and IL12 (**D**) was performed

using real-time PCR analysis after 24 h of treatment, and compared to the control group. Cells treated only with LPS were used as a positive control. The graph represented the mean and SD of three separate experiments. * p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001 compared to untreated control. # p-value ≤ 0.05, ## p-value ≤ 0.01 and ### p-value ≤ 0.001 skatole versus coffee treatment.

Discussion

The main goal of the current study was to assess the potential protective effect of coffee against pro-inflammatory and pro-oxidant conditions triggered by putrefactive compounds released in the presence of an altered intestinal microbiota through the HT-29 human colon cancer cell line model. Due to its ability to express characteristics of mature intestinal cells, the human colon adenocarcinoma cell line HT-29 is effectively used not only to study the biology of human colon cancers, but is also attracting particular attention in studies on food digestion and bioavailability (Verhoeckx, K.; et al. 2015). Notwithstanding the presence of several scientific investigations in the literature with regard to the positive effects of coffee intake on the prevention of colon cancer (Castaldo, L.; et al. 2020 - Bułdak, R.J.; et al. 2018 - Pérez-Burillo, S.; et al. 2019 - Vitaglione, P.; et al. 2012), there is still scarce knowledge concerning the potential anti-inflammatory and antioxidant properties of coffee in the presence of the putrefactive compounds that could be released in gut dysbiosis conditions.

In the present work, in order to mimic the effect of oral, gastric, and intestinal digestion, the INFOGEST protocol was followed (Minekus, M.; et al. 2014). This procedure is widely regarded as one of the most effective protocols to simulate the natural digestion process. In vitro intestinal models represent the gold standard in such investigations; in fact, they can rapidly provide useful information on the impact of food components on health status (Nissen, L.; et al. 2021). Despite the fecal inoculum method representing the most appropriate protocol to replicate in vitro colonic digestion, an increasing number of studies have reported that the combination of bacterial enzymes, such as Pronase E and Viscozyme L, represents a suitable alternative to reproducing intestinal fermentation (Annunziata, G.; et al. 2018 - Eker, M.E.; Karakaya, S. 2020 - Colombo, R.; et al. 2021 - Castaldo, L.; et al. 2021 - Izzo, L.; et al. 2020 - Castaldo, L.; et al. 2021).

In summary, we initially tested the cytotoxicity of skatole on HT-29 cells. The concentration range of skatole chosen to conduct the study ranged from 250 to 1000 μM based on previous studies reporting 1000 μM as the maximum skatole concentration found in human feces (Kurata, K.; et al. 2019). Our data revealed that HT-29 cell viability was affected in a concentration-dependent manner at the higher skatole concentrations tested (750 and 1000 μM), with a significant reduction in cell viability compared to control cells (87% and 78%, respectively). Kurata et al. 2019 evaluated the effects of different skatole concentrations on cell viability using Caco-2 cells, another human colon cancer cell line, showing that skatole promoted apoptosis in these cells in a dose-dependent and time-dependent manner. Their in vivo studies demonstrated that urinary skatole levels in patients significantly decreased after the consumption of probiotic formulations containing *Bifidobacterium* spp. and *Lactobacillus* spp. (Lombardi, F.; et al. 2020).

On the other hand, no cytotoxicity effects were detected in HT-29 cell analysis for espresso coffee samples, but in contrast, a minimal increase in cell viability, up to 20%, was shown for instant and American coffee samples. Moreover, our data suggest that all the assayed coffee samples had an important effect in suppressing ROS formation in HT-29 cells, owing to their antioxidant activity. At the lowest assayed concentration (0.250 mg/mL), the espresso coffee sample appeared to exert a greater reduction in ROS levels than the other coffee brew samples. Moreover, the results highlighted that even at low concentrations (250 and 500 μM), skatole was able to induce an increase in intracellular ROS levels, in particular, at 250 μM of skatole the ROS levels increased by 13%, while at 500 μM of skatole the ROS levels increased by 8%, compared to a control. The data showed that skatole treatment represents a demanding setting for oxidative stress, which promotes raised intracellular ROS levels in HT-29 cells. Interestingly, data obtained in the present scientific study demonstrated that the treatment of HT-29 cells with different types of coffee samples in the presence of skatole significantly decreased the production of ROS compared to untreated cells. Among the different kinds of coffee brews tested, espresso showed the most effective antioxidant activity, reaching a 45% decrease in ROS compared with untreated cells.

In this study, the expression of genes linked to inflammation was also examined. The results showed that skatole exposure triggered an increased expression of pro-inflammatory cytokines and chemokines TNF-α, IL-1β, IL-8, and IL12 in HT-29 cells. These findings are consistent with the literature, which suggests that skatole may have pro-inflammatory effects and reports that this tryptophan metabolite may play a role in the development of colorectal cancer and in the progression and pathogenesis of IBD (Van Nuenen, M.H.; et al. 2004 - Kanazawa, K.; et al. 1996 - Rodríguez-Carrasco, Y.; et al. 2019). Moreover, our data clearly indicated that all three types of coffee studied exhibit anti-inflammatory activity by decreasing the expression levels of cytokines in cells that were pretreated with skatole.

In this context, it has to be highlighted that espresso coffee showed both the highest antioxidant and anti-inflammatory properties among the coffee brews tested. Notably, a possible explanation for these findings could be related to one of our previous investigations, in which we reported that the espresso coffee sample shows a higher polyphenolic content than the other studied coffee samples, as analyzed using a UHPLC-Q-Orbitrap HRMS (Castaldo, L.; et al. 2021). CGAs were the predominant polyphenols quantified in the coffee samples, notably the three CQA isomers, which accounted for 66% to 71% of the total polyphenols. As reported by scientific evidence (Liang, N.; Kitts, D.D. 2016 - Tajik, N.; et al. 2017), CGAs exert a strong antioxidant activity and inhibit the expression of inflammatory factors. Therefore, these results may partly explain the improved reduction in ROS levels and the enhanced anti-inflammatory activity found after treating HT-29 cells with espresso samples in the presence of skatole, indicating that the higher level of polyphenols detected in espresso coffee samples may play an important role in limiting the formation of ROS by exerting anti-inflammatory effects in the presence of putrefactive compounds.

References

- Annunziata, G.; Maisto, M.; Schisano, C.; Ciampaglia, R.; Daliu, P.; Narciso, V.; Tenore, G.C.; Novellino, E. Colon bioaccessibility and antioxidant activity of white, green and black tea polyphenols extract after in vitro simulated gastrointestinal digestion. *Nutrients* **2018**, *10*, 1711.

- Bułdak, R.J.; Hejmo, T.; Osowski, M.; Bułdak, Ł.; Kukla, M.; Polaniak, R.; Birkner, E. The Impact of Coffee and Its Selected Bioactive Compounds on the Development and Progression of Colorectal Cancer In Vivo and In Vitro. *Molecules* **2018**, *23*, 3309.

- Castaldo, L.; Izzo, L.; De Pascale, S.; Narváez, A.; Rodriguez-Carrasco, Y.; Ritieni, A. Chemical Composition, In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Nutraceutical Fennel Waste Extract. *Molecules* **2021**, *26*, 1968.

- Castaldo, L.; Izzo, L.; Narváez, A.; Rodríguez-Carrasco, Y.; Grosso, M.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of Different Coffee Brews Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Foods* **2021**, *10*, 179.

- Castaldo, L.; Lombardi, S.; Gaspari, A.; Rubino, M.; Izzo, L.; Narváez, A.; Ritieni, A.; Grosso, M. In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Spent Coffee Grounds-Enriched Cookies. *Foods* **2021**, *10*, 1837.

- Castaldo, L.; Narváez, A.; Izzo, L.; Graziani, G.; Ritieni, A. In Vitro Bioaccessibility and Antioxidant Activity of Coffee Silverskin Polyphenolic Extract and Characterization of Bioactive Compounds Using UHPLC-Q-Orbitrap HRMS. *Molecules* **2020**, *25*, 2132.

- Castaldo, L.; Toriello, M.; Sessa, R.; Izzo, L.; Lombardi, S.; Narváez, A.; Ritieni, A.; Grosso, M. Antioxidant and Anti-Inflammatory Activity of Coffee Brew Evaluated after Simulated Gastrointestinal Digestion. *Nutrients* **2021**, *13*, 4368.

- Colombo, R.; Ferron, L.; Frosi, I.; Papetti, A. Advances in static in vitro digestion models after COST action Infogest consensus protocol. *Food Funct.* **2021**, *12*, 7619–7636.

- Duary, R.K.; Batish, V.K.; Grover, S. Immunomodulatory activity of two potential probiotic strains in LPS-stimulated HT-29 cells. *Genes Nutr.* **2014**, *9*, 1–16.

- Eker, M.E.; Karakaya, S. Influence of the addition of chia seeds and germinated seeds and sprouts on the nutritional and beneficial properties of yogurt. *Int. J. Gastron. Food Sci.* **2020**, *22*, 100276.

- Hsu, H.; Wen, M. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* **2002**, *277*, 22131–22139.

- Izzo, L.; Rodríguez-Carrasco, Y.; Pacifico, S.; Castaldo, L.; Narváez, A.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of a Red Cabbage Extract Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Antioxidants* **2020**, *9*, 955.

- Kanazawa, K.; Konishi, F.; Mitsuoka, T.; Terada, A.; Itoh, K.; Narushima, S.; Kumemura, M.; Kimura, H. Factors influencing the development of sigmoid colon cancer: Bacteriologic and biochemical studies. *Cancer Interdiscip. Int. J. Am. Cancer Soc.* **1996**, *77*, 1701–1706.

- Kurata, K.; Kawahara, H.; Nishimura, K.; Jisaka, M.; Yokota, K.; Shimizu, H. Skatole regulates intestinal epithelial cellular functions through activating aryl hydrocarbon receptors and p38. *Biochem. Biophys. Res. Commun.* **2019**, *510*, 649–655.

- Liang, N.; Kitts, D.D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* **2016**, *8*, 16.

- Liu, Z.-S.; Chen, P.-W.; Wang, J.-Y.; Kuo, T.-C. Assessment of Cellular Mutagenicity of Americano Coffees from Popular Coffee Chains. *J. Food Prot.* **2017**, *80*, 1489–1495.

- Lombardi, F.; Fiasca, F.; Minelli, M.; Maio, D.; Mattei, A.; Vergallo, I.; Cifone, M.G.; Cinque, B.; Minelli, M. The effects of low-nickel diet combined with oral administration of selected probiotics on patients with systemic nickel allergy syndrome (SNAS) and gut dysbiosis. *Nutrients* **2020**, *12*, 1040.

- Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carriere, F.; Boutrou, R.; Corredig, M.; Dupont, D. A standardised static in vitro digestion method suitable for food–an international consensus. *Food Funct.* **2014**, *5*, 1113–1124.

- Nissen, L.; Valerii, M.C.; Spisni, E.; Casciano, F.; Gianotti, A. Multiunit In Vitro Colon Model for the Evaluation of Prebiotic Potential of a Fiber Plus D-Limonene Food Supplement. *Foods* **2021**, *10*, 2371.

- Pérez-Burillo, S.; Mehta, T.; Esteban-Muñoz, A.; Pastoriza, S.; Paliy, O.; Rufián-Henares, J.Á. Effect of in vitro digestion-fermentation on green and roasted coffee bioactivity: The role of the gut microbiota. *Food Chem.* **2019**, *279*, 252–259.

- Perrone, D.; Farah, A.; Donangelo, C.M. Influence of coffee roasting on the incorporation of phenolic compounds into melanoidins and their relationship with antioxidant activity of the brew. *J. Agric. Food Chem.* **2012**, *60*, 4265–4275.

- Riccio, P.; Sessa, R.; de Nicola, S.; Petruzziello, F.; Trombetti, S.; Menna, G.; Pepe, G.; Maddalena, P.; Izzo, P.; Grosso, M. GATA-1 isoforms differently contribute to the production and compartmentation of reactive oxygen species in the myeloid leukemia cell line K562. *J. Cell. Physiol.* **2019**, *234*, 20829–20846.
- Rodríguez-Carrasco, Y.; Castaldo, L.; Gaspari, A.; Graziani, G.; Ritieni, A. Development of an UHPLC-Q-Orbitrap HRMS method for simultaneous determination of mycotoxins and isoflavones in soy-based burgers. *LWT-Food Sci. Technol.* **2019**, *99*, 34–42.
- Sarnelli, G.; Grosso, M.; Palumbo, I.; Pesce, M.; D'Alessandro, A.; Zaninotto, G.; Annese, V.; Petruzzelli, R.; Izzo, P.; Sepulveres, R. Allele-specific transcriptional activity of the variable number of tandem repeats of the inducible nitric oxide synthase gene is associated with idiopathic achalasia. *United Eur. Gastroenterol. J.* **2017**, *5*, 200–207.
- Tajik, N.; Tajik, M.; Mack, I.; Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *Eur. J. Nutr.* **2017**, *56*, 2215–2244.
- Trombetti, S.; Sessa, R.; Catapano, R.; Rinaldi, L.; Lo Bianco, A.; Feliciello, A.; Izzo, P.; Grosso, M. Exploring the Leukemogenic Potential of GATA-1S, the Shorter Isoform of GATA-1: Novel Insights into Mechanisms

Hampering Respiratory Chain Complex II Activity and Limiting Oxidative Phosphorylation Efficiency. *Antioxidants* **2021**, *10*, 1603.

- Van Nuenen, M.H.; Venema, K.; Van Der Woude, J.C.; Kuipers, E.J. The metabolic activity of fecal microbiota from healthy individuals and patients with inflammatory bowel disease. *Dig. Dis. Sci.* **2004**, *49*, 485–491.

- Verhoeckx, K.; Cotter, P.; López-Expósito, I.; Kleiveland, C.; Lea, T.; Mackie, A.; Requena, T.; Swiatecka, D.; Wichers, H. The impact of food bioactives on health: In vitro and ex vivo models. In *The Impact of Food Bioactives on Health*; SpringerOpen: Berlin, Germany, 2015; pp. 113–124.

- Vitaglione, P.; Fogliano, V.; Pellegrini, N. Coffee, colon function and colorectal cancer. *Food Funct.* **2012**, *3*, 916–922.

- Voloboueva, L.A.; Liu, J.; Suh, J.H.; Ames, B.N.; Miller, S.S. (R)-α-lipoic acid protects retinal pigment epithelial cells from oxidative damage. *Investig. Ophthalmol. Vis. Sci.* **2005**, *46*, 4302–4310.

- Wang, T.; Qin, L.; Liu, B.; Liu, Y.; Wilson, B.; Eling, T.; Langenbach, R.; Taniura, S.; Hong, J. Role of reactive oxygen species in LPS-induced production of prostaglandin E2 in microglia. *J. Neurochem.* **2004**, *2*, 939–947.

3.2. Antioxidant and Anti-Inflammatory Activity of Coffee Brew Evaluated after Simulated Gastrointestinal Digestion

Materials and Methods

Chemicals and Reagents

Phenolic standards (purity >98%) were obtained as follows: 3-CQA, 3,4,-diCQA, gallic acid, caffeic acid *p*-coumaric acid, ferulic acid, quinic acid, and caffeine were ac- quired from Sigma-Aldrich (Saint Louis, MO, USA). Standards used for antioxidants tests were 2,3,5-triphenyltetrazolio chloride (TPTZ), 2,2′-azino-bis-3-ethylbenzthiazoline- 6-sulphonic acid (ABTS), sodium sulfate ($NaSO_4$), potassium persulphate ($K_2S_2O_8$), 6- hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (commonly called Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium thiocyanate (KCNS), calcium chloride di- hydrate ($CaCl_2(H_2O)_2$), monosodium phosphate (NaH_2PO_4), anhydrous ferric chloride ($FeCl_3$), sodium hydroxide (NaOH), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), and sodium acetate ($C_2H_3NaO_3$) were provided from Sigma-Aldrich (Saint Louis, MO, USA). The enzymes and standards used to simulate in vitro GiD were pepsin (≥2500 U/mg solid) from porcine gastric mucosa, α-amylase (1000–3000 U/mg solid) from human saliva, bile salt, pancreatin, Pronase E (bacterial protease from Streptomyces griseus, ≥3.5 U/mg solid), pancreatin (8 × USP) from porcine pancreas, and Viscozyme L were acquired from Sigma-Aldrich (Saint Louis, MO, USA).

Solvents, namely formic acid (FA), methanol (MeOH), hydrochloric acid (HCl), and water (UHPLC-MS grade), were acquired from Merk (Darmstadt, Germany). Deionized water (<18 MX cm resistivity) was acquired from Millipore (Bedford, MA, USA).

Sampling and Coffee Brew Preparation

Coffee bean (*Coffea arabica* L.) samples were acquired from a coffee shop located in Naples, Southern Italy. Coffee beans were roasted using a laboratory roaster (Probat- Sample roaster, Emmerich am Rhein, Germany) for 16 min at a temperature between 195 and 227 ◦C. The roasting degree reached a medium degree on the Agtrom disk of the SCA (tile 55). Then, roasted coffee beans were grounded to 0.4 mm particle size in a coffee grinder (DeLonghi, KG79, Treviso, Italy). The filtered coffee brews were prepared using an American machine (Aigostar Chocolate 30 HIK, Milan, Italy) working with a water temperature of 90 ◦C. Coffee brews were prepared from 50 g of coffee powder and a volume of 600 mL of hot deionized water as declared by the manufacturer. The coffee brews were collected, freeze-dried, and stored until the analysis.

UHPLC and Orbitrap HRMS Analysis

Chromatographic analysis was carried out through an UHPLC (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) prepared with a Quaternary UHPLC pump, a degassing system, and an autosampler device.

Separation of polyphenols and caffeine was performed with a thermostated Kinetex (25 ◦C) column F5 (50 × 2.1 mm, 1.7 μm particle size, Phenomenex, Torrance, USA).

The eluent phase consisted of H_2O (A) and MeOH (B) both prepared at 0.1% of FA. The separation gradient started with 100% A for 1 min, decreased to 20% A in 2 min, and decreased again reaching 0% A in 3 min. Then, the gradient rose up again reaching 100% A in 2 min and was held for 2 min for column re-equilibration. The injection volume was 5 μL, whereas 0.5 mL/min was the flow rate.

Mass spectrometry analysis was performed through a Q-Exactive Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA). An electrospray (ESI) source was simultaneously operated in fast positive/negative ion switching mode, setting two scan events (Full ion MS and All ion fragmentation: AIF) for all investigated compounds.

Full MS/AIF experiments were performed with the settings: automatic gain control (AGC) target, 1e6; mass resolution, 35,000 full width at half maximum (FWHM); microscans, 1; maximum injection time, 200 ms; and scan range, 80–1200 *m/z* for full MS analysis. AIF mode conditions were: maximum injection time 200 ms; mass resolving power to 17,500 FWHM, AGC target, 1e6; scan time, 0.10 s; scan range, 80–1200 *m/z*; isolation window, 5 *m/z*; and retention time to 30 s. The collision energies were varied in the range of 10–60 eV. In both scan events the instrument was set to auxiliary gas, 10; spray voltage, 3.5 kV; sheath gas 45; and capillary temperature to 275 ◦C.

Identification was performed considering exact mass measurements at a mass tolerance of 5 ppm. Data treatment was carried out using Quan/Qual Browser Xcalibur software 3.1.66.19 (Xcalibur, Thermo Fischer Scientific, Waltham, MA, USA).

Simulated GiD

The in vitro digestion process was carried out according to the protocol proposed from INFOGEST network (Minekus, M.; et al. 2014). The simulated fluids (salivary: SSF; gastric: SGF; and intestinal: SIF) were prepared according to the proportion of salts described by Izzo et al. 2020.

In short, to simulate the oral phase, 5 mL of brewed coffee brew was mixed with 975 μL of water, 3.5 mL of SSF, 25 μL of 0.3 M $CaCl_2 (H_2O)_2$, and 0.5 mL of α-amylase enzyme (50 mg of 250 U/mg solid). Then, the solution was incubated in a shaker bath for 2 min at 37 ◦C. Afterward, 7.5 mL of SGF, 695 μL of water, 1.6 mL of pepsin solution (2000 U/mL), and 5 μL of 0.3 M $CaCl_2$ $(H_2O)_2$ were added to the mixture to simulate gastric conditions. The pH of the solution was reduced to 3 with 1 M HCl, and after that, the sample was incubated for 2 h at 37 ◦C. Finally, to simulate the intestinal conditions, 2.5 mL of bile salt solution (65 mg/mL), 11 mL of SIF, 1.3 mL of water, 5 mL pancreatin solution (100 U/mL of trypsin activity), and 40 μL of 0.3 M $CaCl_2 (H_2O)_2$ were added. The pH value of the mixture was increased to 7 using NaOH 1 M. After that, the solution was incubated for 120 min at 37 ◦C. Then, the samples were centrifuged at 5000× *g* at 37 ◦C for 10 min, and the remaining pellets were collected. In order to simulate the colonic stage, the pellets were treated according to a previously described procedure (Castaldo, L.; et al. 2021). Briefly, 5 mL of Pronase E solution (1 mg/mL) was added. The mixture was incubated for 1 h at 37 ◦C. Finally, 5 mL of water and 150 μL of Viscozyme L were added to the mixture and incubated for 16 h at 37 ◦C. After that, the samples were centrifuged at 5000× *g* for 10 min. The supernatants were collected, freeze-dried, and stored until the analysis.

In Vitro Antioxidant Activity

The antioxidant activity of the digested and not-digested coffee brew samples was assessed by using two different free radical scavenging activity methods, including ABTS and DPPH tests, and the ferric ion reducing antioxidant power assay, namely FRAP. Data obtained were expressed as mmol of Trolox equivalents (TE) per gram of sample dry weight (DW). Results were calculated from the calibration curve prepared in triplicate at 6 concentration levels (5–200 μM of Trolox).

FRAP Assay

The FRAP test was carried out according to a methodology previously described (Benzie, I.F.; et al. 1996). In short, the FRAP reagent was prepared by mixing 12.5 mL of acetate buffer (0.3 M, pH 3.6), 1.25 mL of $FeCl_3$ solution (20 mM), and 1.25 mL of TPTZ solution (10 mM). Then, 150 μL of sample was added to 2.85 mL of FRAP reagent. After 4 min, the values of absorbance at 593 nm were immediately recorded.

ABTS Assay

The ABTS test was carried out based on the procedure described by Dini et al. 2020. In short, forty-four microliters of $K_2S_2O_8$ (2.5 mM) were added to aqueous ABTS (2.5 mL; 7 mM). After 16 h at room temperature, the solution was diluted with EtOH to reach an absorbance value of 0.70 (±0.02) at 734 nm. Then, 100 µL of sample was added to 1000 µL of ABTS radical working solution. After 3 min, the values of absorbance were immediately recorded.

DPPH Assay

The DPPH test was performed using the procedure suggested by Dini et al. 2020. Briefly, 4 mg of DPPH was diluted with MeOH until the absorbance value reached 0.9 (±0.02) at 517 nm. Afterward, 1 mL of DPPH radical working solution was added to 0.20 mL of sample. After 10 min, the values of absorbance were immediately recorded.

