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**Plant cell cultures as ingredients
for
3D-(bio)printing of next generation healthy food**

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ABSTRACT

Addressing global challenges such as climate change, reduction of arable land, natural resources erosion, and increasing food demand, requires research on innovative strategies to possibly complement traditional agriculture. Cellular agriculture offers a promising solution in this scenario, by using cell cultures from a wide variety of organisms – animals, plants, and microorganisms – as such to produce agricultural commodities, rather than farmed animals or crops.

The present doctoral project focuses specifically on plant cell agriculture, aiming to formulate novel, value-added food through the help of 3D printing. These novel foods integrate cells from plant suspension cultures and value-added by-products from the agri-food industry. In the perspective of combining such different matrices (i.e., value-added by-products and plant cells), the additive manufacturing approach enables controlled, standardized, and potentially sustainable production of these novel foods. 3D food printing represents an opportunity to expand available food sources by utilizing these unconventional matrices simultaneously valorizing waste materials. Indeed, this technology allows the creation of customized foods in terms of shape, texture, flavor, color, and nutritional content, which can also deceive the palate by providing satisfaction even in the absence of sapid or sweet ingredients, that may be considered unhealthy.

In the present work, to produce cellular biomass, selected microgreen species, were chosen as the starting material for the onset of plant cell cultures. Microgreens are *plantulae* harvested 7 to 15 days after sowing, fully edible for some species, and characterized by phytonutrient concentrations that may reach up to 40 times higher than those found at the adult stage. The selected species included *Ocimum basilicum purpurascens* (var. red rubin), *Perilla frutescens purpurascens* (var. shiso purple), *Beta vulgaris* (var. rubra). Selection criteria prioritized factors such as health benefit and technological value in food manufacturing processes (i.e., bio-accumulation of value-added secondary metabolites belonging either to the class of phenylpropanoids or betalains; antioxidant capacity, radical-scavenging activity, hypolipidemic effects, or antimicrobial properties)

Growth kinetics of the obtained callus cultures were evaluated. To optimize the growth of the *O. basilicum* and *P. frutescens* cultures in terms of dry weight and to modulate metabolites accumulation, different media formulations and physical elicitation strategies based on LED lights at fixed intensity and at different wavelengths (i.e., Blue light 460 nm, 50 $\mu\text{M}/\text{m}^2/\text{s}$; Red light, 660 nm, 50 $\mu\text{M}/\text{m}^2/\text{s}$), and their combination (Red and Blue light, 660 nm + 460 nm, 25 $\mu\text{M}/\text{m}^2/\text{s}$) were explored. The condition resulting in maximum dry matter accumulation was selected. Physical

elicitation using LED light was preferred over chemical elicitation to reduce possible issues risks associated with human consumption.

Pigmented friable callus cultures obtained from *Ocimum basilicum purpurascens* (var. red rubin), and *B. vulgaris* var. rubra, were selected for their potential to accumulate anthocyanins and betalains (betacyanin and betaxanthin), respectively, and evaluated for their response to different media compositions.

After establishing optimal extraction protocols for each species, hydroalcoholic extracts of cultures were analyzed by UV-visible spectroscopy.

Suspension cultures of *Perilla frutescens*, *Ocimum basilicum*, and *Beta vulgaris* were established and characterized in terms of growth kinetics and UV-visible profile. Additionally, a comparative analysis of antioxidant activity and polyphenol content was performed among *P. frutescens* and *O. basilicum* suspension cultures and the starting callus in the best-performing culture conditions, revealing that the suspension cultures exhibited the highest polyphenol accumulation and the greatest antioxidant capacity.

Perilla frutescens var. shiso purple was chosen as the model system for the subsequent experiments due to its rapid growth and UV-visible profile, making it suitable for devising a proof-of-concept, plant cell-based food. To enhance suitability of plant cell cultures to food applications, cell growth media were modified by reducing or replacing traditional synthetic plant growth regulators with relatively low acceptable daily intake (ADI). A different growth medium was evaluated for *Perilla frutescens* callus culture, in absence of 2,4-Dichlorophenoxyacetic acid (2,4-D) and in presence of by-products from the agro-industry such as casein hydrolysate, a by-product of the dairy industry, which provides essential nutrients and growth factors, offering a natural alternative to synthetic components, besides a solution to recycle a side-stream agri-food product. Furthermore, a physical elicitation by Blue LED light was applied in *P. frutescens* suspension culture grown in a modified medium containing low concentrations of potentially toxic plant growth regulators, like 6-Benzylaminopurine (6-BAP), aiming to achieve a metabolic profile comparable to that generated in the culture by the original culture media, while ensuring greater compliance with food safety standards.

Metabolomic analysis highlighted differences between callus and suspension cultures of *Perilla frutescens*, with light emerging as a key factor in metabolite accumulation. LED light exposure, particularly Blue light (Blue light 460 nm, 50 $\mu\text{M}/\text{m}^2/\text{s}$), promoted the biosynthesis of several bioactive compounds. Although certain metabolites were exclusive to each culture, such as rosmarinic acid and tyrosine in the callus, or p-coumaric acid, erythronic acid and vitamin B5 in the suspension, suspension culture generally showed higher overall concentrations. Notably, suspension culture grown under Blue light conditions, more suitable for food purposes, displayed a metabolite

profile rich in secondary compounds of nutraceutical interest, including chlorogenic acid, amino acids, B vitamins.

In the context of the pre-industrialization of prototype food products, cells were encapsulated in pectin-based hydrogels, the latter in view of the application of additive manufacturing (3D-printing). To improve the final product sustainability/circularity and to achieve optimal conditions for hydrogel formation, extracts derived from agri-food by-products and fruit juices were included in the formulation. Extracts of manufacturing value-added by-products from berries (strawberry and blueberry), specifically obtained in citric acid, and a value-added apple juice, were used as the solutions allowing the inclusion of *Perilla frutescens* cells suspension cultures into hydrogels suitable for 3D-printing. The compatibility of the extracts and of the juice with cells was assessed by the preservation of cell integrity and viability. Thereafter the final recipe for the whole cell-containing hydrogel was devised, the compatibility evaluations were conducted on the whole ink, as well. Possible alteration of compounds of interest was evaluated through UV-visible analysis. In addition to the ink specifically designed for the inclusion of plant cell cultures, three further ink recipes were formulated that included agro-industrial by-products, high-value-added fruit juices, honey, carob seed flour (a natural chocolate substitute), banana powder and pectin. These inks were devised to enable the production of two final products: 1) a multilayer bar composed of an external biscuit-like shell, an intermediate layer of white chocolate-like filling, and an internal core of gelée filling for the inclusion of cells and 2) a honey-based sphere with an inner gelée filling containing cells. The formulation of the inks was crucial to ensuring the structural integrity and of the two final products, demonstrating high printability, compatibility with the ingredients and viability of the embedded cells.

The shelf life of the inks was determined through microbiological tests, including assessments of total aerobic mesophilic microflora, yeast and mold counts, as well as physicochemical assessments such as pH measurement over time, water activity, water content, hardness and humidity levels, chemical composition, and water release kinetics. Further analyses involved defining physicochemical characteristics, optimizing printing parameters, and assessing the mechanical texture profile.

The overall results confirmed the good quality of the developed inks, giving a clue on the combination of formulation, technological treatment and storage conditions to guarantee the microbiological stability and chemical-physical integrity of the products over time.

Finally, the two final products were evaluated for their sensory attributes and consumer acceptance through a panel test, a preliminary preference test, and an online questionnaire. The panel test showed that the inclusion of plant cell cultures improved the consistency of the product while the consumer test showed that the product's attractiveness increased significantly after learning about the composition and benefits of these new foods.

In conclusion, the results of this work highlight the potential of plant cell cultures far beyond their traditional application, envisaging possible production approaches for high-value, plant-derived food without fields. In addition, they show the prospect of the integration of plant cell cultures and of agro-industrial by-products in innovative 3D printing food applications.

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1. INTRODUCTION

1.1 The impact of climate change on agriculture

Agriculture has always played a crucial role in society, meeting the basic human need for food while contributing significantly to economic, social and cultural development. Over the centuries to the present day, there have been revolutions and innovations that have greatly improved agricultural practices and, consequently, overall productivity. These advances have been driven by the cultivation of high-yielding varieties, by the use of inorganic fertilizers and pesticides, by the expansion of irrigation systems and the intensive agricultural management (Alam et al., 2023).

However, the unsustainable expansion of agriculture and the prolonged use of water, land and plant genetic resources is having considerable negative impacts, increasing the fragility of ecosystems and humanity itself, with serious environmental, political and economic impacts (Pörtner et al., 2022).

The modern approach of intensive agriculture, characterized by the use of high-performance technologies to maximize crop and livestock production, is indeed the leading cause of climate change (Pareek et al., 2020). Climate change stands as one of the most pressing and transformative challenges of our contemporary world, fundamentally reshaping and, in many cases, actively disrupting Earth's ecosystems. While the Earth has undergone natural climate variations throughout its history, the pace of these changes has accelerated exponentially over the past century, primarily due to human activities (Arora, 2019). These emissions originate from multiple points, and major sources include the production and application of fertilizers and other agrichemicals, that emit CO₂, N₂O and methane (CH₄); the digestive processes of ruminant animals like cows, sheep, and goats that produce CH₄ as a byproduct; the manure from livestock that contains both N₂O and CH₄; the use of fossil fuels within the food production and supply chains, such as for transportation and processing that emits CO₂; the disruption of forests which play a crucial role in absorbing CO₂ (Clark et al., 2020). Collectively, these diverse sources of greenhouse gas (GHG) emissions within the global food system contribute significantly to the environmental footprint of food production. On average, the emissions from the global food system totaled approximately 16 billion tons (Gt) of CO₂ equivalents per year from 2012 to 2017, contributing significantly to GHG emissions from various sources (Clark et al., 2020).

The emission of GHGs into the atmosphere, have led to a significant increase in the global average temperature, rising by 0.9 °C since the 19th century. Projections suggest that this temperature increase could reach 1.5 °C by 2050 or potentially even higher (Arora, 2019). The consequences of this unprecedented temperature rise are manifesting globally in the form of more frequent and severe droughts, floods, erratic precipitation patterns, heatwaves, and other extreme weather events. Rising global temperatures can lead to heat stress in crops and livestock, reducing yields and affecting animal

health. According to the latest report of the Intergovernmental Panel on Climate Change (IPCC) in 2023, global surface temperature has increased by about 1.1°C since 1850-1900 over the period 2011-2020. The IPCC predicts that if current global warming rates persist, global temperatures could increase by an additional 1.5°C between 2030 and 2052 (Lee and Romero 2023).

To mitigate the impacts of climate change, the Paris Agreement of 2015 aims to restrict the rise in the Earth's temperature to either 1.5°C or 2°C above preindustrial levels (Rogelj et al., 2018). However, the road to zero emissions is still a long one.

The consequences of climate change extend to land degradation, which is occurring at an alarming rate and is now considered a major global threat. According to the Global Assessment of Land Degradation and Improvement (GLADA), approximately a quarter of the world's land area can now be classified as degraded. Land degradation affects the lives of 1.5 billion people, with an astonishing 15 billion tons of fertile soil lost each year due to a combination of human activities and climate change (Arora 2019). This phenomenon has led to mass migrations and social and environmental challenges of unprecedented magnitude. In fact, a report published by the United Nations Environment Program in 2017 indicated that 500 million hectares of farmland have been abandoned due to drought and desertification, exacerbating these challenges and underscoring the critical need for action on a global scale (Arora 2019).

The intensifying impact of climate change, particularly through the increasingly frequent occurrence of extreme droughts, poses a significant threat to crop productivity. These droughts, exacerbated by climate change, lead to nutrient immobilization and salt accumulation in soils, rendering them dry, unhealthy, saline, and eventually infertile. The consequence of such barren lands is that they become non-arable over time and are ultimately abandoned by farmers. This not only results in economic losses but also contributes to social issues within affected communities (Arora 2019).

Moreover, climate change has also given rise to more frequent and sporadic floods in recent years. The European Academies' Science Advisory Council (EASAC) reports a 50% increase in extreme events, including floods, over the past decade, occurring at a rate four times higher than two decades ago. These led to the erosion of topsoil and nutrients, resulting in reduced soil productivity for years to come unless proactive remediation strategies are implemented (Arora, 2019).

Rising sea levels and heavy rainfall can also lead to the degradation of agricultural lands in coastal regions. This process has resulted in soil salinity, which in turn places stress on crops by decreasing respiration, photosynthesis, and transpiration. Projected sea level rise could submerge agricultural areas and require the movement of crops to higher elevations, jeopardizing the adaptive capacity of some plants, tree species, and animals. These events can devastate communities and agricultural infrastructure (Pareek et al., 2020).

High intensity agriculture relies on environmentally detrimental fertilizer and pesticides derived from fossil resources. The term "pesticide" encompasses a broad spectrum of chemical compounds, mixtures, and biological agents designed to eliminate or control organisms deemed as pests. Pests can include insects, rodents, nematodes, fungi, weeds, or any other unwanted life forms that pose a threat to human interests (Aktar et al., 2009). The primary consumer of pesticides is the agricultural sector, where they are primarily used to protect crops and enhance yields (EP, 2009). During the "green revolution" that began in the mid-20th century, pesticides played a key role contributing significantly to increasing food production to meet the demands of a growing global population (Jastrzębska et al., 2022). The global use of agricultural pesticides currently stands at a staggering 4.1 million tons, with Europe accounting for 11.6% of this total (FAO, 2017). Pesticide application is measured at an average of 2.63 kg per hectare of cropland worldwide, although significant variations exist between regions and countries (Jastrzębska et al., 2022). The benefits of conventional pesticides in controlling agricultural pests are readily observable and include enhanced crop and livestock yields, improved crop quality, reduced fungal toxins, extended shelf life of produce, and the establishment of retail networks. Pesticides also reduce the need for labor-intensive weeding, minimize soil disturbance, curb pest outbreaks, and help control invasive species. These advantages have boosted economic potential by increasing food production and reducing vector-borne diseases (Aktar et al., 2009). However, as known, the use of pesticides is not without drawbacks. There is now substantial evidence pointing to the potential risks they pose to humans and the environment. Some of these chemicals are hazardous substances, foreign to all living beings, including humans. Exposure to pesticides can occur through dermal contact, ingestion, inhalation, or the consumption of contaminated food and water (Jastrzębska et al., 2022).

A significant problem with pesticides is that a large portion of them fail to reach their intended target organisms and instead end up in the environment, negatively impacting public health, non-target species, and contaminating soil, water, and the atmosphere (Pimentel and Burgess, 2012). To mitigate the damage caused by pesticide contamination, it is essential to switch to safer, non-chemical methods of pest control, including weed control (Jastrzębska et al., 2022). The pervasive impact of climate change, mainly due to intensive agriculture, is unequivocal, but its far-reaching consequences are most evident precisely in the agricultural sector (Figure 1.1). This sector serves as the backbone of global food production and economy. The urgency of addressing climate change cannot be overstated, as its implications reach far beyond environmental concerns, extending into the very foundation of our sustenance and prosperity (Zurek et al., 2022)

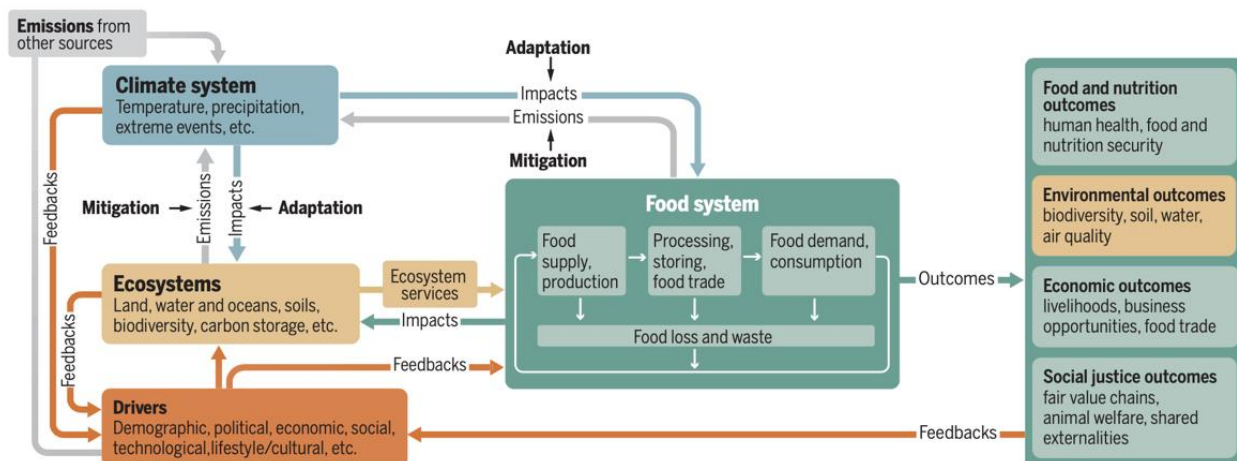


Figure 1.1. How climate change, ecosystems, and food system drivers interact to affect food security (Zurek et al., 2022).

In a context where food demand is constantly increasing due to population growth, it will be increasingly difficult to ensure sufficient food, given the tragic effect of climate change. The 2018 World Food Programme report highlighted a concerning trend: the increase in crop yields per hectare is significantly slower than the rate of population growth. Compounding this issue, data from the Food and Agriculture Organization (FAO) published in 2016 paints a grim picture of the future. If the current trajectory of GHG emissions and climate change persists, we can anticipate a decline in the production of major cereal crops by the year 2100 (Arora 2019).

Reports from FAO underscore the difficulty in achieving the sustainable development goals (SDGs) set for 2030, particularly the goal of eradicating hunger.

World hunger levels have been on the rise since 2014, with an increase in the number of undernourished people from 804 million in 2016 to 824 million in 2017 (Arora 2019).

World food needs are currently projected to increase by 60% in 2050, with only 2% more agricultural land available than today, reaching 40% of the total land area. The current food chain will not be efficient enough to feed the growing population, so new, nutritious, sustainably produced food sources are needed to reduce the negative environmental impacts of agriculture and protect water resources and biodiversity (Nordlund et al., 2020). Several studies have proposed different approaches and one of these solutions could be the implementation of cellular agriculture that may offer a possibly sustainable and eco-friendly approach to farming, emitting fewer greenhouse gases and requiring no land and less water than traditional farming methods (Nordlund et al., 2018).

1.2 Plant cell cultures

1.2.1 Features and scopes of *in vitro* plant tissue culture

Throughout history, humanity has relied on plant resources for nutrition, pharmaceuticals, and agrochemicals. In modern times, promising prospects have been launched for creating plant-based *in vitro* systems (such as callus cultures, cell suspension cultures, and organ cultures) and for genetically modifying these systems to allow massive production of desired plant-based products (Efferth 2019). Plant tissue culture is a technique used in plant biology and biotechnology to grow and propagate plant cells, tissues and organs in a controlled environment outside the plants themselves. This approach is based on the totipotency of plant cells, which possess the ability to either replicate indefinitely in an undifferentiated state or to undertake the differentiation process again. Thanks to these techniques, it's possible to study plant cell behavior, develop new plant varieties, produce plant metabolites of interest, and conduct research in various fields of plant biology and agriculture (Su et al., 2021).

Indeed, plant cell cultures have been essential to unravel plant growth and development, cell physiology, plant genetics, and other aspects of plant biology, including plant propagation by cell multiplication and production of secondary metabolites with industrial, nutritional or medical value. Compared with field-grown material, they offer a continuous production system independent of geographic, climatic or environmental constraints, free of agricultural chemicals and pathogens, with standardized and uniform quality (Efferth et al., 2019). In addition, *in vitro* plant cell culture allows rare or endangered species to be studied, propagated and exploited for industrial purposes without spoiling their natural availability.

To begin *in vitro* culture, small plant explants are excised from a plant tissue of interest, followed by surface sterilization, and placed on appropriate culture media for continuous undifferentiated growth (callus and suspension cultures) or for differentiation in specific organs (e.g., roots or shoots) or to develop a whole plant.

1.2.1.1 Media composition for *in vitro* plant tissue culture

The culture substrate is specially formulated with nutrients to simulate the nutrient conditions of cells in an *in vitro* context and still to promote growth and secondary metabolites accumulation in artificial conditions. This substrate basically consists of an aqueous solution in which mineral salts (both trace and macro-elements), vitamins (such as thiamine, biotin, myo-inositol, nicotinic acid, glycine, and riboflavin), and an organic carbon source, usually sucrose, are dissolved. The Murashige and Skoog (MS) medium (1962) is the most commonly used basic tissue culture medium for plant regeneration from tissues and callus. It is a “high salt” medium, due to its content of K and N salts. The Linsmaier

and Skoog medium (1965) is basically Murashige and Skoog (1962) medium with respect to its inorganic portion, but only inositol and thiamine HCl are retained among the organic components. Gamborg's B-5 medium was devised for soybean callus culture and has lesser amounts of nitrate and particularly ammonium salts than the MS medium. White's medium (White, 1963), which was designed for the tissue culture of tomato roots, has a lower concentration of salts than the MS medium (Beyl et al., 2005). Plant hormones play a key role in growth regulation and are used as phyto regulators. Hormones such as auxins and cytokinins are among the main ones used to drive growth, differentiation and cell multiplication in this *in vitro* environment. Specifically, culture media with high auxin/cytokinin ratios induce root formation (rhizogenesis), while those with low auxin/cytokinin ratios induce shoot formation (caulogenesis). Generally, when auxins and cytokinins are equivalent in the culture medium, callus formation is promoted (callogenesis). Table 1.1 shows the synthetic analogues most commonly used for callus initiation and propagation (Beyl et al., 2005).

Natural plant growth regulators	Synthetic analogues
auxin	2,4-dichlorophenoxyacetic acid (2,4-D)
auxin	naphthalenacetic acid (α -NAA)
cytokinin	6-benzylaminopurine (6-BAP)

Table 1.1. Synthetic plant growth regulators most commonly used for callogenesis.

To ensure successful plant cell culture and maximize the yield of healthy and viable plant cells, maintaining the appropriate pH range within the growth medium is of paramount importance. Plant cells undergoing *in vitro* cultivation exhibit a preference for a slightly acidic pH environment, typically falling within the range of 5.3 to 5.8. It's important to maintain this pH range because deviations from it, such as when pH values drop below 4.5 or rise above 7.0, can impede the growth and development of plant cells *in vitro*.

1.2.1.2 Callus and suspension cultures and plant secondary metabolites

If defined day-night light cycles and growth temperature around $25 \pm 2^\circ\text{C}$ are necessary parameters for whole plant *in vitro* cultures in order to accomplish photosynthesis and growth, callus and organ cultures such as hairy root cultures may require different conditions. In addition, some thrive in a dark environment, while others require well-defined light recipes. Callus cultures can be distinguished between those that develop a rather compact structure and those characterized by a friable texture. The latter are useful for generating suspension single cell cultures, which can then be maintained under liquid conditions, usually allowing higher biomass and secondary metabolite accumulation compared to the initial callus culture with external factors such as light, temperature,

pH and aeration modulating their biosynthesis (Efferth et al., 2019). Figure 1.2 shows a schematic representation of the procedure for the establishment of plant cell suspension culture.

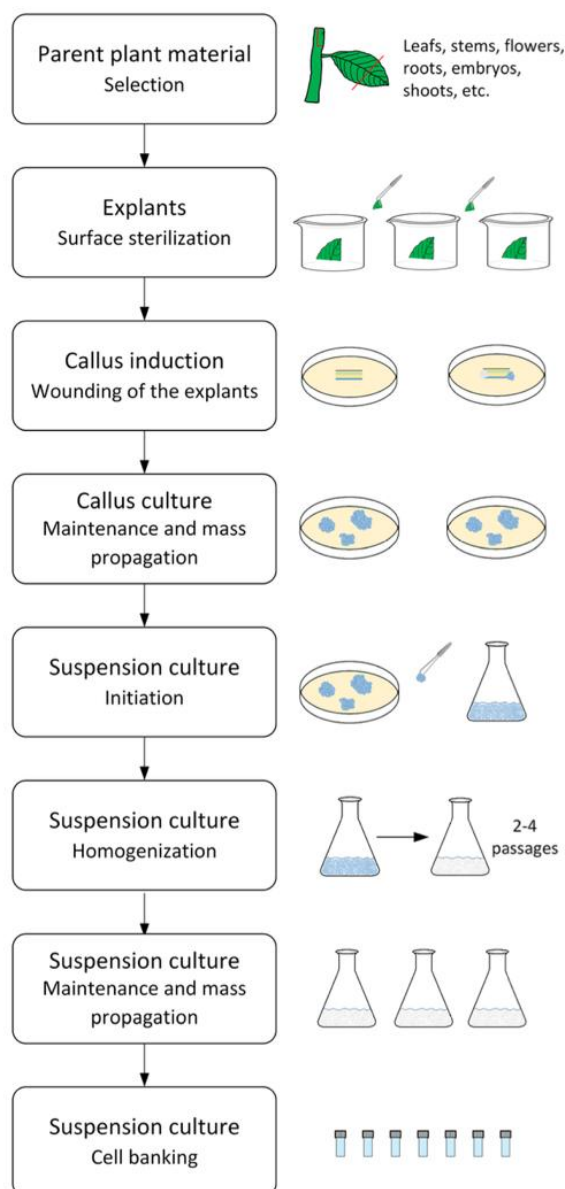


Figure 1.2. Schematic representation of the procedure for the establishment of plant cell suspension culture. (Eibl et al., 2018)

Suspension cell cultures are a type of *in vitro* culture in a liquid substrate and under constant agitation, which allows all nutrients to be evenly distributed and provide excellent aeration for the cells, without letting them settle to the bottom of the container. They provide a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest (Espinosa-Leal et al., 2018).

Callus and suspension culture represent the most widely adopted methods for the production of secondary metabolites (Espinosa-Leal et al., 2018, Davies et al., 2014). The products of plant secondary metabolism have long been used to meet a multitude of different needs. The primary use of these compounds has been as medicinal agents, first empirically and later, beginning in the 19th century, more rationally following the advent of molecule isolation. A secondary metabolite is typically characterized by its diverse and complex chemical structure, which usually includes multiple chiral centers and labile bonds, and this makes its chemical synthesis difficult. Therefore, biologically active molecules are most commonly extracted from their natural sources.

The range of commercially important plant secondary products includes pigments (e.g., anthocyanins and betacyanins), anti-inflammatory anti-microbial agents (e.g., berberine and rosmarinic acid), and anticancer molecules.

Plant secondary metabolites can be divided into four main groups: phenolic and polyphenolic compounds, terpenoids, alkaloids, and sulphur-containing compounds (Guerriero et al., 2018).

Phenolic and polyphenolic compounds, characterized by aromatic or phenolic ring structures, include flavonoids, phenolic acids, and lignans. External stimuli such as microbial infection, ultraviolet radiation, temperature and chemical stressors induce their synthesis.

Flavonoids, the largest group of phenolic compounds, include flavonols (such as quercetin, kampferol and isorhamnetin), flavones (such as apigenin, luteolin and chrysoeriol), anthocyanidins (such as cyanidin, delphinidin and malvidin), chalcones, dihydrochalcones and isoflavones. Many flavonoids, such as anthocyanidins, chalcones and flavones, are plant pigments responsible for the colors of vegetables. Flavonoids found in the human diet show antiviral, anti-inflammatory, antihistamine and antioxidant properties.

Phenolic acids including gallic and caffeic acids, vanillic and cinnamic acids, coumaric acid and others, show antimicrobial and antifungal activity, probably through enzymatic inhibition by their oxidized compounds. Hydroxycinnamic acid derivatives, such as caffeic, chlorogenic, sinapic, ferulic, and p-cumaric acids, possess high antioxidant activity due to inhibition of lipid oxidation and removal of reactive oxygen species. Lignans, in mammals, exhibit antioxidant and (anti)estrogenic properties, which may reduce the risk of certain diseases, including cancer and cardiovascular disease. Among the main terpenoids found in the human diet are carotenoids, tocopherols and tocotrienols, quinones and sterols. Plant carotenoids, including α -carotenes, β -carotenes, xanthophylls and lycopene, constitute fat-soluble pigments that reflect orange, yellow and red colors. Carotenoids acting as vitamin A precursors play a crucial role in human nutrition because they are involved in hormone synthesis, regulation of cell growth and differentiation, and immune responses.

Alkaloids represent a class of basic nitrogen-containing compounds and have always been the subject of great interest because of their physiological and medicinal properties, as in the case of caffeine, nicotine, morphine, atropine, and quinine.

Glucosinolates constitute a broad category of sulfur derivatives of amino acids, play roles related to the activation of enzymes involved in detoxification from carcinogens, and inhibition of enzymes responsible for changes in steroid hormone metabolism, and protection from oxidative damage.

The variety and the combination (phytochemical) of plant compounds beneficial to human health, plays a key role in determining the nutritional quality of plant-derived food (Hounsome et al., 2008).

1.2.2 The cellular agriculture

In recent years, there has been a growing focus on cell agriculture, an innovative concept that is based on the use of cell cultures of various organisms, for food, feed or cosmetic purposes, in a sustainable and efficient manner, representing a significant break with traditional methods based on animal breeding or plant cultivation (Rischer et al., 2020).

As already mentioned specifically for plants, cell cultures refer to the practice of culturing and propagating cells of multicellular organisms in a controlled environment outside the host organism, taking advantage of the cells' ability to proliferate and produce biomolecules.

So far, cell cultures have been used to study cell behavior and physiology, to test drugs and chemicals, to produce proteins and other biomolecules of interest, to conduct disease and cell biology research.

Cellular agriculture has potential significant advantages: reducing the need to raise animals or cultivate plants, helping to decrease the environmental impact associated with livestock and intensive agriculture; increasing resource efficiency by requiring less water, land and food to produce the same amount; tailoring products to specific needs, such as designing foods with specific nutritional compositions (Mattick 2018), lowering animal suffering and altering the nutrient profile of the food (Nyika et al., 2021). It's gaining ground as a promising alternative to traditional agricultural products, aiming to satisfy the potential demand for food and nutrition while reducing the environmental burden. The impact of cellular agriculture will depend on various factors, including the primary source of cells, the capacity for decarbonization energy generation, and the precise environmental footprints associated with production. In this regard, conducting detailed and transparent life cycle analyses (LCAs) is imperative to understand and quantify these effects accurately.

The VTT Technical Research Centre of Finland carried out LCA for plant-based meat (PBM), animal-based meat (ABM) and cell-based meat (CBM). PBM are generally more environmentally sustainable than ABM, in particular the significantly lower potential for eutrophication and land use. However, some parameters such as greenhouse gas emissions and energy consumption for some new plant-based meat products are less sustainable than poultry of animal origin. The water footprint of PBM

varies widely depending on the main protein source, on average, a ton of PBM product consumed 3800 m³ of water, where the increased consumption is due to the processing of meat analogues after the collection of crude protein sources. In addition, the environmental impact of CBM in the laboratory is much debated, with preliminary assessments that present considerable degrees of uncertainty; especially for energy consumption values. The first LCA published in 2011 estimated that meat grown in the laboratory would lead to lower energy consumption (7-45%), greenhouse gas emissions (78-96%), land use (99%) and water use (82-96%) compared to animal-based meat. A subsequent LCA, in 2015, reported less dramatic footprint reductions and established that CBM's energy consumption, acidification potential and potential ozone-depleting impacts could be more harmful than animal meat, especially when compared with poultry production. However, according to experts, CBM, once optimized, will require less resources and emit less waste than meat of animal origin. These reductions are due to the targeted cultivation of tissues, reducing consumable tissues, higher production rates and vertical production systems (Rubio et al., 2020).

The development of cell-based products faces significant challenges, necessitates a profound comprehension of the underlying technologies and active collaboration to surmount a host of challenges, including cost considerations, sensory attributes, and nutritional profiles, the complex regulatory frameworks, ethical considerations, public acceptance, and overcoming technological hurdles. Achieving success in cellular agriculture necessitates a paradigm shift in societal expectations and cultural norms, highlighting the importance of broader cultural evolution (Nyika et al., 2021).

1.2.2.1 Plant cell cultures for food purposes

The possibility to manufacture value-added molecules of pharmaceutical and cosmetic value by plant cell cultures, in a natural and sustainable manner, has brought a new motivation to plant cell culture technology in the last 10 years (Eibl et al., 2018). The main drivers for the use of plant cell cultures instead of whole plants for this kind of production resided mainly in the possibility of being manufactured with lower impacts on the environment, less erosion of natural or endangered species and resources, and independence from location and season.

These reasons may also be valid for using plant cell cultures for food purposes, as well. Indeed, plant cell culture exploitation for cosmetic and drug industrial production has led to the first plant cell culture-based food supplement ingredients, such as Echigena Plus and Teoside 10, to be produced, approved and commercially distributed (Eibl et al., 2018). Fundamental changes in plant-derived food production are indeed necessary to tackle global food insecurity in the current scenario of an increasing global population and the negative environmental impacts of conventional agriculture.

PCC may serve as an important plant-based food production and manufacturing system, complementing, in the future, traditional agriculture.

In recent years, plant-based foods have gained an increasingly prominent position in people's eating habits, driven by awareness of the health benefits associated with a diet rich in fruits, vegetables, legumes and whole grains. In addition, plant-based foods, in some cases can be considered a sustainable alternative, namely, when they require fewer natural resources and produce lower greenhouse gas emissions than meat production. In addition, they help ensure a wider variety of healthy, sustainable and palatable food (Davies et al., 2014).

Plant cell culture technology represents a forward-looking approach providing plant-derived food that may align with the principles of sustainability and resource efficiency, as well (Espinosa-Leal et al., 2018).

Plant cell culture may play a significant role in the future of sustainable production of food, supplementing current food production and providing an alternative for manufacturing value-added and well-balanced food materials (Rischer et al., 2020). In analogy to the radical invention of “cultured meat” but to an even greater extent given the immense significance in secondary metabolites, bioreactor-grown plant cell culture could be exploited as an entirely new food biomass for human consumption (Nordlund et al., 2018).

1.2.2.2 Pioneering plant cell cultures for food production purposes in an era of global changes

In general, the concept of plant cell agriculture implies the alternative process that manufactures food through bacterial, yeast, microalgae, plant or animal cells for food production. Recently, fresh and freeze-dried cell cultures of three berry species (*Rubus chamaemorus L.*, *Rubus saxatilis L.* and *Vaccinium vitis-idaea L.*) have been studied for their nutritional values and potential use as food. These plant cell cultures have shown a color similar to fresh berries, and the presence of the same secondary plant metabolites. The evaluation of nutritional profile showing comparable or higher quantities and qualities than the corresponding fruit. These include high dietary fiber (21-37%), starch (0.3-1.3%), sugars (18-33%), and good quality lipids. In addition, the cultures contain a significant amount of protein (14-19% in dry weight), comparable with the protein content of macroalgae considered "superfoods" (Wells et al., 2017). These plant cell cultures showed balanced amino acid profiles and met amino acid requirements for adults according to WHO recommendations. Their essential amino acid contents were close to or higher than those found in soy protein isolates and concentrates. *In vitro* protein digestion assay showed hydrolysis by digestive enzymes in fresh cells. Lipid analyses indicated that berry cells are rich sources of essential and polyunsaturated fatty acids, contributing to a high-quality lipid composition. In addition, contained higher levels of some

beneficial compounds, such as campesterol, β -sitosterol, and oleanolic acid, than fruits, including seeds. It also showed a significantly higher total dietary fiber content than fruit, with a 37% dietary fiber content in dry weight. The plant cell culture samples also exhibited a fresh odor and flavor, with no significant off-flavors or overly intense taste notes. The sensory evaluation indicated that both fresh and freeze-dried plant suspension cells maintained a neutral and pleasant profile, making them suitable for potential food applications (Table 1.3). These results indicate that plant cell cultures have great potential for use in the food industry and can offer a high-quality nutritional source, as well as possible advantages in terms of sustainability and continuous availability (Nordlund et al., 2018).

Sensory profiling of the fresh and freeze-dried plant cell samples on an intensity scale 0–10 ($n = 2 \times 10$).

	Fresh odour ($p < 0.001$)	Berry-like odour ($p < 0.001$)	Sandy mouthfeel ($p < 0.001$)	Coarse mouthfeel ($p < 0.001$)	Fresh flavour ($p < 0.001$)	Berry-like flavour ($p < 0.001$)	Bitter ($p < 0.001$)	Astringent ($p < 0.001$)
Cloudberry, fresh	6.4 ^a	4.7 ^b	8.4 ^a	8.1 ^a	4.8 ^a	2.3 ^c	2.1 ^c	1.7 ^b
Lingonberry, fresh	7.3 ^a	7.2 ^a	8.8 ^a	9.1 ^a	5.2 ^a	4.2 ^{ab}	3.2 ^{bc}	2.7 ^b
Stoneberry, fresh	6.8 ^a	5.3 ^b	6.9 ^b	4.7 ^b	4.7 ^a	2.4 ^c	2.7 ^{bc}	2.3 ^b
Cloudberry, freeze-dried	4.9 ^b	4.2 ^b	0.6 ^c	0.7 ^c	4.9 ^a	5.3 ^a	2.3 ^c	1.8 ^b
Lingonberry, freeze-dried	4.7 ^b	4.3 ^b	1.5 ^c	1.7 ^c	3.0 ^b	3.4 ^{bc}	8.5 ^a	7.8 ^a
Stoneberry, freeze-dried	4.4 ^b	4.0 ^b	0.7 ^c	0.6 ^c	4.3 ^{ab}	4.2 ^{ab}	4.0 ^b	2.9 ^b

a–c: Means in each column followed by a different letter signify that the samples are statistically significantly different in respect of that variable (Tukey's HSD test; $p < 0.05$).

Table 1.3. Sensory profiling of the fresh and freeze-dried plant cell samples on an intensity scale 0–10 ($n=2 \times 10$). (Nordlund et al., 2018).

In another study carried out by the researchers of VTT (Nohynek et al., 2015) the phenolic composition of the cell culture of the cloudberry (*Rubus chamaemorus*) compared to the fruit was studied. In these cell cultures, phenolic compounds consisted mainly of flavanols, which are not typical of cloudberry fruit and which have aroused much interest due to their beneficial health effects, whereas the berries consisted almost entirely of ellagitannins (80%). The chemical composition of freeze-dried cells included essential fatty acids, such as α linoleic acid, β -sitosterol and vitamin E, and were rich in proteins.

Researchers at the University of Applied Sciences Zurich (ZHAW) used cell culture of *Theobroma cacao* to produce a chocolate model. Theobroma callus cells, cultured at 29 °C on modified Murashige and Skoog medium, were analyzed for polyphenol content (epicatechin, procyanidins B1, B2, C1 and cinnamtannin A2) which were comparable or even up to 40% higher than the starting material, while the caffeine and theobromine alkaloids were reduced up to 100%. In addition, overexpression of the amino acids valine, cysteine and phenylalanine, known to strengthen the immune system, has been detected. From this friable callus was generated the culture in suspension and after about two weeks 300 g of biomass were collected, separated from the culture broth, rinsed and freeze-dried. In vitro cocoa powder has been used to produce three bars of 70% dark chocolate. The chocolate obtained

with cell cultures had a promising sensory profile with an intense fruity and sour aroma comparable to the flavors of citrus and red fruits. The biomass contained both volatile and non-volatile aromatic compounds with a total polyphenol content of 6,69 g/ kg (Eibl et al., 2018).