Total Phenolic Content Assay

The Folin–Ciocalteu method was performed to evaluate the total phenolic content (TPC) value in accordance with the procedure previously described (Izzo, L.; et al. 2020 - Izzo, L.; et al. 2020). In brief, 0.125 mL of Folin–Ciocalteu reagent and 0.50 mL of deionized H_2O were mixed with 0.125 mL of sample.

After 6 min of incubation at room temperature, 1 mL of H_2O and 1.25 mL Na_2CO_3 solution (7.5%) were added to the solution. Finally, after 90 min of incubation, the values of absorbance at 760 nm were immediately recorded. Results were calculated from the calibration curves prepared in triplicate at 6 concentration levels (0.25–0.01 mg/mL of gallic acid).

Cell Culture

The HT-29 human colon carcinoma cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were kept in Dulbecco's Modified Eagle's Medium, high glucose, in the presence of 10% fetal bovine serum, 4 mM glutamine, streptomycin (10 mg/mL), and penicillin (10 U/mL) as previously reported (Sarnelli, G.; et al. 2017). The cell culture was maintained at 37 ◦C and humidified atmosphere (5% CO_2). Moreover, in order to maintain growth in the log phase, cells were subcultured by trypsinization (all reagents from Sigma-Aldrich, Saint Louis, MO, USA). Cells have been routinely checked for mycoplasma contamination with the PCR Mycoplasma Test Kit (AppliChem A3744, Darmstadt, Germany). Only cells negative for mycoplasma contamination were used.

Cell Treatment

Individual stock solution of assayed samples (digested and not-digested coffee brews) at 2 mg/mL were prepared in cell culture media. Therefore, in order to set the optimal experimental conditions to evaluate intracellular ROS levels and to perform cell viability assays and Western blot analysis, HT-29 cells were initially treated with different concentrations of the assayed samples (0.250 to 2 mg/mL) as previously reported (Mojica, B.E.; et al. 2018) for 24, 48, and 72 h.

Analysis of Cell Viability

Cell viability was evaluated through spectrophotometric assay using the thiazolyl blue tetrazolium bromide (MTT) test (Riccio, P.; et al. 2019 - Sodaro, G.; et al. 2018). Briefly, 24 h before treatment, cells were plated into 96-well plates (100 μL cell suspension per well) at a density of 5.5 × 105 cells/mL. Then, cells were treated with the investigated samples. After 24, 48, and 72 h of treatment, 10 μL of the MTT labeling reagent provided by the Cell Proliferation Kit I (Roche, Mannhein, Germany) was added to each well. After 4 h, 100 μL of detergent solubilization buffer 1× (10% SDS in 0.01M HCl) was added to dissolve the insoluble purple formazan products into a colored solution according to the procedure recommended by the manufacturer. Measurement of the soluble formazan product in each well was carried out by photometric reading at 570/690 nm on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Evaluation of Intracellular ROS Level

Intracellular ROS level production was estimated using the fluorescent dye 2′7′- dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Voloboueva, L.A.; et al. 2005). Cells were plated in 96-well black plates (100 μL cell suspension per well) at a density of 5 × 105 cells/mL. After 48 h treatment with different concentrations of the assayed samples (0.250 and 0.500 mg/mL), the cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS). In order to test ROS levels under challenging conditions, after 24 h treatment with 0.250 mg/mL of assayed samples, cells were exposed to 10 ng/mL LPS for an additional 24 h to induce ROS production (Zhou, M.; et al. 2018) - Hsu, H.; et al. 2002). All cell samples were incubated and labelled with 10 μM of H2DCF-DA diluted in Hank's Balanced Salt Solution (HBSS) at 37 ◦C for 20 min in the dark. Then, the dye was removed, and the cells were washed twice with PBS. Cells treated with 100 μM hydrogen peroxide (H_2O_2) were used as positive control. Therefore, fluorescence was measured on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at excitation/emission wavelengths of 485/538 nm.

Protein Extraction and Western Blot Analysis

HT-29 cells were plated into 24-well plates at a density of 1.4 × 105 cells/mL. Cells were treated with the assayed samples at the concentrations of 0.250 and 0.500 mg/mL. After 24 h, cells were exposed further for 24 h to lipopolysaccharide (LPS) to induce inflammation (10 ng/mL). For protein extraction, cells were collected and washed twice with 4 mL of PBS by centrifugation at 1000 rpm for 10 min at 4 ◦C. The pellets were resuspended with 50 μL of RIPA Buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.5

μL of the protein inhibitor cocktail mixture (Sigma-Aldrich), and incubated for 30 min on ice. Then, the whole cell lysates were collected (Brinkhoff, A.; et al. 2018 - Shi, J.; et al. 2017). Protein concentration was measured by spectrophotometric analysis, according to the Bradford method (Carlsson, N.; et al. 2011) with the Bio-Rad protein analysis reagent (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed on 20 μg of total protein extracts. Cell extracts were separated using 4–15% Mini-Protean TGX Stain-Free Precast Gels reagent (Bio-Rad Laboratories) and transferred to membranes using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Antibodies against actin (1:1000 dilution/#SC-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-6 (1:1000 dilution/#ab9324; Abcam, Cambridge, UK), IL-10 (1:1000 dilution/#sc-1783; Santa Cruz Biotechnology), and NF-kB p65 subunit (1:2000 dilution/#ab1604a; Millipore) were diluted in TBT-Tween 20 (TBT-T) buffer containing 5% of milk and applied to mem- branes, followed by overnight incubation at 4 ◦C. The next day, filters were washed three times with TBT-T for 5 min and incubated for 45 min with respective secondary antibodies conjugated to peroxidase. The antigen–antibody complexes were then detected using Clarity Western ECL Substrate (Bio-Rad Laboratories). Quantitative densitometry of bands was carried out by analyzing ChemiDoc system (Bio-Rad Laboratories), and the quantification of the signal was performed by ImageJ as previously reported (Huang, C.; et al. 2007) - Busiello, T.; et al. 2017).

Statistical Analysis

Tukey's test, at the level of significance p-value ≤ 0.05, was used to evaluate differences in antioxidant activity and TPC content between average values of the assayed samples. Statistical differences between untreated control and treated cells were calculated using the two-way analysis of variance (ANOVA) or one-way ANOVA test followed by Dunnett's multiple comparisons test and/or multiple Student's t-tests, where appropriate. The level of significance was set at p-value ≤ 0.05, p-value ≤ 0.01 to be significant (* and **, respectively), and at p-value ≤ 0.001 to be highly significant (***). All analyses were performed in triplicate. Data treatment was performed using Stata 12 software (StataCorp LP, College Station, TX, USA).

Results

Identification of Polyphenol Compounds and Caffeine in the Assayed Samples Using UHPLC-Q-Exactive Orbitrap

UHPLC-Q-Orbitrap HRMS analysis was conducted to identify active molecules (n = 16), including caffeine, CGAs, and hydroxycinnamic acids, in the digested and not- digested samples. Optimal separation of the studied compounds in the assayed samples was achieved by the UHPLC system in a total run time of 13 min. However, the 5- and 4-feruloylquinic acids (FQAs) isomers were quantified as a sum, due to the suboptimal separation. The identification of active compounds was assessed in both Full ion MS and AIF modes. All experiments were conducted in negative ESI− mode, except for caffeine which showed improved fragmentation patterns in positive ESI+ mode. Mass parameters including chemical formula, ion assignment, measured and theoretical mass (*m*/*z*), retention time (RT), and accuracy are shown in Table 2. Isomer identification of *p*-coumaroylquinic acid (*p*-CoQA, *m*/*z* 337.09289), dicaffeoylquinic acid (diCQA, *m*/*z* 515.11950), feruloyl-caffeoylquinic and caffeoyl-feruloylquinic acids (FCQA and CFQA, *m*/*z* 529.13245), and CQA (*m*/*z* 353.08780) was performed by comparing the RT of the peaks with those of the standards and by comparison of fragmentation pattern obtained with data previously reported in the literature.

Table 2. UHPLC-MS parameters of the assayed analytes (n = 16).

Compound	Chemical Formula	Adduct Ion	RT (min)	Measured Mass (*m/z*)	Theoretical Mass (*m/z*)	Accuracy (Δ mg/kg)
Quinic Acid	$C_7H_{12}O_6$	$[M-H]^-$	1.12	191.05531	191.05611	−4.19
5-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.18	353.0879	353.08780	0.03
4-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.19	353.08768	353.08780	−0.34
Caffeic Acid	$C_9H_8O_4$	$[M-H]^-$	3.20	179.03442	179.03498	−3.13
Caffeine	$C_8H_{10}N_4O_2$	[M+H]+	3.21	195.08757	195.08765	−0.41
3-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.22	353.08762	353.08780	−0.51
3-*p*CoQA	$C_{16}H_{18}O_8$	$[M-H]^-$	3.31	337.09232	337.09289	−1.69
5-*p*CoQA	$C_{16}H_{18}O_8$	$[M-H]^-$	3.32	337.0929	337.09289	0.03
3-FQA	$C_{17}H_{20}O_9$	$[M-H]^-$	3.39	367.10309	367.10346	−1.01
4+5-FQA	$C_{17}H_{20}O_9$	$[M-H]^-$	3.40	367.10303	367.10346	−1.17
4,5-CFQA	$C_{26}H_{26}O_{12}$	$[M-H]^-$	3.43	529.13495	529.13245	4.72
Ferulic Acid	$C_{10}H_{10}O_4$	$[M-H]^-$	3.46	193.05017	193.05063	−2.38
p-Coumaric acid	$C_9H_8O_3$	$[M-H]^-$	3.48	163.03934	163.04006	−4.42
3,4-diCQA	$C_{25}H_{24}O_{12}$	$[M-H]^-$	3.50	515.12103	515.11950	2.97
3,5-diCQA	$C_{25}H_{24}O_{12}$	$[M-H]^-$	3.53	515.11993	515.11950	0.83
3,4-FCQA	$C_{26}H_{26}O_{12}$	$[M-H]^-$	3.65	529.13247	529.13245	0.04

Abbreviations: CQA: caffeoylquinic acid; *p*CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acids; diCQA: dicaffeoylquinic acid.

Quantification of Polyphenol Compounds and Caffeine in the Assayed Samples Using UHPLC-Q-Exactive Orbitrap

Brewed coffee samples (digested and not-digested) were investigated using a UHPLC- Q-Orbitrap HRMS method. The quantitative analysis of the target compounds was carried out using calibration curves, and regression coefficients >0.990 were obtained for all analytes. Some important CGAs (n = 11), phenolic acids (n = 4), and caffeine were found in both digested and not-digested samples.

Total CGAs were quantified at concentrations ranging from 68.92 (not-digested samples) up to 81.50 mg/g (digested samples), as shown in Table 3. In the here investigated samples, CQAs were found as the most detected compounds ranging from 43.31% (not- digested samples) to 46.45% (digested samples) of total CGAs. The CQAs were revealed in a concentration range between 29.85 and 37.86 mg/g. Notably, 5-CQA showed to be the more relevant CGA, being quantified in digested and not-digested samples at a mean value of 25.97 and 21.40 mg/g, respectively. Regarding the FQAs isomers, 3- and 4-FQA represented from 30.48 to 30.64% of total CGAs detected in the investigated samples, in a concentration range between 21.11 and 24.84 mg/g. As far as *p*-CoQAs are concerned, 5-pCoQA was the most abundant compound detected in both digested and not-digested samples at a mean value of 7.53 and 7.19 mg/g, respectively. Moreover, diCQAs mainly represented by 3,4- and 3,5-diCQA were detected at a concentration level of 2.61 and 2.23 mg/g in digested and not-digested samples, respectively.

Concerning 3,4-FCQA and 4,5-CFQA isomers, they were found as the less relevant compounds being measured at concentrations ranging from 0.83 up to 1.41 mg/g.

Table 3. CGA (*n* = 11), phenolic acids (*n* = 4), and caffeine content in the assayed samples.

Compounds	Not-Digested Coffee Brew		Digested Coffee Brew	
	mg/g	SD	mg/g	SD
3-CQA	3.40 *	0.43	4.97 *	0.69
4-CQA	5.05 *	0.05	6.92 *	0.06
5-CQA	21.40 *	0.43	25.97 *	0.69
3-CoQA	6.54	0.51	6.27	0.28
5-CoQA	7.19	0.25	7.53	0.96
3-FQA	9.21 *	0.38	10.56 *	0.44
4+5-FQA	11.90 *	0.52	14.28 *	0.53
3,4-diCQA	1.70	0.04	1.88	0.06
3,5-diCQA	0.53 *	0.02	0.74 *	0.01
3-FCQA	1.17 *	0.05	1.41 *	0.01
4-CFQA	0.83 *	0.02	0.98 *	0.01
TOTAL CGA	68.92 *		81.50 *	
Caffeic acid	0.19 *	0.01	1.55 *	0.03
Quinic acid	1.69 *	0.20	2.79 *	0.02
Ferulic acid	2.71 *	0.08	4.12 *	0.07
p-coumaric acid	0.71 *	0.08	1.12 *	0.07
TOTAL phenolic	5.29 *		9.57 *	
Caffeine	19.96 *	0.07	17.68 *	0.27

Differences between groups were statistically analyzed with Tukey's test; * p-value ≤ 0.05 was considered significant. Abbreviations: CQA: caffeoylquinic acid; *p*CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acids; diCQA: dicaffeoylquinic acid.

On the other hand, some important phenolic acids were quantified in the here-assayed samples. Digested samples showed higher total phenolic content (9.57 mg/g) when compared to not-digested samples (5.29 mg/g). Moreover, ferulic acid was found at a concentration level significantly higher than the other assayed phenolic acids, ranging from 2.71 (not-digested samples) up to 4.12 mg/g (digested samples). Apart from those, caffeine was assessed in the herein investigated samples. Not-digested samples showed higher caffeine content (19.96 mg/g) than the digested samples (17.68 mg/g), as shown in Table 3.

Antioxidant Activity and Total Phenolic Content

The antioxidant activity of the digested and not-digested samples was measured and compared using three different tests (FRAP, DPPH, and ABTS). Data were displayed as millimoles of Trolox per 100 g of coffee brew dried matter (mean value and ±SD). The results are reported in Table 4. In all assayed methods, the digested coffee brews showed significantly higher antioxidant capacity (p-value ≤ 0.05) than the not-digested coffee brews. Furthermore, the results revealed that the digested samples showed a two-fold increase in antioxidant activity in both DPPH and ABTS tests (51.4 vs. 22.8 and 89.2 vs. 44.6 mmol Trolox/100 g DW, respectively). As shown in Table 3, the digested samples also showed a significantly higher TPC value (p-value ≤ 0.05) compared to not-digested coffee brew samples. In addition, the blank control resulting from the in vitro digestion experiments was tested, and the results are presented in Table 5. Comparing the FRAP, DPPH, and ABTS data against TPC values measured through the Folin–Ciocalteu test, strong positive correlations were observed (R^2 = 0.892, 0.984, and 0.892 for TPC vs. FRAP, vs. DPPH, and vs. ABTS, respectively).

Table 4. TPC and antioxidant capacity evaluated by FRAP, DPPH, and ABTS of the samples.

Samples	FRAP		DPPH		ABTS		TPC	
	mmol TE/100 g	±SD	mmol TE/100 g	±SD	mmol TE/100 g	±SD	mg GAE/g	±SD
Coffee Brew	67.1 *	3.2	22.8 *	0.6	44.6 *	1.2	97.07 *	1.52
Coffee Brew Digested	72.5 *	0.7	51.4 *	0.3	89.2 *	0.6	106.64 *	2.01

Differences between groups were statistically analyzed with Tukey's test; * *p*-value ≤ 0.05 was considered significant.

Table 5. TPC and antioxidant capacity evaluated by FRAP, DPPH and ABTS of the Blank control.

Samples	FRAP		DPPH		ABTS		TPC	
	mmol TE/100g	±SD	mmol TE/100g	±SD	mmol TE/100g	±SD	mg GAE/g	±SD
Blank control	1.07	0.01	0.39	0.01	1.02	0.01	0.07	0.00

Effect of not-Digested or Digested Coffee on Cell Viability in HT-29 Cells

MTT assay was performed to evaluate the potential cytotoxicity exerted by not-digested and digested coffee extracts in HT-29 cells at different concentrations according to literature data (Mojica, B.E.; et al. 2018). As shown in Figure 14, at concentrations ranging from 0.250 mg/mL to 0.750 mg/mL, both extracts showed similar low effects on cell viability, although it has to be noted that, at the lowest concentration used in this study (0.250 mg/mL), an increase in cell viability was observed in both cases. Conversely, at higher concentrations (1–2 mg/mL) cell viability was significantly reduced for both extracts as compared to untreated control. In this context, it is interesting to note that not-digested coffee samples showed dramatic reduction in cell viability after treatment with 2 mg/mL for 24 h (14%), 48 h (10%), and 72 h (3%). In contrast, exposure to digested samples at the same concentration resulted in a less marked reduction in cell viability, with 77% of viable cells detected at 24 h, 65% at 48 h, and 33% at 72 h (Figure 14A–C), thus suggesting that digested coffee exhibits less cytotoxic effects on HT-29 cells than not-digested coffee samples. To exclude effects mediated by the digestion fluid, the blank control resulting from in vitro digestion was analyzed by MTT assay and compared with untreated cells. Results (Figure 14D) indicated no significant differences between the blank control and untreated cells at 24 h, 48 h, and 72 h of treatment.

Evaluation of Intracellular ROS Level

Changes in intracellular ROS levels in HT-29 cells were evaluated using the H_2DCF- DA assay after 24 h, 48 h, and 72 h exposure to non-toxic doses (0.250 and 0.500 mg/mL) of non-digested and digested coffee samples. Cells treated with 100 μM H_2O_2 were used as positive control. The data summarized in Figure 15 show that treatment with not-digested or digested coffee samples decreased the levels of intracellular ROS levels as compared to untreated cells. Even in this case, different trends of antioxidant activity were detected in not-digested and digested coffee samples, with digested coffee samples showing a more pronounced antioxidant capacity, more evident at the longer exposure times (48 h and 72 h) (Figure 15B,C). In addition, to verify the real effects mediated by the digested coffee and to exclude effects mediated by the digestion fluid, a blank control resulting from in vitro digestion was included in the H_2DCF-DA assay showing no significant differences with untreated cells at each exposure time.

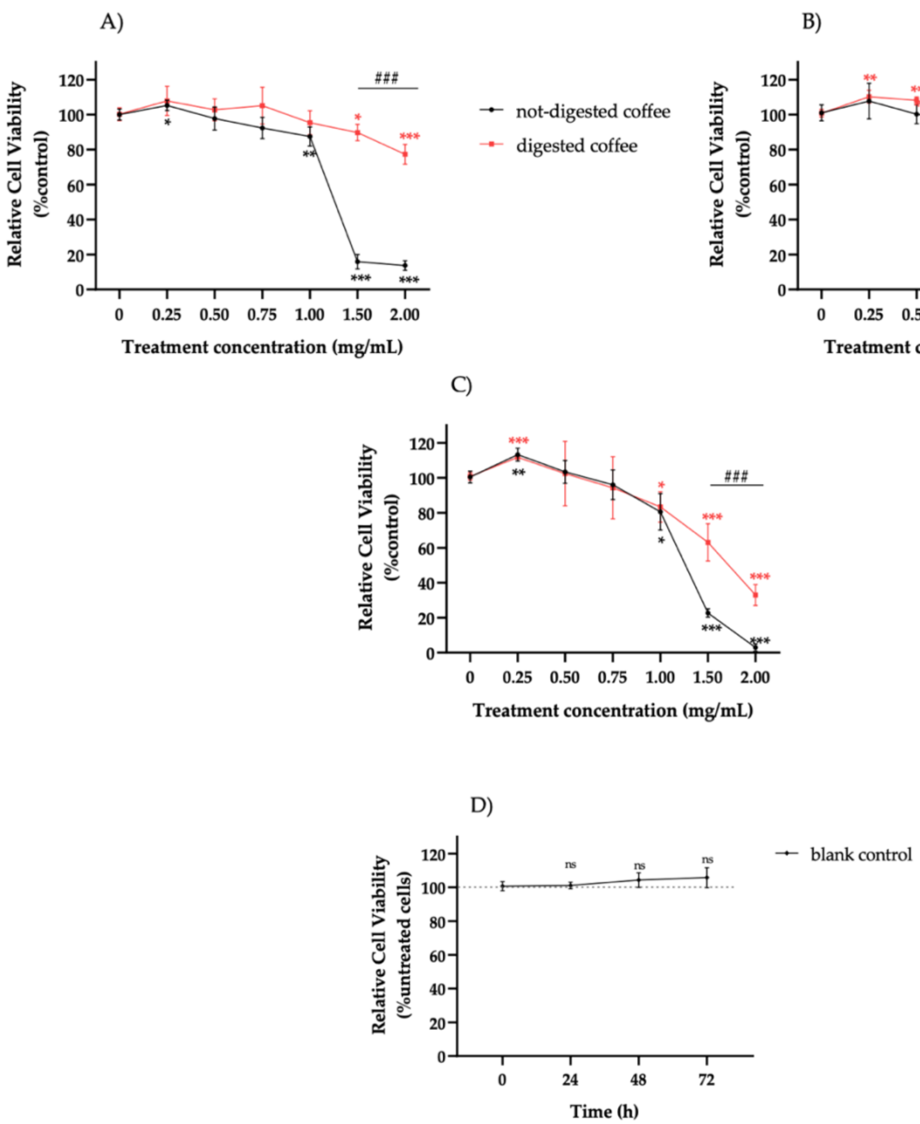


Figure 14. Evaluation of response to digested or not-digested coffee samples in HT-29 cells. The effect of treatment with not-digested or digested coffee extract at 0.25, 0.50, 0.75, 1.00, 1.50, and 2.00 mg /mL on cell viability was estimated by MTT assay after 24 h (**A**), 48 h (**B**), and 72 h (**C**) as compared to

untreated control. The effect of treatment with vehicle (blank control) resulting from in vitro digestion on cell viability was evaluated by MTT assay after 24 h, 48 h, and 72 h (**D**), as compared to untreated cells. No significant differences were observed between blank control and untreated cells. ns: not statistically significant. Mean ± SD of three independent experiments were plotted on the graph. Differences were considered significant when p-value < 0.05 and p-value < 0.01 and highly significant when p-value ≤ 0.001. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus untreated control (calculated as fold-change relative to untreated cells, arbitrarily set at 100%); ### $p < 0.001$ not-digested coffee versus digested coffee.