In another study, Bianconi et al., 2020 demonstrated the industrial potential of a red carrot cell extract for food applications. The extract of suspended cells of *Daucus* carrot grown in the dark in the medium B5 Gamborg, is characterized by large quantities of anthocyanins, higher and more stable than the extracts of the starting material, while the metabolic profiles of both extracts were comparable. In addition, the antioxidant and anti-inflammatory activities of the extract were confirmed in vivo using mice (Gubser et al., 2021).

A proof of concept that plant cell agriculture can be considered an alternative production platform for tackling the challenges faced by some commodities such as coffee, in terms of land use, climate change, and sustainability was demonstrated. Coffee is one of the most consumed beverages in the world and its production is a global market that generates over 30 billion dollars every year. However, coffee cultivation is affected by climatic and environmental factors, such as drought and flooding, which make its production vulnerable. In addition, coffee has a high carbon footprint accounting globally to 33–126 billion kg CO₂ per annum, which is in the range of the annual emissions of countries like Denmark and the Philippines and its trade is dominated only by two varieties, *Arabica* and *Robusta*, that makes production vulnerable in terms of rising temperatures and pests threatening this crop, while many wild coffee species are at risk of extinction. With the demand for coffee increasing, expected to triple by 2050, and predictions that global production will decrease by half in the next 30 years due to the impacts of global climate change, it is apparent that coffee cultivation faces an uncertain future. In this context, cellular agriculture could reduce the environmental impact and support coffee production. The study by Aisala et al., 2023 describes the cell cultivation of coffee (*Coffea arabica*) starting from young leaf explants, cut and incubated on a callus induction medium at 24°C in the dark. Starting from callus, cell suspensions were started and scaled up in bioreactors from which cell biomass was obtained. Cell biomass was collected, washed, frozen, freeze-dried and stored at -20°C until the roasting process. The freeze-dried cells were roasted in a ventilated oven at 225°C, using 25 grams of cells, placed in baking paper bags and roasted in three different conditions (different minutes of roasting). The infusions obtained with different roasting regimes were characterized through chemical and sensorial evaluation approaches and compared with conventional coffee. Unlike conventional coffee, the dominant odor and flavor attributes were similar to burnt sugar and smoke, but with a less pronounced roast. Bitterness and acidity intensities were similar to those of conventional coffee. The results obtained demonstrated the validity of a cellular agriculture approach as an alternative platform for coffee production, providing guidance for future optimization work (Aisala et al., 2023).

1.2.2.3 Safety and sustainability of plant cell culture

The issues related to sustainability and safety of plant cell cultures as food are relevant for both consumers and regulators. From a safety perspective, it is important to consider that plant cell and tissue cultures growth media are typically supplemented with one or more plant growth regulators. Although natural growth regulators found in fruits and vegetables are considered safe to consume at native levels, the synthetic growth regulators pose different risks. Some of them are used as pesticides in agriculture and allowed residual levels in treated products are legally restricted. While synthetic growth regulators remain indispensable in plant cell culture, they are added only in trace amounts at physiological amounts. Hakkinen et al., 2020 have demonstrated that in *Rubus arcticus* cell cultures grown in a medium with synthetic growth regulator 2,4-D (2mg/L) and kinetin (0.5mg/L), only minimal traces of free growth regulators remain in the cells by the time of harvesting. In addition, toxicity assessment by freshwater crustaceans have confirmed that toxicity of the cell culture did not exceed that of traditionally consumed (bilberry) fruit, supporting their safety for food purposes. From a sustainability point of view, the same study by Hakkinen et al., 2020 demonstrated that the 83% - 75% substitution of sucrose (a common carbon-source molecule in plant cell culture and the seventh most expensive culture medium component in Murashige and Skoog-based medium) can be replaced with lactose-rich dairy industry side streams, without significantly changing cell biomass accumulation during culture of arctic bramble and birch cell cultures. Likewise, nutritional composition or sensory properties were not compromised, despite the use of a waste-based medium, which allows for a reduction in the costs, particularly those related to sucrose.

To study plant cell culture's sustainability, the life cycle assessment was determined, considering the case study of cloudberry cell culture. In particular, the global warming potential (GWP), the freshwater eutrophication potential (FEUP), marine eutrophication potential, terrestrial acidification potential (TAP), stratospheric ozone depletion, water consumption and land use were assessed. The study revealed a high environmental impact in terms of GWP, FEUP and TAP (82–93%) due to electricity consumption. Indeed, implying the use of bioreactors, plant cell culture is an energy-intensive process. Nevertheless, using renewable energy sources like wind energy has the potential to reduce the global environmental impact of PCC by 34–81%. Enhancement in the energy efficiency of bioreactor mixing processes and reduction in cultivation time may reduce by 4–47% their environmental impact, as well. In comparison to other novel foods, the environmental impact of the plant cell culture products was demonstrated to be comparable to microalgae-derived food products. The fresh plant cell culture products considered in this study would be similar to the GWP of conventionally grown food products and, with technological advancements, can be highly competitive (Kobayashi et al., 2022).

1.3 Microgreens

In order to establish a plant cell culture, a suitable starting material should be selected, so that the bioactive compounds of interest in the *in vitro* culture can be recovered more likely. In recent years, microgreens are gaining increasing popularity and attention as an emerging food specialty. These are young and tender leaf sprouts cotyledons, characterized by a variety of colors, textures and flavors (Figure 1.3)



Figure 1.3. Microgreens of red radish (*Raphanus sativus* L.) and red basil (*Ocimum basilicum* L.) (Di Gioia 2015)

These vegetables are harvested when the first leaves are fully expanded, but before the real leaves emerge. This immature phase gives them a soft/crisp texture, a vibrant color, high nutrients concentration and a series of quality attributes that enrich the sensory properties of dishes (Mir et al., 2017). Microgreens represent a new category of edible vegetables and are increasingly used as culinary ingredients to enrich salads or as toppings to enhance a wide range of dishes. The most common varieties of microgreens include mustard, cabbage, radish, buckwheat, lettuce and spinach. Their increasing scientific interest is mainly related to the high concentrations of bioactive components compared to mature varieties of vegetables, which makes them particularly beneficial for human health (Mir et al., 2017) being able to promote health and prevent disease (Xiao et al., 2015).

Studies have shown that microgreens may contain much higher levels of vitamins, minerals, and other health-enhancing phytonutrients than mature leaves of the same plants. These include ascorbic acid, carotenoids, phylloquinone, and tocopherols (Xiao et al., 2012). Xiao et al., 2012 have verified the presence of these vitamins, in high concentrations, in 25 microgreens commercially available. The total content of ascorbic acid (vitamin C) ranged from 20.4 to 147.0mg per 100 g fresh weight (FW), while the concentrations of β -carotene, lutein/zeaxanthin and violaxanthin ranged from 0.6 to 12.1, 1.3 to 10.1 and 0.9 to 7mg/100 g fresh weight respectively. The level of phylloquinone (vitamin K) varied from 0.6 to 4.1 μ g/g FW; meanwhile, α -tocopherol and γ -tocopherol (vitamin E) ranged from 4.9 to 87.4 and 3.0 to 39.4mg/100 g fresh weight respectively. Moreover, according to the USDA national nutrient database, the concentration of phylloquinone in mature amaranth, basil and red

cabbage was 1.14, 0.41 and 0.04 µg/g FW respectively, which were much lower than the corresponding microgreens values (4,09, 3,20 and 2,77 µg/g FW, respectively). Garnet amaranth had a much higher ascorbic acid content of 131.6mg/100g FW than mature leaves (11.6-45.3mg/100 g FW). Chinese rose radish, opal basil and opal radish microgreens were also relatively abundant sources of vitamin C with more than 90.0 mg/100 g of fresh weight, equal to 1.5 times the recommended dietary dose. Microgreens of red cabbage contained an average of 11.5mg/100 g FW which is higher than the reported value for mature cabbage (0.044mg/100 g FW). Wasabi, green basil, pea tendrils and microgreens of garnet amaranth are also abundantly concentrated with β-carotene, comparable to that of carrot. Mature spinach contains high amounts of lutein/zeaxanthin (7.2mg/100 g fresh weight), but the microgreen s of acetosella, red cabbage, and amaranth garnet had higher concentrations (Xiao et al., 2012).

In addition to the vitamin benefit mentioned above, another important aspect of microgreens is their significantly higher concentration of bioactive molecules, such as flavonoids and phenolic acids, compared to mature plants.

Red beet (*Beta vulgaris*) stands out among microgreens for its high flavonoid and citric acid content, with anti-inflammatory and anticancer properties, and for its strong antioxidant activity (Balik et al., 2025). Among the main bioactive compounds in *Beta vulgaris* microgreens there are betalains, natural pigments with antioxidant properties and potential health benefits. Betalains are divided into betaxanthins (yellow-orange pigments) and betacyanins (red-purple pigments), both involved in cellular protection against oxidative stress and with possible anti-inflammatory effects.

In particular, betaxanthins reach a peak concentration of 1357.47mg/100g dry matter at 12 days, and then progressively decrease during growth; betacyanins typically increase from 511.15mg/100g dry matter at 9 days to a maximum of 841.85mg/100g dry matter at 15 days, before decreasing in the subsequent growth phases. Total betalains show a similar trend, with a maximum of 1264.83mg/100g dry matter at 15 days, followed by a decline. The total content of these pigments overall significantly ($p < 0.05$) increased in initial days of growth and reached the maximum on day 15 (Acharya et al., 2021). Conversely, in adult red beet, unlike microgreens, the betaxanthin content is 37.6mg/100 g for Vulgaxanthin I and 4mg/100 g for Vulgaxanthin II. Betacyanins have an average concentration of 82 mg/100 g, specifically for betanin it varies between 30 and 60mg/100 g (Frati 2017).

Basil (*Ocimum basilicum*) is a species widely used in the food sector, characterized by a high content of phenolic compounds with antioxidant activity. These compounds, in fact, are able to slow down or inhibit the oxidation of lipids and other biological molecules, acting on oxidative chain reactions and neutralizing free radicals (Lobiuc et al., 2017). Among the main phenolic acids present in basil are rosmarinic acid and caffeic acid, with antiviral, antibacterial, anti-inflammatory and antioxidant activities. In basil microgreens, the contents of caffeic acid and rosmarinic acid are 137mg/100g and

165mg/100g, respectively (Lobiuc et al., 2017). On the other hand, for adult basil, rosmarinic acid accumulates mainly in the leaves, with concentrations varying between 10 and 100mg/100g of dry weight, while the average levels of caffeic acid are equal to 81.5mg/100g of dry weight (DW) (Zare et al., 2021).

Perilla frutescens (also known as shiso or Chinese basil) microgreens, are rich in bioactive compounds with important nutritional properties. They contain high amounts of flavonoids, polyphenols, and Omega-3 fatty acids, as well as vitamins B1, B2, and K, the latter being up to 22 times higher in microgreens compared to the adult counterpart (respectively 16.372 µg/g FW and 0.73 µg/g FW). They are also a good source of beta-carotene (up to 4 times higher in microgreens) and tocopherols (particularly α and γ), which play a key role in antioxidant activity and cellular protection from oxidative stress (Bhaswant et al., 2024, Hashimoto et al., 2020). Moreover, in the study by Lee et al., 2023, *Perilla frutescens* microgreens showed a higher concentration of rosmarinic acid (22,66 mg/g) and total phenolic compounds (2,64 mg gallic acid equivalent (GAE)/g dry weight) in the early growth stages, which then decreased as the plant grew. The levels of rosmarinic acid in dark-grown microgreens increased progressively until day 20, and then decreased in the following days, with the maximum value observed on day 20. DPPH free radical scavenging activity, assessed by ethanol extracts, showed strong antioxidant activity in *Perilla frutescens* microgreens, due to the accumulation of rosmarinic acid and total phenolic compounds, which are well known for their strong antioxidant properties. In contrast, at the adult stage, only 5,64 mg/g of rosmarinic acid has been recorded (Liu et al., 2013). Table 1.4 reports some of the most relevant hydroalcoholic compounds, highlighting the comparison between the molecules of nutritional and pharmaceutical interests present in the adult plant and in microgreens, in favor of the latter stage of growth.

Polar compounds	<i>B. vulgaris</i> microgreens	<i>B. vulgaris</i> adult	<i>P. frutescens</i> microgreens	<i>P. frutescens</i> adult	<i>O. basilicum</i> microgreen	<i>O. basilicum</i> adult
Betaxanthins	1357mg/100g	48mg/100g	-	-	-	-
Betacyanins	841mg/100g	82mg/100g	-	-	-	-
Rosmarinic acid	-	-	2266mg/100g	565 mg/100 g	165mg/100g	6,94 mg/100g

Table 1.4. Comparison of most significant hydroalcoholic compounds between microgreen and adult stage of *B. vulgaris*, *P. frutescens* and *O. basilicum*. Results are presented as mg/100g of dry weight

Furthermore, microgreens represent a promising material for starting a cell culture: being totally edible for many species, the risk of presence of anti-nutritional molecules is minimized in the deriving cell culture. Furthermore, starting from such an added-value molecules-rich tissue, the probability of accumulating useful bioactive molecules in derived cell cultures is accentuated.

1.4 Three-dimensional (3D) food printing

1.4.1 Application and potential of 3D printing in the food industry

3D printing (3DP) finds application in a wide range of solid materials, including plastics, metals, polymers, ceramics, and composites. The 3DP process is controlled by 3DP devices that follow a three-dimensional virtual model created through computer-aided design (CAD) software. Initially, a solid virtual model is created within the CAD software to define the overall shape of the object to be printed. Next, this virtual model is divided into sections to create an instruction file for the 3DP machine. This file specifies the path to follow to deposit or melt the material, along with parameters such as fill density, layer thickness, and print speed (Azam et al., 2018).

In recent times, there has been a growing interest of 3DP in food applications. 3D food printing is an innovative application of 3D printing technology that involves the production of food through the additive deposition of edible materials. Although it is still under development and experimentation, this technology offers exciting perspectives in the field of nutrition and gastronomy. 3D printing has the advantages of time-saving, simple operation and customizable in the whole manufacturing process (Yang et al., 2019). In this technology, food ingredients are liquefied or transformed into paste, acting as "inks" to create overlapping layers that gradually take shape and become complete dishes. This approach opens the way to a greater personalization of foods, allowing the creation of complex shapes difficult to achieve with traditional methods and the possible reduction in waste of ingredients. 3D food printing is considered one promising innovation in food design and production (Zhu et al., 2023). Currently, food materials that can be used for 3DP remain quite limited to sugar, chocolate, cookie paste, pasta, and even meat puree and vegetables with gelling agents. Despite these limitations, interest and research in this field continue to grow, in view of innovative applications in the food industry (Azam et al., 2018b).

So far, the noblest applications of 3DP ranged from creating tailor-made foods for the elderly who have swallowing problems, and for people with dysphagia, a disorder that makes swallowing almost impossible to developing healthy snacks for children based on protein, starch and fiber to meet their specific nutritional need (Chen et al., 2021). Indeed, another advantage of 3DP is to modulate textures and consistencies in order to make food suitable to specific swallowing and chewing necessities and still maintaining tastefulness by deceiving the palate by consistency itself (Bugarin-Castillo et al., 2023).

The demand for customized foods able to meet specialized dietary needs is constantly growing. This request may concern the creation of products for athletes, designed for recovery after training, or foods for pregnant women who need to adapt their nutrient levels, reducing unwanted ingredients and increasing healthy ones, like protein, vitamins and fiber. In addition, older people who face

physiological changes related to aging, such as dysphagia and decreased sensory perception, need special meals, but often the delivered smoothie food is not appetizing. Children also have special dietary needs and are more likely to consume healthy snacks if they are presented in an innovative and fun way (Dankar et al., 2018). 3D food printing offers a solution to these needs, allowing the creation of personalized food not only in terms of nutritional value, but also in shape, size, internal structure and flavor. This may be especially important for promoting a healthy diet, especially among children and adolescents, who often struggle to follow dietary recommendations, such as eating fruits and vegetables. 3DP can help improve nutritional intake and make food more visually attractive, especially for young audiences (Derossi et al., 2018).

One of the main advantages of food 3DP concerns the structure and consistency that can be given to the printed piece and that can provide the palate by providing satisfying taste experience even in the presence of lower quantities of savory ingredients or sugars that can be considered unhealthy. Manipulating the texture allows for the creation of healthier foods, with reduced levels of salt, sugar, or fats (Tejada-Ortigoza and Cuan-Urquiza 2022). For example, 3D food printing can help reduce the sugar content in various products, with the potential to lower the overall sugar intake in the population. Kistler et al., 2021 discovered that a heterogeneous distribution of sucrose between the outer shell and the inner core of 3D-printed food products can increase the perception of sweetness by over 30%. Similarly, 3D-printed chocolate layered with different sugar concentrations has been shown to reduce sugar use by 19% without compromising perceived sweetness or overall liking. (Zhong et al., 2023)

1.4.2 3D Food-printing for agri-food by-products/waste valorization

Sustainable management of food waste is a key challenge for the food industry, as its accumulation leads to negative environmental impacts and waste of resources. However, numerous studies show that by-products can be transformed into value-added food ingredients, improving the nutritional content of the final product and reducing waste (Iuga and Mironeasa, 2020). This approach is particularly amenable to the food 3D printing, where value-added food ingredients can be precisely added to the recipes of printable inks to produce safe, nutritious and attractive food.

In this scenario, a significant obstacle might be the negative perception of consumers regarding food derived from/containing a so-called waste. To address this issue, scientists are developing innovative strategies, including 3D food printing, using raw materials derived from agri-food waste. Recent studies highlight its potential in valorizing by-products and developing new functional foods (Keerthana et al., 2020; Wilson et al., 2020). While 3D food printing offers the advantage of producing foods with customized shapes, this innovative method also addresses consumer concerns

about consuming products derived from waste and by-products, thereby improving acceptability (Jagadiswaran et al., 2021).

In addition, it has been demonstrated that a by-product can contribute to optimize printing conditions by increasing viscosity, requiring slower printing speeds and extrusion, and sensory analysis confirmed the potential of this approach to create functional and value-added foods (Jagadiswaran et al., 2021). In particular, this study presents a sustainable approach to the use of food by-products through 3D printing. A practical example concerns the production of biscuits made from broken wheat (flour) and grape pomace powder, a by-product rich in antioxidants. The addition of grape pomace powder increased the viscosity of the dough, improving printability and allowing the use of lower extrusion speeds. After baking, the biscuits showed good structural stability, and sensory tests confirmed good acceptability, highlighting the potential of this approach to create functional and personalized foods. The concept of "waste into wealth" technology has gained significant attention in various fields and is now being reconsidered for food transformation applications. In this context, the results of this research introduce a new dimension and an additional advantage to 3D food printing, highlighting its potential in sustainable and resource-efficient food production (Muthurajan et al., 2021).

1.4.3 3DP technology applied to plant cells

Recently, the rapid advancement of 3D printing technology has ushered in a new era of possibilities in the creation of printed plant cellular products (Landernau et al., 2022).

In 3D bioprinting, the fundamental concept is to use a biocompatible material containing living cells, known as bioink, to create complex three-dimensional structures (Figure 1.4). The choice of bioink composition is crucial to ensure the desired shape, structural stability after printing and cell viability. Among biomaterials, polymer hydrogels have emerged as a promising choice for three-dimensional cell printing (Landernau et al., 2022) the bioprinting of plant cells implies the use of specially formulated food-inks that contain live cells, often embedded within a pectin matrix, that form cross-link in presence of CaCl_2 . These natural substances serve as a scaffold that helps maintain the integrity of the printed structure and preserve the integrity and viability of the cells (Krasteva et al., 2020). Bioprinting can be performed using several methods, including inkjet bioprinting and micro-extrusion bioprinting. Inkjet bioprinting involves the deposition of small droplets of hydrogels and cells, while microextrusion bioprinting involves the deposition of a liquid solution containing cells through a nozzle by pneumatic or mechanical force. The latter method allows printing highly populated structures of cells on a centimeter scale, which may resemble natural structures. A fundamental aspect in bioprinting is the rheology of bioink. Shear thinning, which results in a decrease in the viscosity of the bioink when applied cutting stress, is crucial to protect cells from cell lysis during extrusion. On

the other hand, the bioink must remain self-supporting during deposition layer by layer, which requires a rheological behavior known as yield stress, representing the point at which the bioink begins to fluidize (Landernau et al., 2022).

Immobilization of plant cells in bioink before printing has been shown also to improve plant cells biosynthetic activity, leading to increased production of secondary metabolites. An example of this is the Vanek et al., 1994 study, which examined the impact of immobilization of *Solanum aviculare* (commonly known as kangaroo apple) cells in several biopolymers such as alginate, pectin, or carrageenan. This study showed how the method and biopolymers used can significantly influence the biological activity of cells compared to free ones (Landerneau et al., 2022).

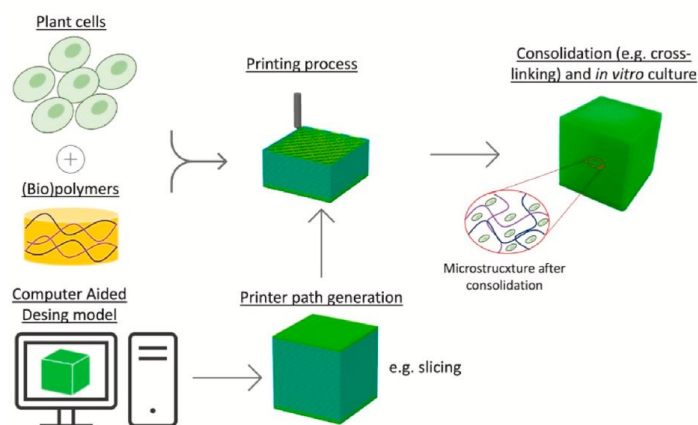


Figure 1.4. Scheme showing 3D bioprinting process of plant cells (Landerneau et al., 2022).

3D bioprinting in the context of plant tissue culture offers interesting opportunities and could have a significant impact in the food, ecological and bioproduction fields.

In the pioneering work by Gelinsky's team, extrusion-based bioprinting was used to cultivate green algae in a spatially organized way, in particular the microalgae *Chlamydomonas reinhardtii*, inside an alginate/methylcellulose bioink (Krujatz et al., 2015; Figure 1.5). This demonstration highlighted the growth of plant cells within the bioink and paved the way for further research on 3D plant bioprinting.

In another study, ink incorporating plant cells of *Daucus carota* in an alginate matrix was prepared at different ratios (1:1, 2:1, 1:2) (Park et al., 2019). Alginate, a natural polysaccharide consisting of a linear copolymer of β -D-mannuronic acid-bound (1, 4) and α -L-guluronic acid residues, minimizes stress on internal cells and has a protective effect during the printing process. In the presence of bivalent ions such as calcium ions, alginate forms the "Eggs-box" model (Figure 1.5). The ink was then extruded in a support bath containing calcium ions and incubated for 1 hour to allow the alginate to reticulate. The structural porosity of the gel made it possible to continuously transfer nutrients and oxygen to fixed plant cells during cultivation.

Other research teams (Vancauwenberghe et al., 2019) have addressed green bioprinting with the aim of producing foods with a consistency similar to that of natural foods. They have tested a new approach to produce food similar to plant tissue using food inks containing plant cells of *Valerianella locusta*, encapsulated in a matrix based on low methoxylated pectin (Figure 1.5). These cubic constructs have been shown to maintain stability up to a height of 1.5 cm. The preparation of the bio-ink consists in incubating the cellular suspension into CaCl_2 buffer solution, then mixing cell/ CaCl_2 suspension into pectin solution (1:1). The bio-ink layer is then extruded by layer at room temperature and incubation in CaCl_2 crosslink solution. This research has successfully tested the concept of 3D printing of living plant cells. It has been shown that the formulation of the bioink at different concentrations of pectin and the 3D printing process have not dramatically altered the cell vitality, which, however, decreases as the concentration of pectin increases due to the higher viscosity of this bioink. The viability of the cells after printing and the consolidation phase was also confirmed, although no long-term cultivation assessment was carried out in this study.

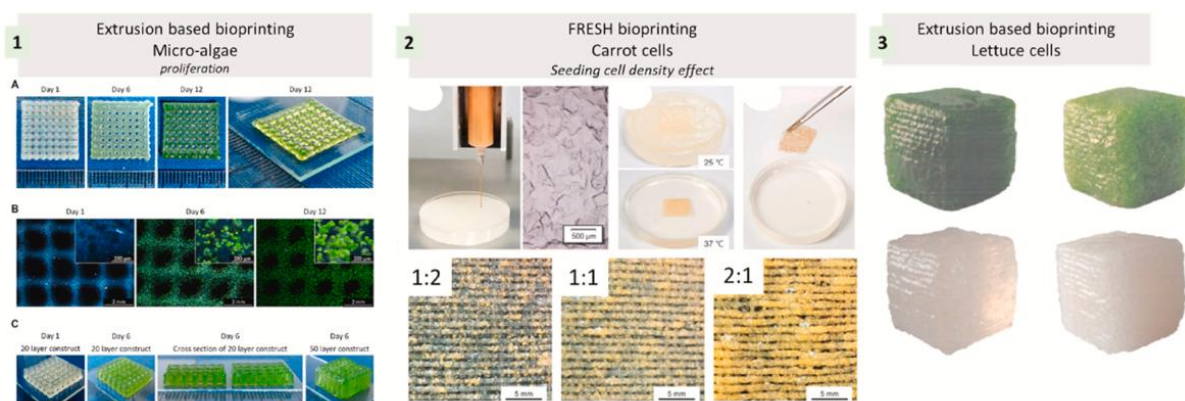


Figure 1.5. Examples of green bioprinting application fields and realisations (Landerneau et al.,2022)

Despite some of these pioneering works were focused on different aims, such as to test the potential of imitating cellular plant tissues using the 3D printing technology, they paved the way to the tailoring of 3DP edible matrices able to embed viable and metabolically active plant cells potentially useful to devise food. Among the different food source options, plant materials are indeed an intriguing choice for 3D printing due to the wealth of bioactive compounds present in plant materials, which offer numerous health benefits.

1.4.4 Pectin-based hydrogel for 3D-(bio)printing

Edible hydrogels play a crucial role in the 3D printing of innovative foods due to their rheological properties, which allow for the formation of complex and customizable structures (Huang et al., 2024). In this context, pectin hydrogels represent a strategic choice for the incorporation of plant cell

cultures and the creation of structured materials, offering both technological and nutritional advantages (Vancauwenberghe et al., 2019). Pectin is a structural polysaccharide present in the primary cell wall and the middle lamella of higher plants (Gawkowska et al., 2018). In addition to being a source of soluble dietary fiber with health benefits, including cholesterol reduction, the ability to chelate heavy metals in the gastrointestinal tract, and a potential anticancer effect, pectin is widely used in the food industry for its gelling properties (Gawkowska et al., 2018). Citrus peels and apple pomace are the main raw materials for pectin production. After a pretreatment to remove contaminating sugars, the extraction takes place in acidified water: the low pH dissociates ionic bonds and hydrolyzes glycosidic ones, reducing the degree of methyl esterification. The result is a pectin with various degrees of methoxylation, classified into low methoxyl pectin (LMP, DM < 50%) and high methoxyl pectin (HMP, DM > 50%) (Byun et al., 2020). The main domain of the pectic macromolecule is homogalacturonan, which represents about 60% of the total pectins. Its structure consists of a linear chain of D-galacturonic acid units, with carboxylic groups esterified with methanol or acetyl groups. High methoxyl pectins (HM) have a degree of methylation (DM) greater than 50%, while low methoxyl pectins (LM) have a DM between 5% and 50% (May 1990).

The gelation mechanism depends on the type of pectin and environmental conditions. HM pectins form gels at pH below 3.5 and in the presence of high sugar concentrations (>55%), thanks to the reduction of repulsive forces between the chains and the formation of hydrogen bonds and hydrophobic interactions. On the contrary, LM pectins gel in the presence of divalent cations, such as Ca^{2+} , through the so-called "egg-box model" (Figure 1.6) in which calcium ions bind between the chains of non-esterified galacturonic acid, creating a three-dimensional network capable of retaining water and solutes (Krongsin et al., 2015). The ability to form gels increases with the presence of more free carboxylic groups, making LM pectins very sensitive even to low concentrations of calcium (Celus et al., 2018). Studies highlight that LM pectins form more viscous and harder gels due to the more effective interaction between non-esterified carboxyl groups and Ca^{2+} ions (Byun et al., 2020). The gelation of LM pectins occurs within a pH range of 2 to 6 (Gawkowska et al., 2018) and can be modulated by varying temperature, sugar concentration, and calcium ion availability. Moreover, it can also occur in the absence or with reduced amounts of Ca^{2+} by lowering the pH to below approximately 3.3 (Gilsenan et al., 2000). pH adjustment reduces the amount of calcium needed for cross-linking, improving the stability and texture of the hydrogel. Increasing the pectin concentration beyond 3 g/L enhances elasticity without altering the gelation temperature, while at lower concentrations, intra-chain bonding reduces gel strength and gelation temperature. The ionic strength also influences the process: the presence of other salts, such as NaCl or KCl, can reduce the electrostatic repulsions between pectin chains, facilitating gel formation in the presence of low concentrations of Ca^{2+} (Cao et al., 2020). The ratio of calcium to pectin affects the consistency of the

gel: a high pectin content with low calcium produces an elastic gel, while higher amounts of calcium generate more fragile structures with possible syneresis (i.e., the tendency of a gel to release liquid over time). In addition to LM pectin, amidated pectin represents another type of pectin of particular interest due to its functional properties/for its improved gelling characteristics. Amidation represents one of the most relevant chemical modifications to improve the Ca^{2+} -dependent gelation capacity of pectin. Compared to non-amidated pectin, amidated pectin requires a lower amount of Ca^{2+} for gel formation, shows greater resistance and stability, thanks to the formation of hydrogen bonds between the amide groups, which favor the cross-linking of the polymeric network, especially in low pH environments. In particular, at $\text{pH} < 3$, amidated pectin gels are described to be significantly more robust than non-amidated pectin gels (Cao et al., 2020).

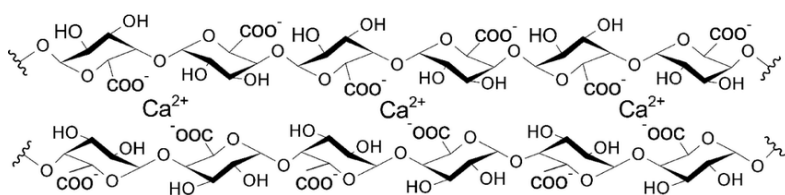


Figure 1.6. Scheme of “egg-box” model (Gawkowska et al., 2018)

Considering the above-mentioned scenario, in order to obtain suitable gelation conditions, an interesting approach may be using citric acid to produce value-added extracts from agro-food waste / by-products or using high-value juices, both to be used as solutions for the integration of pectin in hydrogels with suitable features, either for their pH value or, in the case of juice, for the presence of sugars. The use of these products may also be very useful for enriching the final hydrogel with compounds/molecules of nutritional interest and increasing its sustainability from the perspective of circular bioeconomy by reusing agro-food by-products.

2. MATERIALS AND METHODS

This section outlines the materials and methods used for the preparation of inks for food 3D printing, formulated with by-products from the agri-food industry with or without the inclusion of plant cells. The entire process of producing plant cell biomass from *in vitro* cultures is detailed, including the development of callus, subsequent suspension cultures, characterization, and physical elicitation aimed at enhancing biomass and metabolite accumulation. The integration of the by-products and plant cells into hydrogels suitable for food 3D printing, the formulation of the final inks, and their characterization are also described. Finally, the properties of the formulated inks, including shelf life, printability, and the results of the panel test on the final products, are presented.

2.1 Identification of edible plant species

Firstly, plant species suitable for the onset of the *in vitro* plant cell culture of this work were identified, focusing on edible plants sources of bioactive compounds endowed with both health-related value and useful properties already applied in the food-related manufacturing processes (e.g., antioxidant capacity, radical-scavenging activity, hypolipidemic effects, antimicrobial properties). Further selection criteria included the adaptability to *in vitro* culture to ensure effective culture establishment and growth and the availability of elicitation methods to stimulate and enhance yield in terms of biomass (fresh and dry weight) and metabolite production.

These criteria were established to guide the selection of edible plant species with the potential for *in vitro* culture and high accumulation of useful bioactive compounds. Based on the nutritional and industrial interest and the correlations between these and the adaptability to *in vitro* culture, the following species were identified: *Perilla frutescens* var. shiso purple and *Ocimum basilicum purpurascens* var. red rubin for the bioaccumulation of polyphenols (phenolic acids and anthocyanins with antioxidant properties); *Beta vulgaris* var. rubra and *Amaranthus tricolor* var. red aztec for the main bioaccumulation of betalains (rare bioactives in nature, alternative antioxidants to anthocyanins).

The details of the identified characteristics are reported in Table 2.1.1.

Species	Secondary metabolites of interest	Health-related and industrial nutritional potential of metabolites of interest	Main reference bibliography
<i>Perilla frutescens</i> var. shiso purple	Polyphenols: Rosmarinic acid, Caffeic acid, Anthocyanins (cyanidin and cis-shikonin) Flavonoids (apigenin, scutellarein, luteolin) Organic acids (citric acid) Amino acids Vitamin	Antioxidant, anti-inflammatory, anti-allergic Prevention and treatment of chronic degenerative diseases Inhibition progression of skin tumor in mouse (luteolin) Aromaticity medicinal plant in Asian countries	Lee et al., 2021 Hwang et al., 2014 Meng et al., 2009 Deguchi et al., 2020 Zhong et al., 1994 Ishikura et al., 1983 Nabeta et al., 1983
<i>Ocimum basilicum purpurascens</i> var. <i>purpurascens</i>	Polyphenols: Caffeic acid, Cichoric acid, Rosmarinic acid, Quinic acid Ursolic acid, Anthocyanins (cyanidin, peonidin) Amino acids Vitamin	Antioxidant, anti-inflammatory, neuroprotective Prevention / treatment of chronic degenerative diseases Gastrointestinal, respiratory, and cardiovascular ailments Antifungal, antibacterial, antiviral, insecticidal Eupeptic Analgesic Natural preservative power Aromaticity	Fernandes et al., 2019 Silva et al., 2008 Nazir M. et al., 2020 Nadeem et al., 2019 Nazir S. et al., 2020 Wongsen et al., 2015
<i>Beta vulgaris</i> var. rubra	Betacyanin (Betanin) Betaxanthin Tyrosine, Tyramine, DOPA Catecholamine: Dopamine Amino acids Vitamin	Antioxidant and antiradical Anti-inflammatory Hypolipidemic, hypotensive Hepatoprotective and Neuroprotective Betalain pigments already used in industry (colorants)	Polturak et al., 2018 Akita et al., 2000 Trejo-Tapia et al., 2008 Girod et al., 1991 Rodríguez Monroy et al., 1994
<i>Amaranthus tricolor</i> var. red atztec	Betacyanins and Betaxanthins (Amaranthin and isoamaranthin) Amino acids Vitamin Flavonoids	Natural food colorants Antiradical and antioxidant effects Antioxidant, anti-inflammatory, Antibacterial/antiviral Anticarcinogenic, antitumor promoting	Biswas et al., 2013 Bala et al., 2019

Table 2.1.1. Species of nutritional/industrial interest identified based on the content of bioactive molecules, adaptability to *in vitro* culture, maintenance of the biosynthesis of these molecules *in vitro*, and response to chemical or physical elicitors by literature.

2.2 *In vitro* plant cell culture production

2.2.1 Callus culture initiation

To establish *in vitro* cultures, the juvenile stage of microgreens (within 15 days post-sowing or at the first true leaf stage) was selected, based on literature evidence indicating a significantly higher accumulation of bioactive molecules at this early phase compared to the adult plant. Seeds were obtained from companies specialized in microgreens and ensuring that the seeds were free from chemical treatments. The supplying companies, Sow Seeds (<https://www.sowseeds.co.uk/collections/microgreen-seeds>) and CN Seeds (<https://cn-seeds.co.uk/>) guaranteed a germination rate above 95% and were selected. The tissues selected for callus induction were hypocotyls, cotyledons, and the first true leaf for each chosen genotype. Callus induction was performed on ~5 mm explants obtained from *in vitro* germination on Murashige and Skoog culture medium (MS, Murashige, and Skoog medium, Duchefa) for *Perilla frutescens* var. shiso purple, *Ocimum basilicum purpurascens* var. red rubin and *Beta vulgaris* var. rubra or from hydroponic cultures, for *Amaranthus tricolor* var. red atzech. Initially, the explants were surface-sterilized by rinsing with 1.4% sodium hypochlorite solution (prepared from a 14% stock solution) on a shaker for 15 minutes, followed by three rinses in double-distilled water on a shaker for 10 minutes each. The selected explants, primarily cotyledons and some hypocotyls, especially presenting a reddish color, were then used for callus induction. The callus induction substrates referenced in the literature for the selected species contain micro- and macro-elements and vitamins, utilizing MS basal medium by Murashige and Skoog or LS by Linsmaier and Skoog. These media were supplemented with 30g/L sucrose, various compositions and concentrations of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D, Duchefa), naphthaleneacetic acid (NAA, Duchefa), 6-Benzylaminopurine (6-BAP, Duchefa), and 8g/L Phyto-agar (Duchefa). Media were adjusted to pH 5.7 and autoclaved at 121°C for 20 minutes. The selection of these plant growth regulators took in consideration both their regulated usage and existing experimental data on residual levels in cell cultures. The application of synthetic growth regulators in agriculture is governed by European regulations (https://food.ec.europa.eu/plants_en/Pesticides/eu-pesticides-database/public/?event=homepage&language=EN). *Perilla frutescens*, *Ocimum basilicum*, and *Beta vulgaris* explants were transferred to MS and LS medium, respectively, containing 2,4-D (0,2mg/L) and NAA (0,2mg/L, 1,0mg/L, 2,0mg/L) alone or in combination. Each culture medium was tested for callus induction with at least 10 explants in three replicates per *Petri* dish. Induction was carried out at $23 \pm 2^{\circ}\text{C}$ in the dark. For this species, after four bi-weekly subcultures in the dark, were evaluated: onset of callogenesis (in days), rate of callus induction, color, pigmentation and texture (friable/spongy), absence of roots and/or shoots, uniformity of growth. Callus induction from

Amaranthus tricolor was performed on MS medium supplemented with BAP 2,0mg/L+ NAA 0,25mg/L. *Amaranthus tricolor* explants were placed at 25°C and exposed to white light.

MEDIA	Plant growth regulators (mg/L)		Explant
	2,4-D	NAA	
MS	0,2		Cotyledon
MS	0,2		Hypocotyl
MS	0,2		Leaf
MS	0,2	0,2	Cotyledon
MS	0,2	0,2	Hypocotyl
MS	0,2	0,2	Leaf
MS	0,2	1	Cotyledon
MS	0,2	1	Hypocotyl
MS	0,2	1	Leaf
MS		2	Cotyledon
MS		2	Hypocotyl
MS		2	Leaf
LS	0,2		Cotyledon
LS	0,2		Hypocotyl
LS	0,2		Leaf
LS	0,2	0,2	Cotyledon
LS	0,2	0,2	Hypocotyl
LS	0,2	0,2	Leaf
LS	0,2	1	Cotyledon
LS	0,2	1	Hypocotyl
LS	0,2	1	Leaf
LS		2	Cotyledon
LS		2	Hypocotyl
LS		2	Leaf

Table 2.2.1. Combination of basal media, plant growth regulators and explant used for callus induction of *P. frutescens*, *O. basilicum* and *B. vulgaris*.

Since a friable texture is essential for efficient liquid culture initiation, only friable calli from all species were selected on the medium that maximized both induction and friability for each pigmentation type. Highly proliferative, friable sections of primary callus were transferred to fresh medium every 21 or 28 days and kept in darkness at 23°C ± 2°C and used as a starting material for culture condition optimization or used for the initiation of suspension cell cultures.

2.2.2 Plant cell suspension culture initiation

Starting from callus, small-scale suspension cell cultures were initially established using 250 mL flasks. Approximately 300 mg of friable callus from each species were transferred into different liquid culture media (Table 2.2.2.) chosen on the basis of primary considerations done on the callus cultures and from literature, maintaining a medium-to-flask volume ratio of 1:5. The cultures were incubated under dark conditions at 23°C at 115 rpm (Incubator Shaker Series INNOVA 43)

	<i>P. frutescens</i> apigmented culture	<i>O. basilicum</i> apigmented culture	<i>O. basilicum</i> pigmented culture	<i>B. vulgaris</i> pigmented cultures	<i>A. tricolor</i> apigmented culture
MEDIA	MS 3% + 2,4-D 0.5 mg/L + BAP 1 mg/L	MS 3% + 2,4-D 0.5 mg/L + BAP 1 mg/L	MS 3% + 2,4-D 0.22 mg/L + NAA 1 mg/L	LS 5% + 2,4-D 0.22 mg/L	LS 5% + 2,4-D 0.22 mg/L

Table 2.2.2. Media used for suspension cell cultures.

2.2.2.1 Callus and suspension culture growth kinetic evaluation

For evaluating growth kinetics of the callus cultures obtained from each species, the same amount of callus for each species was distributed on *Petri* dishes in triplicates for each medium composition and/or for each LED elicitation condition

Weekly recordings of biomass weight for each replicate were made using an analytical balance (MonoBloc AB204-S, Mettler Toledo). After each measurement, the sample was transferred in a falcon and stored at -80°C for further analysis. At day 28 from sub-culture, all samples were lyophilized in a freeze dryer (Modulyo Freeze Dryer, Freeze Dryer Model, Edwards) at -60 °C and 0.2 mbar for two overnights. At the end of the freeze-drying process, the dry weight of the samples was recorded. From these data, the dry matter was calculated and expressed as the percentage ratio between dry weight and fresh weight to obtain dry matter calculation.

The growth kinetics of the suspension cell cultures deriving from the callus cultures of the different species were evaluated by measuring fresh weight (g), dry weight (g), and dry matter (%). Cells were harvested at 7-days intervals over a period of 28 days into falcon tubes, centrifuged at 950 g (Centrifuge 5430R, Eppendorf) for 5 minutes to remove the medium and weighed to record the fresh weight, using the analytical balance (MonoBloc AB204-S, Mettler Toledo). The biomass was then lyophilized using in a freeze dryer (Modulyo Freeze Dryer, Freeze Dryer Model, Edwards) at -60 °C and 0.2 mbar for 2 days (overnight) before measuring the dry weight. All experiments were performed in triplicate, in 100mL flasks, (medium-to-flask volume ratio 1:5). A comparison was performed between the UV-visible spectra of the hydroalcoholic extracts obtained from the starting microgreens, the resulting calli, and the subsequent cell suspensions.

2.2.3 Callus culture optimization on different media and in the presence or absence of physical elicitation with Light Emitting Diodes (LED)

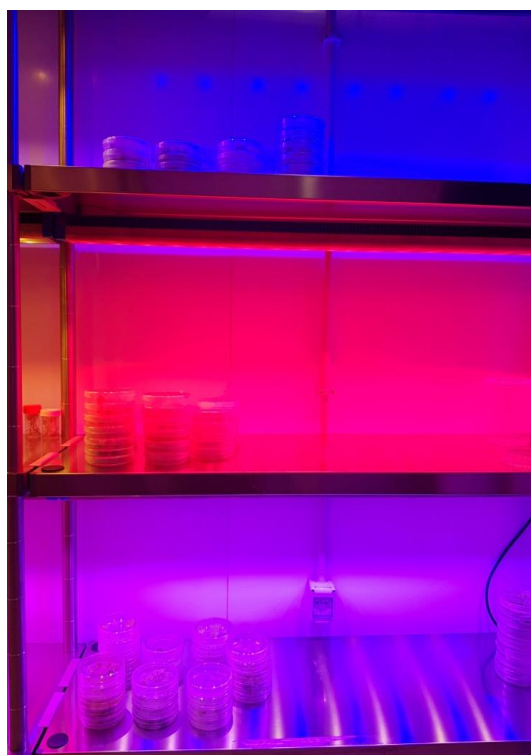
To optimize the growth of friable calli from *Perilla frutescens* and *Ocimum basilicum* in terms of dry weight and metabolite accumulation, four different media formulations and elicitation using LED lights at various wavelengths (Table 2.2.3.) were tested. Physical elicitation using LED light was preferred to reduce the risks associated with the additional use of compounds for elicitation, possibly leaving traces and thus improving safety for human consumption (Gowtham et al., 2024).

Based on a study from Nadeem M. et al., 2019 on *O. basilicum* calli, the choice of different LED light wavelength at a determined intensity (Table 2.2.4) was made by their specific impact on the biosynthesis of phenolic and flavonoid compounds. In particular, blue light (460 nm; proven to be effective in increasing total phenolic content and metabolites such as rosmarinic acid and eugenol), red light (660 nm; shown to improve total flavonoids and anthocyanins, in particular peonidin and cyanidin) were used alone or in combination. As controls, white LED light or a condition of total darkness were used.

Friable calli of *O. basilicum* and of *B. vulgaris*, already pigmented as a consequence of the accumulation of anthocyanins and betalains, respectively, were grown under dark condition without LED light integration to avoid browning and were evaluated exclusively for their response to different medium compositions.

Medium i.d.	Basal medium	Plant growth regulators	Species
T0	Murashige and Skoog (MS)	2,4-D 0,22mg/L	<i>P. frutescens</i> ; <i>O. basilicum</i>
T1	Murashige and Skoog (MS)	2,4-D 0,22mg/L + NAA 2mg/L	<i>P. frutescens</i> ; <i>O. basilicum</i>
T2	Murashige and Skoog (MS)	2,4-D 0,22mg/L+ NAA 1mg/L	<i>P. frutescens</i> ; <i>O. basilicum</i>
T3	Murashige and Skoog (MS)	NAA 2mg/L	<i>P. frutescens</i> ; <i>O. basilicum</i>
LS	Linsmaier and Skoog (LS)	2,4-D 0,22mg/L	<i>B. vulgaris</i>
AMA	Murashige and Skoog (MS)	BAP 2mg/L+ NAA 0,25mg/L	<i>A. tricolor</i>

Table 2.2.3. Composition of the medium used for the cultivation of calli of *O. basilicum*, *P. frutescens*, *B. vulgaris*, and *A. tricolor* for the characterization of biomass accumulation.



Elicitor	Wavelength (nm)	Intensity ($\mu\text{mol}/\text{m}^2/\text{s}$)	Timing	Application
Dark	-	-	24-hrs/24-hrs	Entire cultivation cycle
Red LED	660nm	50 $\mu\text{mol}/\text{m}^2/\text{s}$	24-hrs/24-hrs	Entire cultivation cycle
Blue LED	460nm	50 $\mu\text{mol}/\text{m}^2/\text{s}$	24-hrs/24-hrs	Entire cultivation cycle
Red/Blue LED	660nm+460nm	25 + 25 $\mu\text{mol}/\text{m}^2/\text{s}$	24-hrs/24-hrs	Entire cultivation cycle

Table 2.2.4. Shelves equipped with LED (up panel) and LED elicitation conditions (wavelength, luminous intensity, and timing; down panel) of the calli of *P. frutescens* and *O. basilicum*

To evaluate the effects of LED elicitation on the cultures, growth kinetics of callus cultures were evaluated in triplicate with weekly measurements throughout a 28-day culture cycle. Recordings were made in terms of fresh weight (g), dry weight (g) dry matter (the percentage ratio between dry and fresh weight) for each condition (media composition and/or LED light elicitation). The most effective formulations (medium composition in combination or not with LED light treatment) were identified based on maximum dry matter accumulation. The UV-visible spectra of hydroalcoholic extract obtained upon these optimized conditions were then analyzed.

2.2.4 Study on the replacement or substitution of synthetic plant growth regulators in the culture media

2.2.4.1 Replacement of plant growth regulators and use of LED light in *Perilla frutescens* cell cultures to improve food safety-related issues.

Perilla frutescens callus cultures were maintained in standard medium condition (sucrose 30g/L; 2,4-D 0.22mg/L; agar 8g/L).

Alternative experimental media were devised in order to improve the safety issues related to *Perilla frutescens* cell cultures as food and to replace 2,4-D. This aim was accomplished by testing increasing concentrations of casein hydrolysate (0.5g/L, 1g/L, 2g/L) or yeast extract (1g/L, 2.5g/L, 5g/L) which provide amino acids, peptides, vitamins and growth factors. The pH of the media was adjusted to 5.7 before autoclaving (121°C for 20 minutes). The standard media compositions (and a medium without plant growth regulators or other grow factors) were retained as controls to evaluate growth performance under the modified conditions.

Specifically, 1 gram of callus was sub-cultured, in duplicate, in the modified conditions and maintained at 23°C ± 2°C in complete darkness. Callus growth kinetic was monitored weekly over a 21-day culture cycle by measuring fresh weight. On day 21, samples were harvested to determine dry weight and dry matter and callus cell viability was assessed, by suspending 30mg of fresh callus in liquid medium. The suspension was diluted (1:5) with water, and 0.05 µL of fluorescein diacetate (FDA; Sigma-Aldrich, USA) was added to every 50 µL of resuspended culture. Viability was observed using a Thoma cell counting chamber under an optical microscope (Leitz Wetzlar Labovet, FS) at time 0 (T0).

Hydroalcoholic extract from callus culture, grown under modified conditions, were analyzed with UV-visible spectrophotometry, following the established extraction protocols.

The most effective callus culture condition determining the best dry mass accumulation and UV-visible hydroalcoholic profile was then selected and also used to establish suspension cultures.

Approximately, 300 mg of friable callus culture was transferred into 250 mL flasks containing 50mL fresh modified culture medium (medium-to-flask volume ratio 1:5). For subculture, 10mL of suspension culture were transferred (in duplicate) into 250 mL flasks containing 40mL of the modified medium and incubated either under dark (to replicate the conditions applied to the callus) or upon LED elicitation.

In the case of *P. frutescens* suspension cultures, alternative media compositions not implying the use of growth regulators with a low ADI and, therefore, with higher potential toxicity compared to others

(specifically, 6-BAP 1mg/L) were evaluated in combination with specific wavelength LED light (blue: 460nm; 50 $\mu\text{mol}/\text{m}^2/\text{s}$) application to eventually compensate the substitution.

Fresh weight, dry weight and dry matter were recorded according to the previously established suspension culture protocol. UV-visible spectra of the hydroalcoholic extracts from lyophilized samples were analyzed following the standardized protocols.

2.2.5 Extraction of polar compounds from callus and suspension cultures

In selecting extraction protocols, priority was given to approaches suitable for polar compounds, focusing on polyphenols and betalains of *Perilla frutescens*, *Ocimum basilicum* and *Beta vulgaris*, respectively, and examined for their both health technical/manufacturing properties considered in this work.

Optimized protocols for obtaining hydroalcoholic extracts from the lyophilized sample of each species were defined.

Hydroalcoholic extraction protocols for polyphenols:

1. MeOH (Air Products and Chemicals, USA,); HCl (Hetlab Chemical, USA) 1N (85:15, v/v) (*protocol adapted from Tobgay et al., 2020*)
 - The extraction solvent was prepared by mixing methanol and 1N hydrochloric acid in a volume ratio of 85:15 (v/v).
 - A 30mg sample of lyophilized material was weighed, and 750 μL of solvent was added.
 - The mixture was incubated on a rotator (TKA, Teknolabo A.S.S.1, Milano) for 30 min at room temperature.
 - The sample was centrifuged (Centrifuge 5415R, Eppendorf) at 9300g for 5 minutes at 10°C.
 - After recovering the supernatant, a spectrophotometric analysis (BioSpectrometer, Eppendorf) was performed
2. HCl:H₂O (1:99, v/v) (*protocol adapted from Serrano-Diaz et al., 2014*)
 - The extraction solvent was prepared by mixing double-distilled water and hydrochloric acid in a volume ratio of 1:99 (v/v).
 - A 30mg sample of lyophilized material was weighed, and 750 μL of solvent was added.
 - The mixture was shaken (Thermomixer comfort, Eppendorf) at 500 rpm for 1 hour at 40°C.
 - The sample was centrifuged at 9300g for 5 minutes at 10°C.
 - After recovering the supernatant, a spectrophotometric analysis was performed

3. EtOH:H₂O (70:30, v/v) (Hetalab Chemical, USA) protocol adapted from Standard Operation Procedure available in the laboratory.
- The extraction solvent was prepared by mixing ethanol and double-distilled water in a volume ratio of 70:30 (v/v).
 - A 50mg sample of lyophilized material was weighed, and 1,50mL of solvent was added.
 - The mixture was stirred overnight.
 - The sample was centrifuged at 3500g for 5 minutes at 10°C.
 - The supernatant was recovered.
 - The sample was centrifuged at 9300g for 5 minutes at 10°C.
 - After recovering the supernatant, a spectrophotometric analysis was performed.

Extraction protocols for betalains:

4. C₂H₃NaO₂ 1M, pH 5.4 (*protocol adapted from Akita et al., 2000*)
- A 30mg sample of lyophilized material was weighed, and 1,50mL of sodium acetate was added.
 - The mixture was incubated on a rotator for 1 hour at room temperature.
 - The sample was centrifuged at 9300g for 5 minutes at 10°C.
 - After recovering the supernatant, a spectrophotometric analysis was performed.
5. EtOH:H₂O (40:60, v/v) (*protocol adapted from Delgado-Vargas et al., 2000*)
- The extraction solvent was prepared by mixing ethanol and double-distilled water in a volume ratio of 40:60 (v/v).
 - A 30mg sample of lyophilized material was weighed, and 750μL of solvent was added.
 - The mixture was incubated on a rotator for 1 hour at room temperature.
 - The sample was centrifuged at 9300g for 5 minutes at 10°C.
 - After recovering the supernatant, a spectrophotometric analysis was performed.

Following the identification of the optimal extraction protocols, they were used for the analysis of UV-visible spectra of hydroalcoholic extracts from callus and suspension cultures, and on the ingredients, hydrogels and inks subsequently developed.

2.2.6 Metabolomic analysis

Based on the highest dry mass accumulation, the most effective culture medium and LED-elicitation conditions were selected, and untargeted and targeted metabolomic analyses were performed. Untargeted metabolomic analysis was performed to explore metabolic alterations occurring in response to different experimental conditions.

Hydroethanolic extracts were obtained from freeze-dried biomass (20 mg) by extraction with 70% ethanol (v/v) at a 1:20 w/v ratio, under agitation at room temperature for 48 h in the dark, following a modified protocol from Di Meo et al. (2019). Extracts were centrifuged at 3220 g for 30 min, and ethanol was removed by rotary evaporation at 40 °C. The remaining aqueous phase was freeze-dried and stored at –80 °C until analysis.

For LC-HRMS analysis, 10 mg of dried extract were resuspended in 1 mL of 75% methanol containing 0.1% formic acid and spiked with 1 µg/mL daidzein (Sigma-Aldrich, USA) as internal standard. Samples were centrifuged at 20,000 g for 20 min, and the supernatant was collected and filtered through HPLC-grade filter tubes (0.45 µm pore size). The resulting solution was injected into an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) operating in electrospray ionization mode (HESI). Data acquisition was performed in full-scan MS and data-dependent MS/MS mode, as reported in Frusciante et al. (2022).

Raw data from untargeted metabolomics were processed using Compound Discoverer 3.3 (Thermo Fisher Scientific), following the workflow described in Akhgari et al. (2025). A subset of the most abundant compounds (Top 20) was selected for further annotation, supported by MS2 spectral matching and manual validation using MetFrag (Ruttkies et al., 2016).

For targeted metabolomic analysis, selected metabolites (Table 2.2.5) were identified based on accurate mass, MS/MS fragmentation patterns, and comparison with authentic standards when available. Quantification was performed using Xcalibur Quan software (Thermo Fisher Scientific), by calculating ion peak areas normalized to the internal standard signal (daidzein). Annotation support was obtained from PubChem, KEGG, MetFrag, and MassBank databases.

Metabolite ID	Formula	Monoisotopic	M-H	M+H
Phenylpropanoids				
Elemicin	C ₁₂ H ₁₆ O ₃	208.1099444	207.1027	209.1172
Isoelemicin	C ₁₂ H ₁₆ O ₃	208.1099444	207.1027	209.1172
Myristicin	C ₁₁ H ₁₂ O ₃	192.0786442	191.0714	192.0786
Eugenol	C ₁₀ H ₁₂ O ₂	164.0837296	163.0765	165.0910
Isoeugenol	C ₁₀ H ₁₂ O ₂	164.0837296	163.0765	165.0910
Perilloside E	C ₁₇ H ₂₂ O ₉	370.1263823	369.1191	371.1337
Dillapiole	C ₁₂ H ₁₄ O ₄	222.0892089	221.0819	223.0965
Nothoapiole	C ₁₃ H ₁₆ O ₅	252.0997736	253.1070	253.1070
α-Asarone	C ₁₂ H ₁₆ O ₃	208.1099444	207.1027	209.1172
Estragole	C ₁₀ H ₁₂ O	148.0888150	147.0815	149.0961

Alkaloids				
Neoechinulin A	C ₁₉ H ₂₁ N ₃ O ₂	323.1633769	322.1561	324.1707
1H-indole-3-carboxylic acid	C ₉ H ₇ NO ₂	161.0476785	160.0404	162.0550
Indole-3-carboxaldehyde	C ₉ H ₇ NO	145.0527638	144.0455	146.0600
Phenolic compounds				
Rosmarinic acid	C ₁₈ H ₁₆ O ₈	360.0845175	359.0772	361.0918
Methyl rosmarinic acid	C ₁₉ H ₁₈ O ₈	374.1001675	373.0929	375.1074
Rosmarinic acid-3- <i>O</i> -glucoside	C ₂₄ H ₂₆ O ₁₃	522.1373409	521.1301	523.145
3'-dehydroxyl-rosmarinic acid-3- <i>O</i> -glucoside	C ₂₄ H ₂₅ O ₁₂	505.1346050	504.1273	506.142
Caffeic acid	C ₉ H ₈ O ₄	180.0422587	179.0350	181.0495
Ethyl caffeate	C ₁₁ H ₁₂ O ₄	208.0735589	207.0663	209.0808
Methyl caffeate	C ₁₀ H ₁₀ O ₄	194.0579088	193.0506	195.0652
Vinyl caffeate	C ₁₁ H ₁₀ O ₄	206.0579088	205.0506	207.0652
Caffeic acid-3- <i>O</i> -glucoside	C ₁₅ H ₁₈ O ₉	342.0950822	341.0878	343.1024
Protocatechuic acid	C ₇ H ₆ O ₄	154.0266087	153.0193	155.0339
Protocatechuic aldehyde	C ₇ H ₆ O ₃	138.0316940	137.0244	139.0390
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.0950822	353.0878	355.1024
Vanillic acid	C ₈ H ₈ O ₄	168.0422587	167.0350	169.0495
Isovanillic acid	C ₈ H ₈ O ₄	168.0422587	167.0350	169.0495
Sinapic acid	C ₁₁ H ₁₂ O ₅	224.0684735	223.0612	225.0757
Gallic acid	C ₇ H ₆ O ₅	170.0215233	169.0142	171.0288
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.0579088	193.0506	195.0652
4-coumaric acid	C ₉ H ₈ O ₃	164.0473441	163.0401	165.0546
Coumaroyl tartaric acid	C ₁₃ H ₁₂ O ₈	296.0532173	295.0459	297.0605
4-hydroxyphenyl lactic acid	C ₉ H ₁₀ O ₄	433.8512000	432.8439	434.8585
Sagerinic acid	C ₃₆ H ₃₂ O ₁₆	720.1690349	719.1618	721.1763
Cimidarurine	C ₁₄ H ₂₀ O ₈	316.1158176	315.1085	317.1231
p-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.0316940	137.0244	139.0390
3,4-DHPEA (hydroxy tyrosol)	C ₈ H ₁₀ O ₆ S	154.0629942	153.0557	155.0703
Flavonoids				
Flavones				
Luteolin	C ₁₅ H ₁₀ O ₆	286.0477380	285.0405	287.0550
Luteolin-7- <i>O</i> -glucuronide	C ₂₁ H ₁₈ O ₁₂	462.0798260	461.0726	463.0871
Luteolin-7- <i>O</i> -diglucuronide	C ₂₇ H ₂₆ O ₁₈	638.1119140	637.1046	639.1192
Luteolin 7- <i>O</i> -glucuronide -6"-methyl ester	C ₂₂ H ₂₀ O ₁₂	476.0954761	475.0882	477.1028
Luteolin-5- <i>O</i> -glucoside	C ₂₁ H ₂₀ O ₁₁	448.1005615	447.0933	449.1078
Luteolin-7- <i>O</i> -glucoside	C ₂₁ H ₂₀ O ₁₁	448.1005615	447.0933	449.1078
Apigenin	C ₁₅ H ₁₀ O ₅	270.0528234	269.0455	271.0601
Apigenin-7- <i>O</i> -glucuronide	C ₂₁ H ₁₈ O ₁₁	446.0849114	445.0776	447.0922
Apigenin-7- <i>O</i> -diglucuronide	C ₂₇ H ₂₆ O ₁₇	622.1169994	621.1097	623.1243
Apigenin-7- <i>O</i> -glucoside	C ₂₁ H ₂₀ O ₁₀	432.1056468	431.0984	433.1129
Diosmetin	C ₁₆ H ₁₂ O ₆	300.0633881	299.0561	301.0707
Chrysoeriol	C ₁₆ H ₁₂ O ₆	300.0633881	299.0561	301.0707
Scutellarin	C ₂₁ H ₁₈ O ₁₂	462.0798260	461.0726	463.0871
Scutellarein	C ₁₅ H ₁₀ O ₆	286.0477380	285.0405	286.0477
Scutellarein -7- <i>O</i> -glucuronide	C ₂₁ H ₁₈ O ₁₂	966.3565135	965.3492	967.3638
Negletein	C ₁₆ H ₁₂ O ₅	284.0684735	283.0612	285.0757
Vicenin-2	C ₂₇ H ₃₀ O ₁₅	594.1584703	593.1512	595.1657
Catechin	C ₁₅ H ₁₄ O ₆	290.0790382	289.0718	291.0863
Dihydromyricetin	C ₁₅ H ₁₂ O ₈	320.0532173	319.0459	321.0605
Rutin	C ₂₇ H ₃₀ O ₁₆	610.1533849	609.1461	611.1607
(-)-Epicatechin gallate	C ₂₂ H ₁₈ O ₁₀	442.0899967	441.0827	443.0973
Hesperetin	C ₁₆ H ₁₄ O ₆	302.0790382	301.0718	303.0863
Baicalin	C ₁₅ H ₁₀ O ₅	270.0528234	269.0455	271.0601
Flavanones				
Shisoflavanone A	C ₁₇ H ₁₆ O ₅	300.099775	299.0925	301.1071
Liquiritigenin	C ₁₅ H ₁₂ O ₄	256.0735589	255.0663	257.0808
5,8-dihydroxy-7-methoxyflavanone	C ₁₆ H ₁₄ O ₅	286.0841235	285.0768	287.0914
8-hydroxy-5,7-dimethoxyflavanone	C ₁₇ H ₁₆ O ₅	300.0997736	299.0925	301.1070
Chalcones				

2'.3'-dihydroxy-4'.6'-dimethoxychalcone	C ₁₇ H ₁₆ O ₅	300.0997736	299.0925	301.1070
Aurones				
(Z)-4.6-dimethoxy-7.4'-dihydroxyaurone	C ₁₇ H ₁₄ O ₆	314.0790400	313.07176	315.08632
Anthocyanins				
Shisonin (Perillanin) (cyanidin 3-coumaroyl-glucoside-5-glucoside)	C ₃₆ H ₃₇ O ₁₈ ⁺	757.1979893	756.1907	758.2053
Cis-Shisonin	[Cl ⁻] C ₃₆ H ₃₇ O ₁₇ [O ⁺]	757.1979893	756.1907	758.2053
Cis-malonyl shisonin	C ₃₉ H ₃₉ O ₂₁ ⁺	843.9838325	842.9766	844.9911
Cyanidin 3-O-feruloyl glucoside-5-O-glucoside	C ₄₃ H ₄₉ O ₂₄	949.2613774	948.2541	950.2687
Cyanidin 3-O-caffeoyl glucoside-5-O-glucoside	C ₃₆ H ₃₇ O ₁₉ ⁺	773.1929040	772.1856	774.2002
Cyanidin 3-O-caffeoyl glucoside-5-O-malonyl glucoside	C ₃₀ H ₂₇ O ₁₄ ⁺	611.1400805	610.1328	612.1474
Peonidin 3-O-malonyl glucoside-5-O-p-coumarylglucoside	C ₃₈ H ₄₁ O ₁₇ ⁺	769.2343748	768.2271	770.2417
Coumarins				
Esculetin	C ₉ H ₆ O ₄	178.0266087	177.0193	179.0339
Others				
p-Hydroxybenzaldehyde	C ₇ H ₆ O ₂	122.0367794	121.0295	123.0441
p-Hydroxyacetophenone	C ₈ H ₈ O ₂	136.0524295	135.0452	137.0597
trans-p-Hydroxycinnamic acid	C ₉ H ₈ O ₃	164.0473441	163.0401	165.0546
3'.4'.5'-trimethoxycinnamyl alcohol	C ₁₂ H ₁₆ O ₄	224.1048590	223.0976	225.1121

Table 2.2.5. List of metabolites of interest for *Perilla frutescens* hydroalcoholic extracts.

2.2.7 Antioxidant activity and total polyphenols of plant cell cultures

To evaluate the bioactive potential of the most promising plant cell cultures, both antioxidant activity and total polyphenol content were measured. These analyses were conducted on methanolic extracts obtained from both callus and suspension cultures, under optimal conditions for dry matter accumulation.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to quantify the free radical-scavenging ability of methanolic extracts. Briefly, 1g of freeze-drying sample was treated with 10mL of methanol:H₂O (70:30). The mixture was shaken and incubated in the dark for 30 min at room temperature. Subsequently, the solution was centrifuged at 1000 rpm for 10 min. After centrifugation, the supernatant was separated and recovered by filtration with a syringe equipped with a 20micrometer filter. Forty µL of methanolic extract was added to 1,16mL of 0,06 mM DPPH working solution (Sigma-Aldrich, USA). The mixture was shaken and allowed to stand at room temperature, in the dark, for 15 min. Absorbance was measured at 515 nm (UV-Visible spectrophotometer Shimadzu, Duisburg, Germany) (blank: 1.16 mL of DPPH and 40µl of 70% methanol). The inhibition of free radical DPPH was expressed as DPPH scavenging effect (% inhibition) $I\% = \{(A_0 - A_1)/A_0 \cdot 100\}$, where A1 and A0 are the absorbance values of blank and of tested samples, respectively. Trolox (vitamin E equivalent Antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma Aldrich, USA) was used as a reference standard. The DPPH radical-

scavenging activity was determined from the decrease of absorbance and expressed as equivalents per g of dry matter ($\mu\text{mol Trolox EQ/g}$) using a calibration line of Trolox.

For the dosage of polyphenols, after preparing the calibration curve, the following was added to an Eppendorf tube: 1.25 mL of H_2O ; 78 μL of sample or blank (70% methanol); 78 μL of Folin's reagent. The whole was shaken and incubated in the dark at room temperature for 6-7 minutes. Subsequently, 156 μL of Na_2CO_3 were added. The solution was then vortexed and incubated for 40 minutes in the dark. After incubation, the solutions were transferred to plastic cuvettes and, keeping the sample in the dark, the reading was performed on a spectrophotometer at 725 nm.

2.2.8 Stability test on extracts

In order to verify possible stability issues related to the embedding of the plant cell-derived material into food matrices, the lyophilized suspension culture and the corresponding hydroalcoholic extract chosen to be included into the final hydrogel were incubated at 45 °C for 1 week and at 40 °C for 4 weeks, respectively, monitoring the UV-visible spectrum, color changes (variation of RGB value using Image J (v. 1.53, [National Institutes of Health](#) (NIH), [Bethesda](#), MD, USA)) and pH variation before and after the treatment (See supplementary material).

2.3 Formulation of prototype food products containing plant cells and bioactive molecules from agri-food by-products

2.3.1 Key basic ingredients

Technical solutions were analyzed and evaluated to support the pre-industrialization of prototypes for food products containing cells and bioactive molecules. The study focused on the development of (bio)inks being specifically designed for potential use in 3D food printing technologies.

The basic ingredients were identified from those provided by the Nutri 3D project partners and from other sources based on identified useful chemical-physical characteristics suitable for 3D food printable inks.

In particular, were used:

- Clear, concentrated apple juice (Rigoni di Asiago Srl, Asiago – Italy)
 - Chemical characteristics: °BRIX 70, pH 3,28, acidity (% malic acid): 0.04.
 - Sugar: Fructose: 38,4; glucose: 17,2; sucrose: 7,9; Sorbitol: 3,1
- High-oleic acid oil (Rigoni di Asiago Srl, Asiago – Italy)
- Spray-dried (s.d.) fruit processing by-product powder (strawberries and blueberries) (Rigoni di Asiago Srl, Asiago – Italy).
- Spray-dried (s.d.) apple peel fiber (Rigoni di Asiago Srl, Asiago – Italy).

- Low-methoxyl, non-amidated pectins (LMP) known for their degree of methylation (PRIME 521, Danisco; Copenhagen, Denmark), low ester content – 32-40% – highly reactive to calcium, standardized with sucrose) manufactured from citrus peel with natural color variation from off-white to golden.
- Pectin Amid AF 005 (Herbstreith & Fox GmbH & Co. KG, Germany) low ester content – 30-38%, amidated 7-14% – standardized with calcium citrate to reduce the need for calcium salts, which can produce a bitter flavor in inks formation).
- Flower Honey and Sapin Honey (Mielbio, Rigoni di Asiago Srl, Asiago – Italy)
- Locust bean gum (Rigoni di Asiago Srl, Asiago - Italy)
- Casein hydrolysate (Duchefa Biochemie, Netherlands)
- Citric acid (Duchefa Biochemie, Netherlands)
- Calcium chloride (Duchefa Biochemie, Netherlands)
- Calcium lactate (Sigma-Aldrich, USA)

2.3.2 Recipes of solutions used to facilitate the embedding of cells and value-added molecules from by-products into hydrogels

For the formulation of the inks, solutions that could serve as resuspension ‘medium’ for cells and for introducing other components in optimizing hydrogel formation were evaluated. In particular, the diluted, clear, concentrated apple juice and extracts deriving from blueberry and strawberry value-added by-products obtained in citric acid were considered. These solutions have been selected to optimize gelation, improve the shelf life of the material through pH and, in a circular economy perspective, enrich the finished product with molecules of health interest, such as flavonoids.

In particular:

- Diluted clear, concentrated apple juice: a series of empirical dilutions was prepared and their osmolarity was measured (Loeser™ Freezing Point Osmometer). Specifically, 100μL of apple juice dilution were placed in the sample tube ($\pm 10\%$). The sample tube was dipped into the cooling slot. Supercooling was reached after 1 minute and the needle with the ice crystals was dipped into the sample. When the freezing point was reached, the display showed the measurement value in mosm/kg.

Mixing 4.91mL of clear apple juice concentrate with 95.09mL of double distilled water allowed to reach the osmolarity of 185 mOsm/L, very similar to the 211 mOsm/L of the medium of choice (MS + 3% sucrose + 0.5mg/L 2.4D + 1mg/L BAP) for *P. frutescens* cell suspension cultures (the optimum for plant cell cultures was calculated as 230 - 280 mM by Madhusudhan et al., 1995). The osmolarity

of the diluted juice was considered adequate, also in anticipation of the addition of pectin and by-products extracts, which could further influence the osmotic properties of the solution).

- 1% w/v blueberry by-products s.d. in 5% w/v citric acid solution.
- 1% w/v blueberry by-products s.d. in 0.5% w/v or 5% w/v citric acid solution.
- 1% w/v strawberry by-products s.d. in 0.5% w/v or 5% w/v citric acid solution.

Protocol for the preparation of 1% w/v blueberry or strawberry extracts in 0,5% w/v or 5% w/v citric acid:

- 0,5g or 5g of citric acid powder were dissolved in 100mL of double-distilled water.
- 1g of spray-dried blueberry or strawberry powder was added to 100mL of 0.5% w/v or 5% w/v citric acid solution.
- The powder was dissolved with a magnetic stirrer at 180 rpm for 30 minutes.
- The solution was centrifuged at 6500g at 18°C for 5 minutes.
- Supernatant was collected.

These liquid components constituted the "core/base" of the inks, providing the chemical-physical properties necessary for its formation. Subsequently, pectin was integrated, for the preparation of inks intended for 3D printing.

2.3.3 Inks recipes

To develop ink formulations suitable for incorporating plant cells and/or value-added agri-food by-products, various recipes were created and analyzed. These recipes, based on the products described in section 2.3.1 and on the solutions described in section 2.3.2, led to the production of four added-value inks (namely, Ink 1, Ink 2, Ink 3, Ink 4) aimed at developing two final products:

1. A multilayer bar composed of an external biscuit-like shell, an intermediate layer of dark or white chocolate-like filling, and an internal core of gelée filling for the inclusion of cells
2. A honey sphere with an inner gelée filling that contains cells.

1. Multilayer bar composed by:

Ink 1: formulated to reproduce the consistency and a biscuit-like appearance.

Ink 2: conceived as an intermediate layer with characteristics similar to a white chocolate filling.

Ink 3: designed for the inclusion of plant cells in a jelly-like structure, with properties suitable for their preservation.

2. Honey sphere composed by:

Ink 3: (as above) designed for the inclusion of plant cells in a jelly-like structure, with properties suitable for their preservation.

Ink 4: developed to reproduce a gummy-like outer layer.

To promote gelation, two basic formulations were prepared, each based on pectin (30g/L; LMP or amidated), modifying the methodologies reported by Vancauwenberghe et al., 2017, 2018 and 2019.

- Solution 1: A total of 100mL of diluted juice was prepared by mixing 4.91mL of clear, concentrated apple juice with 95.09mL of double-distilled water. Subsequently, 3g of LMP was added under continuous stirring at 200 rpm, allowing to hydrate pectin for about 20 minutes.
- Solution 2: A total of 100mL of diluted juice was prepared by mixing 4.91mL of clear, concentrated apple juice with 95.09mL of double-distilled water. Subsequently, 3g of amidated pectin and stirring at 300 rpm for 1 hour at room temperature was applied to hydrate pectin.

The final recipes of the inks and the related preparation protocols are reported in the following table (Table 2.3.1.), while the detail of the empirical process leading to the final formulations will be described in detail in the results section.

Inks i.d. and related ingredients	Preparation protocol
Ink 1: Biscuit-like layer of the “multilayer bar”, formulated to reproduce the consistency and appearance of a biscuit-like outer layer. - 1% w:v blueberry by-products extract in 5% w:v citric acid (66,67% v:v) - Apple fiber sd (22,92% w:v) - High-oleic oil (1,60% w:v) - Flower honey (8,01% w:v)	- A 1% w:v blueberry by-products extract in 5% w:v citric was prepared. - Apple fiber (22,92% w:v) was gradually added to 41.6mL (66,67% v:v) of 5% w:v blueberry by-products extract while sifting in small amounts to prevent clumping and then mixed gently with a spatula. - High-oleic oil (1,60% w:v) was added and mixed with a spatula. - Flower honey (8,01% w:v) was added and mixed well with a spatula to complete the recipe. - Printing: the mixture was printed using a 2mL syringe (2ml BD Emerald 1mm diameter) without a needle. - Baking: the printed mixture was baked at 60°C in a thermobalance (MA 50X2ICAWH Moisture Analyzer, Radwag, Poland) for 2 hours (i.e., until it reached a moisture content of 44%).

Ink 2: Molten filling layer of the “multilayer bar”, conceived as an intermediate layer with characteristics similar to a white chocolate filling. -1% w:v blueberry by-products in 0,5% w:v citric acid (14,95% v:v) -Amidated pectin in diluted apple juice (74,74% w:v) -Banana powder (2,84% w:v) -Locust bean gum (7,47% w:v)	- Preparation of Solution 2: 3g of amidated pectin were added to 100mL of diluted apple juice and dissolved for 1 hour at room temperature, stirring at 300 rpm. - A 1% w:v blueberry by-products extract in 5% w:v citric was prepared. - The 1% w:v blueberry by-products extract in citric acid 0.5% w:v (14.95% v:v) was incorporated into Solution 2 (74,74%v:v), while stirring with a magnetic stirrer at 200 rpm. - Banana powder (2,84% w:v) was gradually added to the mixture while stirring at 200 rpm for 10 minutes. - Locust bean gum (7,47% w:v) was added in small increments while stirring at 200 rpm. -Mixing: the mixture was thoroughly mixed with a spatula for 15 minutes until all flour granules were fully dissolved. -Printing: the mixture was printed using a 2mL syringe (2ml BD Emerald 1mm diameter) without a needle.
Ink 3: “Gelée”-like filling layer in the "multilayer bar" and "honey pearl" products, as well as a formulation for cell inclusion, with properties suitable for their viability and integrity preservation. - 1% w/v strawberry by-products extract in 0,5% w:v citric acid Notes: in alternative, 1% w:v blueberry by-products extract in 0,5% w:v citric acid (82,5% v:v, Ink 3b; or diluted clear concentrate apple (82,5% v:v, Ink 3c) were considered as well for the study of the best preservation of cells viability/preservation. -Low-methoxyl, non-amidated pectins (LMP) (3% w:v) -Casein hydrolysate (0,50% w:v) -CaCl₂ 80mM (final concentration of CaCl₂= 11,25mM; 14%v:v)	- 3% w:v LMP was added gradually to the 1% w:v strawberry by-products extract in citric acid 0,5% w:v while stirring for 20 minutes with a magnetic stirrer at 200 rpm, allowing it to completely hydrate. - Casein hydrolysate (0,5% w:v) was added in small amounts to the mixture while stirring at 200 rpm, until complete dissolved. - The stirring speed was increased to 400 rpm and 80mM CaCl₂ solution was added (final concentration of CaCl₂= 11,25mM) - Resting: the mixture was left to rest until air bubbles dissolved . - Printing: the prepared mixture was printed using a 2mL syringe (2mL BD Emerald 1mm diameter) with 25G needle

Ink 4: Shell of the “honey pearl”, developed to reproduce a gummy-like outer layer - 1% w:v blueberry by-products extract in 5% w:v citric acid (85% v:v) - Low-methoxyl, non-amidated pectins (LMP) (10% w:v) - Sapin honey (5% w:v)	- LMP (10% w:v) was gradually added to 42,5mL of the 1% w:v blueberry by-products extract in citric acid 5% w:v (85% v:v) while stirring at 200 rpm, until completely dissolved. -Sapin honey (5% w:v) was added and mixed well using a spatula. - Printing: the mixture was printed using an 2,5mL syringe (Dispenser syringe Eppendorf type, 0,5 mm diameter) into a spherical mold with a diameter of approximately 1 cm. - Baking: the printed mixture was then subjected to a baking process using at 50°C for 50 minutes an incubator with a thermostatic dome (model 715/CT, Bioclass S.r.l., Italy)
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Table 2.3.1. Summary of the final inks formulations and detailed protocols used for their preparation.