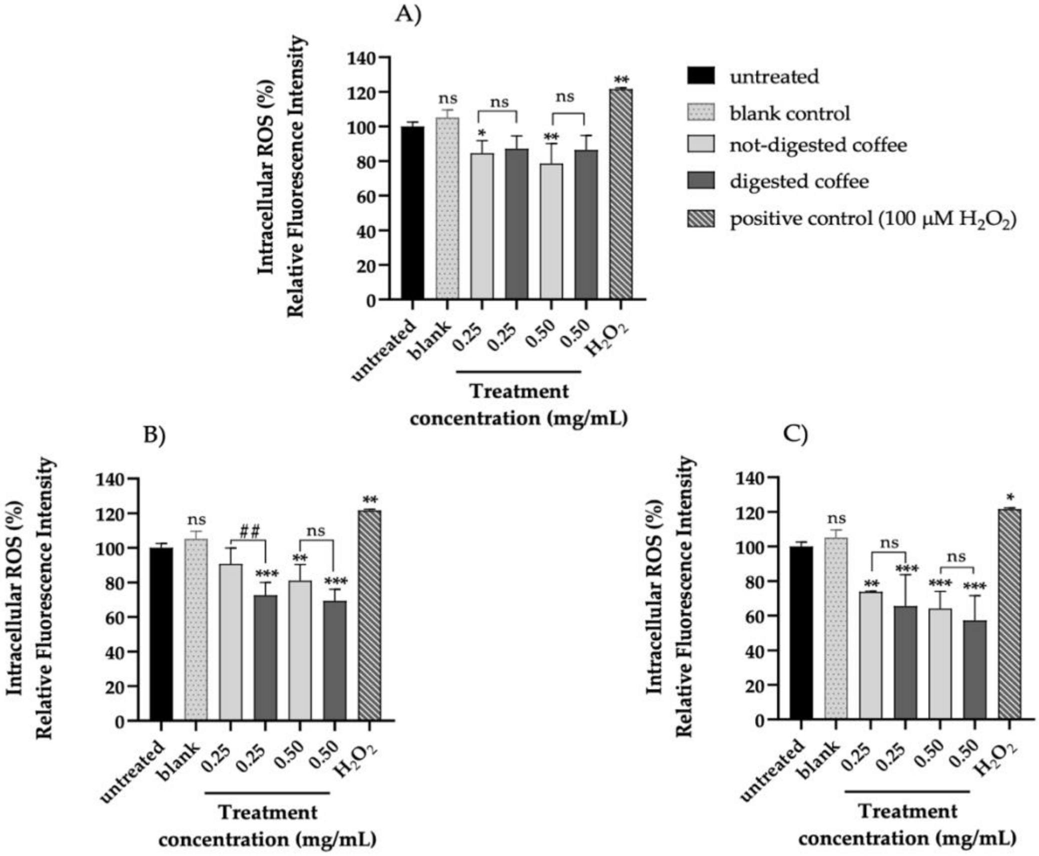


Figure 15. Evaluation of intracellular OS level in HT-29 cells treated with not-digested or digested coffee samples. Intracellular ROS level was assessed by H_2DCF-DA assay on HT-29 cells treated for 24 h (**A**), 48 h (**B**), and 72 h (**C**) with different concentrations of digested and not-digested coffee samples (0.25 and 0.5 mg/mL) and with vehicle (blank control) resulting from in vitro digestion related to untreated cells. Cells treated with 100 μM H_2O_2 were used as positive control. Mean ± SD of three independent experiments were plotted on the graph. Differences were considered significant when p-value ≤ 0.05 and p-value ≤ 0.01 and highly significant when *p*-value ≤ 0.001. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus untreated control (calculated as fold-change relative to untreated cells, arbitrarily set at 100%); ## $p < 0.01$ not-digested

coffee versus digested coffee. No significant differences were observed between blank control and untreated cells at 24h 48h, and 72h. ns: not statistically significant.

Anti-Inflammatory Effects of Not-Digested and Digested Coffee

Western blotting analysis was performed to determine the antioxidant effects of the assayed samples in modulating the expression of the pro-inflammatory protein IL-6 and NF-kB p65 subunit and the expression of the anti-inflammatory protein IL-10. Data obtained from HT-29 cells treated for 24 h with digested and not-digested coffee and then co-exposed to LPS for additional 24 h as pro-inflammatory action are shown in Figure 16. Notably, the results highlighted that the digested coffee samples showed lower expression levels of IL-6 and NF-kB p65 subunit levels than the not-digested samples (1.35 vs. 0.90 arbitrary units and 1.08 vs. 0.90 arbitrary units, respectively), indicating that the digestion process releases molecular compounds with more effective anti-inflammatory activity than the native coffee samples (Figure 16A,B,D,E). Furthermore, to underline the anti-inflammatory effect of the assayed digested coffee sample, expression levels of the anti-inflammatory cytokine IL-10 were examined, resulting to be more enhanced in response to the treatment with digested coffee compared to not-digested coffee samples (Figure 16A,C). To better investigate the antioxidant capacity of not-digested and digested coffee, we also analyzed intracellular ROS levels using the H2DCF-DA assay after 48 h under challenging conditions after LPS treatment. In fact, according to scientific evidence, LPS treatment promotes intestinal inflammation by inducing intracellular ROS production and oxidative injury in HT-29 cells (Zhou, M.; et al. 2018 - Wang, T.; et al. 2004 - Hsu, H.; et al. 2002). Based on these observations, LPS-treated cells, similarly to H_2O_2, showed 30% increased ROS levels compared with the untreated control, thus representing a challenging condition for oxidative stress. After stimulation with LPS, treatments with 0.25 mg/mL of not-digested or digested coffee significantly reduced the levels of ROS in HT-29 cells relative to LPS-stimulated cells by 15% and 30%, respectively. These results confirmed the pro-oxidant status induced by LPS stimulus and suggest that the more protective effects observed for digested coffee treatment are mediated, at least in part, by antioxidant mechanisms of ROS scavenging (Figure 17).

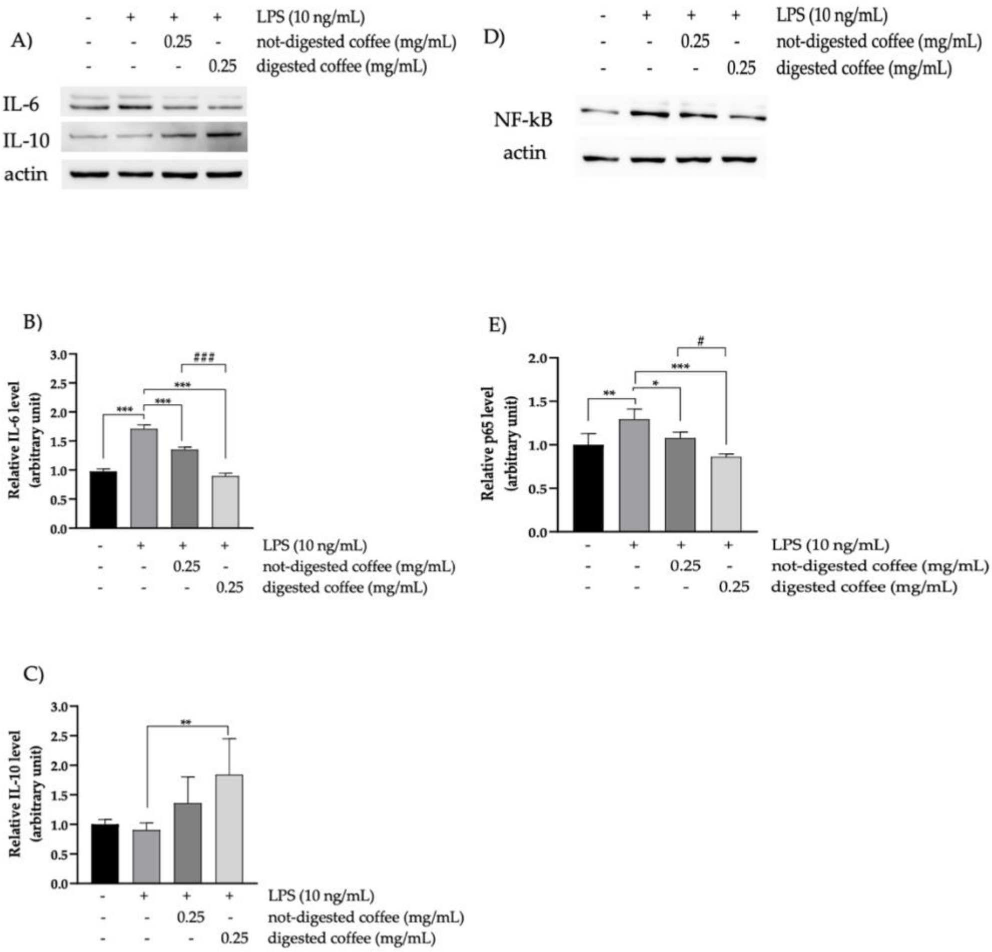


Figure 16. Anti-inflammatory effects of digested coffee samples in LPS-stimulated HT-29 cells. Western blot analysis of the expression levels of interleukin-6 (IL-6) (**A**), interleukin-10 (IL-10) (**A**), and NF-kB (p65 subunit) (**D**) in total cell lysates from untreated control and LPS-stimulated HT-29 cells treated for 48 h with not-digested coffee or digested coffee samples loading. (**B**,**C**,**E**) Densitometric analysis of Western blots. Band intensities were quantified and normalized to actin used as control. All data were analyzed for statistical significance by two-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p-value ≤ 0.05 and p-value ≤ 0.01 and highly significant

when *p*-value ≤ 0.001. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ versus untreated control (calculated as fold-change relative to untreated cells, arbitrarily set at 1); # $p \leq 0.05$, and ### $p \leq 0.001$ not-digested coffee versus digested coffee. Differences were considered significant when *p*-value ≤ 0.05 and highly significant when *p*-value ≤ 0.0001. * *p*-value ≤ 0.05, ** *p*-value ≤ 0.0001 versus LPS-stimulated control; # *p*-value ≤ 0.05, ### $p \leq 0.001$ not-digested coffee versus digested coffee.

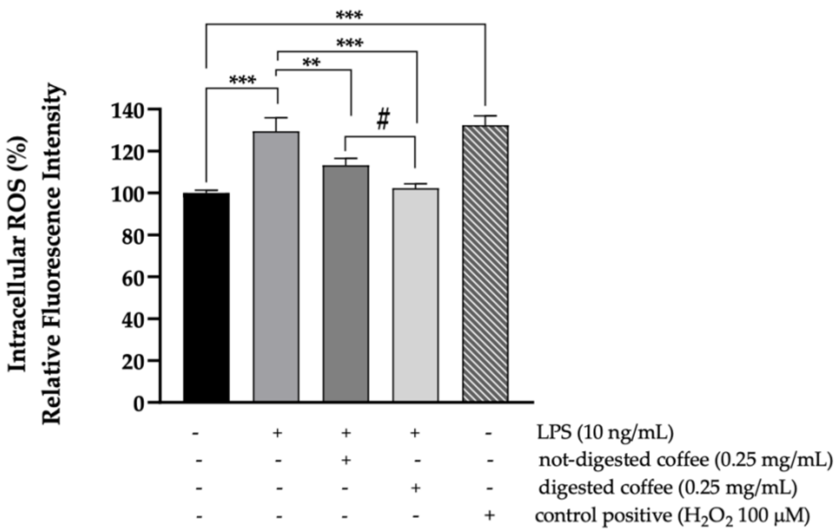


Figure 17. Evaluation of intracellular ROS level in HT-29 cells treated with not-digested and digested coffee after LPS treatments. Intracellular ROS levels were assessed by H_2DCF-DA assay in LPS-stimulated HT-29 cells treated for 48h with digested and not-digested coffee samples (0.25 mg/mL) compared with untreated cells. H_2O_2 (100 µM) was used as positive control. Mean ± SD of three independent experiments were plotted on the graph. Differences were considered significant when *p*-value ≤ 0.05, *p*-value ≤ 0.01 and highly significant when *p*-value ≤ 0.001. ** $p \leq 0.01$, and *** $p \leq 0.001$ versus untreated control (calculated as fold-change relative to untreated cells, arbitrarily set at 100%) and/or LPS-stimulated cells; # $p \leq 0.05$, not-digested coffee versus digested coffee.

Discussion

The present study aimed to provide useful information regarding the bioactivities of coffee brew after simulated GiD. Despite the many scientific works present in the literature concerning the beneficial activities of coffee polyphenols in protecting against colon cancer, the study of bioactivities of compounds released after in vitro digestion has been barely investigated to date. Hence, the main goal of this work was to investigate the antioxidant and anti-inflammatory activities of coffee brews after GiD through the HT-29 human colon cancer cell model. The INFOGEST protocol, recognized as one of the most suitable procedures capable of simulating the physiological digestion process, was performed until the duodenal phase, whereas the associated action of Pronase E (mix of bacterial protease) and Viscozyme L (mix of carbohydrases) was used to mimic the gut microbiota activity since the INFOGEST protocol does not cover the large intestinal phase. Although the use of the fecal inoculum is recognized as the most suitable method to simulate in vitro colonic digestion, an ever-expanding amount of scientific works propose as an effective alternative to reproduce the intestinal fermentation the use of a mix of bacterial enzymes, such as Viscozyme L and Pronase E Annunziata, G.; et al. 2018 - Eker, M.E.; et al. 2020 - Colantuono, A.; et al. 2018 - Colombo, R.; et al. 2021).

Moreover, to better understand the biological activity of coffee, changes in active molecules were also evaluated using a Q-Exactive Orbitrap mass spectrometer. In detail, four phenolic acids, eleven CGAs, and caffeine were identified, quantified, and compared in digested and not-digested coffee brew samples.

Overall, the obtained data highlighted that the filtered coffee brew may represent a rich source of bioactive molecules and the GiD process could affect the monitored active compounds present in a cup of filtered coffee. Moreover, the results clearly indicate that 5-CQA was the predominant active compound found in digested and not-digested coffee samples. Our findings are comparable to those of Farah et al. (Farah, A. 2019) who reported that 5-CQA accounted for 41 to 48% of total CGAs found in different brewed coffee. Concerning total CGAs occurrence in assayed samples, the levels found in the digested coffee brews were higher than the non-digested ones. These data were in accordance with previously published evidence showing increased CGAs content after the colonic phase. Bekerdam et al. (Bekedam, E.K.; et al. 2008) demonstrated that enzymatic hydrolysis using bacterial proteases to mimic the colon stage was able to release CGA and phenolics from coffee samples.

As reported by Pérez-Burillo et al. 2019, melanoidins, which possess significant antioxidant potential, contain in their complex structure a wide range of antioxidant molecules, mainly represented by CGAs and phenolic acids including hydroxycinnamic acids such as ferulic, caffeic, and *p*-coumaric acids (Goya, L.; et al. 2007).

Recently, several scientific studies reported that the coffee melanoidins escape the upper digestion process; however, the activity of gut microbiota could play a role in the release of dietary polyphenols, including CGAs, incorporated into melanoidins. In the present work, the amount of hydroxycinnamic acids found in digested samples was higher than in the not-digested ones. This is in agreement with the literature (Ludwig, I.A.; et al. 2013) suggesting that low-molecular catabolites such as the phenolic acids mentioned above represent the main CGA breakdown metabolites mediated by gut microbiota.

CGAs are well recognized as potent molecules with antioxidant and anti-inflammatory activity (Maalik, A.; et al. 2016). Strong scientific evidence has highlighted the ability of CGAs to delay glucose absorption, modulate glucose and lipid metabolism, thus helping to prevent degenerative and non-degenerative diseases (Kang, N.J.; et al. 2011 - McCarty, M.F. 2005 - Cornelis, M.C.; et al. 2007 - Trombetti, S.; et al. 2021).

On the other hand, antioxidant capacity and TPC values of digested and not-digested samples were also assessed and compared. The obtained data showed that both the TPC values and antioxidant capacity of coffee significantly increased after the simulated gastrointestinal process. Regarding the antioxidant activity, the digested samples showed a two-fold increase, in both DPPH and ABTS tests, compared with the not-digested samples, whereas the TPC value of the digested samples increased about 10% after the colonic stage. Similarly, such phenomena were also observed in our previous study (Castaldo, L.; et al. 2021) which showed that after the simulated digestion process, the antioxidant capacity and TPC value increased in all types of coffee brews assayed, namely espresso, americano, and instant coffee. Moreover, similar data were obtained by Campos-Vega et al. 2015, who demonstrated that both antioxidant capacity and the colon bioaccessibility of polyphenols from spent coffee, rich in CGAs and melanoidins, were significantly higher after colonic fermentation compared to the non-digested samples. Interestingly, results obtained from FRAP, DPPH, and ABTS assays were positively correlated with TPC values, highlighting that these tests could be suitable to provide reliable information about the antioxidant molecules released after the colonic stage.

The antioxidant capacity of the assayed samples was also estimated by assessing changes in intracellular ROS levels in HT-29 cells. In accordance with literature that support a protective effect of coffee bioactive molecules against oxidative stress, our findings showed that coffee has an important effect in preventing ROS production, which is partly due to its antioxidant activity. It is interesting to note that both digested and not-digested samples induced a significant decrease in the levels of intracellular ROS. In particular, when HT-29 human colon cancer cells were treated for 48h with the assayed samples, the digested coffee highlighted more effective antioxidant activity than the not-digested ones, suggesting that the higher number of phytochemicals found in the samples after the digestion process could play an important role in managing ROS production.

On the other hand, no cytotoxicity effects were revealed in HT-29 cells analysis for both not-digested and digested coffee samples tested at a concentration ranging between 0.250 to 0.750 mg/mL. However, at the highest concentrations tested (1 to 2 mg/mL), not-digested coffee samples showed a drastic reduction in cell viability. Nevertheless, the results showed that the gastrointestinal process was able to release compounds that exhibited a less marked reduction in cell viability than not-digested coffee samples. As a whole, our results are in agreement with several previous studies. In fact, Mojica et al. 2018 compared the impact of the different coffee extracts on the growth inhibitory activity of HT-29 cells. The cells were treated with 1 to 50× dilutions of the coffee stock solutions, and the coffee samples were tested without a previous simulated GiD. The authors reported that polyphenolic compounds in different coffee extracts were able to reduce cell growth, revealing that bioactive phytochemicals could positively influence cell survival, avoiding changes in metabolism and mitochondrial structure. Furthermore, Choi et al. 2015 reported that among the bioactive compounds present in coffee, including kahweol, caffeic acid, CGA, and caffeine, only kahweol exhibited significant cytotoxicity in HT-29 cell lines, increasing the expression of caspase-3 and inhibiting the HT-29 cell growth. A further in vitro study (Nam, S.-H.; et al. 2017) on the effect of coffee CGA on HT-29 cells reported that treatment with 1 mM CGA significantly reduced the growth rate of the HT-29 cell by 52%.

LPS, an endotoxin of the cell wall of Gram-negative bacteria, is commonly used as an inflammatory stimulus to induce the production of cytokines (Zhou, M.; et al. 2018) - Shi, J.; et al. 2017). Our data showed reduced protein levels of the pro-inflammatory IL-6 and NF-kB in HT-29 cells treated for 48h with coffee extracts and then exposed to LPS. The presence of CGAs in coffee appears to be fundamental. In fact, previous studies (Liang, N.; et al. 2016 - Farah, A. 2019 - Shin, H.S.; et al. 2015) have highlighted that coffee CGAs in LPS-stimulated Caco-2 cells were able to downregulate the key transcription factors including the pro-inflammatory cytokines IL-6 and tumor necrosis factor-alpha. In addition, our findings reveled increased levels of IL-10, an anti-inflammatory cytokine, after the treatment with digested coffee than with not-digested coffee extract. LPS treatment also is involved in the intestinal inflammation by inducing intracellular ROS production and oxidative injury in several cell models, including HT-29 cells (Zhou, M.; et al. 2018 - Wang, T.; et al. 2004 - Hsu, H.; et al. 2002).

Therefore, we observed that LPS treatment, representing a challenging condition for oxidative stress, induced increased oxidative stress levels in HT-29 cells. Pretreatment with both coffee samples effectively inhibited LPS-induced ROS levels, more efficiently with the digested coffee extract, suggesting that the more effective anti-inflammatory effects observed for this coffee treatment are mediated, at least in part, by antioxidant mechanisms of ROS scavenging.

References

- Castaldo, L.; Narváez, A.; Izzo, L.; Graziani, G.; Ritieni, A. In Vitro Bioaccessibility and Antioxidant Activity of Coffee Silverskin Polyphenolic Extract and Characterization of Bioactive Compounds Using UHPLC-Q-Orbitrap HRMS. *Molecules* **2020**, *25*, 2132.
- Lim, D.; Chang, J.; Ahn, J.; Kim, J. Conflicting effects of coffee consumption on cardiovascular diseases: Does coffee consumption aggravate pre-existing risk factors? *Processes* **2020**, *8*, 438.
- Granby, K.; Fagt, S. Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. *Anal. Chim. Acta* **2004**, *520*, 177–182.
- Cano-Marquina, A.; Tarín, J.; Cano, A. The impact of coffee on health. *Maturitas* **2013**, *75*, 7–21.
- Mure, K.; Maeda, S.; Mukoubayashi, C.; Mugitani, K.; Iwane, M.; Kinoshita, F.; Mohara, O.; Takeshita, T. Habitual coffee consumption inversely associated with metabolic syndrome-related biomarkers involving adiponectin. *Nutrition* **2013**, *29*, 982–987.
- Andersen, L.F.; Jacobs, D.R., Jr.; Carlsen, M.H.; Blomhoff, R. Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. *Am. J. Clin. Nutr.* **2006**, *83*, 1039–1046.

- Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, caffeine, and health outcomes: An umbrella review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156.
- Sánchez-Quesada, C.; Romanos-Nanclares, A.; Navarro, A.M.; Gea, A.; Cervantes, S.; Martínez-González, M.Á.; Toledo, E. Coffee consumption and breast cancer risk in the SUN project. *Eur. J. Nutr.* **2020**, *59*, 3461–3471.
- Di Matteo, G.; Spano, M.; Grosso, M.; Salvo, A.; Ingallina, C.; Russo, M.; Ritieni, A.; Mannina, L. Food and COVID-19: Preventive/co-therapeutic strategies explored by current clinical trials and in silico studies. *Foods* **2020**, *9*, 1036
- Schmit, S.L.; Rennert, H.S.; Rennert, G.; Gruber, S.B. Coffee consumption and the risk of colorectal cancer. *Cancer Epidemiol. Prev. Biomark.* **2016**, *25*, 634–639.
- Kang, N.J.; Lee, K.W.; Kim, B.H.; Bode, A.M.; Lee, H.-J.; Heo, Y.-S.; Boardman, L.; Limburg, P.; Lee, H.J.; Dong, Z. Coffee phenolic phytochemicals suppress colon cancer metastasis by targeting MEK and TOPK. *Carcinogenesis* **2011**, *32*, 921–928.
- Abraham, S. Inhibition of in vivo genotoxicity by coffee. *Food Chem. Toxicol.* **1989**, *27*, 787–792.
- Hou, N.; Liu, N.; Han, J.; Yan, Y.; Li, J. Chlorogenic acid induces reactive oxygen species generation and inhibits the viability of human colon cancer cells. *Anti-Cancer Drugs* **2017**, *28*, 59–65.