2.3.5 Final printing conditions for the 4 inks

The structural integrity of the inks, both with and without plant cells, was initially evaluated through multilayer deposition using manual syringe-based methods. These preliminary tests confirmed the printability of the various formulations while ensuring the preservation of nutritional and functional properties. Once the optimal conditions for manual printing were identified, final tests were performed on the 4 inks using the 3D bioprinter BIO X (CELLINK, Gothenburg, Sweden). Several printing trials were conducted, optimizing parameters such as printing speed, nozzle size, pressure, and filling.

Specifically, the parameters examined included:

1. Nozzle speed;
2. Nozzle size;
3. Extrusion pressure;
4. Final structure infill;
5. Shape and number of layers.

The printing process was carried out using the following constant parameters:

-Room temperature (25 ± 3 °C)

-Dimensions: 20x20x3mm.

-Distance between extruder nozzle tip and printing stage/deposited top layer 0.01 mm

for less than 5 minutes per print, thus preserving the initial nutritional content.

For ink 3 (geleè), additives, such as crystalline nanocellulose (polymer with good shear thinning properties even at low concentrations and able to self-assemble in an aqueous medium) have been occasionally added in order to improve its printing properties.

Table 2.3.2 summarizes the two best printing scenarios obtained from the tests (illustrated in detail in section 3.3.1) and also after further optimization for each ink.

Sample	Speed	Tip	Infill %	Kpa	Shape
Ink 1	10	18 G	20	150	20x20x3mm
Ink 1	20	18 G	20	200	20x20x3mm
Ink 2	10	20	20	150	20x20x3mm
Ink 2	10	22	20	130	20x20x3mm
Ink 3	10	20	20	10	20x20x3mm
Ink 3 (but added with either nanocellulose or with plant cell culture)	10	18	20	70	20x20x3mm
Ink 4	10	18	22	30	20x20x3mm

Table 2.3.2. Defining printing conditions for the four inks

2.4 Embedding of cells into Ink 3

Ink 3 (also in its variants 3b and 3c – see Table 2.3.1) was specifically intended to embed plant cells. The present section describes the methods for: i) the preliminary treatments to include cells (see section 2.4.1); ii) the preparation of the solutions aimed at resuspending cells (see section 2.4.2); iii) the preparation and characterization of the final recipe of Ink 3 used to produce the 3D printed objects (section 2.4.3).

2.4.1 Cells inclusion into Ink 3

P. frutescens suspension culture was sewed through a sterile stainless steel sieve (<100 µm) to select a cell fraction of suitable size to pass through the different nozzles used for 3D printing. This fraction was then centrifuged at 950 g to remove the culture medium. Total cell count was determined using the Thoma chamber under an inverted microscope, while cell viability was quantified by the FDA test (see section 2.3.3.1)

2.4.2 Evaluation of *P. frutescens* plant cell suspension culture viability in value-added by-product extracts and apple juice

The compatibility of by-products extracts or apple juice for cell resuspension was analyzed as a function of the preservation of cell viability at 4 °C. Cells (100 µm-sieved 1×10⁶ cells/mL) were resuspended in:

-Strawberry and blueberry by-product extracts (1% w:v in 0.5% w:v citric acid)

-Clear apple juice concentrate (diluted to 4,91% w/v in double-distilled water)

As a control, cells were resuspended in their standard culture medium (MS with 2,4-D 0.5mg/L and BAP 1mg/L) and cell viability was assessed in all the resuspension solutions by the FDA test (100 μ L of cell suspension was mixed with 0.1 μ L of FDA and incubated for 5 minutes, then diluted 1:8 in the respective solutions. Cell viability was monitored at different time points (2 h, 24 h, 48 h, 120 h, 144 h, 168 h, 192 h) and the experiment was performed in double.

2.4.3 Evaluation of *P. frutescens* plant cell suspension culture viability in the by-product extracts- and high-value apple juice-based hydrogel (Ink3and 3c).

Cell viability was assessed in the complete ink recipe. Combinations of cells and recipe's ingredients were as follows:

- *P. frutescens* cell suspension (1×10^6 cells/mL in culture media) in 1% w/v strawberry processing by-product extracts in 0.5% w/v citric acid, Low-methoxyl, non-amidated pectin (LMP) + casein hydrolysate + CaCl_2
- *P. frutescens* cell suspension (1×10^6 cells/mL in culture media) in clear apple juice concentrate diluted, Low-methoxyl, non-amidated pectin (LMP) + casein hydrolysate + CaCl_2
- *P. frutescens* cell suspension (1×10^6 cells/mL in culture media) in clear apple juice concentrate diluted, Low-methoxyl, non-amidated pectin (LMP) + without casein hydrolysate + CaCl_2

Viability was determined by incubating 250mg of the samples A-D with 500 μ L of Milli-Q water and 1.5 μ L of FDA in the dark for 5 min. Evaluation of viability was performed at different time points (T1: 2 h, T2: 24 h, T3: 48 h, T4: 72 h).

2.4.4 Evaluation of *P. frutescens* plant cell suspension culture viability in the by-product extracts (Ink 3)

Cell viability was assessed in the complete ink recipe. Combinations of cells and recipe's ingredients were as follows:

- SAMPLE A (Control without cells): 1% w:v strawberry by-product extract in 5% w:v citric acid + Low-methoxyl, non-amidated pectins (LMP) + casein hydrolysate + CaCl_2
- SAMPLE B: *P. frutescens* cell suspension (2×10^6 cells/mL) in 1% w:v strawberry by-products extract in 5% w:v citric acid + Low-methoxyl, non-amidated pectins (LMP) + casein hydrolysate+ CaCl_2
- SAMPLE C: *P. frutescens* cell suspension (1×10^6 cells/mL) in 1% w:v strawberry by-products extract in 5% w:v citric acid + Low-methoxyl, non-amidated pectins (LMP) +casein hydrolysate + CaCl_2

-SAMPLE D: *P. frutescens* cell suspension (1×10^6 cells/mL in culture media) in 1% w:v strawberry by-products extract in 5% w:v citric acid + Low-methoxyl, non-amidated pectins (LMP) + casein hydrolysate + CaCl_2

Viability was determined as described in section 2.4.3. A UV-Visible spectrum analysis was conducted to further characterize the ink.

2.5 Characterization of the 3D-printed products

2.5.1 UV-vis spectrum

The UV-Vis spectra of the hydroalcoholic extracts obtained from the printed multilayer bar and the filled honey pearl (150mg of the complete formulation) by MeOH: H_2O (70:30 v/v) or citric acid:water (5:100 w/v) extraction were compared with those from the single ingredients (30 mg) under different storage conditions: vacuum-sealed samples at room temperature or samples stored at 4°C for 7 days).

2.5.2 pH and moisture stability

Stability assessments of the inks were conducted, focusing on pH stability and changes in total moisture content (time 0 to time 5 days). The total moisture of the samples and its variation over time were measured using a MA 50X2ICAWH Moisture Analyzer (Radwag, Poland). For monitoring pH variations over time (0–96 hours), a pH meter (HANNA instrument, pH/ORP, and ORP testers HI70022) was employed. All experiment were performed in duplicate.

2.5.3 Determination of the Shelf-life of 3D Products

To estimate the shelf-life of the produced products, both microbiological and physico-chemical quality were assessed.

To obtain information on the general microbiological status of the products and process conditions, the following microbiological parameters were determined:

- Total aerobic mesophilic microflora (30°C), according to ISO 4833 standard.
- To confirm the morphological identification, the samples were plated on a selective medium for fungi and yeasts (Yeast Glucose Chloramphenicol Agar) at 25°C.

The following physicochemical parameters were also determined:

- pH
- Water activity (a_w)
- Water content

- Hardness
- Moisture (Obtained by weighing after drying in an oven at 105°C for 48 h)

2.5.4 Analytical characterization of inks by FTIR-ATR

The chemical composition of the inks was analyzed by coupling micro-Raman spectroscopy with FTIR spectroscopy in ATR mode (FTIR spectra were recorded by Spectrum 100 Perkin-Elmer FT-IR spectrometer). Simultaneously, based on the chemical composition of the produced inks, the interactions between the ink components were evaluated, along with the potential gelation mechanisms and, depending on the ink composition, the water release behavior over time. Using FTIR-ATR analysis changes in the chemical composition of the inks over time were studied: ink samples (approximately 600mg in weight and 2x1x0.5 cm³ in volume) were analyzed at regular intervals to examine the functional groups interacting with water within the inks.

FTIR analysis was also used to conduct morphological and compositional studies on inks that had been stored for 20 days in the refrigerator at +4°C. These tests were performed through optical microscope photographs of the samples stored for 20 days at 4°C, and by comparing the FTIR-ATR spectra acquired from freshly produced inks and those stored for 20 days at 4°C.

2.5.5 Deswelling behavior and water release over time

The water release behavior of the produced materials was studied over time, depending on the different ink compositions and the various ink formation mechanisms. Specifically, the swelling degree (SD%) and water retention (WR%) values over time were calculated using equations 1 and 2:

$$SD(\%) = \frac{W_{time} - W_{dried}}{W_{dried}} \times 100 \quad (1)$$

$$WR = \frac{W_{time} - W_{dried}}{W_{wet} - W_{dried}}$$

Where:

- Weight at a time is the weight of the ink at a given time.
- Dry weight is the weight of the ink when no further weight changes are recorded after incubation at ambient temperature and humidity (dehydrated ink).
- Hydrated weight is the weight of the ink when fully hydrated.

All data are related to the drying process at a temperature of 25°C and ambient humidity.

The hygroscopic behavior of Ink 3 in the presence of cells (*Perilla frutescens*) was evaluated, studying the effects through morphological, structural analyses, and weight loss measurements. These analyses were performed using optical microscope photographs, FTIR-ATR spectra, and the swelling degree over time of Ink 3, both with and without cells.

2.5.6 Texture Analysis

The texture of the 4 inks was evaluated using a portable single-column texture analyzer (TA.XT Texture Analyser TA.XT plus, Stable Micro System). The mechanical characterization tests were conducted using a cylindrical probe with a flat head, capable of applying uniaxial force to the material and compressing (extruding) the inks through a syringe at a descent speed of 2 mm/sec in a destructive compression test.

The texture analyzer assesses the structural properties by acquiring data on force, distance, and time, which are graphically visualized by specialized software.

There were analyzed:

- Stiction: friction, static friction that occurs between surfaces in the presence of a thin liquid film due to meniscus/viscous effects, to which it is necessary to apply lateral sliding forces. It increases as the roughness of the analyzed surface increases (force value at the start of extrusion, maximum force of the first compression, hardness of the sample)
- Plateau force: the maximum force applied when the probe penetrates the material (force value at plateau value, extrusion resistance).

2.6 Characterization of the biological activities of the 3D printed products after *in vitro* digestion.

These activities were accomplished in collaboration with the “Council for Agricultural Research and Economics” (CREA)-Food and Nutrition. Dr. Marianna Roselli; Dr Raffaella Canali, Dr. Chiara Devirgiliis, Dr. Alberto Finamore, Dr. Barbara Guantario, Dr. Fausta Natella and Dr. Giulia Ranaldi

2.6.1 *In vitro* digestion

The *in vitro* digestion analysis was accomplished in the "worst-case scenario" represented by the multilayer bar, with its mix of hard biscuit-like shell, melting layer and gelatin-like layer containing cells. The multilayer system, with its complex architecture combining a biscuit shell, a melting filler and a gelatinous core containing cells, represents indeed the critical case for the study of digestibility. The multilayer bar was subjected to a standardized *in vitro* digestion protocol (Brodkorb et al., 2019) in which the physicochemical conditions characterizing the different phases of gastrointestinal digestion are simulated. The resulting digests were used to study their biological activities and bioavailability characteristics in cellular intestinal systems.

The samples were named (C2, C3, and C4: replicas of samples obtained with inks 1 + 2 + 3; P3, P5, P6: replicas of samples obtained with inks 1 + 2 + 3 with ink 3 also containing a cell suspension of

Perilla frutescens 1x10⁶ cells/mL), weighed (Table 2.6.1.) and immediately processed by dilution in Simulated Salivary Fluid (SSF) (1g of food/mL of solution) and homogenization with an electric homogenizer, which allowed for a cream-like consistency to be achieved.

A total of 10mL (5g of food + 5mL of SSF) was subsequently taken and frozen at -80 °C. Three C samples (C2, C3, C4) and three P samples (P3, P4, P6) were subjected to the *in vitro* simulated digestion protocol, developed according to Brodkorb et al., 2019. Each of these steps was carried out in the presence of appropriate saline solutions (the compositions of which are reported in Table 2.6.2.), pH values, and digestive enzymes characterizing the different phases.

Saline solution added	Concentration Stock (M)	SSF pH 7 (mL of stock added to prepare 5mL (1.25x))	mM	SGF (pH 3) mL of stock added to prepare 10mL (1.25x)	mM	SIF (pH 7) mL of stock added to prepare 20mL (1.25x)	mM
KCl	0.5	189µl	15.1	172.5µl	6.9	340µl	6.8
KH ₂ PO ₄	0.5	46µl	3.7	22.5µl	0.9	40µl	0.8
NaHCO ₃	1	85µl	13.6	312µl	25	2.125µl	85
NaCl	2			295µl	47.2	480µl	38.4
MgCl ₂ (H ₂ O) ₆	0.15	6.2µl	0.15	10µl	0.12	55µl	0.33
(NH ₄) ₂ CO ₃	0.5	7µl	0.06	125µl	0.5		
HCl	6	11µl	1.1	325µl	15.6	350µl	8.4
CaCl ₂ (H ₂ O) ₂	0.3	30µl	1.5	10µl	0.15	200µl	0.6

Table 2.6.1. Composition of the solutions used. SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF simulated intestinal fluid.

All phases of the procedure were conducted in a thermostatically controlled shaking water bath maintained at 37 °C.

The oral phase was carried out in the presence of amylase 75 U/mL, bringing the pH of the solution to 7 by adding 10 N NaOH and incubating for 10 minutes.

To simulate the gastric phase, after the addition of Simulated Gastric Fluid (SGF) 1:1 (v:v), the samples were incubated for 2 hours in the presence of pepsin 2000 U/mL at pH 3 by adding 6 N HCl. The intestinal digestion phase was initiated with the addition of Simulated Intestinal Fluid (SIF) 1:1 (v:v), pancreatin 100 U/mL, and bile salts 10 mM, after checking the pH value, which was 7. After 2.5 hours of incubation, the digested samples were centrifuged in CENTRICON filters with a molecular cut-off of 3,000 for 1 hour at 4 °C at 4000 rpm. This procedure allowed for the removal of the enzymes used for digestion and any undigested molecules, and the collection of the potentially bio-accessible part of the digests. The entire *in vitro* digestion procedure was repeated with a "blank" (Blk, where the food matrix was replaced with water) to evaluate possible interferences from the constituents of the entire digestive process in subsequent experiments. The eluates obtained through centrifugation were collected, aliquoted, and stored at -80 °C for subsequent analyses. Before freezing, the Brix degrees were measured in each sample, thus obtaining the percentage of present solutes.

2.6.2 Antioxidant activity of the eluates

The antioxidant activity contained in the eluates (after centrifugation in Centricon tubes) obtained after digestion of samples C2, C3, C4 and P3, P5, P6 was subsequently evaluated. The antioxidant activity was evaluated using DPPH, a spectrophotometric assay that utilizes the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, Floegel et al., 2011), which is purple in color. The ability of the molecules to neutralize free radicals, and thus reduce the absorption of DPPH, measures the antioxidant properties of the molecules themselves.

2.6.3 Evaluation of prebiotic activity

The study of the potential prebiotic activity of the products analyzed in the project was conducted through the evaluation of the effect exerted by the products obtained from *in vitro* digestion on the growth of probiotic bacteria, adapting the experimental procedure published in the work of Andrade et al., 2020. Briefly, the procedure is based on replacing the carbon source normally used in the probiotic bacterium's culture medium with the compound to be tested, and on the comparative evaluation of the bacterium's growth on the different carbon sources. The experiments were conducted using the commercial probiotic *Lactocaseibacillus paracasei* CNCMI-1572, widely and successfully employed to restore microbiota balance and to treat intestinal inflammation (Bretto et al., 2022). The probiotic strain, previously cultured in complete medium until the stationary phase, was inoculated at an initial concentration of 1×10^6 cells/mL into the corresponding medium lacking the carbon source (normally represented by glucose), used as a negative control, or into the same medium supplemented with: glucose (conventional carbon source), or synthetic fructo-oligosaccharides (FOS) (control prebiotic), or each of the filtered "digests" obtained from the *in vitro* digestion described in paragraph 1 of the Control (C) or *Perilla frutescens* (P) samples. The blank (Blk), represented by the *in vitro* digestion mix without food, was also included in the analysis. After 24 hours of incubation at 37 °C, bacterial growth was monitored by measuring the optical density at 600 nm, comparing the values obtained for type C and type P samples with the various controls. The experiments were conducted by analyzing the samples C2, P5, C4, and P6.

2.6.4 Effects of digests on the Caco-2 intestinal cell model

Since the intestine represents the first site of impact between food in the diet and the organism, the biological effects of digests were evaluated using an *in vitro* intestinal system represented by the human intestinal cell line Caco-2: an experimental system widely validated for *in vitro* studies of intestinal bioavailability and biological activity (Sambuy et al., 2005). Caco-2 cells are able to spontaneously differentiate after 17-21 days of culture forming an epithelial monolayer consisting

of cells joined by tight junctions, which express the main morphological and functional characteristics typical of the mature enterocyte of the small intestine.

Caco-2 cells were used in these experiments to evaluate possible toxicity effects of digests, preparatory to intestinal transport studies. To this end, cells were differentiated on Transwell porous filters, in which the epithelial monolayer separates two distinct compartments: the apical (AP) and the basolateral (BL) compartments, which in vivo represent the intestinal lumen and the blood flow respectively, as schematically represented in Figure 2.6.1. On these systems it is possible to access and control the two compartments separately and measure the trans-epithelial passage of the substances of interest in different experimental conditions (Ferruzza et al., 2012).

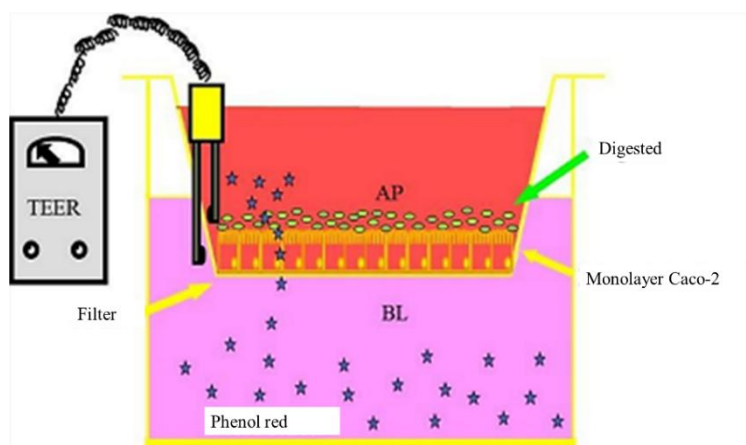


Figure 2.6.1. Schematic representation of the Transwell system. AP: apical compartment; BL: basolateral compartment; TEER: transepithelial electrical resistance

Experiments on Caco-2 cells were performed in a 37 °C thermostated bath in glucose-free saline solution (BSS: 137 mM NaCl, 5.36 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂) buffered at pH 6 with 10 mM 2-(4-morpholino) ethanesulfonic acid (MES) in the AP compartment (corresponding to the intestinal lumen) and at pH 7.4 with 10 mM 4-2-hydroxyethyl-1-piperazinyl-ethanesulfonic acid (HEPES) in the BL compartment (corresponding to the basal lamina), to recreate the pH gradient present in vivo in the small intestine (Thwaites and Anderson, 2007).

To evaluate the potential toxic effects of the digests, the eluates obtained from samples C2, P5 and the blank (Blk) were added to the AP compartment of the Transwell system using a relatively high dilution (1:2) and for a time of 4 h. Based on previous studies carried out in our laboratory, these conditions should be adequate to allow the detection of even molecules transported in an inefficient way. At the end of the treatments, all AP and BL solutions were collected and stored at -80 °C for subsequent analyses. The possible toxic effects of the digests on the integrity of the intestinal barrier were measured with two different permeability assays during and at the end of the 4 h of incubation: the measurement of the Trans-Epithelial Electrical Resistance (TEER) and the transport for 1 h from

AP to BL of the paracellular marker phenol red at the end of 4 h. These parameters indicate the “closed state” of the tight junctions and vary in opposite ways: in case of an increase in permeability of the monolayer, the TEER value decreases and, in parallel, the passage of phenol red increases.

2.7 Sensory Analysis of 3D Products

The sensory analysis activities of the 3D products were carried out in two directions:

- Sensory profile analysis of the products through a Panel Test;
- Consumer acceptance and preference analysis through a Consumer Test.

The sensory tests (Panel Test and preliminary Consumer Test) were designed and conducted by two Panel Leaders trained according to the ISO standards (ISO, 2006; ISO, 2023). Members of the research working group participated as evaluators. For the Panel tests, the Panel Leaders trained a group of 10 researchers specifically on the type of products under analysis. For the preliminary test on consumers, 18 regular consumers of gummy sweets were selected within the working group.

The involvement in the sensory studies was carried out considering safety, voluntary participation, and confidentiality of personal information. Assessors provided their informed consent, according to the General Data Protection Regulation (Regulation (EU) 2016/679).

2.7.1 Panel Test

In the Panel Test, assessors examined the two final products that were compared to a commercial reference commercially available fruit-only bar (Puertosol: Dried apple 60%, dried fruit in variable proportion 25% (apricots; plums; pears; peaches; sucrose; glucose), concentrated apple juice, apple puree, dried lemon) and an organic confectionery product was chosen, namely ‘organic jellies flavored with honey and berries’ (Organic jellies, Kontak, Merate – Italy: cane sugar, glucose syrup (from corn or rice), multi-flower honey, concentrated berry juice, pectin, citric acid, natural flavours).

The Panel Leaders placed two bars and three gummy pearls for each sample in special caps-equipped containers. This provision guarantees that the products' odor is concentrated inside, allowing the judge to evaluate its relative intensity.

The judges performed the sensory test in three replicates on the samples simultaneously to make a direct comparison. Samples were presented blindly in the closed containers identified only by a 2 or 3-digit numeric code (different for each replicate) and according to a randomized and balanced design.

The bars and pearls underwent Descriptive Analysis (ISO, 2016) conducted by a pool (Panel) of 10 judges who objectively assessed their sensory attributes. The judges used an unstructured 10 cm intensity scale, not anchored at the extremes, to rate the samples.

The two test sessions took place in a room designated for sensory analysis and arranged by the ISO 8589 standard (ISO, 2007).

2.7.1.1 Panel Test on Bars

During preliminary sessions, comparisons between samples of the 3D-printed bar developed in the project and the commercially available fruit-only bar allowed for the identification and definition of the following descriptive attributes (with respective evaluation extremes):

- Visual Homogeneity (poor – prominent)
- Visual Defects (not perceivable – highly perceivable)
- Intensity of Berry Aroma (low – intense)
- Intensity of Apple Aroma (low – intense)
- Off-Odors (not perceivable – highly perceivable)
- Sweetness (low – intense)
- Acidity (low – intense)
- Intensity of Apple Flavor (low – intense)
- Flavor Persistence (low – intense)
- Chewiness (low – intense)
- Crumbliness (low – intense)
- Ease of Chewing (low – intense)
- Stickiness (low – intense)
- Off-Flavors (not perceivable – highly perceivable)

2.7.1.2 Panel Test on Pearls

During preliminary sessions on samples of 3D-printed pearls (with and without plant cells) developed in the project, compared to commercially available fruit-only pearls, the following descriptive attributes were identified and defined (with respective evaluation extremes):

- Visual homogeneity (poor – prominent)
- Visual defects (not perceivable – highly perceivable)
- Overall odor Intensity (low – intense)
- Off-Odors (not perceivable – highly perceivable)
- Sweetness (low – intense)
- Acidity (low – intense)
- Overall Flavor Intensity (low – intense)
- Berry Flavor Intensity (low – intense)
- Flavor persistence (low – intense)

- Firmness (low – intense)
- Crumbliness (low – intense)
- Ease of chewing (low – intense)
- Stickiness (low – intense)
- Juiciness (low – intense)
- Off-Flavors (not perceivable – highly perceivable)

The 10 judges were asked to simultaneously carry out the comparison on the three samples, placing, for each attribute, the product with the weakest perception on 0, the one with the most accentuated perception on 10, and the remaining product on an intermediate value, proportional to the other two. The 30 evaluations (10 judges x 3 replicates) were subjected to Analysis of Variance ($P \geq 95\%$) and to the test of the least significant difference (LSD-Test, with $LSD \geq 95\%$).

2.7.2 Online Consumer Test

The panel leaders decided to survey the preferences of potential consumers using an online questionnaire to reach a much larger number of consumers. The survey was kept online for 28 days (from 10 January to 5 February 2024), the time between the first disclosure of the link to the small group of first participants and the failure to receive further responses, 409 survey responses.

The questionnaire was created using the Google Forms platform. It was easily accessible via a web link, which could be sent via email or social networks.

The questions were formulated in the simplest, clearest, and most concise way possible. The order of succession of the questions was carefully chosen to lead the respondent step by step to the end of the survey. Most questions utilized a 5-point Likert scale, with options ranging from "never" to "always", "of little importance" to "essential", "strongly disagree" to "strongly agree", or "not at all" to "extremely".

The questionnaire comprises 22 questions (in addition to the participation consent and the question on the difficulty in completing) and can be completed in less than 20 minutes. It has been structured in the following sections:

- Questionnaire presentation and informed consent.
- Consumer demographics: region of residence, gender, age (by age group), educational qualification, employment, and general economic availability.
- Type of consumer: type of diet (omnivorous, Mediterranean, vegetarian, etc.), frequency of sourcing (including specific products), product information most considered, factors and opinions influencing the choice of a new product.
- Sensitivity and degree of knowledge concerning sustainable food production.

- Willingness to food novelties and consumption habits of the products being tested.
- The palatability of the products is being tested, through observation of the product images, and any change in judgment (and motivation) after the consumer has been made aware of the production methods.
- Propensity to purchase.

Finally, a question on the degree of difficulty in completing the questionnaire to evaluate the clarity of the questions.

The survey was completely anonymous. Responses have been collected and securely stored in aggregate form. Access to the data was limited to a pool of authorized and identified researchers from the National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). Respondents participated voluntarily; they could close the browser at any time and choose not to submit the form. Finally, they were asked to provide electronic informed consent.

2.8 Statistical analysis

Callus culture: For the study of fresh weight, dry weight, and dry matter of callus on the different media T0, T1, T2, or T3 and under the different LED elicitation conditions, data were analyzed with GraphPad Prism 8.4 and analysis of variance was performed by two-way ANOVA test with multiple comparisons and Tukey's post-hoc as a statistical hypothesis. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 , and ***p-value ≤ 0.001).

Suspension culture: The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. The asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 , and ***p-value ≤ 0.001). Fresh weight, dry weight, and dry matter are evaluated.

Panel test: The results were analyzed statistically using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Data are shown as means $\pm$ standard deviation (SD).

In the case of normally distributed data, the statistical processing of the Panel Test data and the online Consumer Test results was conducted using ANOVA analysis with multiple comparisons to highlight differences between the groups. Tukey's post hoc test was used in this case, with a 95% confidence level ($p < 0.05$). The nonparametric Kruskal-Wallis test ($p < 0.05$) followed by Dunn's test for multiple group comparisons was performed to process non-normal data.

A two-tailed paired t-test was used to compare the means of observations on the same parameter before and after a particular activity, with a 95% confidence level ($p < 0.05$).

3. RESULTS

3.1 *In vitro* plant cell culture from selected edible species

3.1.1 Callus culture initiation

Table 3.1.1 summarizes the results of the study carried on for the callus induction from *Perilla frutescens* (PE), *Ocimum basilicum*, that led to apigmented, white (BA) and pigmented, violet (BA2) cultures and *Beta vulgaris* on MS and LS media, supplemented with different concentrations of 2,4-D and NAA, in terms of days needed for callogenesis onset, rate of explants able to induce callogenesis, pigmentation and texture (i.e., friable, spongy). All types of explants were able to form calli. The rate of induction was between 75% and 85% at 4 weeks. Pigmentation and consistency of calluses were maintained over subsequent subcultures.

Media	Plant growth regulators (mg/L)		Explant	Days for callogenesis				% explant with callus				Color and texture				
	2,4-D	NAA		PE	BA	BA 2	BE	PE	BA	BA 2	BE	PE	BA	BA 2	BE	
MS	0,2		Cotyledon	18	17	23	23	80	85	83	79	Wf	Wf	Vf	Of	Rf
MS	0,2		Hypocotyl	17	19	20	21	79	83	81	83	Wf	Of	Vs	Of	Rf
MS	0,2		Leaf	20	21	19	23	77	78	83	81	Wf	Ws	Vf	Of	Rf
MS	0,2	0,2	Cotyledon	17	19	18	21	79	79	85	83	Wf	Of	Vf	Of	Rf
MS	0,2	0,2	Hypocotyl	20	18	19	23	77	78	83	82	Wf	Ws	Vf	Of	Rf
MS	0,2	0,2	Leaf	21	21	21	20	80	85	75	82	Wf	Ws	Vs	Of	Rf
MS	0,2	1	Cotyledon	20	20	23	21	79	82	78	87	Wf	Wf	Vs	Of	Rf
MS	0,2	1	Hypocotyl	17	23	20	18	82	82	83	77	Wf	Of	Vf	Of	Rf
MS	0,2	1	Leaf	19	20	17	17	78	77	75	77	Wf	Of	Vf	Of	Rf
MS		2	Cotyledon	18	18	20	23	85	77	80	80	Wf	Wf	Vf	Of	Rf
MS		2	Hypocotyl	21	17	21	21	83	80	81	85	Wf	Ws	Vf	Of	Rf
MS		2	Leaf	20	19	20	23	83	83	80	83	Wf	Wf	Vf	Of	Rf
LS	0,2		Cotyledon	23	18	21	17	77	79	79	75	Wf	Ws	Vf	Of	Rf
LS	0,2		Hypocotyl	21	17	21	23	79	77	77	78	Wf	Wf	Vf	Of	Rf
LS	0,2		Leaf	23	20	23	20	77	79	75	75	Wf	Wf	Vf	Of	Rf
LS	0,2	0,2	Cotyledon	20	21	21	23	83	77	80	80	Wf	Of	Vf	Of	Rf
LS	0,2	0,2	Hypocotyl	18	22	23	20	77	80	79	80	Wf	Wf	Vs	Of	Rf
LS	0,2	0,2	Leaf	18	17	17	23	80	78	83	76	Wf	Ws	Vf	Of	Rf
LS	0,2	1	Cotyledon	17	19	20	19	85	82	83	80	Wf	Of	Vs	Of	Rf

LS	0,2	1	Hypocotyl	20	18	21	18	83	82	80	83	Wf	Ws	Vs	Of	Rf
LS	0,2	1	Leaf	20	20	18	22	83	80	80	79	Wf	Wf	Vf	Of	Rf
LS		2	Cotyledon	18	21	17	19	80	79	79	82	Wf	Os	Vf	Of	Rf
LS		2	Hypocotyl	17	23	23	23	79	82	82	78	Wf	Wf	Vs	Of	Rf
LS		2	Leaf	19	20	23	20	79	80	78	80	Wf	Wf	Vs	Of	Rf

Table 3.1.1 Media used and percentage of explants that produced callus after 30 days of culture for each species (PE: *Perilla frutescens* var. shiso purple; BA: *Ocimum basilicum purpurascens* var. red rubin (white callus); BA2: *Ocimum basilicum purpurascens* var. red rubin (violet callus); BE: *Beta vulgaris* var. rubra). W: white; O: orange; V: violet; R: red; G: green; s: spongy; f: friable.

After the onset of callogenesis, calli were isolated for each species in each condition and, on the basis of the ability to induce rapidly growing friable calli, the culture media were selected as reported in Table 3.1.2.

Species	Media
<i>P. frutescens</i> var. shiso purple	MS + 2,4-D 0,22mg/L
<i>O. basilicum</i> var. purpurascens	MS + 2,4-D 0,22mg/L
<i>O. basilicum</i> var. purpurascens (violet)	MS + 2,4-D 0,22mg/L, or MS + 2,4-D 0,22mg/L/ NAA 2 mg/L, or MS + 2,4-D 0,22mg/L/ NAA 1mg/L, or MS + NAA 2mg/L
<i>B. vulgaris</i> var. rubra (red)	LS + 2,4-D 0,22mg/L
<i>B. vulgaris</i> var. rubra (orange)	LS + 2,4-D 0,22mg/L

Table 3.1.2. Culture media conditions used for callus growth in the different species.

3.1.2 Callus culture growth kinetics evaluation

The growth kinetics of callus cultures of each species were evaluated in terms of fresh weight, dry weight, and dry matter, with weekly assessments throughout a 28-day culture cycle under dark.

Figures 3.1.1 and 3.1.2 show the growth kinetic for *Perilla frutescens* and *Ocimum basilicum* callus performed in triplicate, describing fresh weight, dry weight, and dry matter along with corresponding mean values and standard deviations.

3.1.2.1 *P. frutescens*

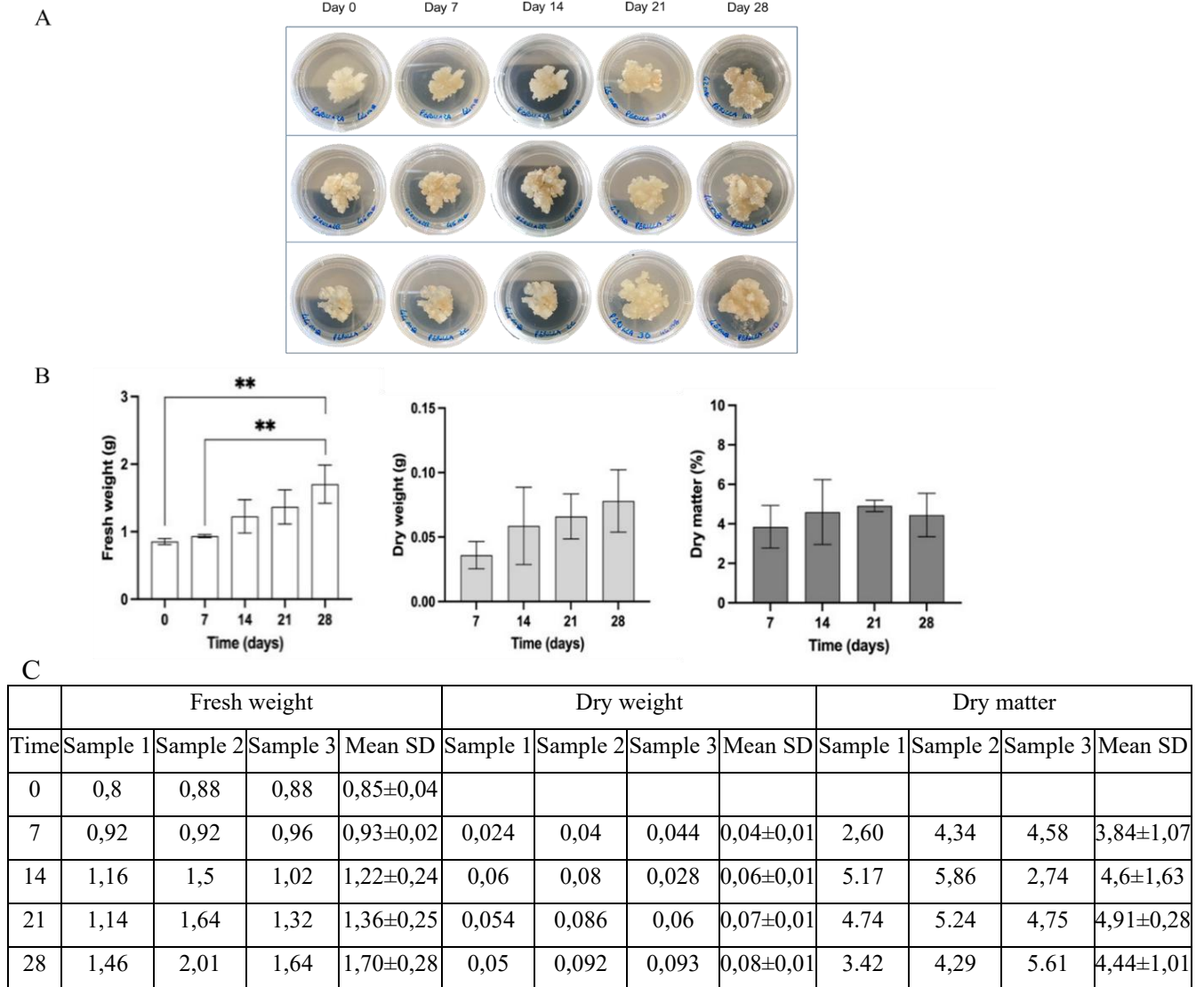


Figure 3.1.1 A) Representative images of *P. frutescens* callus triplicates (n=3) for growth kinetics evaluation. B) Growth kinetics profiles of fresh weight (g), dry weight (g), and dry matter (%) over the subculture period. C) Numerical data and statistical analysis of fresh weight, dry weight, and dry matter for *P. frutescens* callus cultured on T0 medium.

Data were analyzed by two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (compared to control/baseline conditions).

3.1.2.2. *O. basilicum*

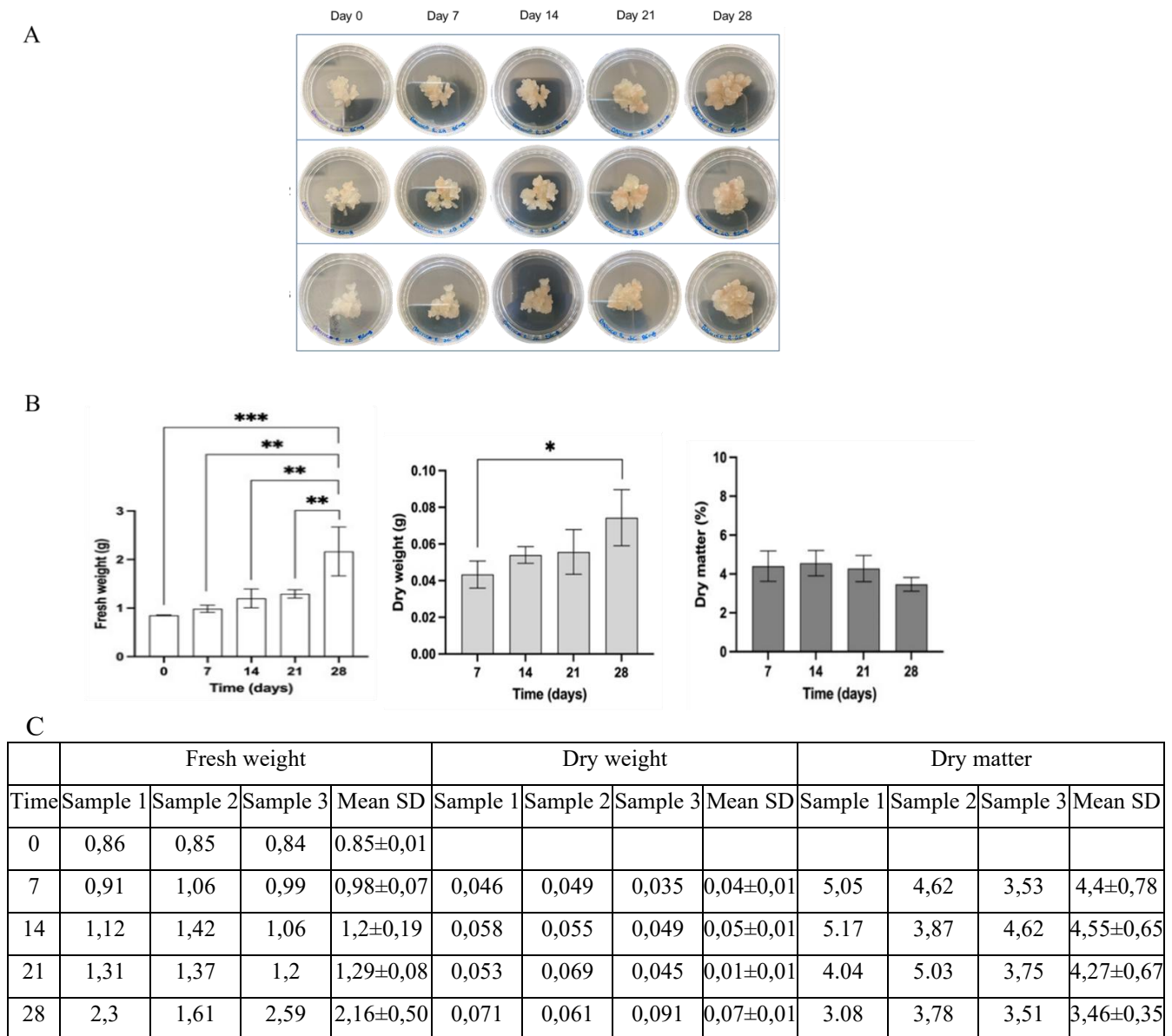


Figure 3.1.2 (A) Representative images of *O. basilicum* callus triplicates (n=3) for growth kinetics evaluation. B) Growth kinetics profiles of fresh weight (g), dry weight (g), and dry matter (%) over the subculture period. C) Numerical data and statistical analysis of fresh weight, dry weight, and dry matter for *O. basilicum* callus cultured on T0 medium. Data were analyzed by two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (compared to control/baseline conditions).

The best harvesting time for dry matter accumulation, in *P. frutescens* and *O. basilicum* is summarized in Table 3.1.3 and reflects the highest absolute value:

Species	Media	Harvesting time
<i>P. frutescens</i> var. shiso purple	MS + 2,4-D 0,22mg/L	21 days
<i>O. basilicum</i> var. purpurascens	MS + 2,4-D 0,22mg/L	14 days

Table 3.1.3. Optimal growth condition in terms of dry matter accumulation in *P. frutescens* and *O. basilicum* identified through growth kinetics analysis.

3.1.3 Callus culture optimization on different media, harvesting time and in the presence or absence of physical elicitation with Light Emitting Diodes (LED)

3.1.3.1 *P. frutescens* (apigmented, white friable callus)

Table 3.1.3 shows mean values of fresh weight (Table 3.1.4A), dry weight (Table 3.1.4.B), and dry matter (Table 3.1.4. C), of *P. frutescens* calli on different media (T0, T1, T2 or T3, see Table 2.2.3 in Materials and Methods section) in combination with different LED elicitation conditions at different cultivation times, at 0, 14, 21 and 28 days.

A)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,24	0,02	3	1,24	0,02	3	1,25	0,03	3	1,27	0,06	3
14	1,73	0,32	3	2,05	0,22	3	1,76	0,16	3	1,71	0,31	3
21	2,17	0,08	3	2,53	0,30	3	1,84	0,38	3	2,17	0,19	3
28	2,79	0,10	3	3,27	0,40	3	2,06	0,43	3	2,93	0,33	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,25	0,02	3	1,25	0,01	3	1,26	0,02	3	1,23	0,01	3
14	1,88	0,11	3	1,69	0,28	3	1,58	0,32	3	1,66	0,25	3
21	2,00	0,27	3	1,95	0,21	3	2,02	0,24	3	2,37	0,04	3
28	2,43	0,28	3	2,31	0,19	3	2,51	0,07	3	2,75	0,08	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,25	0,03	3	1,24	0,04	3	1,25	0,02	3	1,26	0,06	3
14	1,22	0,25	3	1,47	0,63	3	1,35	0,23	3	1,70	0,34	3
21	1,60	0,15	3	1,88	0,47	3	1,89	0,28	3	2,35	0,11	3
28	2,03	0,07	3	2,45	0,45	3	2,27	0,38	3	2,93	0,15	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,24	0,03	3	1,23	0,02	3	1,25	0,02	3	1,28	0,05	3
14	1,46	0,35	3	1,64	0,29	3	1,40	0,24	3	1,70	0,24	3
21	2,16	0,42	3	2,35	0,39	3	2,04	0,37	3	2,50	0,50	3
28	2,43	0,55	3	2,81	0,40	3	2,54	0,29	3	2,86	0,39	3

B)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,06	0,01	3	0,16	0,02	3	0,13	0,04	3	0,16	0,03	3
21	0,17	0,01	3	0,31	0,04	3	0,192	0,04	3	0,21	0,02	3
28	0,13	0,01	3	0,25	0,03	3	0,161	0,03	3	0,41	0,05	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,16	0,03	3	0,14	0,02	3	0,14	0,03	3	0,14	0,02	3
21	0,22	0,03	3	0,38	0,04	3	0,14	0,02	3	0,20	0,01	3
28	0,31	0,04	3	0,17	0,01	3	0,32	0,01	3	0,21	0,01	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,15	0,05	3	0,27	0,12	3	0,17	0,03	3	0,17	0,03	3
21	0,15	0,01	3	0,17	0,04	3	0,37	0,06	3	0,16	0,01	3
28	0,13	0,01	3	0,38	0,07	3	0,32	0,05	3	0,19	0,01	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,16	0,04	3	0,14	0,03	3	0,09	0,02	3	0,18	0,04	3
21	0,19	0,04	3	0,20	0,03	3	0,23	0,04	3	0,26	0,05	3
28	0,17	0,04	3	0,26	0,04	3	0,27	0,03	3	0,40	0,05	3

C)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	3,65	0,06	3	7,93	0,02	3	7,26	1,80	3	9,32	0,02	3
21	8,02	0,01	3	12,48	0,27	3	10,50	0,31	3	9,70	0,17	3
28	4,64	0,07	3	7,57	0,07	3	7,69	0,21	3	14,38	0,23	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	8,35	1,18	3	8,44	0,02	3	8,72	0,03	3	8,66	0,02	3
21	11,06	0,02	3	19,51	0,34	3	6,88	0,14	3	8,49	0,27	3
28	12,53	0,03	3	7,46	0,02	3	12,33	0,15	3	7,59	0,19	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	12,21	1,88	3	18,43	0,03	3	12,37	0,02	3	10,25	0,02	3
21	9,56	0,22	3	9,00	0,01	3	19,50	0,37	3	6,65	0,18	3

28	6,40	0,12	3	15,39	0,24	3	14,40	0,16	3	6,47	0,04	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	10,90	0,03	3	8,42	0,07	3	6,81	0,02	3	10,56	1,22	3
21	8,58	0,13	3	8,62	0,09	3	11,54	0,18	3	10,54	0,11	3
28	7,34	0,26	3	9,27	0,16	3	10,49	0,24	3	14,16	0,03	3

Table 3.1.4. Mean values and standard deviation (SD) of fresh weight(A), dry weight (B), and dry matter (C) for *Perilla frutescens* callus culture across all conditions

The graphs in Figure 3.1.3 A, B, C show the variation in fresh weight, dry weight and dry matter for *P. frutescens* calli, respectively, on T0, T1, T2 or T3 media, 0, 14, 21 and 28 days after sub-culture and under LED elicitation conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests.

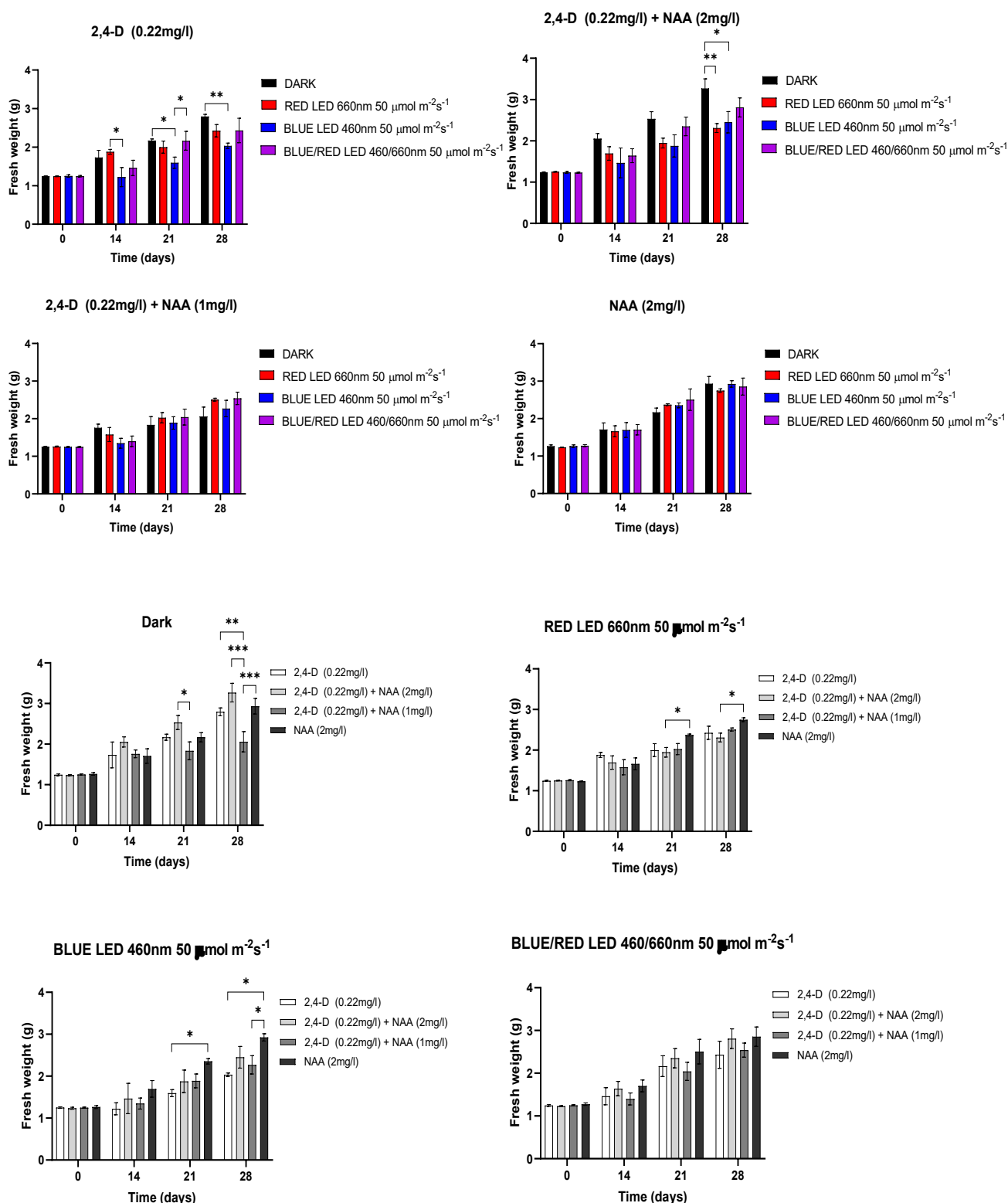


Figure 3.1.3 A. Evaluation of fresh weight of *P. frutescens* calli on T0 medium, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

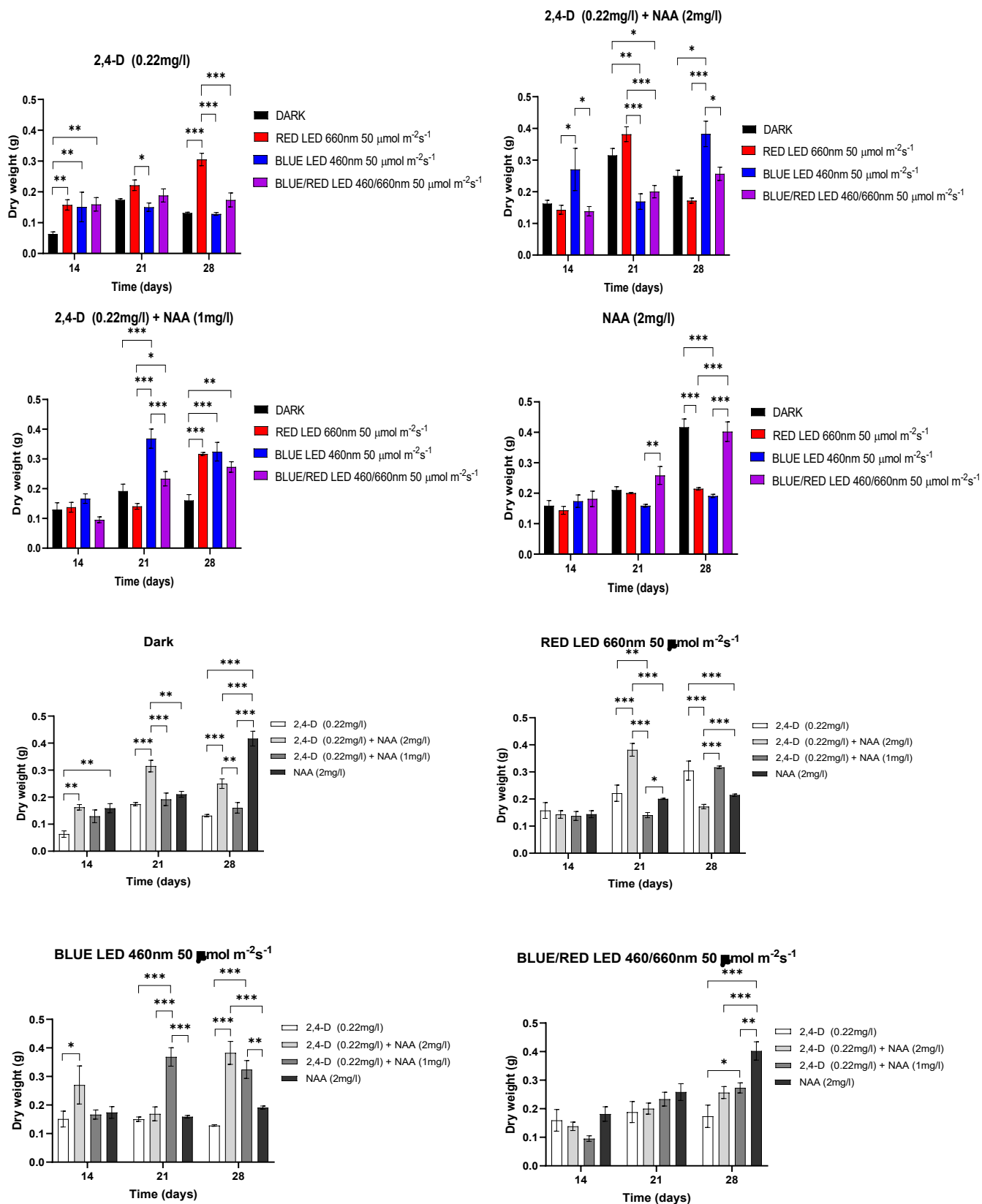


Figure 3.1.3 B. Evaluation of dry weight of *P. frutescens* calli on T0 medium, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

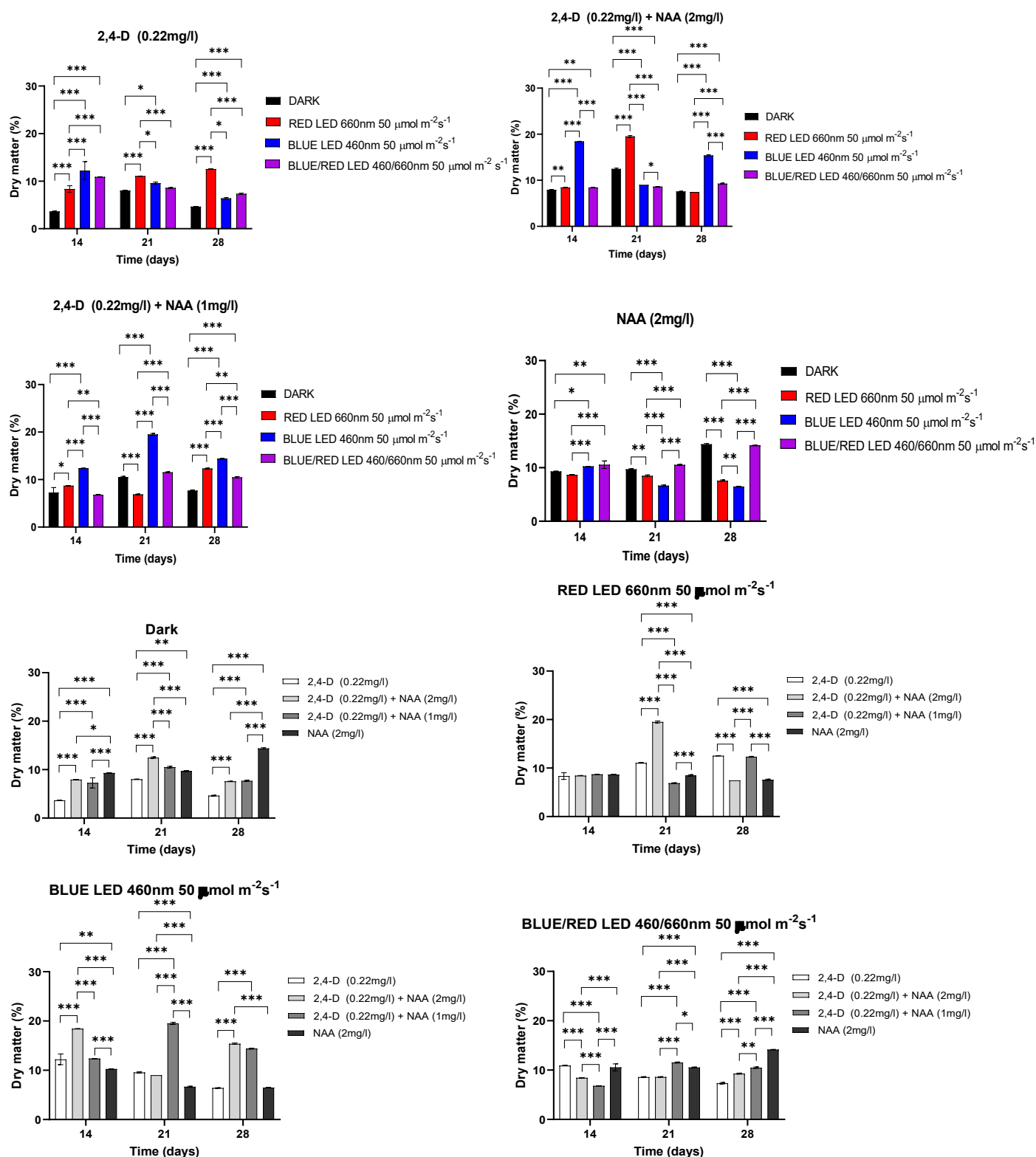


Figure 3.1.3 C. Evaluation of dry matter of *P. frutescens* calli on T0, T1, T2 or T3 media, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

The best condition for the dry matter accumulation in *Perilla frutescens* friable callus under dark was identified in medium T3 with a maximum at 28 days from sub-culture.

Growth on the T1 medium and harvesting on day 21, was identified as the best condition for the accumulation of dry matter accumulation in *Perilla frutescens* friable callus under Red LED light.

Under blue LED, on day 21, the accumulation of dry matter of calli grown on the T2 medium record the highest statistically relevant values compared to the other media. As a consequence, this condition was identified as the best to accumulate dry matter in *Perilla frutescens* friable callus under Blue LED light. This condition was preferred to harvesting on day 28, after growth on T1 or T2 due to the shorter culture time.

Harvesting on day 28, after growth on medium T3 showed the highest value for dry matter accumulation under Red-Blue light.

3.1.3.2 *O. basilicum* (apigmented, white friable callus)

Table 3.1.6 shows mean values of fresh weight (Table 3.1.5A), dry weight (Table 3.1.5.B), and dry matter (Table 3.1.5. C), of *O. basilicum* calli on different media (T0, T1, T2 or T3, see Table 2.2.3 in Materials and Methods section) in combination with different LED elicitation conditions at different cultivation times, at 0, 14, 21 and 28 days.

A)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,24	0,04	3	1,21	0,01	3	1,28	0,04	3	1,25	0,04	3
14	2,10	0,93	3	2,22	0,82	3	2,41	0,50	3	1,53	0,13	3
21	2,40	0,70	3	2,92	1,52	3	2,92	0,61	3	1,86	0,43	3
28	2,54	0,78	3	3,73	2,67	3	3,18	0,81	3	2,09	0,39	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,23	0,02	3	1,26	0,01	3	1,26	0,02	3	1,25	0,04	3
14	1,19	0,53	3	1,52	0,57	3	1,36	0,28	3	1,00	0,37	3
21	1,85	0,23	3	2,31	0,68	3	1,85	0,35	3	1,53	0,17	3
28	1,93	0,54	3	2,62	0,71	3	2,21	0,51	3	1,76	0,22	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,25	0,02	3	1,24	0,01	3	1,22	0,01	3	1,28	0,01	3
14	1,59	0,30	3	1,67	0,17	3	1,32	0,04	3	1,54	0,24	3
21	1,62	0,43	3	1,71	0,30	3	1,45	0,23	3	1,62	0,32	3

28	1,92	0,52	3	1,82	0,49	3	1,56	0,28	3	1,74	0,46	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	1,24	0,04	3	1,25	0,03	3	1,25	0,03	3	1,26	0,03	3
14	2,50	0,78	3	2,26	0,43	3	1,70	0,26	3	1,92	0,41	3
21	2,91	1,07	3	2,10	0,72	3	1,50	0,35	3	1,68	0,35	3
28	3,55	1,75	3	2,23	0,80	3	1,75	0,28	3	1,98	0,29	3

B)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,167	0,074	3	0,249	0,092	3	0,193	0,040	3	0,015	0,001	3
21	0,134	0,039	3	0,180	0,094	3	0,090	0,019	3	0,117	0,027	3
28	0,201	0,062	3	0,170	0,122	3	0,185	0,047	3	0,306	0,058	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,131	0,059	3	0,077	0,029	3	0,147	0,030	3	0,072	0,021	3
21	0,160	0,020	3	0,203	0,060	3	0,173	0,032	3	0,100	0,011	3
28	0,103	0,029	3	0,184	0,050	3	0,178	0,041	3	0,041	0,005	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,109	0,021	3	0,233	0,024	3	0,096	0,003	3	0,121	0,019	3
21	0,216	0,058	3	0,147	0,033	3	0,196	0,031	3	0,129	0,025	3
28	0,205	0,056	3	0,191	0,055	3	0,142	0,026	3	0,143	0,038	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,150	0,047	3	0,013	0,003	3	0,103	0,016	3	0,169	0,036	3
21	0,128	0,047	3	0,119	0,041	3	0,186	0,043	3	0,159	0,033	3
28	0,255	0,126	3	0,145	0,052	3	0,189	0,030	3	0,098	0,014	3

C)

Time (days)	Dark T0			Dark T1			Dark T2			Dark T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	7,58	0,32	3	11,35	0,35	3	8,39	0,37	3	1,56	0,47	3
21	5,41	0,16	3	6,17	0,06	3	3,29	0,18	3	6,36	0,25	3
28	7,06	0,09	3	4,41	0,17	3	5,79	0,02	3	14,70	0,21	3
Time (days)	Red T0			Red T1			Red T2			Red T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N

14	11,62	0,52	3	5,48	0,35	3	10,58	0,26	3	7,72	3,52	3
21	8,43	0,33	3	8,58	0,49	3	9,39	0,06	3	6,67	0,20	3
28	5,68	0,29	3	7,08	0,10	3	8,46	0,43	3	2,31	0,07	3
Time (days)	Blue T0			Blue T1			Blue T2			Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	6,53	0,42	3	13,41	0,48	3	7,40	0,45	3	7,48	0,35	3
21	13,43	0,45	3	5,76	4,99	3	13,35	0,14	3	7,53	0,46	3
28	10,75	0,09	3	10,54	0,40	3	9,25	0,17	3	8,64	0,32	3
Time (days)	Red-Blue T0			Red-Blue T1			Red-Blue T2			Red-Blue T3		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	5,80	0,18	3	0,67	0,09	3	6,60	0,48	3	8,56	0,47	3
21	4,75	0,30	3	5,67	0,22	3	12,49	0,16	3	9,64	0,26	3
28	7,38	0,19	3	6,44	0,09	3	10,45	0,34	3	4,54	0,44	3

Table 3.1.5. Mean values and standard deviation (SD) of fresh weight(A), dry weight (B), and dry matter (C) for *Ocimum basilicum* callus culture across all conditions

The graphs in Figure 3.1.4 A, B, C show the variation in fresh weight, dry weight and dry matter for *O. basilicum* calli, respectively, on T0, T1, T2 or T3 media, 0, 14, 21 and 28 days after sub-culture and under LED elicitation conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests

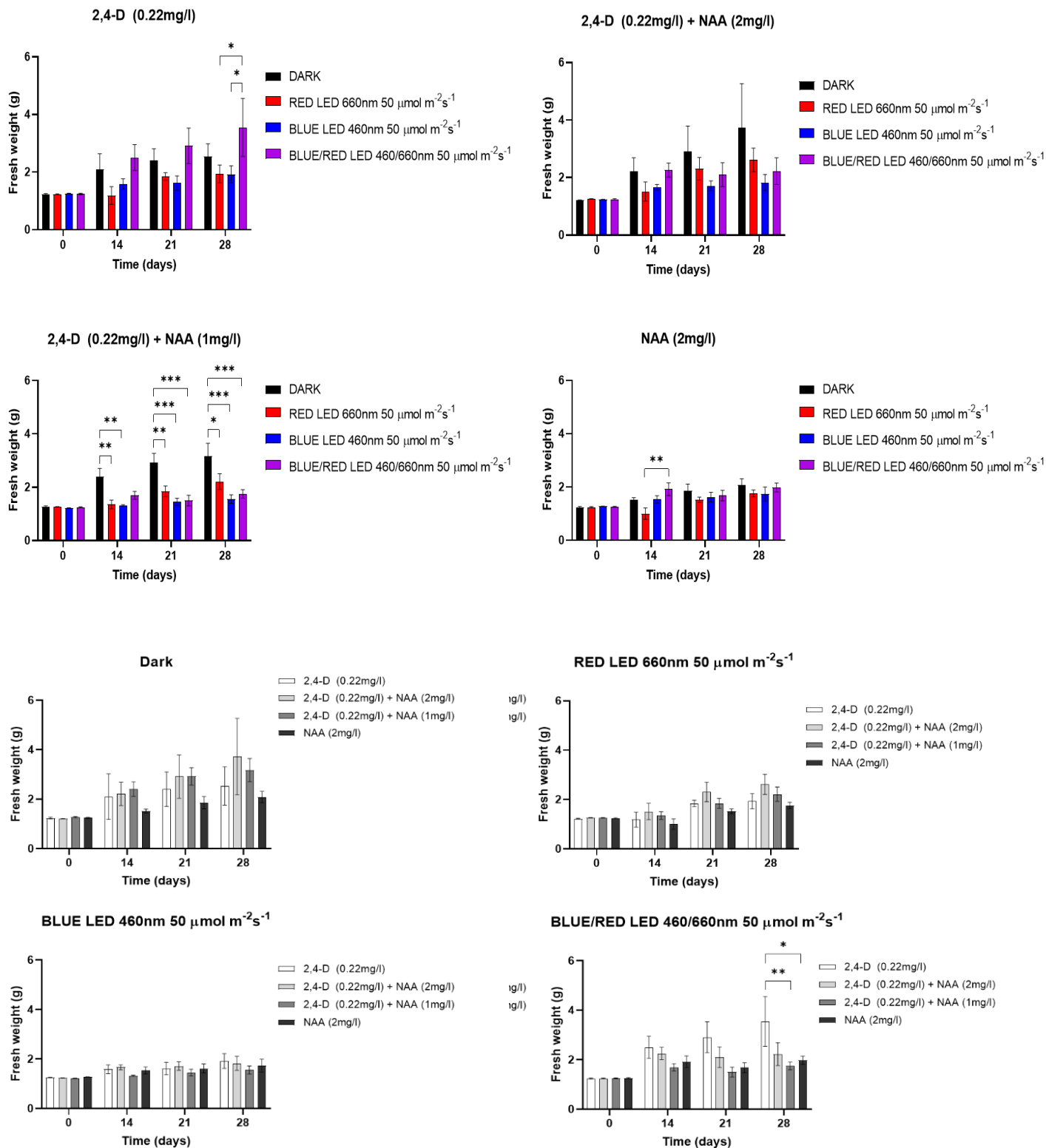


Figure 3.1.4 A. Evaluation of fresh weight of *O. basilicum* calli on T0 medium, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

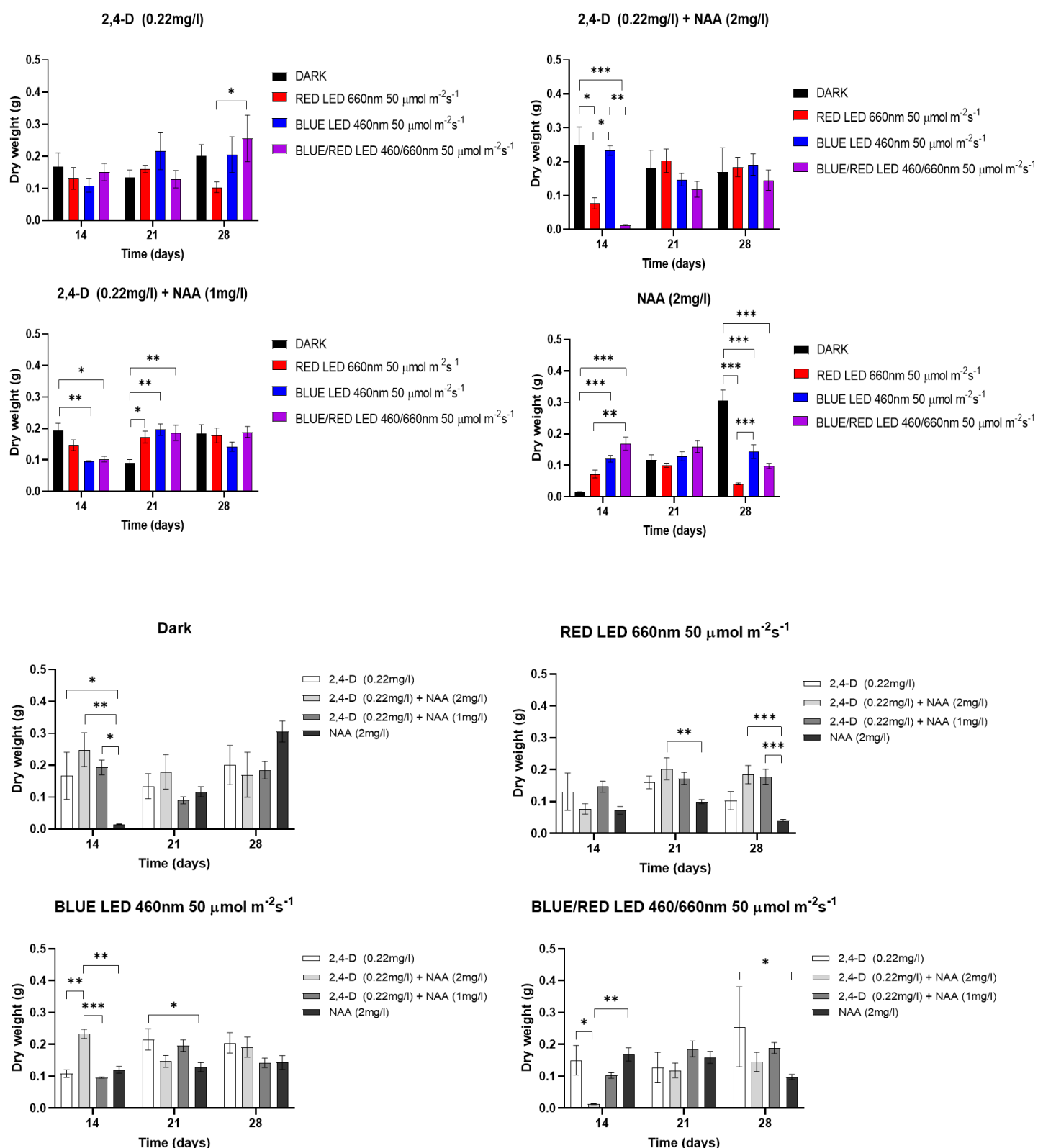


Figure 3.1.4 B. Evaluation of dry weight of *O. basilicum* calli on T0 medium, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

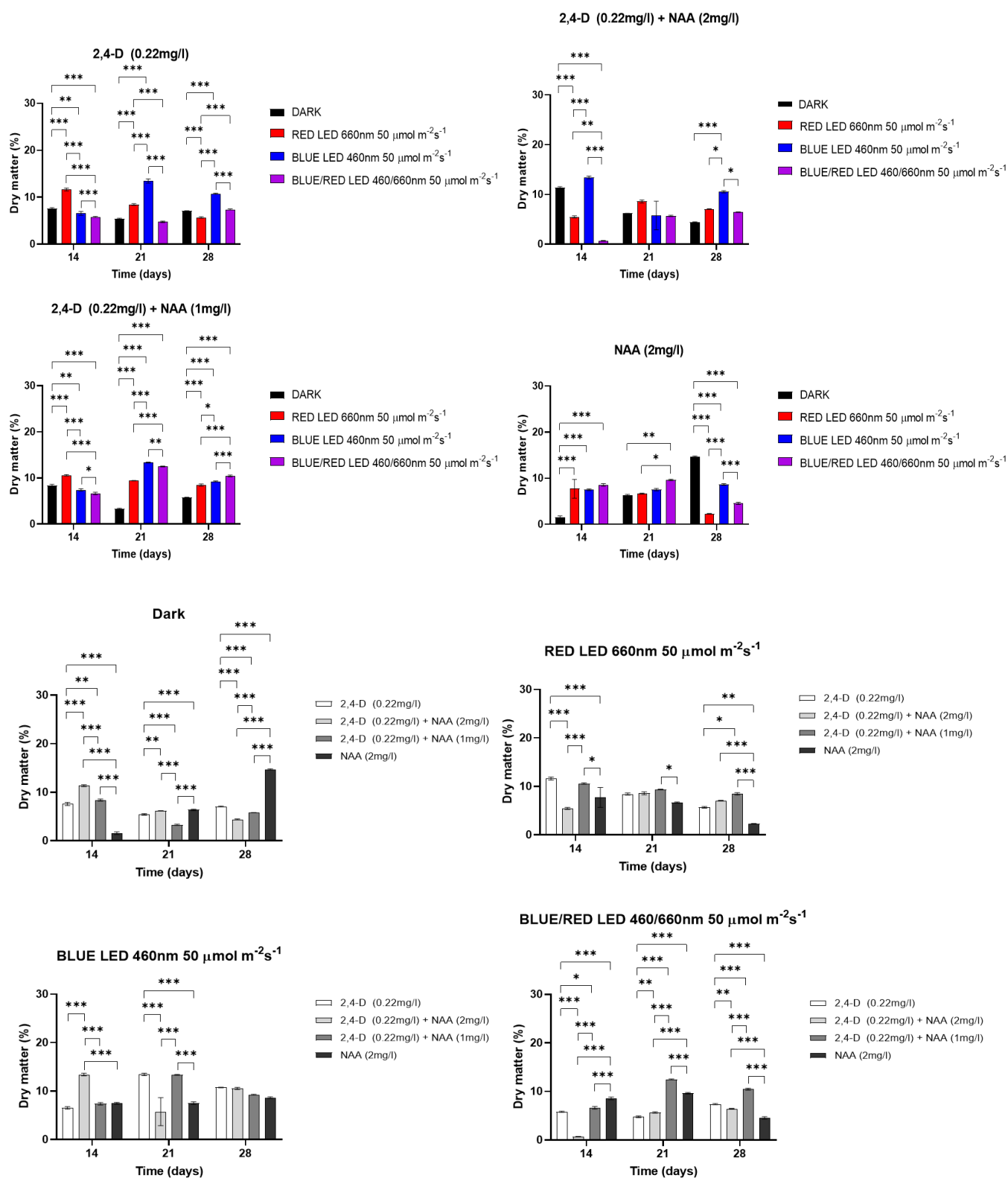


Figure 3.1.4 C Evaluation of dry matter of *O. basilicum* calli on T0, T1, T2 or T3 media, in different LED elicitation and harvesting time conditions. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

Under Dark T3 medium showed to be the most statistically relevant in promoting biomass accumulation, with 14,70% of dry matter accumulation at 28 days.

Under Red LED, 14 days after sub-culture, T0 medium, containing only 2,4-D, induced the highest dry matter content (11,62%) among all conditions, followed by T2 medium (10,58%) and was selected as the optimal medium.