- Sarriá, B.; Martínez-López, S.; Mateos, R.; Bravo-Clemente, L. Long-term consumption of a green/roasted coffee blend positively affects glucose metabolism and insulin resistance in humans. *Food Res. Int.* **2016**, *89*, 1023–1028.
- Jia, H.; Aw, W.; Egashira, K.; Takahashi, S.; Aoyama, S.; Saito, K.; Kishimoto, Y.; Kato, H. Coffee intake mitigated inflammation and obesity-induced insulin resistance in skeletal muscle of high-fat diet-induced obese mice. *Genes Nutr.* **2014**, *9*, 389.
- Rebello, S.A.; Chen, C.H.; Naidoo, N.; Xu, W.; Lee, J.; Chia, K.S.; Tai, E.S.; van Dam, R.M. Coffee and tea consumption in relation to inflammation and basal glucose metabolism in a multi-ethnic Asian population: A cross-sectional study. *Nutr. J.* **2011**, *10*, 61.
- Giovannucci, E. Metabolic syndrome, hyperinsulinemia, and colon cancer: A review. *Am. J. Clin. Nutr.* **2007**, *86*, 836S–842S
- Galeone, C.; Turati, F.; La Vecchia, C.; Tavani, A. Coffee consumption and risk of colorectal cancer: A meta-analysis of case–control studies. *Cancer Causes Control.* **2010**, *21*, 1949–1959.
- Gan, Y.; Wu, J.; Zhang, S.; Li, L.; Cao, S.; Mkandawire, N.; Ji, K.; Herath, C.; Gao, C.; Xu, H. Association of coffee consumption with risk of colorectal cancer: A meta-analysis of prospective cohort studies. *Oncotarget* **2017**, *8*, 18699
- Sinha, R.; Cross, A.J.; Daniel, C.R.; Graubard, B.I.; Wu, J.W.; Hollenbeck, A.R.; Gunter, M.J.; Park, Y.; Freedman, N.D. Caffeinated

and decaffeinated coffee and tea intakes and risk of colorectal cancer in a large prospective study. *Am. J. Clin. Nutr.* **2012**, *96*, 374–381.

- De Marco, L.M.; Fischer, S.; Henle, T. High molecular weight coffee melanoidins are inhibitors for matrix metalloproteases. *J. Agric. Food Chem.* **2011**, *59*, 11417–11423.
- Wang, H.-Y.; Qian, H.; Yao, W.-R. Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chem.* **2011**, *128*, 573–584.
- Moreira, A.S.; Nunes, F.M.; Domingues, M.R.; Coimbra, M.A. Coffee melanoidins: Structures, mechanisms of formation and potential health impacts. *Food Funct.* **2012**, *3*, 903–915.
- Perrone, D.; Farah, A.; Donangelo, C.M. Influence of coffee roasting on the incorporation of phenolic compounds into melanoidins and their relationship with antioxidant activity of the brew. *J. Agric. Food Chem.* **2012**, *60*, 4265–4275.
- Mullen, W.; Nemzer, B.; Stalmach, A.; Ali, S.; Combet, E. Polyphenolic and hydroxycinnamate contents of whole coffee fruits from China, India, and Mexico. *J. Agric. Food Chem.* **2013**, *61*, 5298–5309.
- Castaldo, L.; Lombardi, S.; Gaspari, A.; Rubino, M.; Izzo, L.; Narváez, A.; Ritieni, A.; Grosso, M. In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Spent Coffee Grounds-Enriched Cookies. *Foods* **2021**, *10*, 1837.

- Naveed, M.; Hejazi, V.; Abbas, M.; Kamboh, A.A.; Khan, G.J.; Shumzaid, M.; Ahmad, F.; Babazadeh, D.; FangFang, X.; Modarresi-Ghazani, F. Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed. Pharmacother.* **2018**, *97*, 67–74.
- Tajik, N.; Tajik, M.; Mack, I.; Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *Eur. J. Nutr.* **2017**, *56*, 2215–2244.
- Hernández-Arriaga, A.M.; Oomah, B.D.; Campos-Vega, R. Microbiota source impact in vitro metabolite colonic production and anti-proliferative effect of spent coffee grounds on human colon cancer cells (HT-29). *Food Res. Int.* **2017**, *97*, 191–198.
- Bakuradze, T.; Lang, R.; Hofmann, T.; Stiebitz, H.; Bytof, G.; Lantz, I.; Baum, M.; Eisenbrand, G.; Janzowski, C. Antioxidant effectiveness of coffee extracts and selected constituents in cell-free systems and human colon cell lines. *Mol. Nutr. Food Res.* **2010**, *54*, 1734–1743.
- Saura-Calixto, F.; Serrano, J.; Goñi, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492–501.
- Castaldo, L.; Izzo, L.; Narváez, A.; Rodríguez-Carrasco, Y.; Grosso, M.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of Different Coffee Brews Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Foods* **2021**, *10*, 179.

- Stalmach, A.; Steiling, H.; Williamson, G.; Crozier, A. Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch. Biochem. Biophys.* **2010**, *501*, 98–105.
- Spencer, J.P.; Chowrimootoo, G.; Choudhury, R.; Debnam, E.S.; Srai, S.K.; Rice-Evans, C. The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Letters* **1999**, *458*, 224–230.
- Olthof, M.R.; Hollman, P.C.; Katan, M.B. Chlorogenic acid and caffeic acid are absorbed in humans. *J. Nutr.* **2001**, *131*, 66–71.
- Vitaglione, P.; Fogliano, V.; Pellegrini, N. Coffee, colon function and colorectal cancer. *Food Funct.* **2012**, *3*, 916–922.
- Aljahdali, N.; Gadonna-Widehem, P.; Anton, P.M.; Carbonero, F. Gut Microbiota Modulation by Dietary Barley Malt Melanoidins. *Nutrients* **2020**, *12*, 241.
- Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carriere, F.; Boutrou, R.; Corredig, M.; Dupont, D. A standardised static in vitro digestion method suitable for food–an international consensus. *Food Funct.* **2014**, *5*, 1113–1124.
- Annunziata, G.; Maisto, M.; Schisano, C.; Ciampaglia, R.; Daliu, P.; Narciso, V.; Tenore, G.C.; Novellino, E. Colon bioaccessibility and antioxidant activity of white, green and black tea polyphenols extract after in vitro simulated gastrointestinal digestion. *Nutrients* **2018**, *10*, 1711.

- Bekedam, E.K.; Schols, H.A.; Van Boekel, M.A.; Smit, G. Incorporation of chlorogenic acids in coffee brew melanoidins. *J. Agric. Food Chem.* **2008**, *56*, 2055–2063.
- Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.
- Brinkhoff, A.; Sieberichs, A.; Engler, H.; Dolff, S.; Benson, S.; Korth, J.; Schedlowski, M.; Kribben, A.; Witzke, O.; Wilde, B. Pro-inflammatory Th1 and Th17 cells are suppressed during human experimental endotoxemia whereas anti-inflammatory IL-10 producing T-cells are unaffected. *Front. Immunol.* **2018**, *9*, 1133.
- Busiello, T.; Ciano, M.; Romano, S.; Sodaro, G.; Garofalo, O.; Bruzzese, D.; Simeone, L.; Chiurazzi, F.; Fiammetta Romano, M.; Costanzo, P. Role of ZNF224 in cell growth and chemoresistance of chronic lymphocitic leukemia. *Hum. Mol. Genet.* **2017**, *26*, 344–353.
- Campos-Vega, R.; Vázquez-Sánchez, K.; López-Barrera, D.; Loarca-Piña, G.; Mendoza-Díaz, S.; Oomah, B.D. Simulated gastrointestinal digestion and in vitro colonic fermentation of spent coffee (*Coffea arabica* L.): Bioaccessibility and intestinal permeability. *Food Res. Int.* **2015**, *77*, 156–161.
- Carlsson, N.; Borde, A.; Wölfel, S.; Åkerman, B.; Larsson, A. Quantification of protein concentration by the Bradford method in the

presence of pharmaceutical polymers. *Anal. Biochem.* **2011**, *411*, 116–121.

- Castaldo, L.; Izzo, L.; De Pascale, S.; Narváez, A.; Rodriguez-Carrasco, Y.; Ritieni, A. Chemical Composition, In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Nutraceutical Fennel Waste Extract. *Molecules* **2021**, *26*, 1968.
- Choi, D.W.; Lim, M.S.; Lee, J.W.; Chun, W.; Lee, S.H.; Nam, Y.H.; Park, J.M.; Choi, D.H.; Kang, C.D.; Lee, S.J. The cytotoxicity of kahweol in HT-29 human colorectal cancer cells is mediated by apoptosis and suppression of heat shock protein 70 expression. *Biomol. Ther.* **2015**, *23*, 128.
- Colantuono, A.; Ferracane, R.; Vitaglione, P. Potential bioaccessibility and functionality of polyphenols and cynaropicrin from breads enriched with artichoke stem. *Food Chem.* **2018**, *245*, 838–844.
- Colombo, R.; Ferron, L.; Frosi, I.; Papetti, A. Advances in static in vitro digestion models after COST action Infogest consensus protocol. *Food Funct.* **2021**, *12*, 7619.
- Cornelis, M.C.; El-Sohemy, A. Coffee, caffeine, and coronary heart disease. *Curr. Opin. Lipidol.* **2007**, *18*, 13–19.
- Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A.J. An Environmentally Friendly Practice Used in Olive Cultivation Capable of Increasing Commercial Interest in Waste Products from Oil Processing. *Antioxidants* **2020**, *9*, 466.

- Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A. Effects of Trichoderma biostimulation on the phenolic profile of extra-virgin olive oil and olive oil by-products. *Antioxidants* **2020**, *9*, 284.
- Eker, M.E.; Karakaya, S. Influence of the addition of chia seeds and germinated seeds and sprouts on the nutritional and beneficial properties of yogurt. *Int. J. Gastron. Food Sci.* **2020**, *22*, 100276.
- Farah, A. *Coffee: Consumption and Health Implications*; Royal Society of Chemistry: London, UK, 2019.
- Goya, L.; Delgado-Andrade, C.; Rufián-Henares, J.A.; Bravo, L.; Morales, F. Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Mol. Nutr. Food Res.* **2007**, *51*, 536–545.
- Hsu, H.; Wen, M. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* **2002**, *277*, 22131–22139.
- Huang, C.; Becker, M.F.; Keto, J.W.; Kovar, D. Annealing of nanostructured silver films produced by supersonic deposition of nanoparticles. *J. Appl. Phys.* **2007**, *102*, 054308.
- Izzo, L.; Castaldo, L.; Narváez, A.; Graziani, G.; Gaspari, A.; Rodríguez-Carrasco, Y.; Ritieni, A. Analysis of Phenolic Compounds in Commercial Cannabis sativa L. Inflorescences Using UHPLC-Q-Orbitrap HRMS. *Molecules* **2020**, *25*, 631.

- Izzo, L.; Pacifico, S.; Piccolella, S.; Castaldo, L.; Narváez, A.; Grosso, M.; Ritieni, A. Chemical Analysis of Minor Bioactive Components and Cannabidiolic Acid in Commercial Hemp Seed Oil. *Molecules* **2020**, *25*, 3710.
- Izzo, L.; Rodríguez-Carrasco, Y.; Pacifico, S.; Castaldo, L.; Narváez, A.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of a Red Cabbage Extract Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Antioxidants* **2020**, *9*, 955.
- Lee, W.-S.; Shin, J.-S.; Jang, D.S.; Lee, K.-T. Cnidilide, an alkylphthalide isolated from the roots of Cnidium officinale, suppresses LPS-induced NO, PGE2, IL-1β, IL-6 and TNF-α production by AP-1 and NF-κB inactivation in RAW 264.7 macrophages. *Int. Immunopharmacol.* **2016**, *40*, 146–155.
- Liang, N.; Kitts, D.D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* **2016**, *8*, 16
- Ludwig, I.A.; Paz de Peña, M.; Concepción, C.; Alan, C. Catabolism of coffee chlorogenic acids by human colonic microbiota. *Biofactors* **2013**, *39*, 623–632.
- Maalik, A.; Bukhari, S.M.; Zaidi, A.; Shah, K.H.; Khan, F.A. Chlorogenic acid: A pharmacologically potent molecule. *Acta Pol. Pharm.* **2016**, *73*, 851–854.

- McCarty, M.F. A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk. *Med. Hypotheses* **2005**, *64*, 848–853.
- Mojica, B.E.; Fong, L.E.; Biju, D.; Muharram, A.; Davis, I.M.; Vela, K.O.; Rios, D.; Osorio-Camacena, E.; Kaur, B.; Rojas, S.M. The impact of the roast levels of coffee extracts on their potential anticancer activities. *J. Food Sci.* **2018**, *83*, 1125–1130.
- Nam, S.-H.; Ko, J.-A.; Jun, W.; Wee, Y.-J.; Walsh, M.K.; Yang, K.-Y.; Choi, J.-H.; Eun, J.-B.; Choi, J.; Kim, Y.-M. Enzymatic synthesis of chlorogenic acid glucoside using dextransucrase and its physical and functional properties. *Enzym. Microb. Technol.* **2017**, *107*, 15–21.
- Pérez-Burillo, S.; Mehta, T.; Esteban-Muñoz, A.; Pastoriza, S.; Paliy, O.; Rufián-Henares, J.Á. Effect of in vitro digestion-fermentation on green and roasted coffee bioactivity: The role of the gut microbiota. *Food Chem.* **2019**, *279*, 252–259.
- Riccio, P.; Sessa, R.; de Nicola, S.; Petruzziello, F.; Trombetti, S.; Menna, G.; Pepe, G.; Maddalena, P.; Izzo, P.; Grosso, M. GATA-1 isoforms differently contribute to the production and compartmentation of reactive oxygen species in the myeloid leukemia cell line K562. *J. Cell. Physiol.* **2019**, *234*, 20829–20846.
- Sarnelli, G.; Grosso, M.; Palumbo, I.; Pesce, M.; D'Alessandro, A.; Zaninotto, G.; Annese, V.; Petruzzelli, R.; Izzo, P.; Sepulveres, R. Allele-specific transcriptional activity of the variable number of

tandem repeats of the inducible nitric oxide synthase gene is associated with idiopathic achalasia. *United Eur. Gastroenterol. J.* **2017**, *5*, 200–207.

- Shi, J.; Shan, S.; Li, H.; Song, G.; Li, Z. Anti-inflammatory effects of millet bran derived-bound polyphenols in LPS-induced HT-29 cell via ROS/miR-149/Akt/NF-κB signaling pathway. *Oncotarget* **2017**, *8*, 74582.
- Shin, H.S.; Satsu, H.; Bae, M.-J.; Zhao, Z.; Ogiwara, H.; Totsuka, M.; Shimizu, M. Anti-inflammatory effect of chlorogenic acid on the IL-8 production in Caco-2 cells and the dextran sulphate sodium-induced colitis symptoms in C57BL/6 mice. *Food Chem.* **2015**, *168*, 167–175.
- Sodaro, G.; Blasio, G.; Fiorentino, F.; Auberger, P.; Costanzo, P.; Cesaro, E. ZNF224 is a transcriptional repressor of AXL in chronic myeloid leukemia cells. *Biochimie* **2018**, *154*, 127–131.
- Trombetti, S.; Cesaro, E.; Catapano, R.; Sessa, R.; Lo Bianco, A.; Izzo, P.; Grosso, M. Oxidative Stress and ROS-Mediated Signaling in Leukemia: Novel Promising Perspectives to Eradicate Chemoresistant Cells in Myeloid Leukemia. *Int. J. Mol. Sci.* **2021**, *22*, 2470.
- Voloboueva, L.A.; Liu, J.; Suh, J.H.; Ames, B.N.; Miller, S.S. (R)-α-lipoic acid protects retinal pigment epithelial cells from oxidative damage. *Investig. Ophthalmol. Vis. Sci.* **2005**, *46*, 4302–4310.
- Wang, T.; Qin, L.; Liu, B.; Liu, Y.; Wilson, B.; Eling, T.; Langenbach, R.; Taniura, S.; Hong, J. Role of reactive oxygen species in LPS-induced

production of prostaglandin E2 in microglia. *J. Neurochem.* **2004**, *2*, 939–947.

- Zhou, M.; Xu, W.; Wang, J.; Yan, J.; Shi, Y.; Zhang, C.; Ge, W.; Wu, J.; Du, P.; Chen, Y. Boosting mTOR-dependent autophagy via upstream TLR4-MyD88-MAPK signalling and downstream NF-κB pathway quenches intestinal inflammation and oxidative stress injury. *EBioMedicine* **2018**, *35*, 345–360.

3.3. Effect of Dewaxed Coffee on Gastroesophageal Symptoms in Patients with GERD: A Randomized Pilot Study

Materials and Methods

Chemicals and Reagents

Formic acid (FA), methanol (MeOH), and water (H_2O) were acquired from Carlo Erba reagents (Milan, Italy). Polyphenol standards (purity > 98%) including caffeine, quinic acid, ferulic acid, *p*-coumaric acid, caffeic acid, 5-caffeoylquinic acid (5-CQA), and 3,5-dicaffeoylquinic acid (3,5-diCQA) were purchased from Sigma-Aldrich (Milan, Italy). For each standard, a stock solution at a concentration of 1 mg/mL was prepared in methanol. Working standard solutions were obtained by serial dilution and were stored at −20 °C until use.

Sampling

Standard Coffee (SC) and Dewaxed Coffee (DC) pods were obtained from Kimbo Caffè S.p.A. Pods are packed with a paper filter covering for use in a non-grinding espresso machine. The result is an espresso, which has a beautiful crema. Dewaxed coffee is an intense and aromatic blend, with a limited content of waxes and caffeine. The waxes of the cortical part of the grain are removed with a dewaxing process that uses an organic solvent (dichloromethane), which also extracts part of the caffeine.

Roasted and ground coffee, with medium-roasted coffee beans, a blend of carefully selected fine coffees, Arabica (80%), and Robusta (20%) coffee beans from South America were chosen for both typologies of pods. Samples were stored at room temperature in their original individual packaging prior to analysis.

Sample Preparation

Bioactive compounds were extracted in accordance with the procedure reported by (Castaldo, L.; et al. 2021) with some changes. In short, 1 g of powder sample was extracted with 20 mL of mixture H_2O:EtOH (75:25 *v*/*v*). The samples were vortexed (ZX3; VEPL Scientific, Usmate, Italy) for 1 min, sonicated (LBS 1; Zetalab srl, Padua, Italy) for 15 min, and stirred for 15 min. Then, the mixture was centrifuged for 5 min at 5000 rpm, the supernatant collected, and the pellet re-extracted another time. Finally, the two-supernatants were collected, filtrated through a 0.22 μm filter, and appropriately diluted in methanol until (1:10) further analysis.

UHPLC Q-Orbitrap HRMS

Polyphenolic profile was carried out by using an Ultra High-Pressure Liquid Chromatograph (UHPLC, Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Quaternary UHPLC pump working at 1250 bar, a degassing system, and an autosampler device. Chromatographic separation was performed with a thermostated (T = 25 °C) Kinetex 1.7 μm F5 (50 × 2.1 mm, Phenomenex, Torrance, CA, USA) column. The mobile phase consisted of water (A) and methanol (B) both containing 0.1% FA in. The injection volume was 1 μL. The gradient elution program was as follows: an initial 100% A, decreased to 60% A in 1 min, to 20% A in 1 min, and to 0% B in 3 min. The gradient was held for 4 min at 0% A, increased to 100% A in 2 min, and another 2 min for column re-equilibration at 100%. The total run time was 13 min. The flow rate was set at 500 μL/min. The UHPLC (Thermo Fischer Scientific, Waltham, MA, USA) system was coupled to a Q-Exactive Orbitrap mass spectrometer equipped with an electrospray (ESI) source. The mass spectrometer was operated in both positive and negative ion mode by setting a full ion MS. Full ion MS experiments were carried out with the settings: spray voltage 3.5 kV; capillary temperature 320 °C; S-lens RF level 60; sheath gas pressure 18; auxiliary gas 3; auxiliary gas heater temperature 350 °C; scan range 80–1200 *m/z*; microscans 1; mass resolution 35,000 full width at half maximum (FWHM); maximum injection time 200 ms; and automatic gain control (AGC) target 1×10^6. For accurate mass measurement, identification was carried out at a mass tolerance of 5 ppm. Data analysis and processing were performed by using Xcalibur software, v. 3.1.66.10 (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA) (Izzo, L.; et al. 2020).

Identification of Bioactive Compounds in Coffee Pods Samples through UHPLC-Q-Orbitrap HRMS

Identification of bioactive compounds (n = 14) including chlorogenic, hydroxycinnamic acids and caffeine in standard and dewaxed coffee pods was performed by using UHPLC-Q-Orbitrap HRMS analysis. The identification of bioactive compounds was carried out in Full ion MS mode. All experiments were set in negative ESI- mode, except for caffeine which showed an improved pattern in positive ESI+ mode. Satisfactory chromatography separation of analytes was achieved in a runtime of 13 min. For feruloylquinic acids (FQAs) isomers, 4-FQAand 5-FQA acids, quantification was reported as the sum because poor abundance prevents a good separation. Identification of isomers which includes CQA (*m/z* 353.08780), *p*-CoQA (*m/z* 337.09289), FCQA (*m/z* 367.10346), diCQA (*m/z* 515.11950) was carried out comparing the retention time with the standards and also by comparison of patterns previously reported in the literature (Castaldo, L.; et al. 2021). Table 6 shows all the mass parameters referred to the studied compounds, such as chemical formula, theorical and measured mass (*m/z*), adduct ion, retention time, and accuracy. Determination of the predominant CGAs and caffeine was carried out by using UHPLC-Q-Orbitrap HRMS analysis. Eight concentration levels were used for building the calibration curves of target analytes, and the correlation coefficients obtained were >0.99. For the semi-quantification purpose, a representative standard from the same group was used. In fact, for 3 and 5-*p*CoQA; 3, 4 and 5-FQA; 3 and 5-CQA; and 4,5-CFQA and 3,4-FCQA isomers, no standards were available.

Table 6. UHPLC-MS parameters of the assayed analytes (*n* = 14).

Compound *	Chemical Formula	Adduct Ion	RT * (min)	Measured Mass (*m*/*z*)	Theoretical Mass (*m*/*z*)	Accuracy (Δ mg/kg)
Quinic Acid	$C_7H_{12}O_6$	$[M-H]^-$	1.12	191.05531	191.05611	4.18
3-*p*CoQA	$C_{16}H_{18}O_8$	$[M-H]^-$	2.84	337.09232	337.09289	−1.69
3-FQA	$C_{17}H_{20}O_9$	$[M-H]^-$	3.03	367.10367	367.10346	−0.57
Caffeic Acid	$C_9H_8O_4$	$[M-H]^-$	3.07	179.03426	179.03498	4.02
5-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.09	353.08813	353.08780	−0.93
4-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.10	353.08901	353.08780	−3.42
3-CQA	$C_{16}H_{18}O_9$	$[M-H]^-$	3.12	353.08852	353.08780	−2.03
Caffeine	$C_8H_{10}N_4O_2$	$[M+H]^+$	3.20	195.08751	195.08765	0.72
p-Coumaric acid	$C_9H_8O_3$	$[M-H]^-$	3.25	163.03926	163.04006	4.91
5-*p*CoQA	$C_{16}H_{18}O_8$	$[M-H]^-$	3.27	337.09389	337.09289	−2.97
3,4-diCQA	$C_{25}H_{24}O_{12}$	$[M-H]^-$	3.28	515.12036	515.11950	−1.67
4 + 5-FQA	$C_{17}H_{20}O_9$	$[M-H]^-$	3.34	367.10303	367.10346	4.72
Ferulic Acid	$C_{10}H_{10}O_4$	$[M-H]^-$	3.38	193.05017	193.05063	−2.38
3,5-diCQA	$C_{25}H_{24}O_{12}$	$[M-H]^-$	3.45	515.12036	515.11950	−1.67

* Abbreviations: CQA: Caffeoylquinic; *p*CoQA: *p*-Coumaroylquinic acid; FQA: Feruloylquinic acid; diCQA: Dicaffeoylquinic acid, RT: retention time.