Under Blue LED, day 21, the accumulation of dry matter increased for the T2 medium, recording the highest statistically relevant values compared to the other media. As a consequence, this condition was identified as the best to accumulate dry matter in *O. basilicum* friable callus under Blue LED light. At day 28, no statistically significant differences are observed in biomass accumulation in the four different media

Under Red-Blue Light, at day 21, the T2 medium seems to be the best in inducing the accumulation of dry matter, followed by the T3 medium. At day 28, medium T2 continues to exhibit the highest dry matter content, although at lower level than before, followed by the T0.

3.1.3.3 *O. basilicum* (pigmented, violet friable callus)

A violet, friable callus (Figure 3.1.5) originated from *O. basilicum*, and already showing pigmentation due to the accumulation of anthocyanins, was simply characterized in terms of growth kinetics under different media compositions (T0, T1, T2, T3) for possible optimization, with weekly recordings of fresh weight, dry weight and dry matter over the entire 28-day long sub-culture (Figure 3.1.6 and Table 3.1.6).

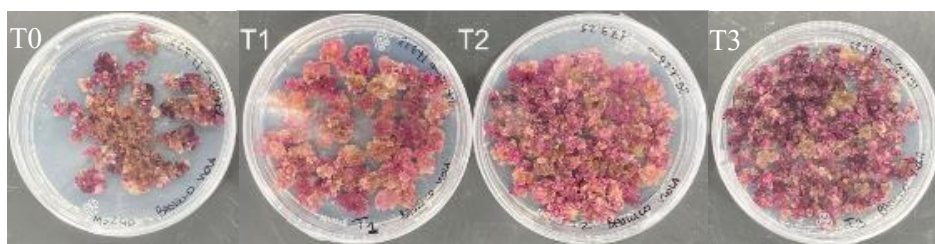


Figure 3.1.5. *O. basilicum* friable violet callus, grown on different media (from left to right: T0, T1, T2, T3)

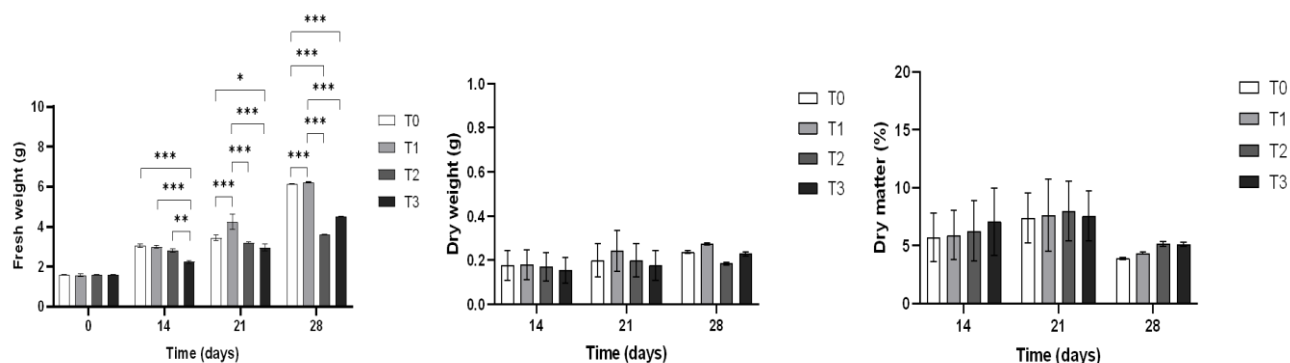


Figure 3.1.6 Evaluation of fresh weight, dry weight and dry matter of *O. basilicum* (violet) calli on T0, T1, T2 or T3 media and in different harvesting time. The analysis of variance was carried out using two-way ANOVA with multiple comparisons and Tukey's post-hoc tests. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

A(T0)

	Fresh weight (g)				Dry weight (g)				Dry matter (%)			
Time (days)	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ S	Sample 1	Sample 2	Sample 3	Mean $\pm$ S
0	1,61	1,56	1,60	1,59 $\pm$ 0,02								
14	3,18	3,08	2,86	3,04 $\pm$ 0,09	0,15	0,302	0,07	0,18 $\pm$ 0,06	4,75	9,78	2,63	5,72 $\pm$ 2,12
21	3,31	3,29	3,60	3,45 $\pm$ 0,152	0,17	0,345	0,08	0,20 $\pm$ 0,07	5,23	9,58	2,38	7,41 $\pm$ 2,17
28	6,12	6,13		6,13 $\pm$ 0,01	0,245	0,232		0,24 $\pm$ 0,01	4,00	3,78		3,89 $\pm$ 0,11

B(T1)

	Fresh weight (g)				Dry weight (g)				Dry matter (%)			
Time (days)	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ S
0	1,48	1,544	1,67	1,57 $\pm$ 0,06								
14	3,02	3,08	2,85	2,98 $\pm$ 0,07	0,154	0,307	0,07	0,18 $\pm$ 0,06	5,08	9,97	2,69	5,91 $\pm$ 2,14
21		4,61	3,88	4,24 $\pm$ 0,36	0,209	0,418	0,10	0,24 $\pm$ 0,09	4,53	10,75		7,64 $\pm$ 3,11
28	6,22	6,24		6,22 $\pm$ 0,01	0,278	0,270		0,27 $\pm$ 0,04	4,48	4,25		4,36 $\pm$ 0,11

C(T2)

	Fresh weight (g)				Dry weight (g)				Dry matter (%)			
Time (days)	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ S
0	1,60	1,62	1,54	1,58 $\pm$ 0,02								
14	2,91	2,60	2,90	2,83 $\pm$ 0,10	0,14	0,29	0,07	0,17 $\pm$ 0,06	5,03	11,26	2,52	6,27 $\pm$ 2,59
21		3,16	3,24	3,20 $\pm$ 0,04	0,17	0,34	0,08	0,20 $\pm$ 0,07	5,44	10,59		8,03 $\pm$ 2,58
28	3,58	3,61		3,59 $\pm$ 0,01	0,19	0,18		0,18 $\pm$ 0,01	5,35	4,96		5,16 $\pm$ 0,12

D(T3)

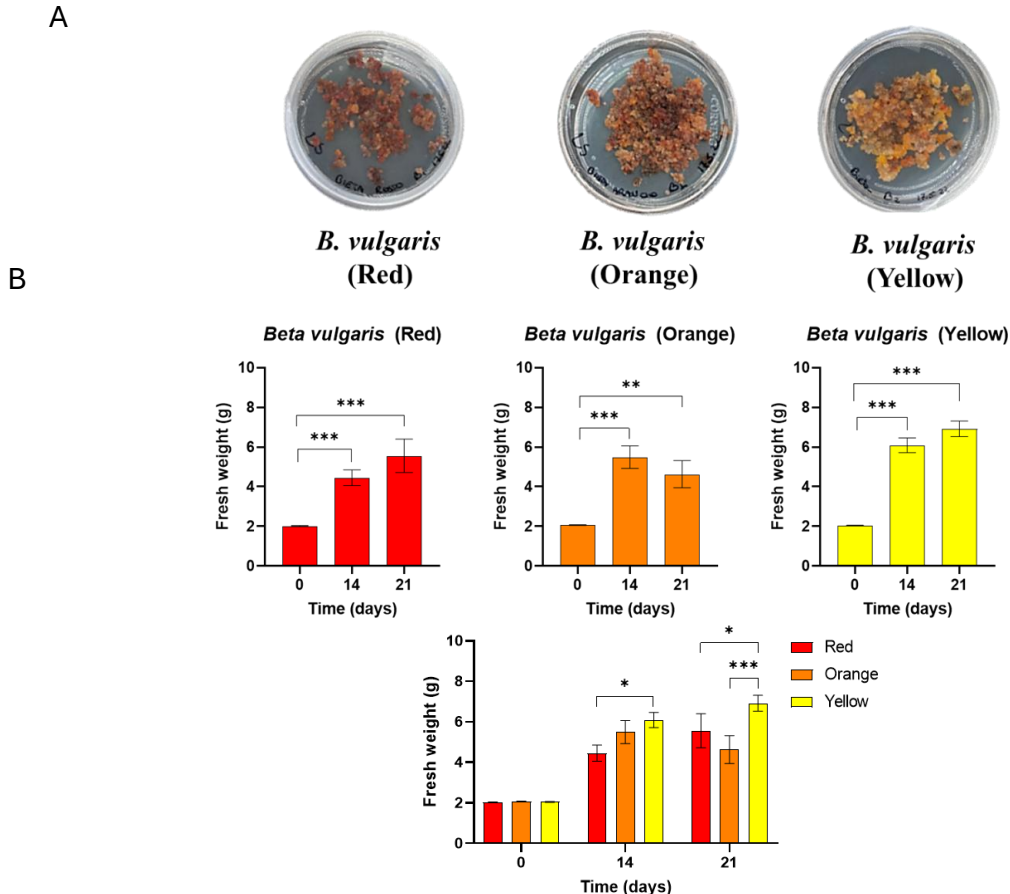
	Fresh weight (g)				Dry weight (g)				Dry matter (%)			
Time (days)	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ SD	Sample 1	Sample 2	Sample 3	Mean $\pm$ S
0	1,62	1,62	1,54	1,59 $\pm$ 0,03								
14	2,30	2,11	2,33	2,25 $\pm$ 0,07	0,13	0,27	0,07	0,15 $\pm$ 0,06	5,78	12,58	2,85	7,01 $\pm$ 2,88
21		2,81	3,12	2,96 $\pm$ 0,16	0,15	0,30	0,08	0,18 $\pm$ 0,07	5,40	9,72		7,56 $\pm$ 2,15
28	4,50	4,53		4,52 $\pm$ 0,02	0,24	0,22		0,23 $\pm$ 0,01	5,31	4,92		5,12 $\pm$ 0,19

Table 3.1.6. Replicate value, mean values and standard deviation (SD) of fresh weight, dry weight, and dry matter for *O. basilicum* callus culture across all conditions: T0(A), T1(B), T2(C), T3(D).

Although the analysis of fresh weight shows increase along the 28 days sub-culture for all the media, the most statistically significant value in terms of fresh weight was observed in media T0 and T1. Instead, dry matter content did not exhibit statistically significant differences among the media. Consequently, all four culture conditions were maintained for further analysis, with the aim of identifying the most effective medium based on spectrophotometric evaluation through UV-visible analysis.

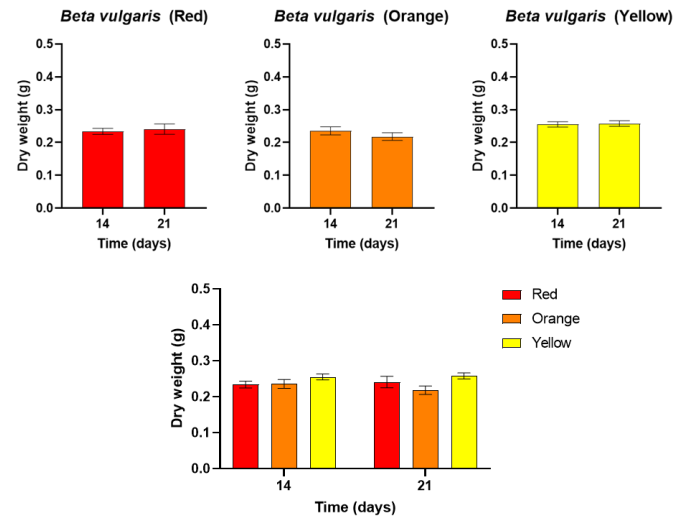
3.1.3.4 *B. vulgaris*

Calli from *Beta vulgaris* var. rubra (BR) were grown on Linsmaier & Skoog (LS) medium supplemented with 2,4-D (0.22mg/L). The accumulation of betalains (betacyanins and betaxanthins) conferred red, orange, and also yellow colors to the cultures (BR_{red}, BR_{orange} and BR_{yellow}), as shown in figure 3.1.7 A. Callus growth was assessed under dark conditions, in terms of fresh weight, dry weight and dry matter (Figure 3.1.7 B, C and D). The maximum values of fresh weight for the red and yellow callus were found at 21 days, whereas the highest dry matter was recorded 14 days after subculture. In contrast, orange callus showed the highest fresh weight at 14 days, whereas dry matter reached its maximum 21 days from subculture. For all three callus culture, day 14 was selected as the optimal time point for sub-culture to avoid browning.



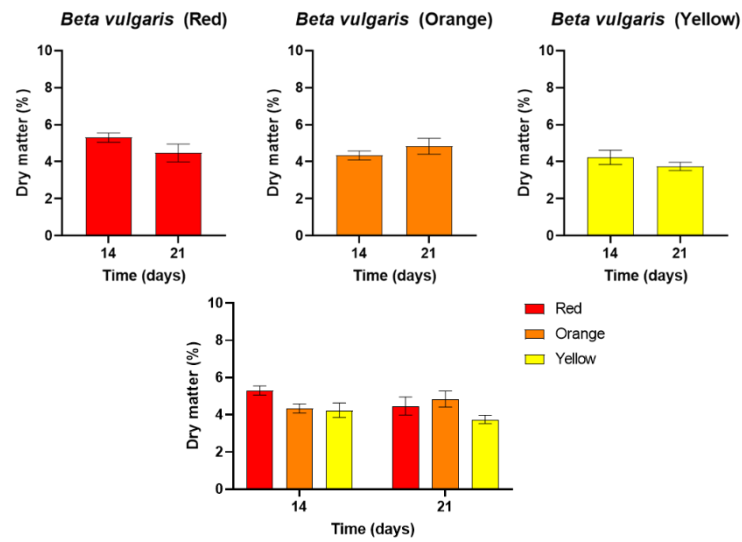
Time (days)	<i>B. vulgaris</i> Red			<i>B. vulgaris</i> Orange			<i>B. vulgaris</i> Yellow		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	2,02	0,05	6	2,07	0,05	6	2,05	0,04	6
14	4,46	0,69	3	5,50	0,98	3	6,09	0,64	3
21	5,56	1,45	3	4,63	1,19	3	6,92	0,68	3

C



Time (days)	<i>B. vulgaris</i> Red			<i>B. vulgaris</i> Orange			<i>B. vulgaris</i> Yellow		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	0,23	0,02	3	0,24	0,02	3	0,26	0,01	3
21	0,24	0,03	3	0,22	0,02	3	0,26	0,01	3

D



Time (days)	<i>B. vulgaris</i> Red			<i>B. vulgaris</i> Orange			<i>B. vulgaris</i> Yellow		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
14	5,30	0,44	3	4,34	0,43	3	4,24	0,68	3
21	4,47	0,84	3	4,84	0,75	3	3,75	0,39	3

Figure 3.1.7. A) Representative images for *B. vulgaris* red, orange and yellow during callus growth kinetics. Growth kinetics profiles of B) fresh weight (g) C) dry weight (g) D) and dry matter (%) over the subculture period. Numerical data and statistical analysis of fresh weight, dry weight, and dry matter for *B. vulgaris* callus red (c), orange (d) and yellow (e) cultured on LS medium. Data were analyzed by two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (compared to control/baseline conditions).

3.1.3.5. Summary of the best culture conditions identified for the friable callus deriving from *P. frutescens*, *B. vulgaris*, *O. basilicum*

Statistical evaluation of biomass accumulation enabled the identification for the optimal dry matter accumulation according to media, harvesting time and LED conditions (when performed) for *P. frutescens*, *O. basilicum* (white and violet), and *B. vulgaris* (red, orange and yellow), as summarized in Table 3.1.7:

Species	Media	Elicitation	Time of harvesting
<i>P. frutescens</i>	MS + NAA 2mg/L	Dark	28 days
<i>P. frutescens</i>	MS + 2,4-D 0,22mg/L + NAA 2mg/L	Red LED	21 days
<i>P. frutescens</i>	MS + 2,4-D 0,22mg/L + NAA 1mg/L	Blue LED	21 days
<i>P. frutescens</i>	MS + NAA 2mg/L	Red/Blue LED	28 days
<i>O. basilicum</i>	MS + NAA 2mg/L	Dark	28 days
<i>O. basilicum</i>	MS + 2,4-D 0,22mg/L	Red LED	14 days
<i>O. basilicum</i>	MS + 2,4-D 0,22mg/L + NAA 1mg/L	Blue LED	21 days
<i>O. basilicum</i>	MS + 2,4-D 0,22mg/L + NAA 1mg/L	Red/Blue LED	21 days
<i>O. basilicum</i> (violet)	MS + 2,4-D 0,22mg/L + NAA 1mg/L	Dark	21 days
<i>B. vulgaris</i> (red)	LS + 2,4-D 0,22mg/L	Dark	14 days
<i>B. vulgaris</i> (orange)	LS + 2,4-D 0,22mg/L	Dark	14 days
<i>B. vulgaris</i> (yellow)	LS + 2,4-D 0,22mg/L	Dark	14 days

Table 3.1.7. Optimal growth conditions in terms of dry matter accumulation identified for *P. frutescens*, *B. vulgaris* and *O. basilicum* (white and violet), on different media and LED conditions through growth kinetic analysis and statistical validation (two-way ANOVA, Tukey's post-hoc test), as described in the previous sections.

3.1.4 Extraction of polar compounds from callus cultures

Optimal extraction protocols from callus matrix, as detailed in the Materials and Methods section, were identified for each species. The UV-visible analysis of extracts, reported in Figure 3.1.8, highlighted significant differences among the protocols.

For *P. frutescens*, the HCl:H₂O 1:99 v/v buffer allowed to obtain a higher absorbance at 283nm and 313nm, whereas EtOH:H₂O 70:30 v/v buffer optimally extracted the compounds associated with the peak at 398 nm. For the *O. basilicum* friable callus, the MeOH:HCl 1N 85:15 v/v protocol exhibited enhanced absorbance (O.D) at 283nm and 314nm, indicating greater efficiency in the extraction of phenolic compounds. For the *O. basilicum* callus, the MeOH:HCl 1N 85:15 v/v buffer provided a greater absorbance at 290nm and 325nm, while both MeOH:HCl 1N 85:15 v/v and HCl:H₂O 1:99 v/v demonstrated equivalent efficacy for the peak at 525nm. For *B. vulgaris*, C₂H₃NaO₂ (sodium acetate) 1M at pH 5.4 maximally extracted compounds associated with the peak at 280nm, whereas for the peaks at 477nm and 535nm, both the EtOH:H₂O 40:60 v/v buffer and C₂H₃NaO₂ 1M at pH 5,4 allowed equivalent absorbance.

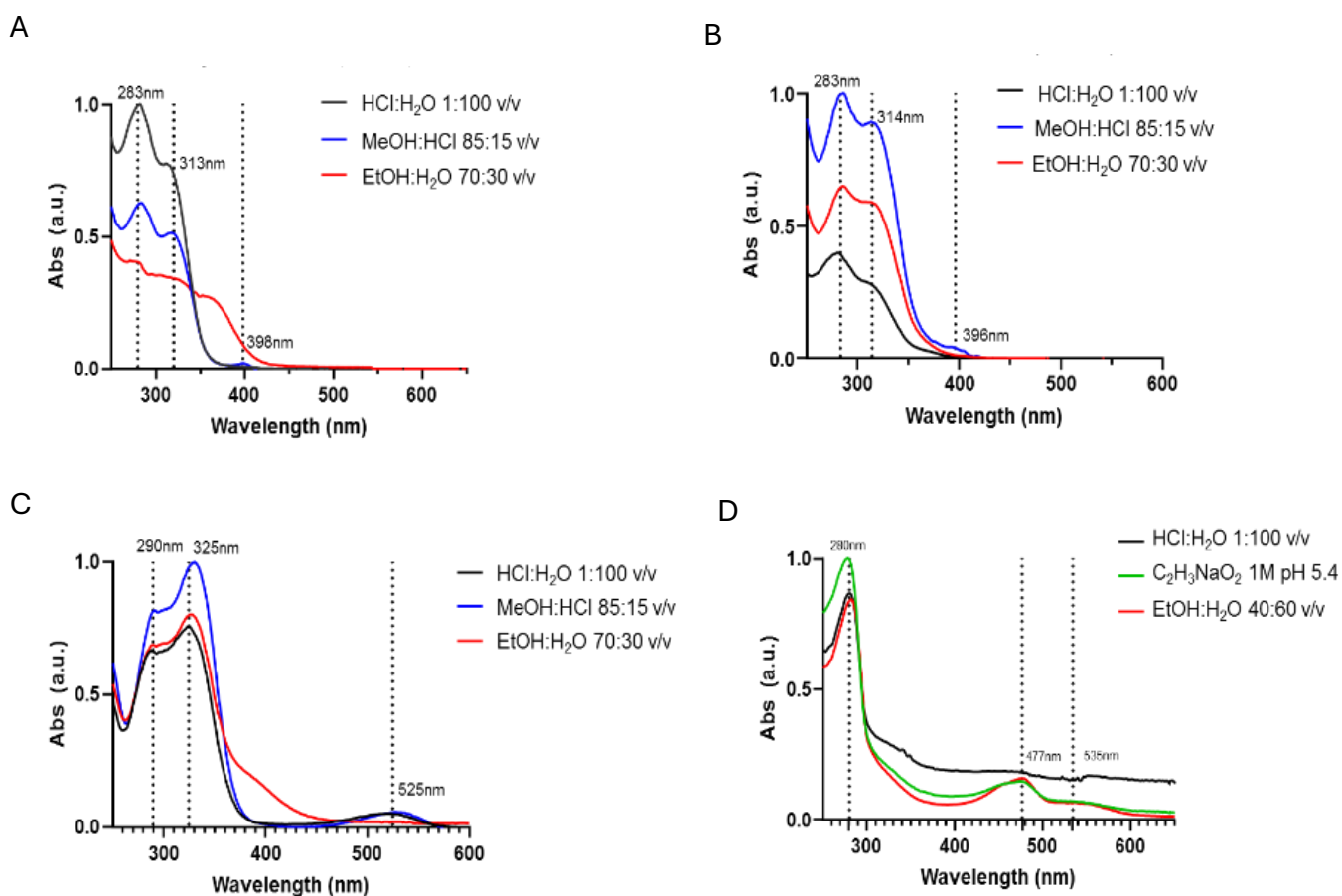


Figure 3.1.8. Comparative evaluation of extraction protocols for *P. frutescens* (A), *O. basilicum* (B), *O. basilicum* violet (C), and *B. vulgaris* (D), assessing UV-visible absorption spectra to identify optimal solvent systems for phenolic compound extraction.

The protocols that were identified as the protocols of choice for the different calli are listed in Table 3.1.8:

Species	Extraction protocols
<i>P. frutescens</i>	HCl:H ₂ O 1:99 v/v
<i>O. basilicum</i>	MeOH:HCl 1N 85:15 v/v
<i>O. basilicum</i> (violet)	MeOH:HCl 1N 85:15 v/v
<i>B. vulgaris</i>	EtOH:H ₂ O 40:60 v/v and C ₂ H ₃ NaO ₂ 1M

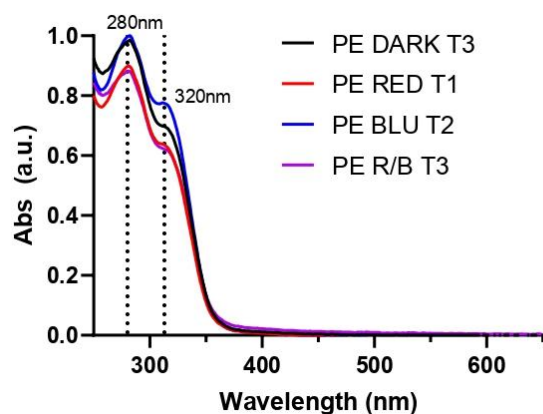
Table 3.1.8. Table summarizes specific extraction protocol established for each species

3.1.5 UV-visible analysis of extracts from optimized callus cultures

After identifying the optimal extraction protocols for callus cultures, UV-visible analysis was performed as a qualitative screening to assess the culture condition determining the best overall UV-visible absorbance related to specialized metabolite classes of interest (i.e., either polyphenols or betacyanins/betaxanthins) under the best culture condition identified for each species in terms of dry matter accumulation. To better compare the different conditions, spectra were represented in arbitrary units (a.u.) (i.e., units normalized to one, used to represent the ratio of a measured value to the maximum or reference value), set equal to 1. The real absorbance values are reported in the tables associated to each spectrum.

3.1.5.1 *P. frutescens* white friable calli

In *P. frutescens* (extracted in HCl:H₂O 1:99, v/v), the absorbance values were comparable across the four best conditions for dry matter accumulation. Specifically, at 280nm absorbance values (O.D.) were comparable between Blue T2 and Dark T3. However, Blue light-T2 medium condition showed the highest absorbance at 313nm, indicating a marginally better performance under this treatment. For this reason, it was selected as the optimal condition (Figure 3.1.9). These wavelengths correspond to specific classes, including flavanones at 283nm (Bijlsma et al., 2022), but also aromatic amino acids at 280nm, like tryptophan, tyrosine, and phenylalanine (Prasad et al., 2017) and citric acid (Krishnaswamy et al., 2019); hydroxycinnamic acids at 316nm, likely attributable to caffeic and rosmarinic acid, based on literature data (Carocho et al., 2016) or chlorogenic acid at 326nm (Velkoska-Markovska, L., et al 2018).

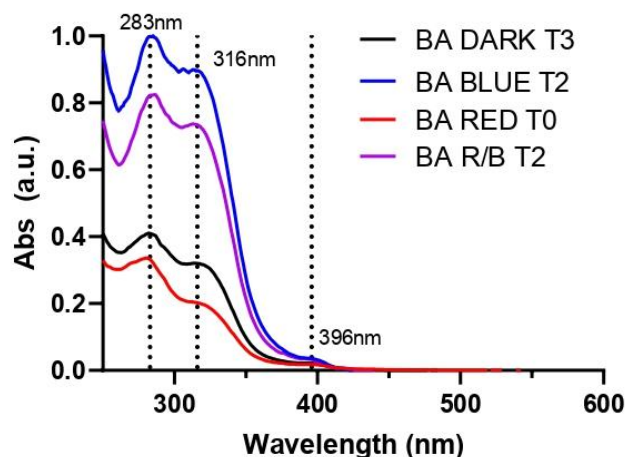


<i>P. frutescens</i> Abs $\pm$ SD				
Wavelength	Dark T3	Red T1	Blue T2	R/B T3
280nm	4,18 $\pm$ 0,1	3,827 $\pm$ 0,85	4,25 $\pm$ 0,44	3,754 $\pm$ 0,245
313nm	2,842 $\pm$ 0,73	2,557 $\pm$ 0,543	3,145 $\pm$ 0,16	2,536 $\pm$ 0,174

Figure 3.1.9. Comparison of UV-visible spectra of calli of *P. frutescens* under conditions of highest dry matter accumulation as a function of culture medium and LED elicitation. Abs: absorbance, SD: standard deviation. Note that the Abs values in the table are related to a dilution 1:10 of the extract.

3.1.5.2 *O. basilicum* white and violet friable calli

The UV-visible analysis at the maximum dry matter accumulation revealed that for *O. basilicum* (extracted in MeOH: HCl 1N 85:15, v/v), Blue LEDs under T2 medium conditions resulted in the highest absorbance, followed by the combination with the same medium under R/B LEDs (Figure 3.1.10). Absorption peaks are similar to *P. frutescens*, with the peak at 283nm correspond to flavanones, peak at 316nm of hydroxycinnamic acids, likely attributable to caffeic and rosmarinic acid and flavonols at 396nm (Jomovà et al 2019).

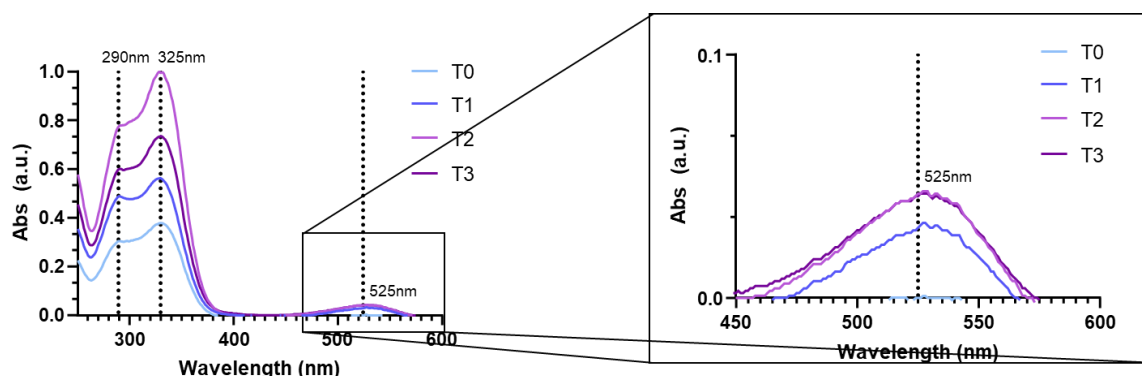


<i>O. basilicum</i> Abs $\pm$ SD				
Wavelength	Dark T3	Red T0	Blue T2	R/B T2
283nm	1,225 $\pm$ 0,364	0,993 $\pm$ 0,463	2,461 $\pm$ 0,62	4,18 $\pm$ 0,1
316nm	0,957 $\pm$ 0,114	0,605 $\pm$ 0,259	2,19 $\pm$ 0,371	2,842 $\pm$ 0,73
396nm	0,029 $\pm$ 0,002	0,018 $\pm$ 0,001	0,033 $\pm$ 0,002	0,035 $\pm$ 0,01

Figure 3.1.10. Comparison of UV-visible spectra of calli of *O. basilicum* under conditions of highest dry matter accumulation as a function of culture medium and LED elicitation. Abs: absorbance, SD: standard deviation. Note that the Abs values in the table are related to a dilution 1:10 of the extract.

Hydroalcoholic extraction of the *O. basilicum* pigmented callus, under optimal dry matter accumulation conditions (21days for all media), was performed with MeOH:HCl 1N 85:15 (v/v). The UV-visible spectral analysis of the hydroalcoholic extract has shown in addition to the characteristic peaks of flavanones (290nm) and hydroxycinnamic acids (323nm), previously identified in the *O. basilicum* (white) friable callus, an additional peak at 518nm, characteristic of anthocyanins (Figure 3.1.11). The highest absorbance value at 290nm and 323nm was recorded in T1 and T2 media, whereas the 518nm peak exhibited greater value in T2 and T3 media. Based on these findings, T2 medium was identified as the most suitable for the accumulation of both phenolic compounds and anthocyanins. The detected absorption peaks correspond to flavan-3-ols (Arasaretnam 2018),

flavanons, likely attributable to eriodictyol and naringenin (Bijlsma et al., 2022), as well as rosmarinic acid and caffeic acid at 325nm (Carocho et al., 2016) and anthocyanin at 525nm (Saha et al., 2019).

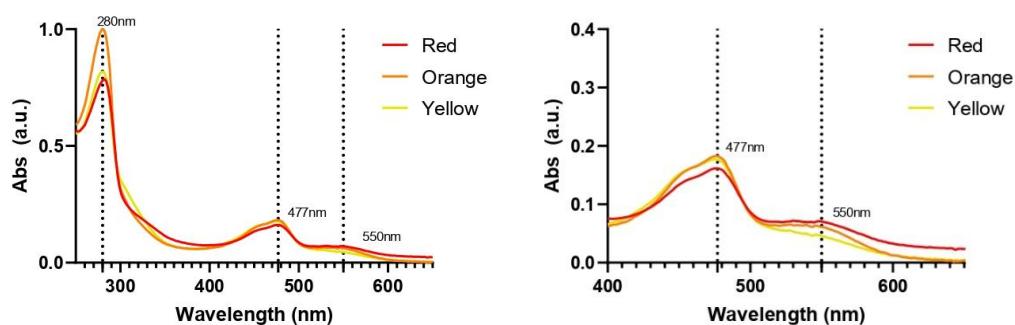


<i>O. basilicum</i> (violet) Abs $\pm$ SD				
Wavelength	T0	T1	T2	T3
290nm	0,305 $\pm$ 1,39	0,49 $\pm$ 0,55	0,80 $\pm$ 0,77	0,60 $\pm$ 0,178
325nm	0,371 $\pm$ 0,163	0,557 $\pm$ 0,64	0,98 $\pm$ 0,98	0,724 $\pm$ 0,12
525nm	0,001 $\pm$ 0,01	0,03 $\pm$ 0,02	0,043 $\pm$ 0,06	0,042 $\pm$ 0,23

Figure 3.1.11. UV-visible spectrum of hydroalcoholic extracts from *O. basilicum* (violet) friable callus, highlighting anthocyanin absorption at 518nm. Abs: absorbance, SD: standard deviation. Note that the Abs values in the table are related to a dilution1:0 of the extract.

3.1.5.3 *B. vulgaris* yellow, orange and red friable calli

The UV-visible analysis of the hydroalcoholic extract from *B. vulgaris* samples (using EtOH: H₂O 40:60 v/v protocol) revealed absorption peaks at 280nm and 535nm, corresponding to betacyanins, particularly those with Cyclo-Dopa residues at 280nm (Sadowska-Bartosz et al., 2021) and at 477nm for betaxanthins (Kugler et al., 2007). The spectral profiles were substantially equivalent across the yellow and red callus types apart from the 280nm absorbance, which was higher in the callus exhibiting orange pigmentation (Figure 3.1.12)



Wavelength	Red	Orange	Yellow
280nm	8,06 $\pm$ 1,82	10,34 $\pm$ 0,05	8,46 $\pm$ 0,39
477nm	1,67 $\pm$ 0,60	1,88 $\pm$ 0,01	1,83 $\pm$ 0,01
550nm	0,73 $\pm$ 0,16	0,66 $\pm$ 0,01	0,52 $\pm$ 0,02

Figure 3.1.12. UV-visible spectrum obtained from extraction of red, orange and yellow friable calli of *B. vulgaris*. Abs: absorbance, SD: standard deviation. Note that real values are reported in the corresponding Table.

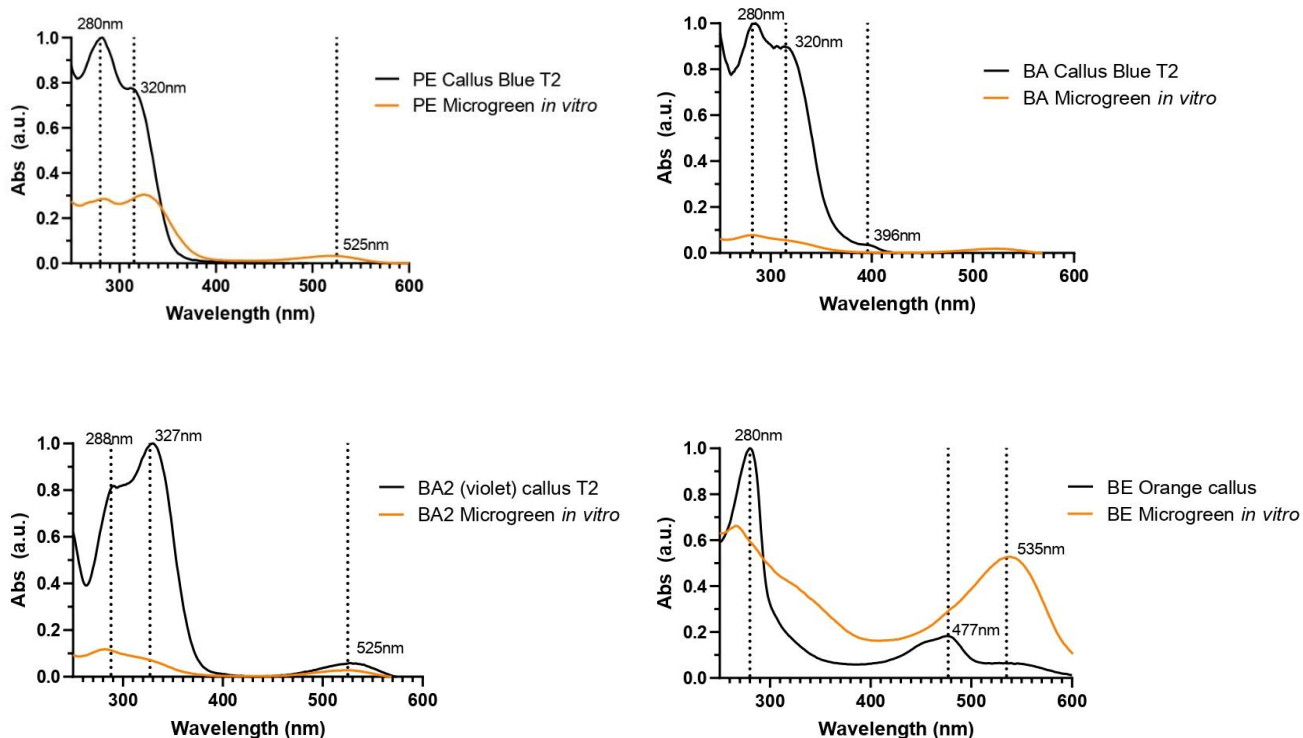
Table 3.1.9 summarizes the conditions that result in the most favorable UV-visible spectra, exhibiting the highest absorbance profile for each species.

Species	Media	Elicitation	Time of harvesting
<i>P. frutescens</i>	MS + 2,4-D 0,22mg/L+ NAA 1mg/L	Blue LED	21 days
<i>O. basilicum</i>	MS + 2,4-D 0,22mg/L+ NAA 1mg/L	Blue LED	21 days
<i>O. basilicum</i> (violet)	MS + 2,4-D 0,22mg/L+ NAA 1mg/L	Dark	21 days
<i>B. vulgaris</i> (orange)	LS + 2,4-D 0,22mg/L	Dark	14 days

Table 3.1.9. Summary of optimal condition for each callus species based on UV-visible spectral analysis.

3.1.5.4 Comparison between callus culture and microgreens

The comparative UV-visible spectral analysis of friable callus cultures of *P. frutescens* and *O. basilicum* (white and violet) under optimal conditions demonstrated also a significant increase in absorbance (O.D.) compared to microgreens (i.e., the starting material for the callus cultures onset), which themselves exhibit higher secondary metabolite concentration than adult plants (Figure 3.1.13). In *B. vulgaris*, the extract from callus showed an increase absorbance in correspondence of the 280nm peak compared to the microgreen. However, no improvement was observed at 535nm peak, where the UV-visible absorbance is higher for microgreens extracts compared to callus culture.



Wavelength	<i>P. frutescens</i> microgreen	<i>P. frutescens</i> callus culture dil 1:10
280nm	1,21±0,07	4,25±0,06
320nm	1,28±0,04	3,14±0,02

Wavelength	<i>O. basilicum</i> microgreen	<i>O. basilicum</i> callus culture dil 1:10
280nm	0,22±0,04	2,95±0,03
320nm	0,14±0,02	2,64±0,02
396nm	0,008±0,001	0,105±0,001

Wavelength	<i>O. basilicum</i> microgreen	<i>O. basilicum</i> (violet) callus culture dil 1:10
288nm	0,22±0,02	1,59±0,06
327nm	0,14±0,02	1,96±0,08
525nm	0,05±0,01	0,11±0,01

Wavelength	<i>B. vulgaris</i> microgreen	<i>B. vulgaris</i> callus culture dil 1:10
280nm	0,62±0,03	1,03±0,05
477nm	0,30±0,03	0,18±0,02
535nm	0,54±0,01	0,06±0,01

Figure 3.1.13. UV-visible spectra profile and absorbance (Abs) value (table) comparing microgreens (starting material) and callus culture of the four species. Values reported in the spectra and in the related tables are the real values of undiluted extracts, unless otherwise specified. When applied, dilutions are indicated in the corresponding graphs or tables.