Study Design

In this single-center pilot study, a short-term nutritional intervention was performed. The four-week randomized, cross-over design was comprised of two weeks of Standard Coffee (SC) consumption and two weeks of Dewaxed Coffee (DC) consumption, separated by a two-week washout period. Randomization was performed in blocks of four using a computer-generated list, with a non-concealed allocation.

Patients were asked to follow their habitual diet during the whole study period (Figure 18A-B). On day one, patients received the assigned coffee (56 coffee pods of SC or DC) and were advised to consume a maximum of four pods a day. After two weeks (14 days) patients returned and received the second type of coffee (DC or SC, respectively). Adherence to coffee consumption (Number of consumed coffee pods: 0, 1, 2, 3, 4), presence of typical GERD symptoms (heartburn and regurgitation), and antacid assumption were assessed by patient entries into a tick-box diary for both study periods. Patient compliance with diary filling in was monitored and noncompliant patients were counseled. A complete clinical evaluation of Gastrointestinal Symptoms (PAGI-SYM and IBS-SSS) (Francis, C.Y.; et al. 1997 - Trudeau, E.; et al. 2004) and quality of life (PAGI_QoL) (Revicki, D.A.; et al. 2004) was performed at baseline (B) and after both intervention periods at weeks two and four (SC and DC).

The PAGI-SYM (Figure 19) and PAGI-QoL (Figure 20) are standardized, and validated questionnaires were used to evaluate the severity of symptoms and the quality of life, respectively, in patients with upper gastrointestinal disorders (including GERD) over the 14 days preceding the visit (Izzo, L.; et al. 2020 - Francis, C.Y.; et al. 1997 - Trudeau, E.; et al. 2004). Both PAGI-SYM and PAGI-QoL subscales and total rating are scored on a scale from zero (no symptoms/lowest Qol) to five (very severe symptoms/highest Qol) (Fraser, A.; et al. 2005).

The IBS symptom severity scale (IBS-SSS, Figure 21) is a validated five-question survey investigating the severity of abdominal pain and distension and the dissatisfaction with bowel habits over the 10 days preceding the visit (Trudeau, E.; et al. 2004). A change of 50 is adequate to detect a clinical improvement. The full versions of the questionnaires are reported at the end of this section.

Firstly, the symptom-free days (Heartburn and regurgitation) and antacid assumption over both two-week treatment periods (SC and DC) were compared. Then, the evaluation of change from the baseline PAGI-SYM (Patient Assessment of Upper Gastrointestinal Symptom Severity Questionnaire-Symptoms Severity Index), PAGI-QoL (PAGI-Quality of Life) and IBS-SSS scores were assessed. A visual description of the study design is shown in Figure 22.

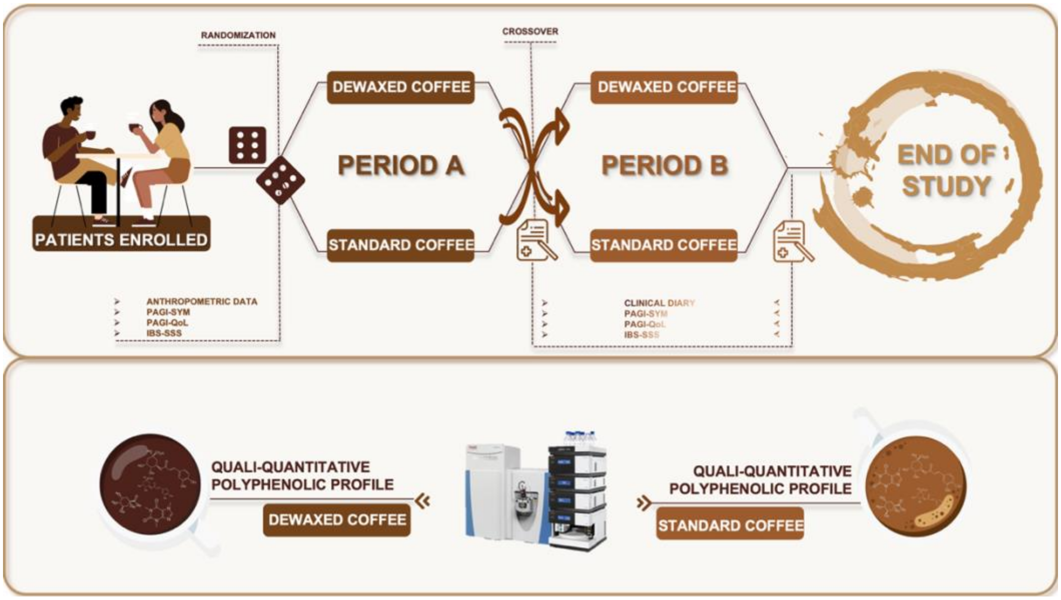


Figure 22. Visual description of study design. IBS-SSS, Irritable Bowel Syndrome—Symptom Severity Scale; PAGI-QoL, Patient Assessment of Upper Gastrointestinal Disorders-Quality of Life; PAGI-SYM, PAGI-Symptoms Severity Index.

Subjects

In total, 40 patients with a clinical and instrumental diagnosis of GERD (16 F) were recruited from the gastroenterology outpatient clinic of the University Hospital "Federico II" of Naples. The eligibility criteria were as follows: male and females aged 18–65 years, presence of typical GERD symptoms, and report of at least a one-year history of heartburn and/or regurgitation occurring at least 50% of the time following coffee consumption (Papakonstantinou, E.; et al. 2015). The exclusion criteria included: pregnancy;

breastfeeding; alcohol or drug abuse; any organic gastrointestinal disease; any malignancy; any kind of organic, systemic, metabolic or autoimmune disease; history of major gastrointestinal surgery; use of proton pump inhibitor (PPI) within two weeks before screening; use of H2-blocker, prokinetics or antacids within three days before screening; or any other condition considered to be inappropriate for the study. The study was approved by the Federico II ethical committee (prot. number 70/21), and all patients gave their written consent to participate.

Data Analysis

A symptom-based assessment was performed to assess treatment efficacy (Mouli, V.P.; Ahuja, V. 2011 - Moayyedi, P.; et al. 2000). The frequencies of symptom-free days (heartburn and regurgitation % from patient diary) were assessed at weeks two and four and were compared between SC and DC to verify the primary efficacy endpoint. To assess the secondary efficacy endpoint, any change of GERD-related symptoms, lower gastrointestinal symptoms and quality of life (QoL) were also evaluated using the PAGI-SYM, the IBS-SSS, and the PAGI-QoL questionnaires scores, respectively. A physical examination and an assessment of vital signs were performed at the initial appointment and at the end of both study periods.

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Science IBM SPSS version 25 statistical software package (Chicago, IL, USA). Continuous variables are described as mean ± standard deviation (SD), while categorical variables are described as number and frequencies. Fisher exact test, *t*-test and one-way ANOVA followed by Bonferroni post-test were used, respectively, when appropriate. All tests were two-tailed with a confidence interval of 95%. Significance was expressed at a *p*-value < 0.05.

Results

Quantification of Bioactive Compounds in Coffee Pods Samples through UHPLC-Q-Orbitrap HRMS

As shown in Table 7, thirteen analytes were identified, quantified, or semi-quantified in the coffee pods samples. CQAs were the most abundant investigated compounds in the coffee pods samples, with a concentration level ranging between 7.316 (DC) and 6.721 mg/g (SC). In particular, 5-CQA was the most predominant CQA in the assayed coffee pods samples, ranging from 2.928 (SC) to 3.121 (DC) mg/g powder. In the coffee pods samples investigated here, FQAs represented 5.4% (DC) to 6.6% (SC) of total CGAs with a concentration level ranging between 0.397 and 0.447 mg/g. Regarding, diCQA, concentration levels ranging between 0.107 and 0.114 mg/g represented 1.4% (DC) to 1.7% (SC) of total CGAs. Finally, *p*CoQA were found at a concentration range of 0.580 and 0.456 for DC and SC, respectively. Apart from CGAs, caffeine was quantified at a concentration level of 5.691 mg/g and 11.091 for DC and SC, respectively.

Table 7. Chlorogenic acids and other bioactive compounds (n = 14) content in standard and dewaxed coffee pods samples. Data are displayed as average value (mg/g) and standard deviation (±SD).

Compound *	Dewaxed Coffee		Standard Coffee	
	mg/g	±SD	mg/g	±SD
Quinic Acid	0.672	0.049	0.684	0.033
3-*p*CoQA	0.509 [a]	0.001	0.404 [b]	0.002
3-FQA	0.094	0.003	0.103	0.005
Caffeic Acid	0.022 [a]	0.001	0.015 [b]	0.002
5-CQA	3.132 [a]	0.016	2.928 [b]	0.017
4-CQA	1.034 [a]	0.012	0.928 [b]	0.013
3-CQA	0.932 [a]	0.005	0.728 [b]	0.013
Caffeine	5.691 [a]	0.07	11.091 [b]	0.11
p-Coumaric acid	NF		NF *	
5-*p*CoQA	0.071 [a]	0.007	0.053 [b]	0.003
3,4-diCQA	0.083	0.001	0.086	0.002
4 + 5-FQA	0.303 [a]	0.024	0.344 [b]	0.008
Ferulic Acid	0.440 [a]	0.094	0.420 [b]	0.033
3,5-diCQA	0.025 [a]	0.000	0.028 [b]	0.001

* Abbreviations: CQA: Caffeoylquinic; *p*CoQA: *p*-Coumaroylquinic acid; FQA: Feruloylquinic acid; diCQA: Dicaffeoylquinic acid; SC: standard coffee pods; DC: dewaxed coffee pods, NF: Not found. Tukey's test was used to evaluate differences between SC and DC samples considering p-value less than 0.05 as significant. a, b Different letters show a significant difference ($p < 0.05$) between SC and DC samples.

A randomized Pilot Study

The demographic and baseline characteristics of all patients (n = 40) are summarized in Table 8. The assessment of the percentage of symptom-free days experienced by patients during SC and DC periods showed a significant reduction of symptom frequency when consuming DC as compared to SC, with a similar number of coffees consumed during the two periods (2.7 ± 0.6 vs. 2.8 ± 0.8 for SC and DC respectively, p = not significant). In particular, the analysis of patient diaries proved a significant increase in both heartburn-free days and regurgitation-free days during DC compared to SC (Table 9). These findings were further supported by the observation that patients had a significant increase of antacid-free days during DC compared to SC (Table 9). Figure 23 summarizes the individual trends for GERD-related symptoms and clearly illustrates that, after DC, a significant improvement of heartburn, regurgitation and a reduced needing of antacid assumption was reported by a majority of patients.

The overall gastrointestinal symptoms assessment showed a significant reduction in both upper and lower gastrointestinal symptoms. In particular, the total PAGI-SYM score reveals a significant improvement of upper gastrointestinal symptoms after ingestion of DC compared to SC (Table 10). Going even more in detail, the analysis of PAGI-SYM subscales demonstrated a meaningful improvement of nausea (0.64 ± 0.64 vs. 0.29 ± 0.32; $p < 0.01$), postprandial fullness (1.69 ± 0.86 vs. 1.07 ± 0.6; $p < 0.01$), abdominal bloating (2.51 ± 1.15 vs. 1.46 ± 1.05; $p < 0.01$), upper (2.1 ± 1.19 vs. 1.09 ± 0.83; $p < 0.01$)

and lower (1.36 ± 1.01 vs. 0.82 ± 0.74; $p < 0.01$) abdominal pain, heartburn, and regurgitation (1.88 ± 0.92 vs. 0.92 ± 0.56; $p < 0.01$) after the two week DC period compared to the SC period (Figure 24).

Table 8. Demographic and baseline characteristics of patients. Values are means ± SD unless otherwise indicated; *n* = 40 patients.

Patients	T0
Age (years)	41.5 ± 12
Sex *n* (%)	F 16 (40)
Weight (kg)	75.3 ± 15.9
Height (m)	1.7 ± 0.1
BMI (kg/m^2)	25.5 ± 4
Smoke *n* (%)	13 (32.5)
Physical Activity *n* (%)	19 (47.5)

Table 9. Symptoms and Antacid-Free Days during SC and DC Treatment Periods. Data are presented as percentages (%).

	SC	DC	*p*-Value
Heartburn-free days, %	50.18 ± 17.46	79.82 ± 10.84	$p < 0.05$
Regurgitation-free days, %	64.46 ± 14.87	82.68 ± 12.83	$p < 0.05$
Antacid-free days, %	62.5 ± 22.22	87.5 ± 11.29	$p < 0.05$

Abbreviations: DC: Dewaxed Coffee; SC: Standard Coffee.

Table 10. PAGI-SYM, PAGI-QoL and IBS-SSS total scores changes in basal conditions and during SC and DC Treatment Periods. Data are presented as mean ± SD.

	B	SC *	DC *	*p*-Value *
PAGI-SYM	1.6 ± 0.75	1.7 ± 0.72	0.9 ± 0.48	$p < 0.01$
PAGI-QoL	1.3 ± 0.73	1.2 ± 0.81	0.8 ± 0.64	$p < 0.01$
IBS-SSS	196.9 ± 71.61	215.65 ± 68.51	149.75 ± 56.97	$p < 0.01$

B, basal conditions; DC, Dewaxed Coffee; IBS-SSS, Irritable Bowel Syndrome—Symptom Severity Scale; PAGI-QoL, Patient Assessment of Upper Gastrointestinal Disorders-Quality of Life; PAGI-SYM, PAGI-Symptoms Severity Index; SC, Standard Coffee. * (DC vs. SC).

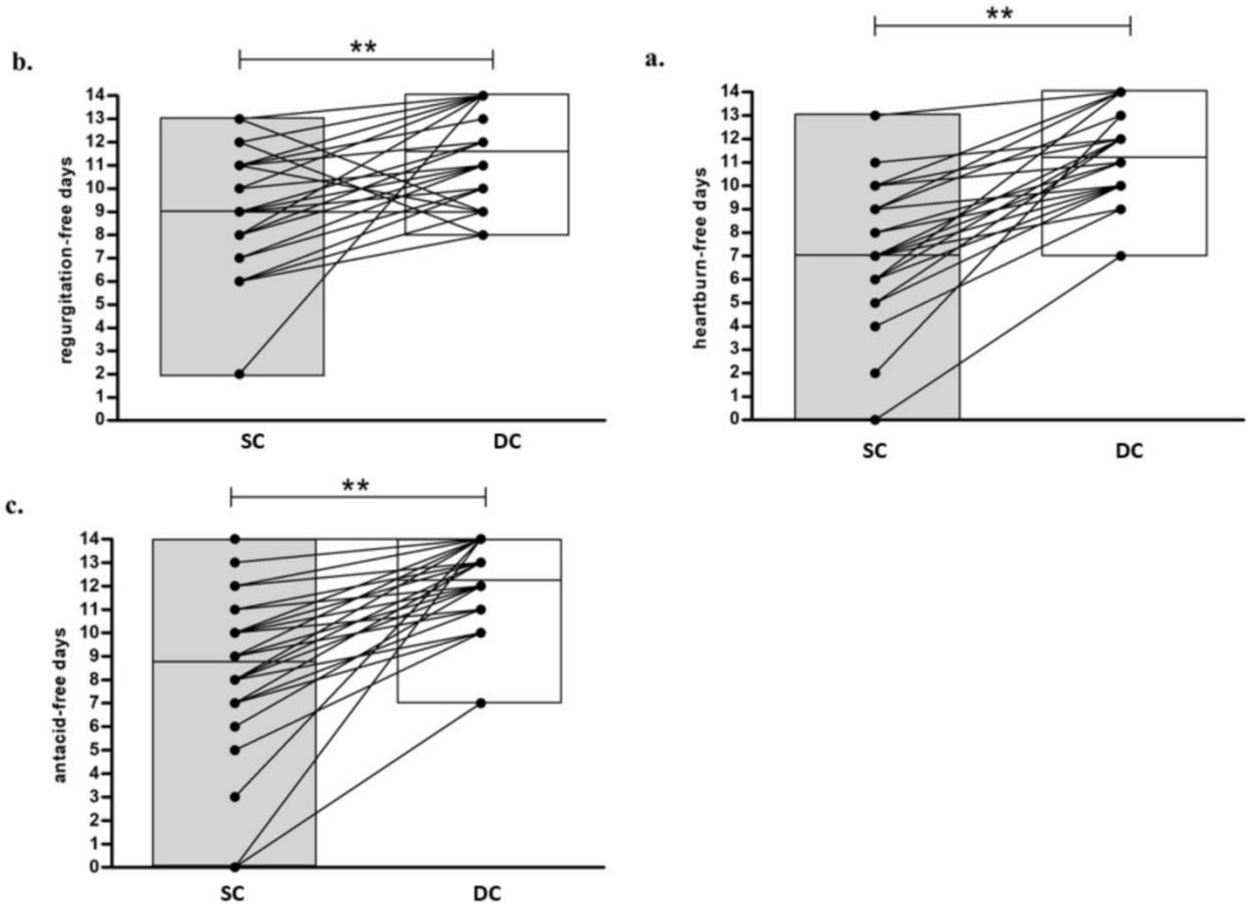


Figure 23. Evaluation of heartburn-free days (**a**), regurgitation-free days (**b**) and antacid-free days (**c**) during both treatment periods (n = 40). DC, Dewaxed Coffee; SC, Standard Coffee. ** $p < 0.01$.

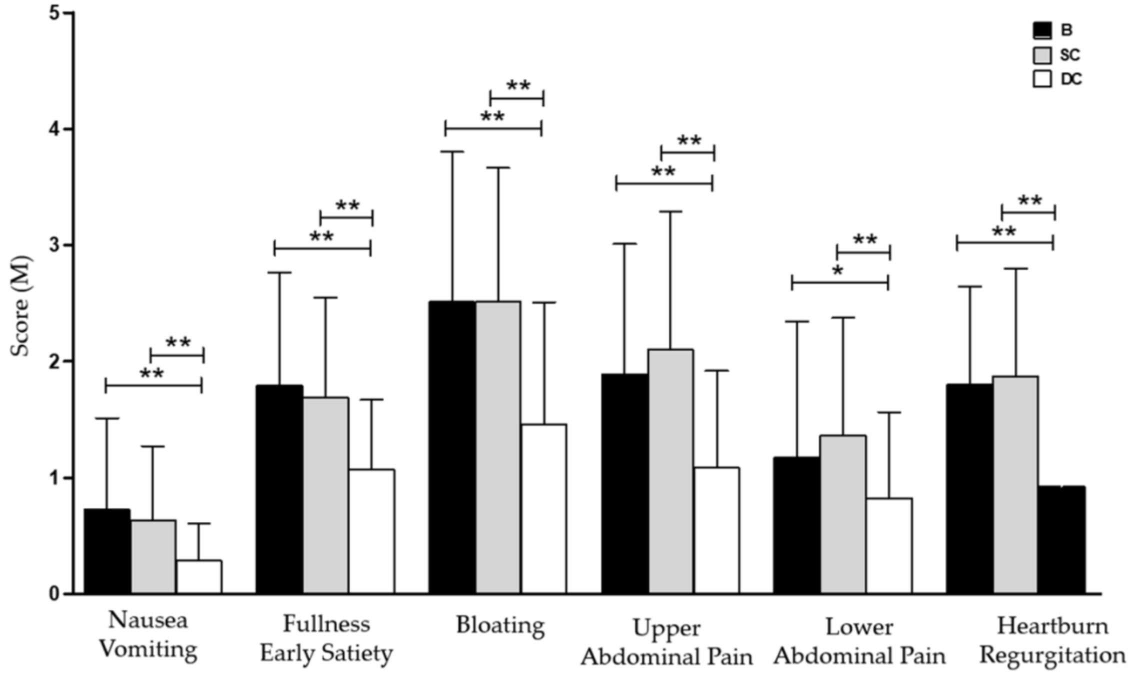


Figure 24. PAGI-SYM subscales score at basal condition and after both treatment periods. Data are presented as mean ± SD (n = 40). B, Basal Conditions; DC, Dewaxed Coffee; SC, Standard Coffee. * $p < 0.05$; ** $p < 0.01$.

Furthermore, IBS-SSS score analysis demonstrated a significant reduction of lower gastrointestinal symptoms after DC compared to SC (Table 10). In both cases, the differences shown above were similar to those obtained when comparing the DC period with baseline conditions.

The PAGI-QoL scores analysis showed a significant improvement of quality of life after the two-week DC period compared to the SC period (Table 10). In particular, a meaningful difference in subscales for diet and food habits (1.65 ± 0.55 vs. 0.67 ± 0.39; $p < 0.01$), psychological wellbeing and distress (2.17 ± 0.58 vs. 1.1 ± 0.67; $p < 0.01$), daily activity (1.15 ± 0.61 vs. 0.75 ± 0.54; $p < 0.01$) and clothing (1.38 ± 0.25 vs. 0.5 ± 0.41; $p < 0.05$) were observed (Figure 25). Here too, the differences found comparing the DC period with basal conditions were similar to those obtained comparing the DC and SC periods.

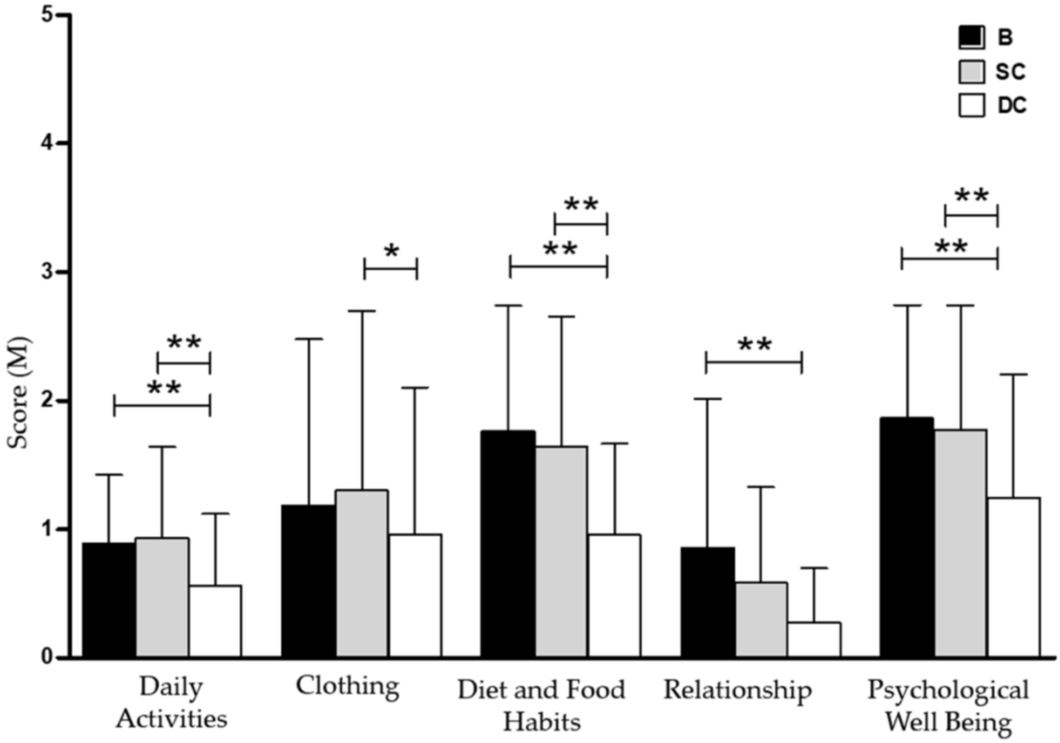


Figure 25. PAGI-QoL subscales score at basal condition and after both treatment periods. Data are presented as mean ± SD (n = 40). B, Basal Conditions; DC, Dewaxed Coffee; SC, Standard Coffee. * $p < 0.05$; ** $p < 0.01$.

Discussion

Until now, scarce scientific evidence for putative coffee components affecting gastric acid secretion in humans is reported in the literature (Rubach, M.; et al. 2014). Previous studies already speculated that variations in coffee type and processing might be important in the genesis of coffee-related upper gastrointestinal symptoms, yet no significant difference has emerged (Papakonstantinou, E.; et al. 2015 - Brazer, S.R.; et al. 1995 - Mehta, R.S.; et al. 2020). Although the mechanism of action is not completely understood yet, it has been hypothesized that modifying roasting conditions could reduce stomach-irritating compounds, namely caffeine, chlorogenic acids (CGAs), and N-alkanoyl-5-hydroxytryptamides (C5HTs) (Rubach, M.; et al. 2010). Caffeine is frequently investigated as the main responsible molecule in inducing GERD symptoms (Nwokediuko, S. 2009) - Zhang, Y.; Chen, S.-H. 2013). Interestingly, a recent ongoing prospective US cohort study demonstrated a minimal change in upper gastrointestinal symptoms upon stratification by caffeine status among caffeinated beverages (coffee, soda, and tea) and a major association between decaffeinated tea and GERD symptoms (Feldman, M.; Barnett, C. 1995). In line with these findings, an older experimental study demonstrated a worsening of upper gastrointestinal symptoms after caffeinated coffee consumption, but not after caffeinated tap water consumption, suggesting a feasible involvement of other unknown components of coffee in inducing GERD symptoms (Folstar, P.; et al. 1980).

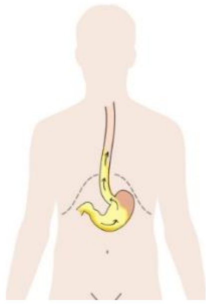
Overall, the results indicate that the analyzed coffee pods may represent a considerable source of CGAs and other important bioactive compounds. Concerning the CGAs in assayed coffee brew samples, the concentrations found in dewaxed coffee pods were slightly higher ($p < 0.05$) when compared to those in standard coffee pods with a concentration of 5.10 and 4.59 mg/g, respectively. According to data reported in the literature, the contents of CGAs in coffee present a large variability and are influenced by many factors, such as the variety of coffee, the roast degrees, and the brewing method used. It has been reported that, in medium roasts, a 60% loss of CGA has been observed and up to 100% loss in a dark roast. The optimal roasting condition for coffee is medium above which there is a significant reduction of bioactive compounds (Song, J.L.; et al. 2018 - Bastian, F.; et al. 2021 - Derossi, A.; et al. 2018). In general, the most studied CGAs in coffee are the three main CQA isomers, whereas diCQAs and FQAs have been barely investigated. Several investigations have reported the capability of CGAs to positively modulate important biological status, maintain health, and exert a pivotal role in the reduction of risk of a variety of diseases (Wianowska, D.; Gil, M. 2019 - Tajik, N.; et al. 2017). Our findings confirm that coffee pods even after the dewaxed process, maintain a considerable source of CGAs and other important bioactive compounds correlated with the reduction of risk of a variety of diseases.

Patients with GERD often implicate coffee in causing or worsening reflux symptoms such as heartburn and regurgitation, thus leading to coffee avoidance (Van der Stegen, G. 1979 - Rentz, A.; et al. 2004 - Papakonstantinou, E.; et al. 2015 - Rubach, M.; et al. 2014). Furthermore, the lack of defined and standardized guidelines leads physicians to frequently recommend limiting coffee consumption in patients with GERD. Previous research has already tried to identify an existing coffee type or product that is less likely to trigger typical reflux symptoms in coffee-sensitive individuals, without any significant results (DiBaise, J.K. 2003). Dewaxing is an innovative procedure in which the waxy layer is removed from unroasted coffee together with a small amount of caffeine with an organic solvent. Although our study is limited by the absence of a caffeine controlled interventional arm, we believe that our main findings showed that, in a large well-selected population of coffee-sensitive patients with GERD, chronic DC consumption:

(1). Was associated with an increase of symptom-free days and antacid-free days compared to SC;

(2). Led to a reduction of both upper (PAGI-SYM) and lower (IBS-SSS) gastrointestinal symptoms compared to SC;

(3). Improved gastrointestinal-related quality of life (PAGI-QoL) compared to SC. While still preliminary, data obtained from the present pilot study provide promising evidence for the efficacy of DC consumption in patients with GERD. Particularly, DC seems to be better tolerated, does not compromise the quality of life, and does not affect gastrointestinal well-being in coffee-sensitive patients with GERD.



	HEARTBURN	REGURGITATION
DAY 1	YES ☐ NO ☐	YES ☐ NO ☐
DAY 2	YES ☐ NO ☐	YES ☐ NO ☐
DAY 3	YES ☐ NO ☐	YES ☐ NO ☐
DAY 4	YES ☐ NO ☐	YES ☐ NO ☐
DAY 5	YES ☐ NO ☐	YES ☐ NO ☐
DAY 6	YES ☐ NO ☐	YES ☐ NO ☐
DAY 7	YES ☐ NO ☐	YES ☐ NO ☐
DAY 8	YES ☐ NO ☐	YES ☐ NO ☐
DAY 9	YES ☐ NO ☐	YES ☐ NO ☐
DAY 10	YES ☐ NO ☐	YES ☐ NO ☐
DAY 11	YES ☐ NO ☐	YES ☐ NO ☐
DAY 12	YES ☐ NO ☐	YES ☐ NO ☐
DAY 13	YES ☐ NO ☐	YES ☐ NO ☐
DAY 14	YES ☐ NO ☐	YES ☐ NO ☐

Figure 18A. Patient diary during the 14 treatment days. Annotation for heartburn, regurgitation symptoms

	NEED TO TAKE ANTACIDS	N. COFFEE
DAY 1	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 2	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 3	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 4	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 5	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 6	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 7	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 8	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 9	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 10	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 11	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 12	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 13	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
DAY 14	YES ☐ NO ☐	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐

Figure 18B. Patient diary during the 14 treatment days. Annotation for need to take antacids correlated to coffee intake.

PAGI-SYM QUESTIONNAIRE

For each symptom, put an "X" next to the number that best describes how *severe* the symptom has been during the past 2 weeks.

0: ***NO SYMPTOM***
1: If the symptom has been ***VERY MILD***
2: If the symptom has been ***MILD***
3: If the symptom has been ***MODERATE***
4: If the symptom has been ***SEVERE***
5: If the symptom has been ***VERY SEVERE***

SYMPTOMS	**0**	**1**	**2**	**3**	**4**	**5**
1. *NAUSEA* (feeling sick to your stomach as if you were going to vomit or throw up)						
2. *RETCHING* (heaving as if to vomit, but nothing comes up)						
3. *VOMIT*						
4. Stomach *FULLNESS*						
5. *NOT ABLE* to finish a normal-sized meal						
6. Feeling *EXCESSIVELY FULL* after meals						
7. *LOSS OF APPETITE*						

8. *BLOATING* (feeling like you need to loosen your clothes)						
SYMPTOMS	**0**	**1**	**2**	**3**	**4**	**5**
9. Stomach or belly visibly *LARGER*						
10. Upper abdominal (above the navel) *PAIN*						
11. Upper abdominal (above the navel) *DISCOMFORT*						
12. Lower abdominal (below the navel) *PAIN*						
13. Lower abdominal (below the navel) *DISCOMFORT*						
14. *HEARTBURN* (burning pain rising in your chest or throat) during *THE DAY*						
15. *HEARTBURN* (burning pain rising in your chest or throat) when *LYING DOWN*						
16. Feeling of DISCOMFORT inside your chest during *THE DAY*						
17. Feeling of DISCOMFORT inside your chest at night (during *SLEEP TIME*)						
18. *RIGURGITATION* or *REFLUX* (fluid or liquid from your stomach coming up into your throat) during the *DAY*						
19. *RIGURGITATION* or *REFLUX* (fluid or liquid from your stomach coming up into your throat) when you lying down						
20. *BITTER, ACID* or *SOUR TASTE* in your mouth						

Figure 19. PAGI-SYM Questionnaire.

PAGI-QoL QUESTIONNAIRE

Please indicate with an "X" how often your gastrointestinal disturbances have affected the following conditions.

0: ***NONE OF THE TIME***
1: ***A LITTLE OF THE TIME***
2: **SOME OF THE TIME**
3: **A GOOD BIT OG THE TIME**
4: **MOST OF THE TIME**
5: **ALL OF THE TIME**

CONDITION	0	1	2	3	4	5
1. Have you had *TO DEPEND* on others to do your daily activities?						
2. Have you *AVOIDED* performing your daily activities?						
3. Have you had *DIFFICULTY CONCENTRATING*?						
4. Has it taken you *LONGER THAN USUAL* to perform your daily activities?						
5. Have you felt *TIRED*?						
6. Have you lost the *DESIRE TO PARTECIPATE* in social activities such as visiting friends or relatives?						
7. Have you been *WORRIED* about having stomach symptoms in public?						
8. Have you *AVOID* performing physical activities or sports?						

9. Have you *AVOID* traveling?						
10. Have you felt *FRUSTRATED* about not being able to do what you wanted to do?						
CONDITION	**0**	**1**	**2**	**3**	**4**	**5**
11. Have you felt *CONSTRICTED* in the clothes you wear?						
12. Have you felt *FRUSTRATED* about not being able to dress as you wanted to?						
13. Have you felt *CONCERNED* about what you can and cannot eat?						
14. Have you *AVOIDED* certain types of foods?						
15. Have you *RESTRICTED EATING* at restaurant or at someone's home?						
16. Have you felt *LESS ENJOYMENT* in food than usual?						
17. Have you felt *CONCERNED* that a change in your food habits could trigger your symptoms?						
18. Have you felt *FRUSTRATED* about not being able to choose the food you wanted to?						
19. Have you felt *FRUSTRATED* about not being able to choose the type of beverage you wanted to?						
20. Has your *RELATIONSHIP WITH YOUR SPOUSE OR PARTNER* been disturbed?						
21. Has your *RELATIONSHIP WITH YOUR CHILDREN OR RELATIVES* been disturbed?						
22. Has your *RELATIONSHIP WITH YOUR FRIENDS* been disturbed?						
23. Have you been in a *BAD MOOD*?						
24. Have you felt *DEPRESSED*?						
CONDITION	**0**	**1**	**2**	**3**	**4**	**5**

25. Have you felt *ANXIOUS*?						
26. Have you felt *ANGRY*?						
27. Have you felt *IRRITABLE*?						
28. Have you felt *DISCOURAGED*?						
29. Have you felt *STRESSED*?						
30. Have you felt *HELPLESS*?						

Figure 20. PAGI-QoL Questionnaire.

IBS-SSS

1a – Do you currently (over the last 10 days or so) suffer from *ABDOMINAL (TUMMY) PAIN*?

YES ☐ NO ☐

1b – If yes, how severe is your *ABDOMINAL (TUMMY) PAIN* ?

0

10

1c – Please enter the number of the days that you get the pain in every 10 days. For example, if you enter 4 it means that you get pain 4 out of 10 days. If you get pain every day enter 10

Number of the days with pain: ________

2a - Do you currently (over the last 10 days or so) suffer from *ABDOMINAL DISTENSION** (bloating, *swollen or tight tummy)*
* (women, please ignore distension related to your periods)

YES ☐ NO ☐

2b – If yes, how severe is your abdominal distension/tightness from 0 to 10?

0

10

3 – How satisfied are you with your *BOWEL HABIT* (stool frequency / shape, difficulty/pain in defecation...) from 0 to 10?

0

10

4 – Please indicate (from 0 to 10) with a cross on the line below how much your *IRRITABLE BOWEL SYNDROME* is affecting or interfering with your life in general

0

10

Figure 21. IBS-SSS.

References

- Bastian, F.; Hutabarat, O.S.; Dirpan, A.; Nainu, F.; Harapan, H.; Emran, T.B.; Simal-Gandara, J. From Plantation to Cup: Changes in Bioactive Compounds during Coffee Processing. *Foods* **2021**, *10*, 2827.
- Brazer, S.R.; Onken, J.E.; Dalton, C.B.; Smith, J.W.; Schiffman, S.S. Effect of different coffees on esophageal acid contact time and symptoms in coffee-sensitive subjects. *Physiol. Behav.* **1995**, *57*, 563–567.
- Castaldo, L.; Lombardi, S.; Gaspari, A.; Rubino, M.; Izzo, L.; Narváez, A.; Ritieni, A.; Grosso, M. In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Spent Coffee Grounds-Enriched Cookies. *Foods* **2021**, *10*, 1837.
- Derossi, A.; Ricci, I.; Caporizzi, R.; Fiore, A.; Severini, C. How grinding level and brewing method (Espresso, American, Turkish) could affect the antioxidant activity and bioactive compounds in a coffee cup. *J. Sci. Food Agric.* **2018**, *98*, 3198–3207.
- DiBaise, J.K. A randomized, double-blind comparison of two different coffee-roasting processes on development of heartburn and dyspepsia in coffee-sensitive individuals. *Dig. Dis. Sci.* **2003**, *48*, 652–656.
- Feldman, M.; Barnett, C. Relationships between the acidity and osmolality of popular beverages and reported postprandial heartburn. *Gastroenterology* **1995**, *108*, 125–131.

- Folstar, P.; Schols, H.A.; Van der Plas, H.C.; Pilnik, W.; Landheer, C.A.; Van Veldhuizen, A. New tryptamine derivatives isolated from wax of green coffee beans. *J. Agric. Food Chem.* **1980**, *28*, 872–874.
- Francis, C.Y.; Morris, J.; Whorwell, P.J. The irritable bowel severity scoring system: A simple method of monitoring irritable bowel syndrome and its progress. *Aliment. Pharmacol. Ther. Clin. Risk Manag.* **1997**, *11*, 395–402.
- Fraser, A.; Delaney, B.; Moayyedi, P. Symptom-based outcome measures for dyspepsia and GERD trials: A systematic review. *Off. J. Am. Coll. Gastroenterol.* **2005**, *100*, 442–452.
- Izzo, L.; Rodríguez-Carrasco, Y.; Pacifico, S.; Castaldo, L.; Narváez, A.; Ritieni, A. Colon bioaccessibility under in vitro gastrointestinal digestion of a red cabbage extract chemically profiled through UHPLC-Q-Orbitrap HRMS. *Antioxidants* **2020**, *9*, 955.
- Mehta, R.S.; Song, M.; Staller, K.; Chan, A.T. Association between beverage intake and incidence of gastroesophageal reflux symptoms. *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 2226–2233.
- Moayyedi, P.; Forman, D.; Braunholtz, D.; Feltbower, R.; Crocombe, W.; Liptrott, M.; Axon, A.; Leeds HELP Study Group. The proportion of upper gastrointestinal symptoms in the community associated with Helicobacter pylori, lifestyle factors, and nonsteroidal anti-inflammatory drugs. *Am. J. Gastroenterol.* **2000**, *95*, 1448–1455.

- Mouli, V.P.; Ahuja, V. Questionnaire based gastroesophageal reflux disease (GERD) assessment scales. *Indian J. Gastroenterol.* **2011**, *30*, 108–117.
- Nwokediuko, S. Gastroesophageal reflux disease: A population based study. *Gastroenterol. Res.* **2009**, *2*, 152.
- Papakonstantinou, E.; Kechribari, I.; Sotirakoglou, K.; Tarantilis, P.; Gourdomichali, T.; Michas, G.; Kravvariti, V.; Voumvourakis, K.; Zampelas, A. Acute effects of coffee consumption on self-reported gastrointestinal symptoms, blood pressure and stress indices in healthy individuals. *Nutr. J.* **2015**, *15*, 26.
- Revicki, D.A.; Rentz, A.M.; Tack, J.; Stanghellini, V.; Talley, N.J.; Kahrilas, P.; De La Loge, C.; Trudeau, E.; Dubois, D. Responsiveness and interpretation of a symptom severity index specific to upper gastrointestinal disorders. *Clin. Gastroenterol. Hepatol.* **2004**, *2*, 769–777.
- Rubach, M.; Lang, R.; Bytof, G.; Stiebitz, H.; Lantz, I.; Hofmann, T.; Somoza, V. A dark brown roast coffee blend is less effective at stimulating gastric acid secretion in healthy volunteers compared to a medium roast market blend. *Mol. Nutr. Food Res.* **2014**, *58*, 1370–1373.
- Rubach, M.; Lang, R.; Skupin, C.; Hofmann, T.; Somoza, V. Activity-guided fractionation to characterize a coffee beverage that effectively down-regulates mechanisms of gastric acid secretion as compared to regular coffee. *J. Agric. Food Chem.* **2010**, *58*, 4153–4161.

- Song, J.L.; Asare, T.S.; Kang, M.Y.; Lee, S.C. Changes in bioactive compounds and antioxidant capacity of coffee under different roasting conditions. *Korean J. Plant Resour.* **2018**, *31*, 704–713.
- Tajik, N.; Tajik, M.; Mack, I.; Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *Eur. J. Nutr.* **2017**, *56*, 2215–2244.
- Trudeau, E.; Marquis, P.; Kahrilas, P.; Stanghellini, V.; Talley, N.J.; Tack, J.; Revicki, D.A.; Rentz, A.M.; Dubois, D. Cross-cultural development and validation of a patient self-administered questionnaire to assess quality of life in upper gastrointestinal disorders: The PAGI-QOL©. *Qual. Life Res.* **2004**, *13*, 1751–1762.
- Wianowska, D.; Gil, M. Recent advances in extraction and analysis procedures of natural chlorogenic acids. *Phytochem. Rev.* **2019**, *18*, 273–302.
- Zhang, Y.; Chen, S.-H. Effect of coffee on gastroesophageal reflux disease. *Food Sci. Technol. Res.* **2013**, *19*, 1–6.

3.4. In Vitro Bioaccessibility and Antioxidant Activity of Polyphenolic Compounds from Spent Coffee Grounds-Enriched Cookies

Materials and Methods

Reagents and Materials

Polyphenol standards (purity > 98%) were purchased as follows: 4-CQA, 3,4-diCQA, *p*-coumaric acid, quinic acid, ferulic acid, caffeic acid, and gallic acid from Sigma-Aldrich (Milan, Italy). The reagents used in simulated GiD, including α-amylase from human saliva, protease from Streptomyces griseus (Pronase), pepsin from porcine gastric mucosa, bile salts, Viscozyme L, pancreatin from porcine pancreas, potassium chloride (KCl), sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), ammonium carbonate ($NaHCO_3)_2$), sodium hydroxide (NaOH), and calcium chloride dihydrate ($CaCl_2(H_2O)_2$), were provided from Sigma-Aldrich (Milan, Italy). The standards used in antioxidant assays, including 2,3,5-triphenyltetrazolio chloride (TPTZ), Trolox, 2′2-azino-bis-3- ethylbenzthiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium persulfate ($K_2S_2O_8$), and ferric chloride ($FeCl_3$), were acquired from Sigma-Aldrich (Milan, Italy). Formic acid (FA), water (H_2O), and methanol (MeOH) were provided from Carlo Erba reagents (Milan, Italy). Deionized H_2O was obtained from a Milli-Q water system (Millipore, Darmstadt, Germany).

Sampling and Coffee Preparation

Colombian roasted coffee (*C. arabica* L.; medium degree) beans (n = 10) were acquired from a local supermarket in Italy. The roasted coffee samples were finely ground by using a coffee grinder (Bosch Elettrodomestici, TSM6A013B, Milan, Italy) to obtain a composite sample. Coffee was prepared through an American coffeemaker (Aigostar Chocolate 30HIK, Aigostar S.r.L., Milan, Italy), and then, SCG was recovered from the filter and dried in a laboratory oven until the moisture of the material reached a level between 12% and 14.5%. Finally, the samples were stored at room temperature.

Spent Coffee Grounds-Enriched Cookies Preparation

Spent coffee grounds-enriched cookies (SCGc) were prepared following the recipe reported previously by Colantuono et al. 2016. Briefly, dough was prepared with 5 g of SCG, 40 g of wheat flour, 10 g of butter, 17.5 g of sugar, 0.2 g of sodium chloride, 0.8 g of bicarbonate, and 20 g of H2O. Control cookies (CTc) were prepared using wheat flour in substitution of the SCG. The doughs were molded into circular shapes of a diameter of 4 cm and 5 mm in height. After that, the cookies (samples and controls) were baked simultaneously at 205 ◦C for 10 min in an oven. Then, the samples were cooled and stored at room temperature in bags of polyethylene until the analysis.

Polyphenols and Caffeine Extraction

Polyphenols and caffeine extraction was carried out according to the procedure previously reported by Gonçalves et al. 2019. Briefly, 300 mg of samples were suspended in 25 mL of H_2O:EtOH 75:25 (*v*/*v*), stirred for 15 min at 300× *g*, and then sonicated for 15 min. After that, the mixture was centrifuged at 4200× *g* for 5 min, and the supernatant was collected and employed for the total phenolic content (TPC), antioxidant activity determination, and spectrometric characterization.

High Molecular Weight Melanoidins Content

High molecular weight melanoidins quantification was assessed following the procedure previously reported by De Cosío-Barrón et al. 2020. In short, Amicon Ultra-4-cell model regenerated cellulose with 10 kDa of nominal molecular mass were used to ultrafiltrate 4 mL of extract (5 mg/mL). The samples were subjected to ultrafiltration for 80 min at 5000× *g*. The retentates were washed three times with 4 mL of water. HMWM was quantified by weighing the freeze-dried retentate resulted after dialysis. The obtained results were displayed as g/100 g.

Ultra-High-Performance Liquid Chromatography and Orbitrap High-Resolution Mass Spectrometry Analysis

The separation of the investigated analytes was obtained using an ultra-high-performance liquid chromatography (UHPLC; Dionex Ultimate 3000, Thermo

Fischer Scientific, Waltham, MA, USA), provided by an autosampler device, a degassing system, a Quaternary UHPLC pump, and a Kinetex column F5 (50 mm × 2.1 mm, 1.7 μm particle size, Phenomenex, Torrance, USA) thermostated at 25 ◦C. The mobile phases (phase A: water; and phase B: methanol) were both prepared at 0.1% of formic acid. The separation gradient program started as follows: initial 0% B for 1 min and then rose up to 80% B in 2 min. Afterward, the gradient increased again to 100% B in 3 min. Then, the gradient returned to the equal % B in 2 min and was maintained for 2 min for column re-equilibration.