3.1.5.5. Comparison of UV-visible profile and dry matter under the optimal conditions identified for each species

Figure 3.1.14 presents a comparative analysis of the UV-visible spectral profiles and dry matter accumulation under the optimal experimental condition identified for each species (see Table 3.1.11). Notably, the UV-visible spectrum of *P. frutescens* extract exhibits a higher O.D. at 280nm, indicating a higher bioaccumulation of flavanones, organic acids and amino acids (Figure 3.1.14A). Conversely, *O. basilicum* violet shows an increased absorbance at 327nm. In terms of dry matter accumulation, *P. frutescens* demonstrates higher scores, compared to the others, with statistically significant differences (Figure 3.1.14B). For these reasons, *Perilla frutescens* was selected as the model system for further experiments due to rapid growth rate and high metabolite production, making it an ideal proof-of-concept for assessing the feasibility of novel food applications.

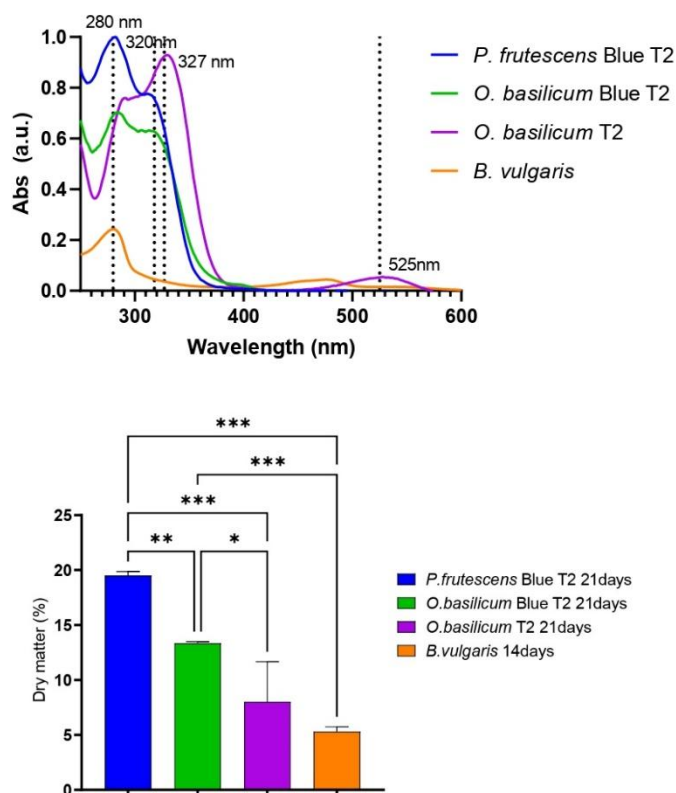


Figure 3.1.14. Comparison in terms of UV-visible analysis (A) and Dry matter content (B) among the best conditions obtained for each species. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001).

3.1.6 Suspension culture

Cell suspension cultures derived from callus cultures of all the considered species, maintained under controlled conditions (darkness, 23 °C, 115 rpm), preserved the pigmentation profile of the original calli over time. Suspension culture of *Perilla frutescens*, *Ocimum basilicum* and *Beta vulgaris* exhibited a high degree of cellular homogeneity and the formation of stable suspension composed of small, isodiametric cells (Figure 3.1.15). The use of 6-BAP for *P. frutescens* and *O. basilicum* (MS 2,4-D 0,5mg/L + BAP 1mg/L) enhanced these characteristics, promoting uniform cell morphology and reduced cell size, in agreement with Woo et al., 2021. In the case of *B. vulgaris*, the same concentration of 2,4-D (0,22mg/L), used during callogenesis and subculture was maintained in the liquid medium, and the addition of 6-BAP was not required. The only adjustment was an increase in sucrose concentration from 30 g/L to 50 g/L, to prevent browning and enhance cellular proliferation. Growth kinetics of these species were evaluated through fresh weight (g), dry weight (g), and dry matter content (%) as for the callus cultures.

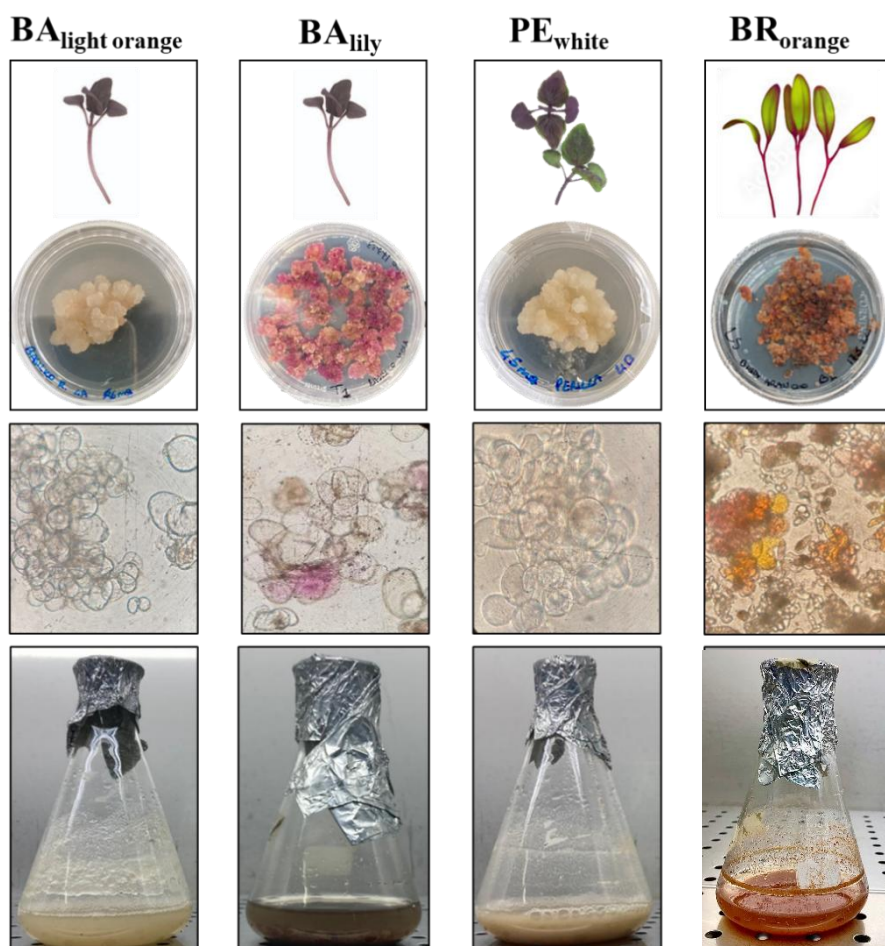


Figure 3.1.15. Friable calli as starting material and deriving suspension cultures in flasks for lab-scale suspension cell culture (from top to bottom panel and from left to right: *O. basilicum* white and violet, *P. frutescens* and *B. vulgaris*; microscopic photo of cells in suspension (20X magnification, central panel).

3.1.6.1 Definition of the best suspension culture conditions for each species in terms of media and harvesting time

3.1.6.1.1 *P. frutescens*

The cell suspension culture derived from *Perilla frutescens* exhibited a peak in fresh weight between 21 and 28 days after sub-culture. Despite the increase in fresh weight, no statistically significant differences were observed in dry weight and dry matter content throughout the subculture period (Figure 3.1.16).

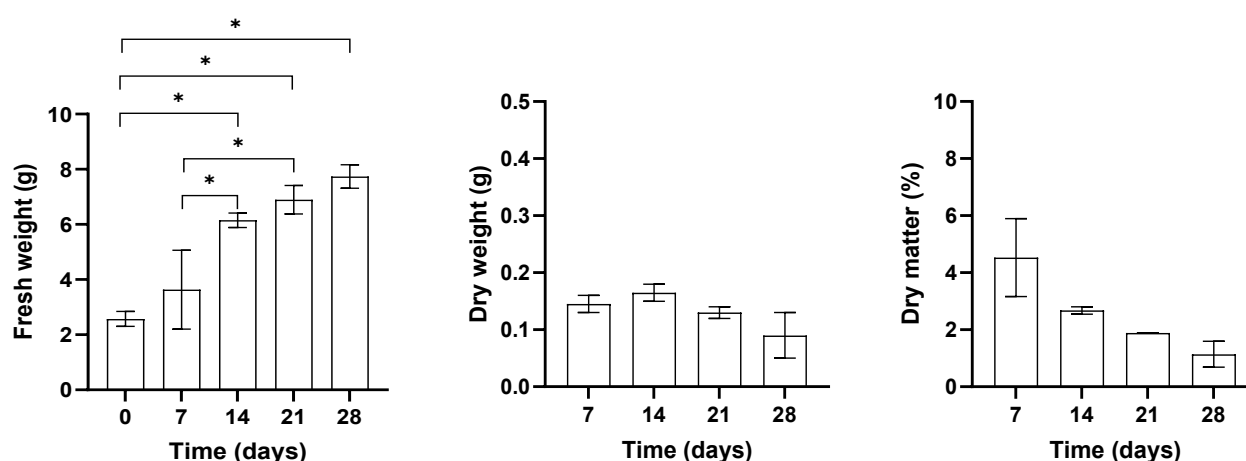


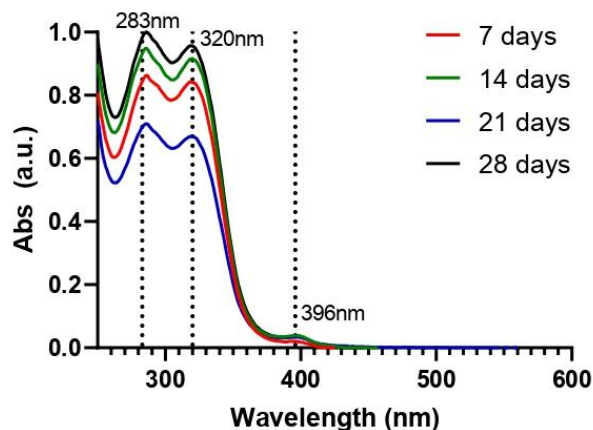
Figure 3.1.16. Study of the growth kinetics of *P. frutescens* by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%). The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001).

	Fresh weight (g)			Dry weight (g)			Dry matter (%)		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
0	2,30	2,84	2,57±0,38						
7	5,06	2,21	3,63±2,02	0,16	0,13	0,15±0,02	3,16	5,89	4,53±1,93
14	6,41	5,89	6,15±0,36	0,18	0,15	0,17±0,02	2,81	2,54	2,68±0,18
21	7,41	6,38	6,90±0,73	0,14	0,12	0,13±0,01	1,88	1,88	1,89±0,01
28	7,31	8,16	7,74±0,60	0,05	0,13	0,1±0,05	0,68	1,59	1,14±0,64

Table 3.1.10. Study of the growth kinetics of *P. frutescens* suspension by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%).

The UV-visible analysis (Figure 3.1.17) conducted at day 7, 14, 21 and 28 after sub-culture allowed the identification of the optimal harvest time for polyphenols accumulation, which turned out to be day 28 after sub-culture. The UV-visible spectral profiles of hydroalcoholic extracts from lyophilized suspension cultures throughout the sub-culture cycle (7–28 days) remained qualitatively consistent

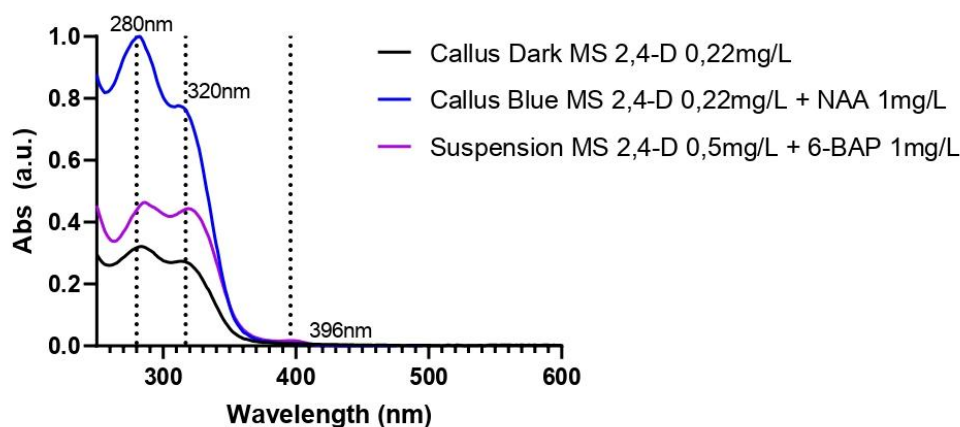
with those of the original callus, maintaining the characteristic absorbance peaks at 283nm, 320nm, and 396nm typically described in literature as corresponding to flavanones, organic acid (citric acid) and amino acid at 283nm, hydroxycinnamic acids (caffeic, rosmarinic and chlorogenic acid) at 320nm and flavonols at 396nm.



	Abs <i>P. frutescens</i> suspension culture			
Wavelength	7 days	14 days	21 days	28 days
283nm	16,68	18,4	13,80	19,42
320nm	16,61	18,09	13,22	18,88
396nm	0,40	0,76	0,67	0,74

Figure 3.1.17. UV-visible spectrum of *P. frutescens* suspension cell culture growth kinetics

The optimal UV-visible spectra of *P. frutescens* suspension culture under dark turned out to be at 28 days after sub-culture, as already mentioned. This spectrum was compared with that of callus culturing optimum under dark (i.e., MS 2,4-D 0,22mg/L, T0 medium, control – see Table 3.1.2) and with that of callus culturing optimum (Blue LED, MS 2,4-D 0,22mg/L + NAA 1mg/L, T2 medium – see Table 3.1.9) (Figure 3.1.18). *P. frutescens* suspension cultures (Dark, MS 2,4-D 0,5mg/L + BAP 1mg/L medium) exhibited an enhanced UV-visible spectral profile compared to the callus culture in the same dark growth condition, but overall lower absorbance compared with the callus cultures grown under Blue LED light on T2 medium



	Abs <i>P. frutescens</i> dil. 1:10		
Wavelength	Callus Dark T0	Callus Blue T2	Suspension Dark 2,4-D 0,5mg/L + BAP 1mg/L
280nm	1,35	4,25	1,86
320nm	1,13	3,14	1,88
396nm	0,03	0,04	0,07

Figure 3.1.18. UV-visible spectra comparison of *P. frutescens* suspension culture at 28 days after sub-culture with Dark T0 and Blue T2 callus culture. The absorbance values reported in the table refer to 1:10 diluted samples.

3.1.6.1.2 *O. basilicum*

O. basilicum cell suspension culture derived from callus culture exhibited a progressive increase in fresh weight, reaching its peak from day 14 to day 21 after sub-culture. From day 14 onward, a statistically significant reduction in dry weight was observed (Figure 3.1.19).

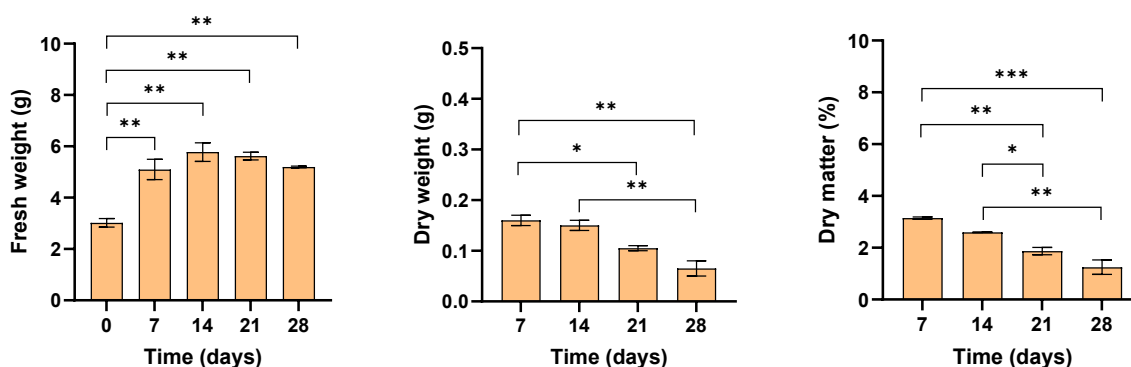
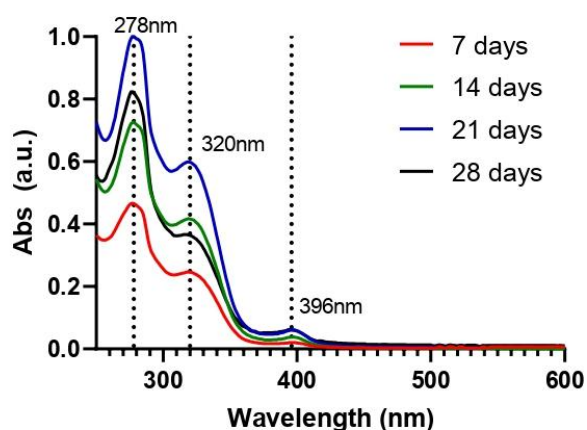


Figure 3.1.1.19. Study of the growth kinetics of *O. basilicum* suspension by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%). The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001).

Time (days)	Fresh weight (g)			Dry weight (g)			Dry matter (%)		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
0	2,85	3,19	3,02±0,23						
7	4,70	5,50	5,1±0,56	0,15	0,18	0,16±0,01	3,19	3,09	3,14±0,07
14	5,41	6,14	5,78±0,52	0,14	0,16	0,15±0,01	2,58	2,60	2,6±0,01
21	5,47	5,77	5,62±0,21	0,11	0,10	0,11±0,01	2,01	1,73	1,87±0,19
28	5,16	5,23	5,2±0,05	0,05	0,08	0,07±0,02	0,96	1,53	1,25±0,39

Table 3.1.11. Study of the growth kinetics of *O. basilicum* suspension by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%).

The analysis of the UV-visible spectrum enabled the determination of the optimal conditions for the accumulation of polyphenols, which peaked at 21 days (Figure 3.1.20).



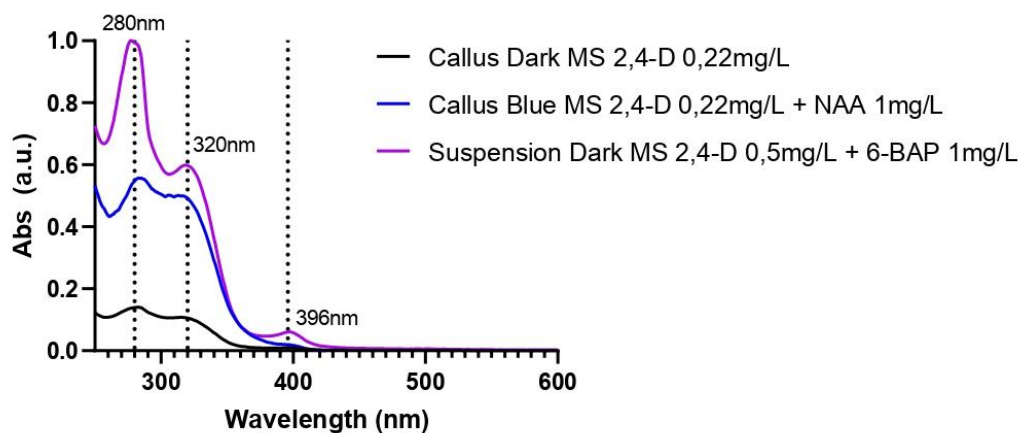
	Abs <i>O. basilicum</i> suspension culture			
Wavelength	7 days	14 days	21 days	28 days
278nm	6,254	9,749	13,436	11,037
320nm	3,293	5,593	8,031	4,89
396nm	0,273	0,51	0,815	0,822

Figure 3.1.20. UV-visible spectrum on the growth kinetics of *O. basilicum* suspension cell culture. The absorbance values reported in the table refer to 1:10 diluted samples.

The UV-visible spectra of hydroalcoholic extracts from suspension cultures harvested at the peak of dry matter accumulation (21 days, in dark condition) were compared with the corresponding callus culture cultivated under dark conditions in T0 medium (i.e., the best growing condition of the callus under dark - see Table 3.1.2 and that of callus culturing optimum (i.e., grown under Blue LED light on T2 medium – see Table 3.1.8) (Figure 3.1.23).

The spectral profiles of extracts from suspension cultures throughout the subculture cycle (7–28 days) remained qualitatively consistent with those of the original callus, preserving characteristic

absorbance peaks at 278nm, 320nm, and 396nm (Figure 3.1.21). Notably, an increase in peak intensity at 280nm was observed, indicating an enhanced accumulation of aromatic or phenolic compounds in the suspension cultures compared to both culture conditions of calli.



	Abs <i>O. basilicum</i> dil 1:10		
Wavelength	Callus Dark T0	Callus Blue T2	Suspension Dark 2,4-D 0,5mg/L + BAP 1mg/L
280nm	0,7435	2,892	6,718
320nm	0,568	2,64	4,01
396nm	0,0525	0,105	0,407

Figure 3.1.21. Comparison of UV-visible spectra of extracts of *O. basilicum* obtained from callus grown in the dark in optimum condition, callus cultured under the best LED and medium composition and the suspension cultures at the higher accumulation of secondary metabolites time point under dark. The absorbance values reported in the table refer to 1:10 diluted samples.

The establishment of a stable and homogeneous suspension culture for *O. basilicum* violet culture proved to be challenging due to inconsistent dispersed growth and to the presence of hard cell aggregates.

3.1.6.1.3 *B. vulgaris*

B. vulgaris callus with orange pigmentation, due to its most favorable accumulation of betanins and betacyanins at 280nm, betaxanthins at 477nm and 535nm, was selected for initiating the cell suspension culture. Specifically, two distinct *B. vulgaris* cultures, namely BR6 and BR8, were established from the same starting callus, differing slightly in pigmentation (BR6 exhibiting a brighter orange color, while BR8 a lighter orange compared to each other).

The BR6 cell suspension culture reached the peak of fresh weight at day 14 post sub-culture, followed by a no-statistically significant decline by day 21. Despite the increase in fresh weight, no significant variation in dry weight or dry matter content was observed throughout the subculture period (Figure

3.1.22). Conversely, the BR8 suspension culture exhibited peak of fresh weight at 21 days post-subculture, while dry matter progressively declined over time (Figure 3.1.23).

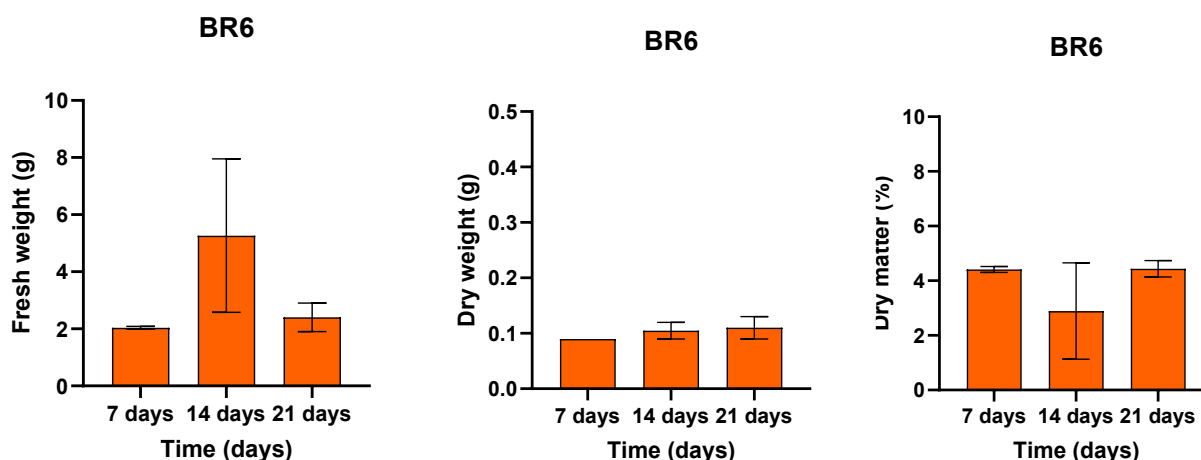


Figure 3.1.22. Study of the growth kinetics of *B. vulgaris* suspension culture (BR6) by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%). The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

Time (days)	Fresh weight (g)			Dry weight (g)			Dry matter (%)		
	Sample 1	Sample 2	Mean $\pm$ SD	Sample 1	Sample 2	Mean $\pm$ SD	Sample 1	Sample 2	Mean $\pm$ SD
7	2,09	1,99	2,04 $\pm$ 0,07	0,09	0,09	0,09	4,30	4,55	4,41 $\pm$ 0,15
14	7,95	2,58	5,27 $\pm$ 3,8	0,09	0,12	0,11 $\pm$ 0,02	1,13	4,65	2,89 $\pm$ 2,49
21	1,9	2,9	2,40 $\pm$ 0,21	0,13	0,09	0,11 $\pm$ 0,02	4,73	4,13	4,44 $\pm$ 0,42

Table 3.1.12. Study of the growth kinetics of *B. vulgaris* suspension culture (BR6) by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%).

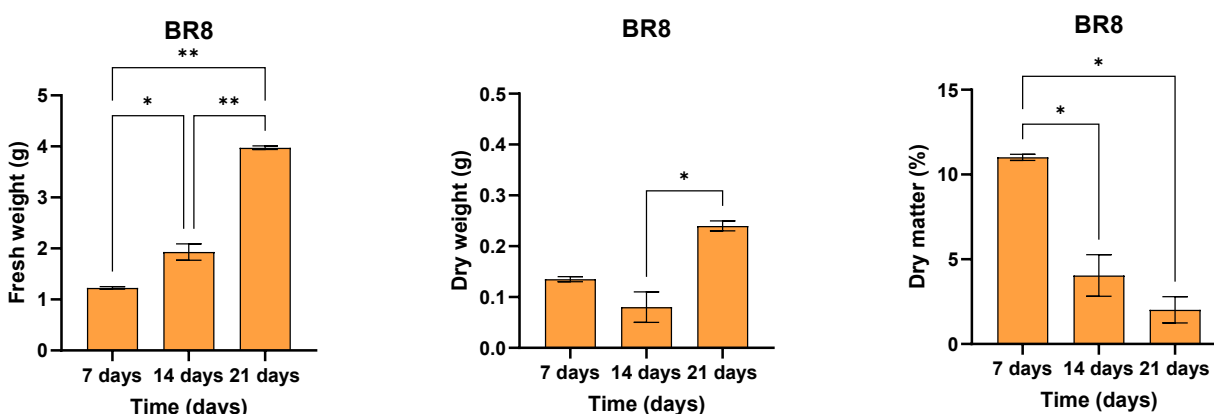


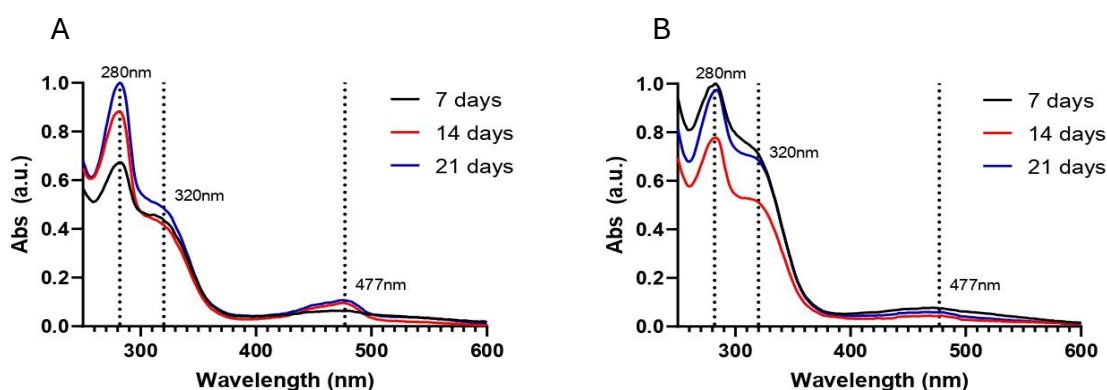
Figure 3.1.23. Study of the growth kinetics of *B. vulgaris* suspension culture (BR8) by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%). The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001)

comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001).

Time (days)	Fresh weight (g)			Dry weight (g)			Dry matter (%)		
	Sample 1	Sample 2	Mean $\pm$ SD	Sample 1	Sample 2	Mean $\pm$ SD	Sample 1	Sample 2	Mean $\pm$ SD
7	1,25	1,20	1,23 $\pm$ 0,03	0,14	0,13	0,14 $\pm$ 0,01	11,2	10,83	11 $\pm$ 0,3
14	2,09	1,77	1,93 $\pm$ 0,22	0,11	0,05	0,08 $\pm$ 0,04	5,26	2,82	4,04 $\pm$ 1,72
21	3,94	4,01	3,98 $\pm$ 0,04	0,25	0,23	0,24 $\pm$ 0,01	2,79	1,24	2,02 $\pm$ 1,09

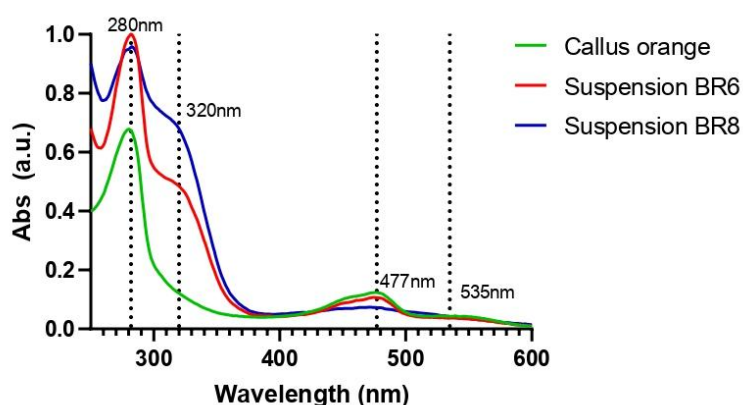
Table 3.1.13. Study of the growth kinetics of *B. vulgaris* suspension culture (BR8) by measuring the parameters fresh weight (g), dry weight (g) and dry matter (%).

The UV-visible spectra of the hydroalcoholic extracts for samples collected throughout the subculture cycle (7 – 21 days), revealed spectral profiles qualitatively similar to those of the starting callus, with characteristic absorption peaks at 280nm and 477nm, along with an additional peak at 320nm, indicative of hydroxycinnamic acids. In BR6 cell culture, extracts obtained at 21 days exhibited peaks with a higher absorbance value, suggesting enhanced metabolite accumulation (Figure 3.1.24A). In contrast, BR8 extracts at 7 and 21 days showed comparable spectral profiles, with slightly higher absorbance observed in the extract at 7 days (Figure 3.1.24B). These spectra were compared both to each other and to that of the starting callus culture (Figure 3.1.26) highlighted key differences: both cultures exhibited similar absorbance at 280nm (associated with betaxanthins and betacyanins), while BR6 extracts showed a greater absorbance at 477nm (betaxanthins). Conversely the BR8 extracts have a higher hydroxycinnamic acids peak at 320nm. Both suspension cultures spectra exhibit higher absorbance compared to callus spectrum (Figure 3.1.25).



	Abs <i>B. vulgaris</i> (BR6)			Abs <i>B. vulgaris</i> (BR8)		
Wavelength	7 days	14 days	21 days	7 days	14 days	21 days
280nm	0,66	0,88	1,51	1,44	0,77	0,95
320nm	0,43	0,41	0,73	1,03	0,51	0,68
477nm	0,06	0,09	0,16	0,11	0,04	0,05

Figure 3.1.24. UV-visible spectrum along the growth kinetics of *B. vulgaris* suspension culture. BR6 (A) and BR8 (B) suspension cell cultures. The absorbance values reported in the table refer to 1:10 dilute samples.



Wavelength	Abs <i>B. vulgaris</i>		
	Callus orange	BR6 21days	BR8 7days
280nm	1,03	1,51	1,44
320nm	0,18	0,73	1,03
477nm	0,18	0,16	0,11
535nm	0,06	0,05	0,06

Figure 3.1.25. Comparison of the absorbances of extracts of *B. vulgaris* suspension culture (BR6 and BR8) and callus under the best condition. The absorbance values reported in the table refer to 1:10 diluted samples.

A. tricolor, despite being able to produce a highly friable callus, did not generate sufficient suspension biomass (data not shown) and, therefore, was not considered for further analysis. This species had been, indeed, initially considered in parallel with *B. vulgaris* to ensure cell culture-based betacyanin production from at least one of the two. Given the successful establishment of *B. vulgaris* cultures and their betacyanin production, the exclusion of *A. tricolor* did not compromise the objectives of this study.

3.1.7 Metabolomic analysis of *P. frutescens* cultures under different growth conditions

An untargeted metabolomic approach was applied to the hydroethanolic extracts of *Perilla frutescens* callus culture in the four most promising conditions previously identified. A total of 3,326 m/z features were identified. The results, summarized in Table 3.1.13, include the 19 most abundant metabolites, highlighting the bioactive compounds with the highest accumulation levels. Figure 3.1.26 presents the heatmap of the 19 identified differential metabolites, while figure 3.1.27 displays a histogram highlighting the most abundant compounds among them.

Name	Dark		Red		Blue		Red-Blue	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD
N-Caffeoylputrescine	281,459	1,402	147,982	1,667	193,276	3,092	186,824	6,536
Leucine	46,323	1,207	26,910	0,807	20,811	0,442	34,086	0,469
MDN-0171	31,495	0,369	29,579	0,334	30,781	0,256	26,169	0,246
Acetophenone (fragment of tyrosine)	25,530	0,864	24,126	0,409	20,314	0,252	29,054	1,197
Le-pyrrolopyrazine B	28,819	0,370	28,736	0,523	20,308	0,531	21,696	0,311
Plumerubroside	25,806	0,507	18,296	0,845	12,420	0,109	27,268	0,947
Pyrrolam B	18,856	0,355	18,460	0,494	15,624	0,472	22,396	0,507
L-Pyroglutamic acid	21,178	0,532	8,942	0,118	11,023	0,076	8,001	0,315
L-Glutamine	20,896	0,977	15,751	1,188	11,761	0,154	9,365	3,712
Grossamide	12,602	0,094	12,670	0,609	16,140	0,727	8,097	0,430
Cefdinir-LIKE	14,158	0,420	6,769	0,091	6,401	0,071	9,569	0,114
Acetophenone (FRAGMENT)	14,252	0,102	3,593	0,192	5,772	0,353	5,337	0,211
CURCULIGOSIDE- LIKE C22H26O11 M+HAC-H	13,850	0,140	8,561	0,030	6,886	0,093	8,177	0,152
Pyrrolam B	11,619	0,708	13,550	0,451	10,841	0,536	11,534	0,430
Tyramine	9,619	0,151	5,133	0,174	4,493	0,014	11,261	0,329
2,3-Octadiene-5,7- diyn-1-ol	8,716	0,309	9,620	0,199	8,175	0,344	8,892	0,153
Agrocybyne A	8,716	0,309	9,620	0,199	8,175	0,344	8,892	0,153
Foligorubine A	8,595	0,377	8,364	0,096	9,464	0,163	8,655	0,209
Chlorogenic acid	8,595	0,377	8,364	0,096	9,464	0,163	8,655	0,209

Table 3.1.13. List of 19 most abundant metabolites identified by untargeted metabolomics in *Perilla frutescens* (hydrophylic extract). Quantification is based on peak area.