A Q-Exactive mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) combined with an electrospray (ESI) source allowed the acquisition in negative/positive ion mode fast polarity-switching mode, setting two scan events full ion MS and all ion fragmentation (AIF). The following parameters were set in full MS experiments: maxi- mum injection time, 200 ms; automatic gain control (AGC) target, 1 × 106; scan range, 80–1200 *m*/*z*; microscans, 1; mass resolution, 35,000 full width at half maximum (FWHM); scan time, 0.10 s; retention time to 30 s; and isolation window to 5 *m*/*z*. The collision ener- gies (CEs) were optimized considering values varied in the range 10–60 eV. Identification was based on exact mass measurements with a mass error < 5 ppm in both full ion MS and AIF mode. Data processing was carried out through Quan/Qual Browser Xcalibur software 3.1.66.19 (Thermo Fischer Scientific, Waltham, USA).

In Vitro Gastrointestinal Digestion

The SCGc and CTc samples were subjected to the in vitro GiD process following the developed protocol recently created by the INFOGEST network. The simulated salivary (SSF), gastric (SGF), and intestinal (SIF) fluids were developed in accordance with procedure reported by Minekus et al. 2014.

In order to simulate the oral condition, 500 mg of the grinded samples were mixed with 500 μL of the α-amylase solution, 3.5 mL of SSF, 25 μL of 0.3 M of calcium chloride solution, and 0.975 mL of H2O. Then, the pH of the solution was adjusted to 7 with sodium hydroxide 1 M and incubated at 37 ◦C for 2 min. The gastric phase was simulated by adding to the mixture 0.685 mL of H2O, 5 μL of 0.3 M of calcium chloride solution, and 1.6 mL of pepsin solution. Afterward, the pH of the solution was adjusted to 3 with HCL 1 M and incubated at 37 ◦C for 2 h. Then, 1.3 mL of H2O, 5 mL of pancreatin solution, 2.5 mL of bile salt solution, and 40 μL 0.3 M calcium chloride solution were added to the mixture in order to recreate the intestinal condition. After, the pH of the solution was adjusted to 7 with sodium hydroxide 1 M and incubated at 37 °C for 120 min. Finally, to simulate the activity of gut microbiota, the samples were subjected to the previously described protocol (Fogliano, V.; et al. 2011). In brief, the pH of the solution was adjusted to 8 with sodium hydroxide 1 M, and then, 5 mL of Pronase solution at a concentration level of 5 mg/mL was added. The samples were incubated for 1 h at 37 °C. Then, 150 μL of Viscozyme L and 5 mL of water were added to the mixture. The pH of the solution was adjusted to 4 with HCl 1 M and incubated to 16 h at 37 °C.

An aliquot of the supernatant (1 mL) was recovered at the end of each phase of in vitro GiD and replaced by the appropriate fluid phase in order to assess the changes in polyphenol bioaccessibility and antioxidant activity during the different stages of the GiD.

1.8. Determination of the Antioxidant Activity

The antioxidant activity of the cookies (digested and not-digested) and SCG material was evaluated by using three different assays, including DPPH, FRAP, and ABTS tests.

DPPH Assay

The determination of DPPH assay was carried out following the procedure reported by Dini et al. 2020. In short, 1 mg of DPPH standard was dissolved in methanol until reaching a value of absorbance of 0.90 (±0.01) at 517 nm. Afterward, 200 μL of sample extract were added to 1 mL of DPPH solution. The absorbance value after 10 min was immediately recorded.

FRAP Assay

The FRAP method was conducted according to the procedure described by Izzo et al. 2020. As reported, the FRAP solution was prepared by adding 2.5 mL of acetate buffer, 0.25 mL of TPTZ in HCl, and 0.25 mL of a 20 mM of $FeCl_3$ solution. Afterward, 150 μL of sample extract were added to 2.85 mL of FRAP solution. The absorbance value at 593 nm after 4 min was immediately recorded.

ABTS Assay

The determination of ABTS activity was assessed by using the procedure reported by Dini et al. 2020. In short, 5 mL of ABTS (7 mM) were mixed to 88 µL of $K_2S_2O_8$ (2.5 mM) and kept at room temperature for 16 h. Afterward, the EtOH was used to dilute the ABTS solution until reaching a value of absorbance of 0.70 (±0.01) at 734 nm. Then, 100 µL of sample extract were added to 1000 µL of ABTS solution. The absorbance value after 3 min was immediately recorded.

Determination of Total Phenolic Content

The Folin–Ciocalteu procedure was performed to assess the TPC content in accordance with the methodology described previously by Izzo et al. 2020. Briefly, 125 µL of the Folin– Ciocalteu reagent were added to 500 µL of H_2O and mixed with 125 µL of sample extract. The mixture was incubated for 6 min at room temperature. Then, 1.25 mL of $NaCO_3$ solution (7.5%) and 1 mL of H_2O were added. The absorbance value after 90 min at 760 nm was immediately recorded.

Statistics and Data Analysis

Tukey's test was used to evaluate differences between SCGc and control samples considering *p*-value less than 0.05 as significant. All analysis were conducted in triplicate and the results expressed as average ± standard deviation (SD). Data processing was carried out through Stata 12 software (StataCorp LP, College Station, TX, USA).

Results

High Molecular Weight Melanoidins Content

The HMWM content in SCG and SCGc samples was carried out through the ultra- filtration technique. As far as SCG was concerned, the HMWM were quantified at a concentration range from 10.15 to 11.47g/100 g, with an average value of 10.80 g/100 g. Moreover, HMWM levels found in SCGc samples ranged from 0.75 to 0.84 g/100 g, with an average value of 0.78 g/100 g.

Identification of Polyphenol Compounds and Caffeine in the Assayed Samples Using UHPLC-Q-Exactive Orbitrap

Identification of individual hydroxycinnamic acids (n = 4), CGAs (n = 9) and caffeine in the SCG, SCGc, and CTc samples was carried out through UHPLC-Q-Orbitrap high-resolution mass spectrometry. Good separation of the assayed analytes was achieved in 13 min. Nevertheless, the isomers 4-FQA and 5-FQA were quantified together (4+5-FQA), caused by insufficient chromatographic separation. The chemical formula, ion assignment, retention time (RT), and measured and theoretical mass for the studied analytes are reported in Table 11. The structural isomers *p*-CoQA (*m*/*z* 337.09289), CQA (*m*/*z* 353.08780), and diCQA (*m*/*z* 515.11950) were identified by comparing the obtained fragmentation pattern with data previously reported (Mullen, W.; et al. 2013) and the RT of standards with the obtained peaks.

Table 11. UHPLC-MS parameters of the assayed analytes (n=14)

Compound	Chemical Formula	Adduct Ion	RT (min)	Measured Mass (*m/z*)	Theoretical Mass (*m/z*)	Accuracy (Δ mg/kg)
Quinic Acid	$C_7H_{12}O_6$	[M−H]-	1.12	191.05531	191.05611	-4.19
5-CQA	$C_{16}H_{18}O_9$	[M−H]-	3.18	353.08790	353.08780	0.03
4-CQA	$C_{16}H_{18}O_9$	[M−H]-	3.19	353.08768	353.08780	-0.34
Caffeic Acid	$C_9H_8O_4$	[M−H]-	3.20	179.03442	179.03498	-3.13
Caffeine	$C_8H_{10}N_4O_2$	[M+H]+	3.20	195.08757	195.08765	-0.41
3-CQA	$C_{16}H_{18}O_9$	[M−H]-	3.22	353.08762	353.08780	-0.51
3-*p*CoQA	$C_{16}H_{18}O_8$	[M−H]-	3.31	337.09232	337.09289	-1.69
5-*p*CoQA	$C_{16}H_{18}O_8$	[M−H]-	3.32	337.09290	337.09289	0.03
3-FQA	$C_{17}H_{20}O_9$	[M−H]-	3.39	367.10309	367.10346	-1.01
4+5-FQA	$C_{17}H_{20}O_9$	[M−H]-	3.40	367.10303	367.10346	-1.17
Ferulic Acid	C10H10O4	[M−H]-	3.46	193.05017	193.05063	-2.38
p-Coumaric acid	C9H8O3	[M−H]-	3.48	163.03934	163.04006	-4.42
3,4-diCQA	$C_{25}H_{24}O_{12}$	[M−H]-	3.50	515.12103	515.11950	2.97
3,5-diCQA	$C_{25}H_{24}O_{12}$	[M−H]-	3.53	515.11993	515.11950	0.83

Abbreviations: CQA, Caffeoylquinic; *p*CoQA: *p*-Coumaroylquinic acid; FQA, Feruloylquinic acid; diCQA, Dicaffeoylquinic acid

Quantification of Polyphenol Compounds and Caffeine in the Assayed Samples Using UHPLC-Q-Exactive Orbitrap

The predominant CGAs, some important phenolic acids, and caffeine were quantified in SCG, SCGc, and CTc samples by using a high-resolution Orbitrap mass analysis. Calibration curves with ten concentration levels (regression coefficient > 0.99) were carried out for the quantitative determination of the found molecules.

Up to thirteen different polyphenolic compounds were quantified in the assayed samples, as reported in Table 12. Total CQA represented from 84.8 to 85.8% of total CGA detected in SCG and SCGc samples, respectively. Moreover, 5-CQA showed to be the most relevant CGA, being quantified in SCG and SCGc samples at a mean value of 1163.9 and 81.6 mg/kg, respectively. Referring to FQAs isomers, 3- and 4+5-FQA represented from 7.9 (SCG) to 8.4% (SCGc) of total CGA found in the here-investigated samples. As far as diCQAs concerned, 3,5-diCQA was the compound quantified at the highest concentration in both SCG and SCGc samples, at an average value of 135.9 and 8.3 mg/kg, respectively. Furthermore, *p*CoQA isomers mainly represented by 3- and 5- *p*CoQA were detected as the minor CGAs found in both SCG and SCGc samples at a mean value of 4.75 and 0.26 mg/kg, respectively. Moreover, some bioactive phenolic acids (ferulic acid, caffeic acid, quinic acid, and *p*-coumaric acid) were assessed in the analyzed samples. These important molecules represented from 0.42 to 0.46% of total polyphenolic compounds found in samples. Caffeic acid was the most common one, ranging from 0.5 (SCGc) up to 7.2 (SCG) mg/kg. Apart from polyphenols, caffeine was also evaluated in the here-assayed samples. As shown in Table 2, SCG samples displayed a caffeine concentration up to 1193.89 mg/kg, whereas the content detected in SCGc samples was 64.60 mg/kg.

Table 12. Chlorogenic acids (*n* = 9), phenolic acids (*n* = 4), and caffeine content in spent coffee grounds, spent coffee grounds-enriched cookies, and control cookies samples. Data are displayed as average value (mg/kg) and standard deviation.

Compound	**SCG** Average (mg/kg)±SD	**SCGc** Average (mg/kg)±SD	**CTc** Average (mg/kg)±SD
3-CQA	405.9±31.9	25.3±2.1	0.2±0.0
4-CQA	521.7±38.3	31.9±3.3	nd
5-CQA	1163.9±58.4	81.6±6.6	0.3±0.0
3-*p*CoQA	3.2±0.1	0.2±0.0	nd
5-*p*CoQA	6.3±0.2	0.3±0.0	nd
3-FQA	29.8±0.9	1.6±0.1	nd
4+5-FQA	176.5±11.5	11.1±0.2	0.1±0.0
3,4-diCQA	22.4±1.6	1.3±0.0	nd
3,5-diCQA	135.9±9.3	8.3±0.1	nd
p-Coumaric acid	0.2±0.0	0.1±0.0	nd
Ferulic acid	0.8±0.0	0.1±0.0	nd
Caffeic acid	7.2±0.3	0.5±0.0	nd
Quinic acid	2.1±0.1	0.1±0.0	nd
Caffeine	1193.9±62.3	64.6±7.8	nd
Total CGAs	2465.6±19.5	161.6±2.3	0.6±0.0

Abbreviations: CQA, Caffeoylquinic; *p*CoQA, *p*-Coumaroylquinic acid; FQA, Feruloylquinic acid; diCQA, Dicaffeoylquinic acid; CGA, Chlorogenic acid; SCG, spent coffee ground; SCGc, spent coffee grounds-enriched cookies; CTc, control cookies. Tukey's test was used to evaluate differences between SCGc and CTc samples considering p-value less than 0.05 as significant.

In Vitro Bioaccessibility of Coffee Polyphenols

Simulated GiD was performed in order to evaluate the SCGc polyphenols bioaccessibility and variation in antioxidant activity. In each step of the GiD, the content in TPC was assessed by using the Folin–Ciocalteu method.

As highlighted by Table 13, SCGc samples showed significantly (p-value < 0.05) higher TPC values than CTc samples in each step of the GiD. Moreover, along the simulated GiD, the colonic stage (Pronase plus Viscozyme L stages) showed the highest TPC value, 167.7 and 116.8 mg GAE/100 g for SCGc and CTc samples, respectively. In particular, the potential polyphenol bioaccessibility in the colonic stage reported by SCGc samples was 96.7%, whereas for CTc samples, it was 88.7%.

The antioxidant activity of the not-digested and digested samples was assessed using three different tests: DPPH, FRAP, and ABTS assays. Table 14 displays the results as mmol of Trolox equivalent (TE) per kilogram of the sample (average value and SD).

Concerning the antioxidant activity measured in not-digested samples, SCGc showed higher antioxidant activity than CTc samples in all evaluated spectrophotometric tests. In particular, not-digested SCG samples showed a percentage of increase in antioxidant activity of 12.4%, 12.9%, and 24.7% for DPPH, FRAP, and ABTS respectively, when compared to not-digested CTc.

On the other hand, the variation in antioxidant activity release during the simulated GiD of the SCGc samples was also evaluated. Digested samples (SCGc and CTc) showed significantly lower antioxidant activity (p-value < 0.05) than the not-digested samples through all the simulated GiD stages. However, compared to CTc samples, data highlighted that the SCGc samples showed a higher antioxidant activity in all simulated GiD phases. Moreover, the colonic stage (considered as Pronase plus Viscozyme L stages) showed the highest antioxidant activity along the simulated GiD.

Table 13. TPC value in not-digested samples and during the simulate GiD

Sample	**Digestion Stage**	TPC mg GAE/100g±Sl
SCGc	Not digested	174.4±6.5
	Oral stage	48.3±3.6
	Gastric stage	22.1±4.3
	Duodenal stage	72.5±2.1
	Pronase	76.6±7.3
	Viscozyme L.	91.1±9.4
	Total colonic stage	167.7±8.3
Control	Not digested	131.6±5.1
	Oral stage	32.4±2.2
	Gastric stage	12.5±1.3
	Duodenal stage	59.2±3.2
	Pronase	57.7±4.9
	Viscozyme L.	59.1±3.5
	Total colonic stage	116.8±4.2
SCG	Not digested	1067.2±57.3

Abbreviations: SCG, spent coffee ground; SCGc, spent coffee grounds-enriched cookies; CTc, control cookies. Tukey's test was used to evaluate differences between SCGc and CTc samples considering *p*-value less than 0.05 as significant.

Table 14. Antioxidant activity in not-digested samples and during the simulated gastrointestinal digestion, evaluated by DPPH, FRAP, and ABTS assays.

Sample	Digestion Stage	DPPH	FRAP	ABTS
SCGc	Not digested	13.6±0.4	10.2±0.3	19.4±0.5
	Oral satge	2.6±0.1	2.0±0.1	3.5±0.3
	Gastric stage	2.1±0.1	1.3±0.1	2.3±0.1
	Duodenal stage	5.4±0.2	3.3±0.2	3.6±0.4
	Pronase	5.5±0.3	2.8±0.1	7.4±0.5
	Viscozyme L.	7.7±0.3	4.6±0.3	6.1±0.3
	Total colonic stage	12.4±0.3	7.4±0.2	13.5±0.4
Control	Not digested	11.2±0.4	8.6±0.3	16.2±0.6
	Oral satge	1.2±0.1	0.9±0.1	2.8±0.3
	Gastric stage	0.6±0.0	0.4±0.0	1.7±0.1
	Duodenal stage	2.1±0.1	0.9±0.1	2.4±0.2
	Pronase	1.8±0.1	1.1±0.2	4.8±0.4
	Viscozyme L.	4.1±0.3	2.6±0.3	3.3±0.3
	Total colonic stage	5.9±0.2	3.7±0.3	8.1±0.3
SCG	Not digested	186.4±12.7	156.7±13.4	203.9±9.5

Abbreviations: SCG, spent coffee ground; SCGc, spent coffee grounds-enriched cookies; CTc, control cookies. Tukey's test was used to evaluate differences between SCGc and CTc samples considering p-value less than 0.05 as significant.

Discussion

In this study, a comprehensive characterization of polyphenols compounds and caf- feine contained in SCG and SCG-enriched cookies was carried out using an UHPLC-Q-Exactive Orbitrap instrument. In detail, nine predominant CGAs, four phenolic acids, and caffeine were assessed in SCG as well as SCGc samples.

Overall, the results indicate that SCG material may represent an important source of bioactive compounds, such as high content of polyphenols, melanoidins, and caffeine. Regarding the CGAs content found in assayed samples, the total concentration displayed by SCG samples was 2465.6 mg/kg. These levels showed a two-fold increase compared to SCG samples previously analyzed by Angeloni et al. 2020 who reported total CGAs concentration up to 1299.8 mg/kg. The monitored CGAs were only three (5-CQA, 3-, and 5-diCQA) of nine CGAs studied in the analyzed SCG samples, which may explain the different levels observed. In addition, these variabilities in the concentration of CGAs could be attributed to the influence of many factors, including origin of SCG, brewing procedures, and roasting degree, which plays a fundamental role in the presence of CGAs in this studied coffee by-product (Tfouni, S.A.; et al. 2014). Furthermore, the data obtained clearly showed that 5-CQA was the most prevalent CGA in the analyzed SCG samples, accounting for 84% of total CGAs. A wide variability was observed by Campos et al. [5], who reported a concentration range of 5-CQA from 397 to 2642 mg/kg in SCG samples. In the last decade, a broad number of epidemiological and experimental studies have linked CGA habitual intake to specific biological effects involved in preventing degenerative diseases and maintaining human health status (Tajik, N.; et al. 2017 - Kanno, T.; et al. 2013). This is due to the several properties that have been reported for these active molecules, playing a fundamental role in modulating lipid and glucose metabolism, helping to handle a wide range of disorders such as diabetes, hepatic steatosis, obesity, and cardiovascular disease as well (McCarty, M.F. 2005 - Liang, N.; Kitts, D.D. 2016).

As regards the melanoidins content in the assayed SCG samples, the levels found (~11 g/100 g) in the investigated samples were lower when compared to SCG samples previously analyzed, reporting a concentration range between 13 to 25 g/100 g (Mesías, M.; Delgado-Andrade, C. 2017). Melanoidins are well recognized as important heterogeneous compounds found in SCG material able to exert biological activities (Wang, H.-Y.; et al. 2011). According to Moreira et al. 2012, melanoidins have strong antioxidant activity, mainly due to the presence of CGA residue incorporated into them during the coffee-roasting process.

Concerning the caffeine occurrence in SCG material, our results revealed high concentrations in this mentioned alkaloid reaching up to 1193.89 mg/kg. Similar high levels were reported by Cruz et al. 2012, in which caffeine was found in espresso SCG material at concentrations ranging from 800 to 1400 mg/kg. Available evidence showed that moderate caffeine consumption (<400 mg per day) appears to be related to potentially positive effects in healthy adults, helping to reduce the incidence of various chronic diseases and improving mental and physical performances (Cakir, O.K.; et al. 2017).

On the other hand, SCGc prepared in this study at 7.5% of SCG provided 780 mg of melanoidins, 16.2 mg of CGAs, 6.5 mg of caffeine, and 0.08 mg of phenolic acids per 100 g of SCG-enriched cookies. This formulation guaranteed microbiological and chemical safety (hydroxymethylfurfural and acrylamide) of the product according to Martinez-Saez et al. 2017 who evaluated the application of SCG in the formulation of SCG- enriched cookies. Moreover, the authors reported that the taste, texture, colour, and overall acceptance of SCG-enriched cookies were comparable to commercial cookies. The main aim of this study was to evaluate the bioaccessibility of polyphenols from SCG-enriched baked food as well as their antioxidant activity displayed during an in vitro GiD to evaluate the application of SCG material as an innovative ingredient in the development of healthy bakery products. In the last decades, SCG material has been successfully employed to produce new foods and beverages, including spirits, pastry, cereal, and confectionery (Galanakis, C.M.; et al. 2015). As reported, the products developed with SCG were appropriate for particular nutritional needs due to the high dietary fiber content and low glycemic and caloric index.

Overall, our results showed that the TPC value and antioxidant activity significantly increased ($p < 0.05$) in biscuits formulated with SCG. Different findings have been previously reported in baked biscuits enriched with dietary fiber extracted from SCG (Vázquez-Sánchez, K.; 2018). The monitored TPC value in the enriched cookies was similar to the control. The authors revealed that dietary fiber extracted from SCG retained only 50% of phenolic compounds present in SCG material, resulting in a non-significant contribution of dietary fiber extracted from SCG to the antioxidant properties and TPC level of the formulated cookies.

The in vitro GiD was performed following the protocol recently developed by the INFOGEST network until the duodenal phase, whereas Viscozyme L and Pronase were used to simulate the activity of gut microbiota. Pronase is a commercial mixture of several bacteria nonspecific exo- and endoproteases, while Viscozyme L is a multi-enzyme complex containing a broad range of carbohydrases, such as arabanase, β-glucanase, xylanase, and cellulase. Previous articles have reported that the combined use of these commercial products in in vitro studies represents an effective alternative to the conventional use of the fecal inoculum to reproduce gut microbial metabolism (Fogliano, V.; et al. 2011 - Graziani, G.; et al. 2021).

The here-obtained results showed that both the TPC values and the antioxidant activity of SCGc samples exhibited significantly higher values than CTc samples during each step of the simulated GiD. Compared to CTc samples, the highest percentage of increase in antioxidant activity and TPC value was observed during the colonic phase in SCGc samples. In particular, the TPC value observed in SCGc samples was increased by about 30% at the end of the colonic phase, whereas SCGc samples displayed a two- fold increase in antioxidant activity after the colonic phase than the CTc samples. These outcomes suggested that the gut microbiota might be able to release phenolic compounds from SCGc samples with enhanced antioxidant activity, resulting in positive implications in improving and maintaining human health.

The increased value of TPC and antioxidant activity in SCGc samples after the colonic stage may be due to the presence of melanoidins in SCGc formulation. Many scientific studies have suggested that melanoidins have an important role in the gastrointestinal tract (Vitaglione, P.; et al. 2012). It has been reported that the melanoidins escape digestion, acting as fiber– antioxidant complex, to be fermented by the enzymes of the gut microbiota, releasing low-molecular-weight phenolic compounds linked to them (Borrelli, R.C.; et al. 2002). Previous scientific works demonstrated that the combined activity of Viscozyme and Pronase was able to mimic the enzymatic hydrolysis of intestinal microbiota, releasing phenolic compounds from the coffee melanoidins (Castaldo, L.; et al. 2021- Castaldo, L.; et al. 2020). The released CGAs could exert a local antioxidant effect in protection against colon cancer, modulating colonic population and providing a wide range of benefits after absorption through the epithelial cells. Moreover, as suggested by Bertolino et al. 2019, the increased TPC value highlighted in the SCGc samples after the colonic stage could be caused also by the several biotransformations that involve the phenolic compounds during the fermentation process, including deprotonation of the hydroxyl residue present on the aromatic rings of the CGAs.

Although the evaluation of the in vitro bioaccessibility of polyphenols extracted from baked foods enriched with SCG material after simulated GiD has been evaluated in previous works, the activity of the gut microbiota needed to be clarified. Indeed, the protocol used by Martinez-Saez et al. 2017 excluded the colonic stage in the analysis, whereas our results highlighted the critical role of this biological site in the release of antioxidant compounds, suggesting their potential advantages for human health.

On the other hand, based on the traditional daily consumption of biscuits and considering that SCG is present in low percentages in the formulated cookies, it is unrealistic to achieve concentrations of bioactive compounds capable of generating a health effect only with SCGc consumption. Moreover, another limitation of this work is the novel food status of SCG in the European Union. According to the web-based list, there are no applications pending for used SCG in baked food products (European Union – accessed on 2021).

References

- Angeloni, S.; Nzekoue, F.K.; Navarini, L.; Sagratini, G.; Torregiani, E.; Vittori, S.; Caprioli, G. An analytical method for the simultaneous quantification of 30 bioactive compounds in spent coffee ground by HPLC-MS/MS. *J. Mass Spectrom.* **2020**, *55*, e4519.
- Bertolino, M.; Barbosa-Pereira, L.; Ghirardello, D.; Botta, C.; Rolle, L.; Guglielmetti, A.; Borotto Dalla Vecchia, S.; Zeppa, G. Coffee silverskin as nutraceutical ingredient in yogurt: Its effect on functional properties and its bioaccessibility. *J. Sci. Food Agric.* **2019**, *99*, 4267–4275.
- Borrelli, R.C.; Visconti, A.; Mennella, C.; Anese, M.; Fogliano, V. Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.* **2002**, *50*, 6527–6533.
- Cakir, O.K.; Ellek, N.; Salehin, N.; Hamamcı, R.; Keleş, H.; Kayalı, D.G.; Akakın, D.; Yüksel, M.; Özbeyli, D. Protective effect of low dose caffeine on psychological stress and cognitive function. *Physiol. Behav.* **2017**, *168*, 1–10.
- Campos-Vega, R.; Loarca-Pina, G.; Vergara-Castaneda, H.A.; Oomah, B.D. Spent coffee grounds: A review on current research and future prospects. *Trends Food Sci. Technol.* **2015**, *45*, 24–36.
- Castaldo, L.; Izzo, L.; De Pascale, S.; Narváez, A.; Rodriguez-Carrasco, Y.; Ritieni, A. Chemical Composition, In Vitro Bioaccessi- bility and Antioxidant Activity of Polyphenolic Compounds from Nutraceutical Fennel Waste Extract. *Molecules* **2021**, *26*, 1968.

- Castaldo, L.; Izzo, L.; Narváez, A.; Rodríguez-Carrasco, Y.; Grosso, M.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of Different Coffee Brews Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Foods* **2021**, *10*, 179.
- Castaldo, L.; Narváez, A.; Izzo, L.; Graziani, G.; Ritieni, A. In Vitro Bioaccessibility and Antioxidant Activity of Coffee Silverskin Polyphenolic Extract and Characterization of Bioactive Compounds Using UHPLC-Q-Orbitrap HRMS. *Molecules* **2020**, *25*, 2132.
- Clifford, M.N.; Jaganath, I.B.; Ludwig, I.A.; Crozier, A. Chlorogenic acids and the acyl-quinic acids: Discovery, biosynthesis, bioavailability and bioactivity. *Nat. Prod. Rep.* **2017**, *34*, 1391–1421.
- Colantuono, A.; Ferracane, R.; Vitaglione, P. In vitro bioaccessibility and functional properties of polyphenols from pomegranate peels and pomegranate peels-enriched cookies. *Food Funct.* **2016**, *7*, 4247–4258.
- Cornelis, M.C.; El-Sohemy, A. Coffee, caffeine, and coronary heart disease. *Curr. Opin. Lipidol.* **2007**, *18*, 13–19.
- Cruz, R.; Cardoso, M.M.; Fernandes, L.; Oliveira, M.; Mendes, E.; Baptista, P.; Morais, S.; Casal, S. Espresso coffee residues: A valuable source of unextracted compounds. *J. Agric. Food Chem.* **2012**, *60*, 7777–7784.
- De Cosío-Barrón, A.C.G.; Hernández-Arriaga, A.M.; Campos-Vega, R. Spent coffee (*Coffea arabica* L.) grounds positively modulate indicators

of colonic microbial activity. *Innov. Food Sci. Emerg. Technol.* **2020**, *60*, 102286.
- Del Castillo, M.D.; Iriondo-DeHond, A.; Martinez-Saez, N.; Fernandez-Gomez, B.; Iriondo-DeHond, M.; Zhou, J.-R. Applications of recovered compounds in food products. In *Handbook of Coffee Processing By-Products*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 171–194.
- Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A. Effects of Trichoderma biostimulation on the phenolic profile of extra-virgin olive oil and olive oil by-products. *Antioxidants* **2020**, *9*, 284.
- Dini, I.; Graziani, G.; Fedele, F.L.; Sicari, A.; Vinale, F.; Castaldo, L.; Ritieni, A.J. An Environmentally Friendly Practice Used in Olive Cultivation Capable of Increasing Commercial Interest in Waste Products from Oil Processing. *Antioxidants* **2020**, *9*, 466.
- European Union. Summary of Applications and Notifications. Available online: https://ec.europa.eu/food/safety/novel_food/authorisations/summary-applications-and-notifications_en (accessed on 22 July 2021).
- Fogliano, V.; Corollaro, M.L.; Vitaglione, P.; Napolitano, A.; Ferracane, R.; Travaglia, F.; Arlorio, M.; Costabile, A.; Klinder, A.; Gibson, G. In vitro bioaccessibility and gut biotransformation of polyphenols

present in the water-insoluble cocoa fraction. *Mol. Nutr. Food Res.* **2011**, *55*, S44–S55.

- Fogliano, V.; Morales, F.J. Estimation of dietary intake of melanoidins from coffee and bread. *Food Funct.* **2011**, *2*, 117–123.
- Galanakis, C.M.; Martinez-Saez, N.; del Castillo, M.D.; Barba, F.J.; Mitropoulou, V.S. Patented and commercialized applications. In *Food Waste Recovery*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 337–360.
- Graziani, G.; Gaspari, A.; Di Vaio, C.; Cirillo, A.; Ronca, C.L.; Grosso, M.; Ritieni, A. Assessment of In Vitro Bioaccessibility of Polyphenols from Annurca, Limoncella, Red Delicious, and Golden Delicious Apples Using a Sequential Enzymatic Digestion Model. *Antioxidants* **2021**, *10*, 541.
- Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, caffeine, and health outcomes: An umbrella review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156. [CrossRef]
- International Coffee Organization—(ICO). Available online: https://www.ico.org/documents/cy2020-21/cmr-0221-e.pdf (accessed on 22 July 2021).
- Ismail, T.; Akhtar, S.; Riaz, M.; Ismail, A. Effect of pomegranate peel supplementation on nutritional, organoleptic and stability properties of cookies. *Int. J. Food Sci. Nutr.* **2014**, *65*, 661–666.

- Iwai, K.; Narita, Y.; Fukunaga, T.; Nakagiri, O.; Kamiya, T.; Ikeguchi, M.; Kikuchi, Y. Study on the postprandial glucose responses to a chlorogenic acid-rich extract of decaffeinated green coffee beans in rats and healthy human subjects. *Food Sci. Technol. Res.* **2012**, *18*, 849–860.
- Izzo, L.; Pacifico, S.; Piccolella, S.; Castaldo, L.; Narváez, A.; Grosso, M.; Ritieni, A. Chemical Analysis of Minor Bioactive Components and Cannabidiolic Acid in Commercial Hemp Seed Oil. *Molecules* **2020**, *25*, 3710.
- Izzo, L.; Rodríguez-Carrasco, Y.; Pacifico, S.; Castaldo, L.; Narváez, A.; Ritieni, A. Colon Bioaccessibility under In Vitro Gastrointestinal Digestion of a Red Cabbage Extract Chemically Profiled through UHPLC-Q-Orbitrap HRMS. *Antioxidants* **2020**, *9*, 955.
- Kaczmarczyk, M.M.; Miller, M.J.; Freund, G.G. The health benefits of dietary fiber: Beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. *Metabolism* **2012**, *61*, 1058–1066.
- Kanno, Y.; Watanabe, R.; Zempo, H.; Ogawa, M.; Suzuki, J.-i.; Isobe, M. Chlorogenic acid attenuates ventricular remodeling after myocardial infarction in mice. *Int. Heart J.* **2013**, *54*, 176–180.
- Kovalcik, A.; Obruca, S.; Marova, I. Valorization of spent coffee grounds: A review. *Food Bioprod. Process.* **2018**, *110*, 104–119.
- Liang, N.; Kitts, D.D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* **2016**, *8*, 16.

- López-Barrera, D.M.; Vázquez-Sánchez, K.; Loarca-Piña, M.G.F.; Campos-Vega, R. Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators in vitro. *Food Chem.* **2016**, *212*, 282–290.
- Lucas-González, R.; Viuda-Martos, M.; Pérez-Alvarez, J.A.; Fernández-López, J. In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges. *Food Res. Int.* **2018**, *107*, 423–436.
- Magalhães, L.M.; Machado, S.; Segundo, M.A.; Lopes, J.A.; Páscoa, R.N. Rapid assessment of bioactive phenolics and methylxan- thines in spent coffee grounds by FT-NIR spectroscopy. *Talanta* **2016**, *147*, 460–467.
- Makita, C.; Chimuka, L.; Cukrowska, E.; Steenkamp, P.; Kandawa-Schutz, M.; Ndhlala, A.; Madala, N. UPLC-qTOF-MS profiling of pharmacologically important chlorogenic acids and associated glycosides in Moringa ovalifolia leaf extracts. *S. Afr. J. Bot.* **2017**, *108*, 193–199.
- Martinez-Gomez, A.; Caballero, I.; Blanco, C.A. Phenols and Melanoidins as Natural Antioxidants in Beer. Structure, Reactivity and Antioxidant Activity. *Biomolecules* **2020**, *10*, 400.
- Martinez-Saez, N.; García, A.T.; Pérez, I.D.; Rebollo-Hernanz, M.; Mesías, M.; Morales, F.J.; Martín-Cabrejas, M.A.; Del Castillo, M.D.

Use of spent coffee grounds as food ingredient in bakery products. *Food Chem.* **2017**, *216*, 114–122.

- McCarty, M.F. A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk. *Med. Hypotheses* **2005**, *64*, 848–853.
- Mesías, M.; Delgado-Andrade, C. Melanoidins as a potential functional food ingredient. *Curr. Opin. Food Sci.* **2017**, *14*, 37–42.
- Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carriere, F.; Boutrou, R.; Corredig, M.; Dupont, D. A standardised static in vitro digestion method suitable for food–an international consensus. *Food Funct.* **2014**, *5*, 1113–1124.
- Moreira, A.S.; Nunes, F.M.; Domingues, M.R.; Coimbra, M.A. Coffee melanoidins: Structures, mechanisms of formation and potential health impacts. *Food Funct.* **2012**, *3*, 903–915.
- Mullen, W.; Nemzer, B.; Stalmach, A.; Ali, S.; Combet, E. Polyphenolic and hydroxycinnamate contents of whole coffee fruits from China, India, and Mexico. *J. Agric. Food Chem.* **2013**, *61*, 5298–5309.
- Murthy, P.S.; Naidu, M.M. Sustainable management of coffee industry by-products and value addition—A review. *Resour. Conserv. Recycl.* **2012**, *66*, 45–58.
- Olthof, M.R.; Hollman, P.C.; Katan, M.B. Chlorogenic acid and caffeic acid are absorbed in humans. *J. Nutr.* **2001**, *131*, 66–71.

- Pérez-Burillo, S.; Mehta, T.; Esteban-Muñoz, A.; Pastoriza, S.; Paliy, O.; Rufián-Henares, J.Á. Effect of in vitro digestion- fermentation on green and roasted coffee bioactivity: The role of the gut microbiota. *Food Chem.* **2019**, *279*, 252–259.
- Pérez-Burillo, S.; Rajakaruna, S.; Pastoriza, S.; Paliy, O.; Rufián-Henares, J.Á. Bioactivity of food melanoidins is mediated by gut microbiota. *Food Chem.* **2020**, *316*, 126309.
- Ramón-Gonçalves, M.; Gómez-Mejía, E.; Rosales-Conrado, N.; León-González, M.E.; Madrid, Y. Extraction, identification and quantification of polyphenols from spent coffee grounds by chromatographic methods and chemometric analyses. *J. Waste Manag.* **2019**, *96*, 15–24.
- Reis, S.F.; Rai, D.K.; Abu-Ghannam, N. Apple pomace as a potential ingredient for the development of new functional foods. *Int. J. Food Sci. Technol.* **2014**, *49*, 1743–1750.
- Šaponjac,V.T.;C´etkovic´,G.;Cˇanadanovic´-Brunet,J.;Pajin,B.;Djilas,S.;Petrovic´,J.;Loncˇarevic´,I.;Stajcˇic´,S.;Vulic´, J.Sour cherry pomace extract encapsulated in whey and soy proteins: Incorporation in cookies. *Food Chem.* **2016**, *207*, 27–33.
- Saura-Calixto, F.; Serrano, J.; Goñi, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492–501.

- Spencer, J.P.; Chowrimootoo, G.; Choudhury, R.; Debnam, E.S.; Srai, S.K.; Rice-Evans, C. The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Lett.* **1999**, *458*, 224–230.
- Stalmach, A.; Steiling, H.; Williamson, G.; Crozier, A. Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch. Biochem. Biophys.* **2010**, *501*, 98–105.
- Tajik, N.; Tajik, M.; Mack, I.; Enck, P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *Eur. J. Nutr.* **2017**, *56*, 2215–2244.
- Tfouni, S.A.; Carreiro, L.B.; Teles, C.R.; Furlani, R.P.; Cipolli, K.M.; Camargo, M.C. Caffeine and chlorogenic acids intake from coffee brew: Influence of roasting degree and brewing procedure. *Int. J. Food Sci. Technol.* **2014**, *49*, 747–752.
- Valdés, L.; Cuervo, A.; Salazar, N.; Ruas-Madiedo, P.; Gueimonde, M.; González, S. The relationship between phenolic compounds from diet and microbiota: Impact on human health. *Food Funct.* **2015**, *6*, 2424–2439.
- Vázquez-Sánchez, K.; Martinez-Saez, N.; Rebollo-Hernanz, M.; Del Castillo, M.D.; Gaytán-Martínez, M.; Campos-Vega, R. In vitro health promoting properties of antioxidant dietary fiber extracted from spent coffee (Coffee arabica L.) grounds. *Food Chem.* **2018**, *261*, 253–259.

- Vitaglione, P.; Fogliano, V.; Pellegrini, N. Coffee, colon function and colorectal cancer. *Food Funct.* **2012**, *3*, 916–922.
- Wang, H.-Y.; Qian, H.; Yao, W.-R. Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chem.* **2011**, *128*, 573–584.
- Williamson, G.; Dionisi, F.; Renouf, M. Flavanols from green tea and phenolic acids from coffee: Critical quantitative evaluation of the pharmacokinetic data in humans after consumption of single doses of beverages. *Mol. Nutr. Food Res.* **2011**, *55*, 864–873.
- Zhang, X.; Chen, F.; Wang, M. Antioxidant and antiglycation activity of selected dietary polyphenols in a cookie model. *J. Agric. Food Chem.* **2014**, *62*, 1643–1648.

Chapter 4
Reflections and Perspectives

The examination of the potential health benefits linked to coffee components as outlined in this doctoral thesis provides intriguing insights into the intersection of food science, nutrition, and human health. The effects of different coffee brews on bioactive compounds release were investigated, as well as subsequent antioxidant and anti-inflammatory activities after simulated gastrointestinal digestion (GiD). Among the coffee brews analyzed, espresso coffee exhibited superior antioxidant and anti-inflammatory properties, possibly due to its higher polyphenolic content. Thus, the use of GiD to assess the release of bioactive compounds provides valuable information on the metabolic processes through which coffee constituents are metabolized by our bodies, leading to potential health advantages. The discoveries highlighting the favorable impacts of coffee on oxidative stress, inflammation, and gut dysbiosis underscore its potential not only as a morning beverage but also as a dietary element with genuine health-promoting characteristics. Furthermore, the investigation into modifying roasting conditions to decrease stomach-irritating compounds opens up possibilities for enhancing the gastrointestinal tolerability of coffee, thus increasing its accessibility to individuals with gastric disease. Additionally, the innovative application of spent coffee grounds (SCG) in food products, such as SCG-enriched cookies, not only addresses environmental concerns related to coffee waste but also presents a creative solution to enhance the nutritional value of everyday snacks.

Looking forward, there is significant potential for further innovation in the coffee industry, particularly with regards to the development of functional beverages and food products. Building upon the findings of this research, future studies could explore the underlying mechanisms that contribute to the health benefits of coffee components, potentially uncovering novel therapeutic applications. Furthermore, as public awareness of sustainability concerns continues to increase, there is an opportunity to explore new approaches to coffee cultivation, processing, and waste management. This could involve the adoption of sustainable practices at every stage of the coffee supply chain, ranging from the initial cultivation to the final consumption, in order to minimize environmental impact while maximizing health advantages. Additionally, the incorporation of new functional ingredients, as suggested in the study, brings possibilities for creating coffee products that provide diverse consumer preferences and dietary requirements. By integrating botanical extracts or repurposing waste materials that are abundant in beneficial substances, manufacturers can develop offerings that not only deliver a pleasing taste but also contribute to overall health and well-being.

In light of these points, the future of coffee research and product development displays promise in addressing the dual challenges of promoting human health and environmental sustainability. By embracing a comprehensive approach and leveraging scientific advancements, the coffee industry can continue to evolve in ways that benefit both consumers and the planet.

Chapter 5
Conclusions

Coffee, a widely consumed beverage, is renowned not only for its delightful flavor but also for the potential health benefits it offers through its bioactive compounds. From our investigations, we have demonstrated that coffee possesses anti-inflammatory effects and the ability to alleviate oxidative stress, even in the presence of high levels of putrefactive compounds. This underscores its potential in improving health conditions by regulating the risk of colorectal inflammation. Results have indicated that coffee samples inhibit intracellular reactive oxygen species (ROS) and pro-inflammatory pathways, potentially conferring protective effects against cancer transformation in human colon cells. Using simulated GiD presents a novel approach to evaluating the effects of bioactive compounds on the gut, particularly in the context of chronic ROS injury and inflammatory stimuli, where these compounds can exert their beneficial health effects. Additionally, direct treatment with skatole, a putrefactive compound, induces cytotoxicity in human colon cancer cells in a concentration-dependent manner.

However, simultaneous treatment with coffee and skatole reduces ROS production and suppresses the upregulation of pro-inflammatory cytokines induced by skatole exposure. Our data indicate that direct treatment with skatole induced cytotoxicity in the HT-29 human colon cancer cell line in a concentration-dependent manner, resulting in a significant reduction in cell viability with respect to control cells. Moreover, the simultaneous treatment of HT-29 cells with coffee and skatole was able to decrease ROS production compared to control cells. Furthermore, HT-29 cells treated with skatole showed increased expression levels of the pro-inflammatory cytokines and chemokines TNF-α, IL-1, IL-8, and IL12.

Finally, our data demonstrate that all three types of coffee analyzed exhibited anti-inflammatory activity by hampering the up-regulation of pro-inflammatory cytokines induced by skatole exposure. These findings highlight that coffee could exert anti-inflammatory activity and mitigate oxidative stress in the presence of high levels of putrefactive compounds, suggesting that coffee consumption may improve health conditions by modulating the risk of colorectal inflammation.

Furthermore, in the realm of research and development focused on formulating products that not only provide a gratifying sensory experience but also deliver health benefits, a correlation study was conducted on the consumption of standard coffee and dewaxed coffee, as well as the symptoms of Gastroesophageal Reflux Disease (GERD). A randomized pilot study has demonstrated that consuming dewaxed coffee pods significantly alleviates both upper and lower gastrointestinal symptoms in GERD patients. This improvement in symptoms, encompassing nausea, abdominal bloating, and pain, implies that dewaxed coffee pods may enhance the quality of life for individuals with functional gastrointestinal symptoms. Nevertheless, further and more extensive randomized trials are required to validate these results and gain a better understanding of the relationship between waxes, caffeine content, and gastroesophageal symptoms. If confirmed, dewaxed coffee pods could present a promising option for reintroducing coffee consumption in GERD patients, potentially reducing the need for dietary restrictions.

As regards results from the investigation of byproduct, spent coffee grounds (SCG), which are typically disposed of as waste, are now being acknowledged for their copious presence of valuable bioactive compounds like polyphenols, melanoidins, and caffeine, with a particular emphasis on chlorogenic acid (CGA) and 5-CQA. These compounds not only enhance the flavor of baked goods but also provide various health advantages, thus making SCG a valuable resource for fortifying food.

Scientific investigations have revealed that the significant caffeine content in SCG indicates its pharmacological activity, which could potentially contribute to its health-promoting effects. Furthermore, results achieved have indicated that the incorporation of SCG into baked goods enhances the bioaccessibility of polyphenols, particularly during the colonic phase following simulated gastrointestinal digestion (GiD). This suggests that SCG-enriched baked goods may release antioxidant compounds in the colon, thereby amplifying their health-promoting effects. Additionally, by incorporating SCG into food products, the coffee industry can not only enhance the value of coffee waste but also mitigate environmental impact, thereby providing consumers with enriched food options that have increased biological activity.

Caro Professore Ritieni,

Mi rivolgo a Lei con profonda commozione e gratitudine. Nonostante la sua assenza fisica, la sua presenza e il suo insegnamento rimarranno per sempre nel nostro cuore e nella nostra memoria.

La sua dedizione alla professione e la passione con cui ha insegnato hanno lasciato un'impronta indelebile in ognuno di noi. Grazie alle Sue lezioni e alla Sua guida, ho potuto acquisire non solo conoscenze accademiche, ma anche valori umani e professionali che mi accompagneranno per tutta la vita.

Riflettendo sul percorso che ho compiuto, riconosco il Suo contributo fondamentale. Grazie a Lei posso dire con orgoglio di aver conseguito un titolo in più, ma soprattutto di essere cresciuta come persona e come professionista.

Le sarò eternamente grata per tutto ciò che ha fatto per me. La Sua figura rimarrà un punto di riferimento e un esempio di eccellenza per sempre.

Con sincero affetto e stima,
Sonia