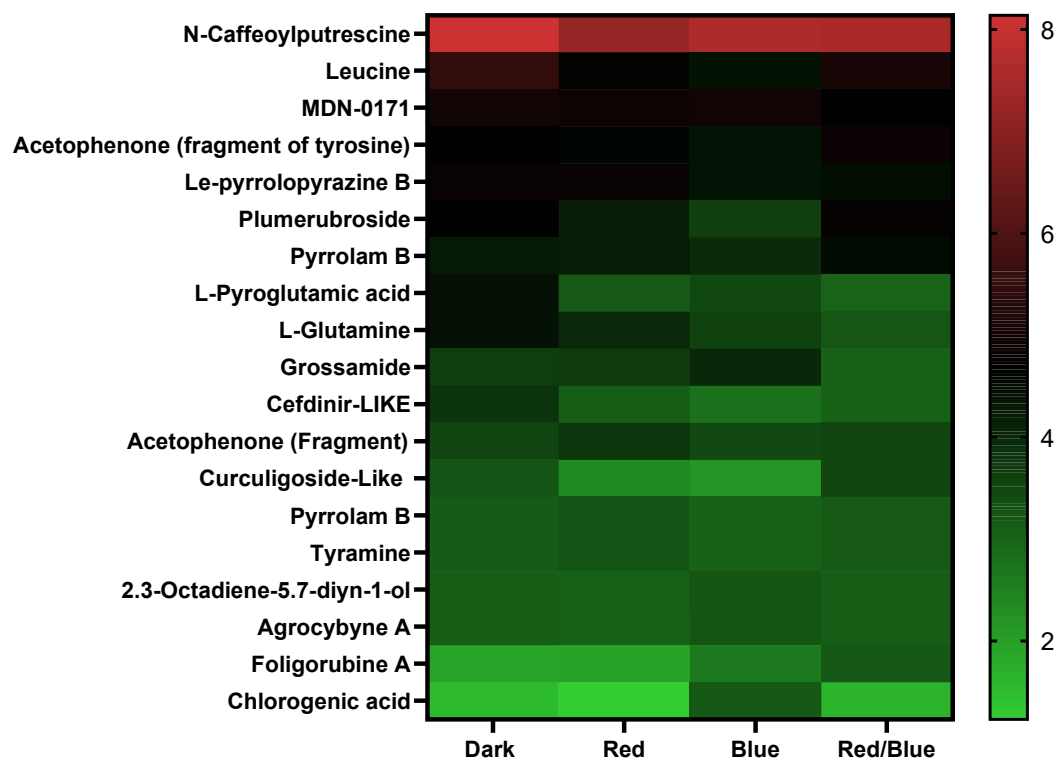


Figure 3.1.26. Heatmap of untargeted LC-HRMS analysis of metabolites across the four different light and media conditions in *P. frutescens* callus cultures. Data are expressed as log₂ of the mean from three technical replicates, calculated from relative abundances normalized to the internal standard (Fold IS, Daidzein). Red and green colors refer to significantly up- and down-regulated metabolites, respectively

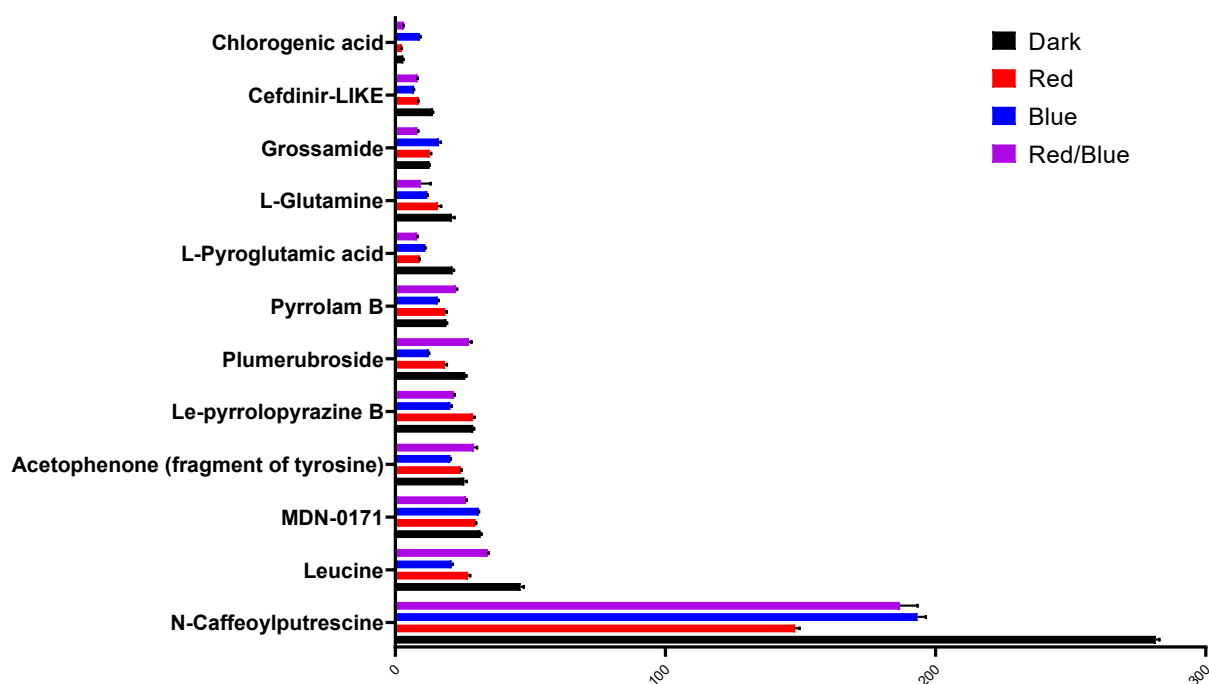


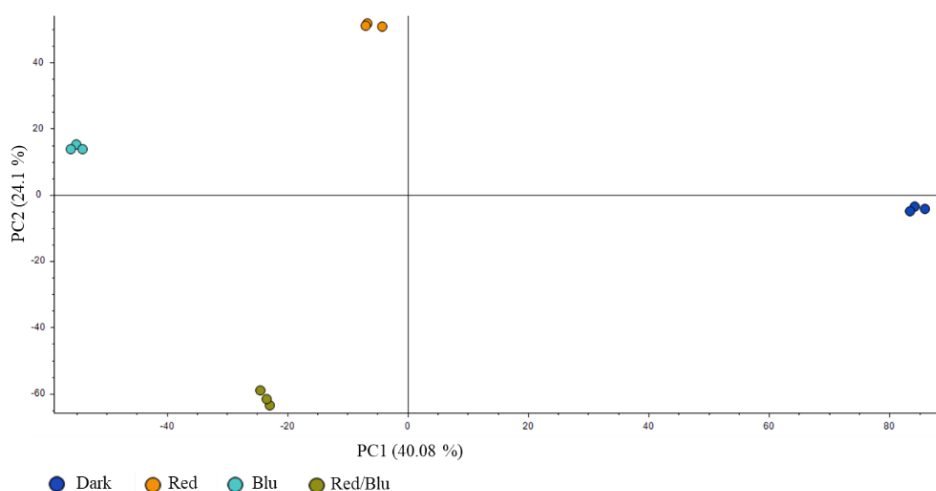
Figure 3.1.27. Histogram showing the most abundant compounds among the top 19 metabolites detected through untargeted metabolomics.

From the untargeted metabolomic analysis of callus cell cultures, several metabolites emerged. The most abundant compound is N-caffeoylputrescine, with maximum concentrations detected in the

culture grown on dark condition, followed in decreasing order by blue, red/blue (R/B) and red. Among the phenolic acids, chlorogenic acid stands out, particularly abundant in the culture in blue conditions, followed by grossamide, a lignanamide also mainly concentrated in the blue condition. With regard to the amino acid profile, the most represented compounds are leucine, L-pyroglutamic acid (cyclic derivative of glutamate), and glutamine, with higher levels in the cultures grown in dark conditions. Among the phenolic derivatives, acetophenone, secondary product of tyrosine, represents the second most abundant compound after leucine, with peaks in the red/blue condition. In red light conditions, a marked accumulation of tyramine, another derivative of tyrosine, is evident.

A compound of particular note is MDN-0171, an analogue of medermicin, an antibiotic known for its antimicrobial properties. MDN-0171 represents the third most abundant metabolite after N-caffeoylputrescine and leucine, with comparable concentrations in *Perilla frutescens* cultures grown in blue light and dark conditions. The analysis also highlighted the presence of compounds structurally similar to Cefdinir, a β -lactam antibiotic, with greater abundance observed in cultures in dark conditions. Among the terpenoids, plumerubroside, an iridoid with potential antioxidant applications, stands out, more abundant in the Red/Blue condition. Finally, for the alkaloid class, pyrrolam B, a pyrrolizidine alkaloid, stands out, with the highest abundance also under red/blue.

Calli cultured under the four most favorable conditions for dry matter accumulation (i.e., medium + LED light elicitation + harvesting time) through metabolomic analysis highlighted a significant accumulation of the bioactive compound chlorogenic acid specifically in calli grown in T2 medium under blue LED light elicitation (Figure 3.1.28). Principal component analysis (PCA) clearly identified as separate the cell cultures grown under the four conditions.



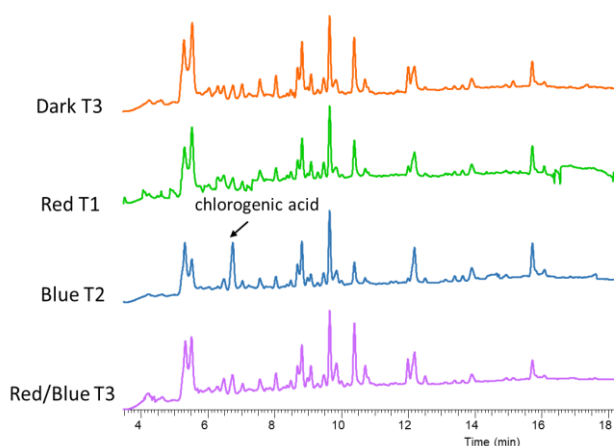


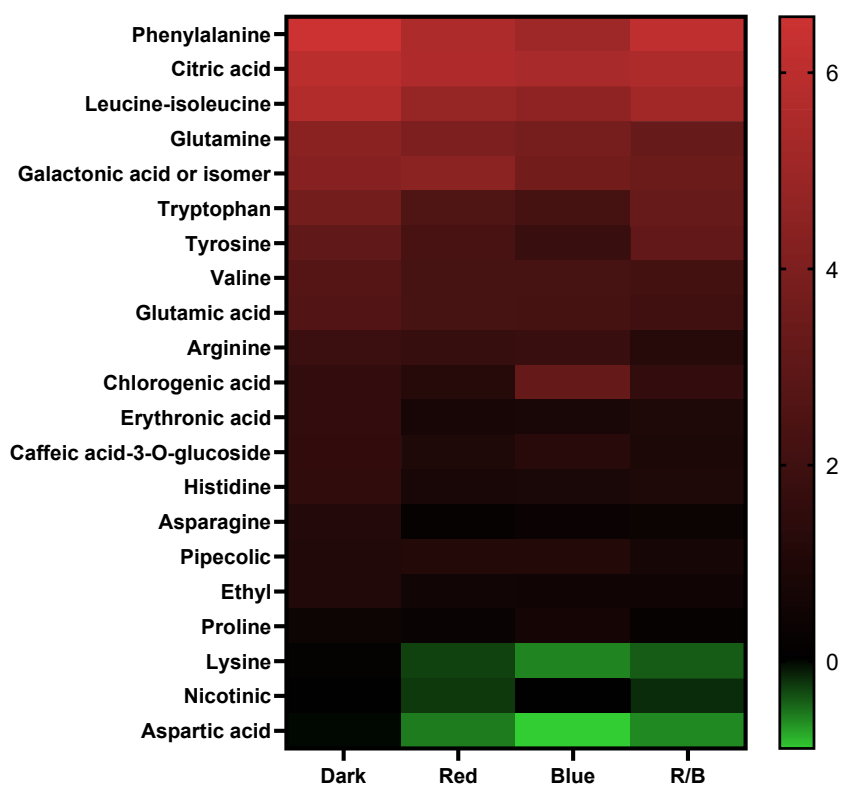
Figure 3.1.28. Metabolomic analysis of *P. frutescens* hydroalcoholic extracts by HPLC-DAD-ESI-HRMS followed by untargeted analysis (XCMS software). HPLC-DAD (full scan) of representative samples and principal component analysis (PCA) of untargeted metabolomics data.

For the targeted metabolomic analysis, a custom database was prepared, including a selection of compounds of interest, such as organic acids, vitamins, and phenylpropanoids, to guide the characterization of the hydroalcoholic extract of *Perilla frutescens*. The LC-HRMS analysis results are reported in Table 3.1.14, which lists all the identified and quantified metabolites across the four experimental conditions: dark, red, blue, and red/blue. Notably, the majority of these compounds showed higher accumulation levels in cell cultures grown under dark and red/blue light conditions. Figure 3.1.29 presents the heatmap of the identified differential metabolites, while figure 3.1.30 displays a histogram highlighting the most abundant compounds among them.

	Dark		Red		Blue		Red/Blue	
Name	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Phenylalanine	94.854	1.858	46.496	1.936	33.279	1.502	68.056	3.026
Citric acid	63.376	1.655	48.050	1.341	42.129	0.307	46.827	0.982
Leucine-isoleucine	52.285	1.528	27.772	0.913	23.736	0.279	35.831	0.641
Glutamine	21.785	0.210	16.125	0.781	13.586	0.017	9.839	0.458
Galactonic acid or isomer	19.612	0.125	22.274	0.254	12.300	0.245	10.936	0.375
Tryptophan	12.960	0.085	5.663	0.131	4.559	0.061	9.819	0.122
Tyrosine	8.367	0.194	5.043	0.129	3.477	0.019	8.648	0.222
Valine	6.511	0.127	4.758	0.148	4.733	0.068	4.492	0.075
Glutamic acid	6.156	0.247	4.727	0.218	4.669	0.086	4.075	0.266
Arginine	3.654	0.175	3.278	0.181	3.431	0.136	2.278	0.027
Chlorogenic acid	3.057	0.106	2.283	0.016	9.545	0.394	3.068	0.032
Erythronic acid	3.043	0.110	1.671	0.030	1.715	0.063	1.963	0.073
Caffeic acid-3- <i>O</i> -glucoside	2.969	0.045	1.980	0.081	2.454	0.060	1.902	0.044
Histidine	2.950	0.076	1.717	0.082	1.765	0.024	1.990	0.024
Asparagine	2.174	0.043	1.163	0.076	1.270	0.016	1.281	0.023
Pipecolic acid	2.127	0.005	2.220	0.058	2.207	0.005	1.595	0.045
Ethyl caffeate	2.095	0.045	1.468	0.014	1.409	0.022	1.399	0.022
Proline	1.332	0.039	1.243	0.099	1.564	0.041	1.129	0.037
Lysine	1.100	0.018	0.819	0.030	0.672	0.008	0.758	0.017
Nicotinic acid (VitB3)	0.995	0.064	0.842	0.024	1.025	0.118	0.874	0.024
Aspartic acid	0.976	0.025	0.690	0.080	0.540	0.014	0.661	0.037
Spermidine	0.925	0.044	0.846	0.030	0.912	0.014	1.040	0.038
Riboflavine (VitB2)	0.787	0.006	0.510	0.021	0.554	0.013	0.376	0.006

Threonine	0.766	0.022	0.520	0.033	0.621	0.016	0.488	0.040
Rosmarinic acid	0.508	0.006	0.057	0.003	0.022	0.001	0.068	0.003
Caffeic acid	0.414	0.009	0.152	0.005	0.340	0.005	0.151	0.008
Glucaric acid	0.407	0.012	0.423	0.027	0.363	0.014	0.364	0.016
Vicenin-2	0.370	0.026	0.210	0.003	0.198	0.005	0.386	0.013
Quinic acid	0.302	0.072	0.274	0.047	0.443	0.033	0.247	0.054
Catechin	0.121	0.044	0.038	0.004	0.069	0.010	0.052	0.002
Diosmetin	0.075	0.005	0.077	0.004	0.080	0.003	0.076	0.004
p-Hydroxy- benzoic acid	0.059	0.015	0.085	0.093	0.047	0.013	0.033	0.019
Shisoflavanone A	0.043	0.001	0.011	0.002			0.002	
Hesperetin	0.035	0.001	0.015	0.001	0.021	0.003	0.071	0.002
Esculetin	0.034	0.036	0.048	0.003	0.166	0.006	0.112	0.014
Ribose 2-deoxy	0.029	0.004	0.012	0.006	0.018	0.010	0.019	0.002
Isovanillic acid	0.014				0.009	0.006	0.012	0.002
3'-Dehydroxyl-rosmarinic acid-3-O-glucoside	0.009	0.002			0.004			
Perilloside E	0.009	0.001	0.004	0.000	0.005	0.001	0.007	0.001
4-Coumaric acid	0.002	0.000	0.004	0.001	0.002	0.002	0.004	0.001
Rutin					0.001		0.003	0.000
Vanillic acid			0.014	0.002	0.013	0.011	0.012	0.011

Table 3.1.14. Targeted metabolic analysis of *Perilla frutescens* callus culture hydrophilic extract. Data are expressed as relative abundances compared to the internal standard (Fold IS, Daidzein) and represent averages of three technical replicates for each condition.



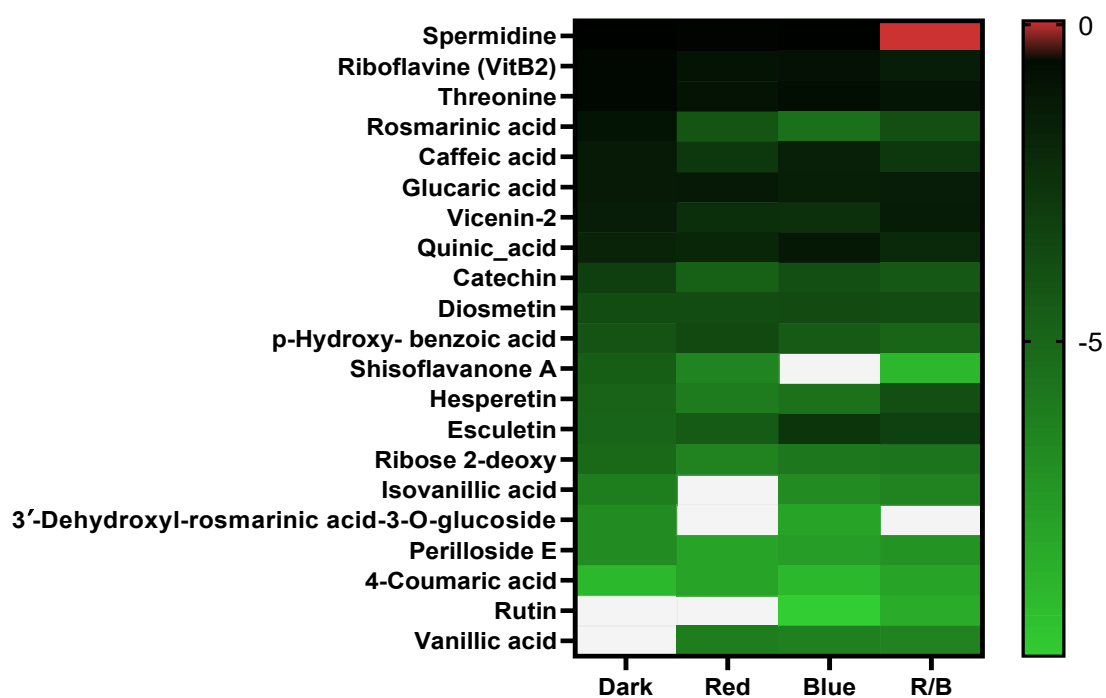


Figure 3.1.29. Heatmap of targeted LC-HRMS analysis of metabolites across the four different light and media conditions in *P. frutescens* callus cultures. Data are expressed as log₂ of the mean from three technical replicates, calculated from relative abundances normalized to the internal standard (Fold IS, Daidzein). Red and green colors refer to significantly up- and down-regulated metabolites, respectively.

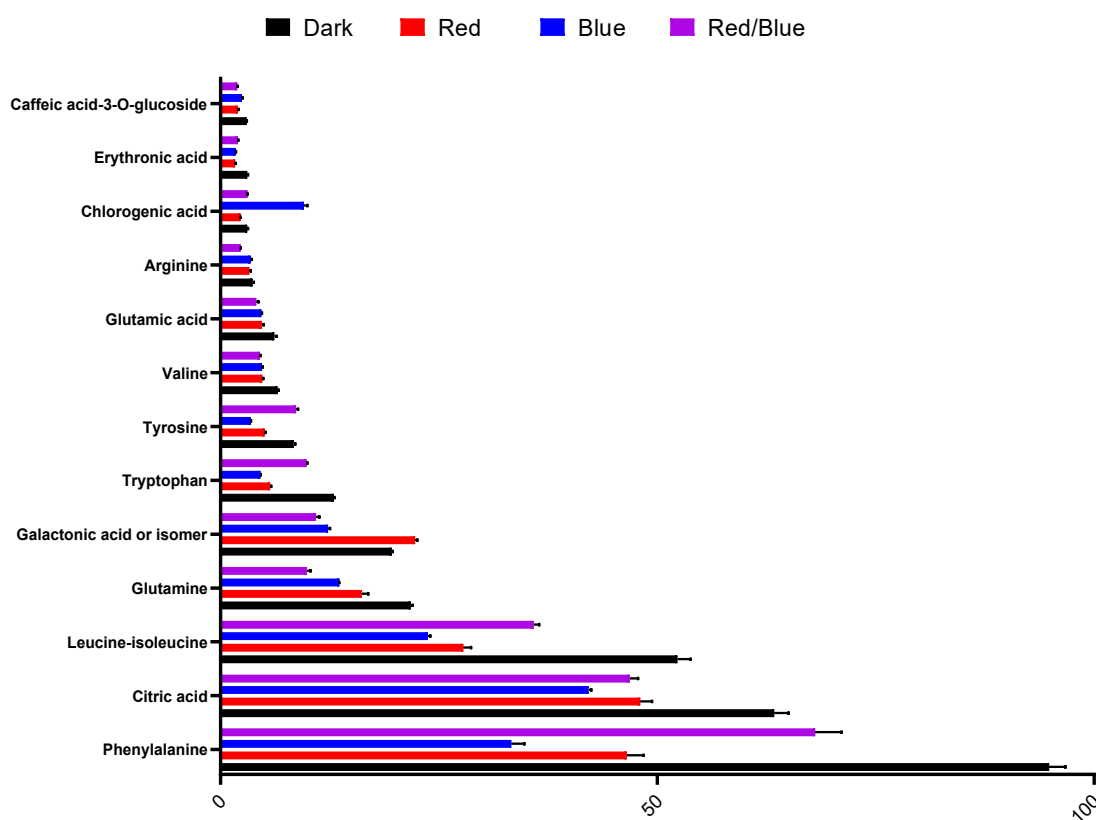


Figure 3.1.30. Histogram showing the most abundant compounds among the metabolites detected through targeted metabolomics.

From the targeted analysis of metabolites present in cell cultures, a clear predominance of some organic and phenolic compounds emerges, with significant variations depending on the light conditions and the growth medium. Among organic acids, citric acid stands out as the most abundant, especially in cultures maintained in dark conditions, followed by a high presence also in those exposed to red light. Galactonic acid is also significant, showing the highest concentration in red light conditions, while chlorogenic acid is more expressed in cultures grown under blue light.

Other phenolic acids and derivatives show a more marked accumulation in dark conditions, including erythronic acid, caffeic 3-O-glucoside, rosmarinic acid, caffeic acid and its ethyl ester (ethyl caffeate). The amino acid profile reveals that phenylalanine is the most abundant amino acid overall, with a significant peak in cultures grown in the dark, followed by a high presence also in the red/blue condition. Following this, leucine and isoleucine are also highly accumulated in the dark followed by the red/blue condition. Glutamine and tryptophan also show a prevalence in the dark, followed by the red/blue condition, while tyrosine is mainly concentrated under red/blue light. Finally, in the vitamin compartment, a clear predominance of riboflavin (vitamin B2) is observed in dark condition, while nicotinic acid (vitamin B3) is more abundant in cultures grown under blue light.

An untargeted metabolomic approach was applied to the hydroethanolic extracts of *Perilla frutescens* suspension culture under different conditions: *P. frutescens* blue T2 (best condition obtained from callus culture), *P. frutescens* dark T2 (control) and *P. frutescens* grown under standard condition (2,4-D + BAP). The results, summarized in Table 3.1.15, include the 19 most abundant metabolites, highlighting the bioactive compounds with the highest accumulation levels. Data are expressed as relative abundances compared to the internal standard (Fold IS, Daidzein) and represent averages of three technical replicates for each condition. Quantification is based on peak area.

Name	PE Blue T2		PE Dark MS2,4D-BAP		PE DARK T2	
	AVG	SD	AVG	SD	AVG	SD
Feruloylputrescine	163,909192	0,908557298	213,1213308	2,237042302	99,01283966	1,623251762
N-Caffeoylputrescine	96,83681305	1,925146089	139,4365374	3,505164746	87,37745013	2,072739606
L-Phenylalanine	60,98470667	0,38039987	71,42507255	2,32286041	112,9323849	3,337992617
p-Coumaroylputrescine	78,23047598	1,178447167	62,56665124	0,889859209	66,71515001	1,037842341
Tyramine	55,72203753	0,489451821	65,62191221	2,087114054	25,07460709	1,107510974
L-Norleucine	45,44119696	0,510738308	49,83368943	0,94284978	34,9310682	0,876974591
Citric acid	6,636415565	0,060153272	20,32310113	0,476832757	33,21881298	0,818610404
DL-Glutamine	23,83874182	0,320762308	4,138341056	0,088117828	1,882741623	0,071634169
gamma-Glutamyltyramine	11,67054208	0,062895109	22,41666388	0,078188758	11,71230284	0,252357698
NP-008309	10,99856765	0,06004495	16,52140235	0,401114388	17,9598316	0,766339828
trans-Cinnamoyl beta-D-glucoside	15,01369227	0,150692634	0,116115078	0,00037716	3,643800664	0,079112161
D-(+)-Mannose	10,37915445	0,209679245	14,59953683	0,283013082	2,830587149	0,023559089
L-Pyroglutamic acid	13,95586004	0,113860199	0,9903761	0,095585699	1,046423479	0,012376003
Hopantenic acid	10,8582246	0,31279289	0,060968031	0,003830379	2,73162688	0,029708059
1-hexadecyl-2-amino-2-deoxy-sn-glycerol	9,900684702	0,261807538	8,96403619	0,23686214	9,99635836	0,360887546
Citric acid	3,296994679	0,050951275	6,230008419	0,367104293	10,44836164	0,280245331

2,4,6-Octatriyn-1-ol	7,071229705	0,057235622	9,153868045	0,080294191	6,186668045	0,163894574
1-hexadecyl-2-amino-2-deoxy-sn-glycerol	8,496866548	0,221444937	7,420616596	0,175654939	8,339451879	0,352614217
4-Methylumbelliferone	6,467510141	0,10936276	8,158367093	0,250780304	3,806365783	0,022565381
Chlorogenic acid	8,021372972	0,219752985	5,303520225	0,105943689	4,624714991	0,194941297

Table 3.1.15. List of 19 most abundant metabolites identified by untargeted metabolomics in *Perilla frutescens* suspension cultures (hydrophylic extract). Quantification is based on peak area.

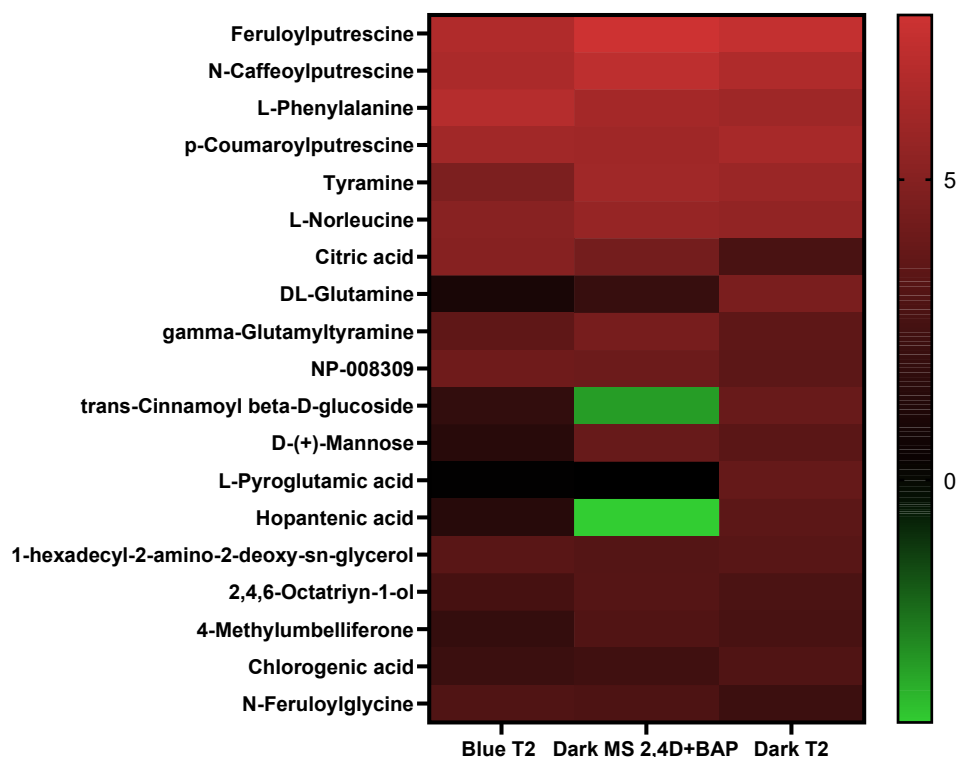


Figure 3.1.31. Heatmap of untargeted LC-HRMS analysis of metabolites across the four different light and media conditions in *P. frutescens* suspension cultures. Data are expressed as log₂ of the mean from three technical replicates, calculated from relative abundances normalized to the internal standard (Fold IS, Daidzein). Red and green colors refer to significantly up- and down-regulated metabolites, respectively.

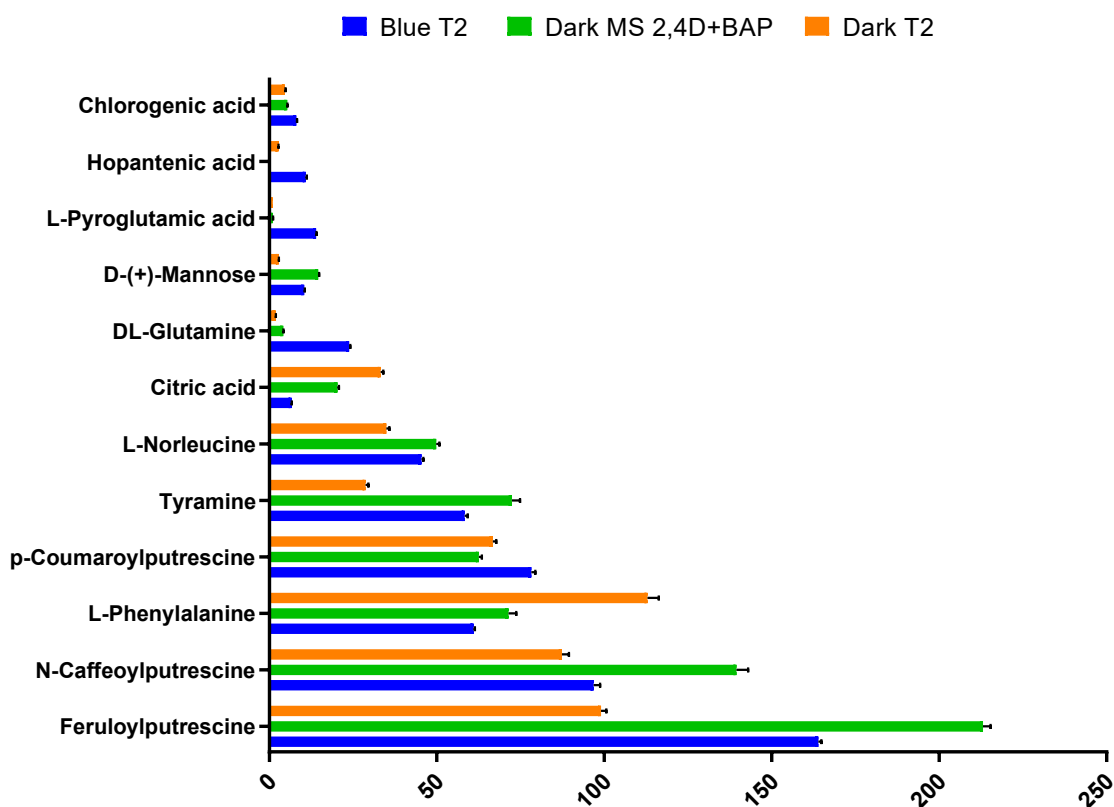


Figure 3.1.32. Histograms show the most abundant compounds among the metabolites detected through untargeted metabolomics.

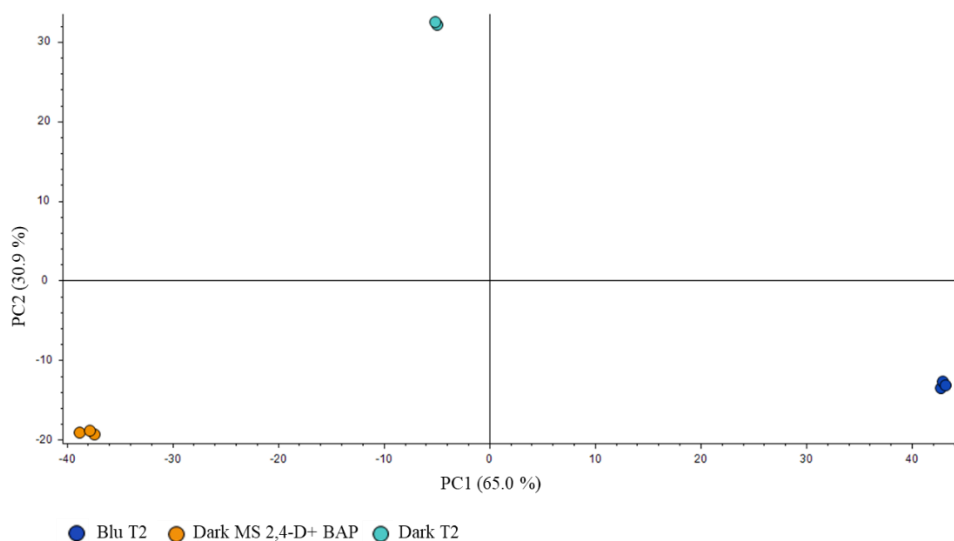


Figure 3.1.33. Metabolomic analysis of *P. frutescens* hydroalcoholic extracts by HPLC-DAD-ESI-HRMS followed by untargeted analysis (XCMS software). HPLC-DAD (full scan) of representative samples and principal component analysis (PCA) of untargeted metabolomics data.

Untargeted analysis of cell suspensions revealed a distinct metabolic profile compared to callus, with specific variations depending on light conditions. In particular, in standard conditions in the absence

of light (MS with 2,4-D + BAP), a marked accumulation of feruloylputrescine and N-caffeoylputrescine (the two most abundant compounds), as well as tyramine and D-mannose, was observed. Elicitation with blue light instead determined an increase in p-coumaroylputrescine, D-glutamine, pyroglutamic acid, hopantenic acid and chlorogenic acid. Finally, in darkness with T2 medium, an increase in phenylalanine and citric acid was observed.

For the targeted metabolomic analysis of *P. frutescens* suspension culture, a custom database was prepared, including a selection of compounds of interest, such as organic acids, vitamins, and phenylpropanoids, to guide the characterization of the hydroalcoholic extract. The LC-HRMS analysis results are reported in Table 3.1.16, which lists all the identified and quantified metabolites across the three experimental conditions (previously described for the untargeted analysis). Data are expressed as relative abundances compared to the internal standard (Fold IS, Daidzein) and represent averages of three technical replicates for each condition. Figure 3.1.31 presents the heatmap of the identified differential metabolites, while figure 3.1.32 displays a histogram highlighting the most abundant compounds among them.

Name	PE Blue T2		PE Dark MS2,4D-BAP		PE DARK T2	
	AVG	SD	AVG	SD	AVG	SD
spermidine	2,946991737	0,395438117	13,8647204	0,629552884	2,79989388	0,225173594
arginine	28,94746147	0,664911931	23,2632588	0,331362195	13,3747443	0,363415984
asparagine	13,11018294	4,169207596	7,11024425	0,309309143	12,8807335	6,451562996
aspartic acid	0,369866742	0,090971541	0,38012568	0,081479056	0,56450518	0,024903915
cysteine	0	0	0,07415851	0,01803225	0	0
glutamic acid	40,03075115	1,323583753	11,5225368	0,523019368	16,5046524	0,958654961
glutamine	35,27731002	21,92684781	13,6407556	6,267679613	5,39043381	3,730245896
glycine	0	0	0	0	0	0
histidine	11,19542199	4,614053375	17,9888335	2,898182981	10,8312477	6,917326212
leucine-isoleucine	103,7326225	115,8153819	261,823657	4,892631933	185,996351	9,582788519
lysine	2,196345925	2,10306734	5,18983428	3,082536218	1,54605031	1,973916037
phenylalanine	287,5817408	73,38866957	352,850532	12,63918315	551,454003	25,14255421
pipecolic acid	3,53321316	0,536726957	5,15159475	0,253158383	2,84192171	0,899687637
proline	3,45570525	0,108616778	5,24407981	0,23544086	1,88479572	0,688087113
pyruvic acid	131,0715115	2,873529974	13,4531113	0,533714113	9,25241867	0,120876612
threonine	4,988027785	0,256307697	4,21894921	0,231372717	2,54488555	0,13464144
tryptophan	47,70438705	0,238782078	34,7806347	0,569875022	78,6864948	0,80857142
tyrosine	21,98087105	0,233923005	24,3777173	0,448335408	29,0231019	3,538215145
valine	18,99456929	0,979520869	31,9864551	0,519366482	15,4096801	0,410925708
Esculetin	0,091889051	0,083288169	0,03965851	0,044493569	0,09744068	0,168772215
diosmetin	0,474761598	0,040709811	0,45186019	0,039244532	0,49113906	0,001874839
Hesperetin	0	0	0	0	0	0
Rutin	0	0	0	0	0	0
Citric acid	47,07260237	0,767187964	128,836332	1,521539494	210,254262	4,055338195
erythronic acid	11,27429852	4,517798774	17,9556406	2,897675818	10,8977765	6,759229557
galactonic acid or isomer	7,393133447	0,051281466	5,07709771	0,089327725	4,29448152	0,21026346
glucaric acid	1,255964981	0,050369027	0,80170375	0,047706155	1,17815109	0,035493593
isovanillic acid	0	0	0	0	0	0
quinic acid	0,854599577	0,056392814	0,39804025	0,034327025	0,27305493	0,022932123
sinapic acid	0,011893933	0,003852935	0,00214882	0,003721873	0	0
vanilic acid	0,094201326	0,071402688	0,02102856	0,021367814	0,03280927	0,056827334
3'-dehydroxyl-	0	0	0,00536742	0,009296659	0,00670797	0,011618551

rosmarinicacid-3-O-glucoside						
4-coumaric acid	0,04120013	0,071360718	0,01369018	0,023712088	0,02153597	0,037301402
Caffeic acid	1,646596857	0,042642834	0,80395570	0,037433482	0,40848467	0,013446119
Caffeic acid-3-O-glucoside	2,888644235	0,082539029	10,8265655	0,45686735	4,36421238	0,067612863
Chlorogenic acid	16,94102675	0,427971847	12,1157498	0,142186641	9,63323177	0,245884644
p-Hydroxybenzoic acid	0,391353259	0,275698975	0	0	0,06068151	0,086754864
p-coumaric acid	2,048342422	0,055244484	3,45575918	0,091135392	3,13222641	0,044671775
shikimic acid	0,017123574	0,004650694			0,00907273	0,001841486
ribose_2-deoxy-	0	0			0	0
nicotinic acid (VitB3)	0,028732185	0,033110065			0,12635777	0,001171942
pantothenic acid (VitB5)	13,8718128	0,123317989			5,38819122	0,590724849
riboflavin (VitB2)	4,190715554	0,057621536			6,14255388	0,100632679

Table 3.1.15. Targeted metabolic analysis of *Perilla frutescens* suspension culture hydrophilic extract. Data are expressed as relative abundances compared to the internal standard (Fold IS, Daidzein) and represent averages of three technical replicates for each condition.

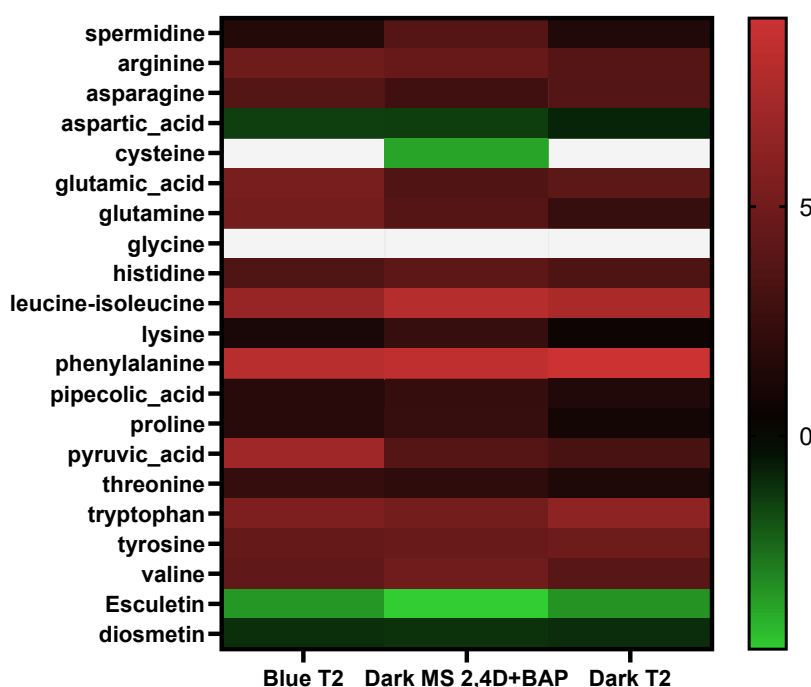
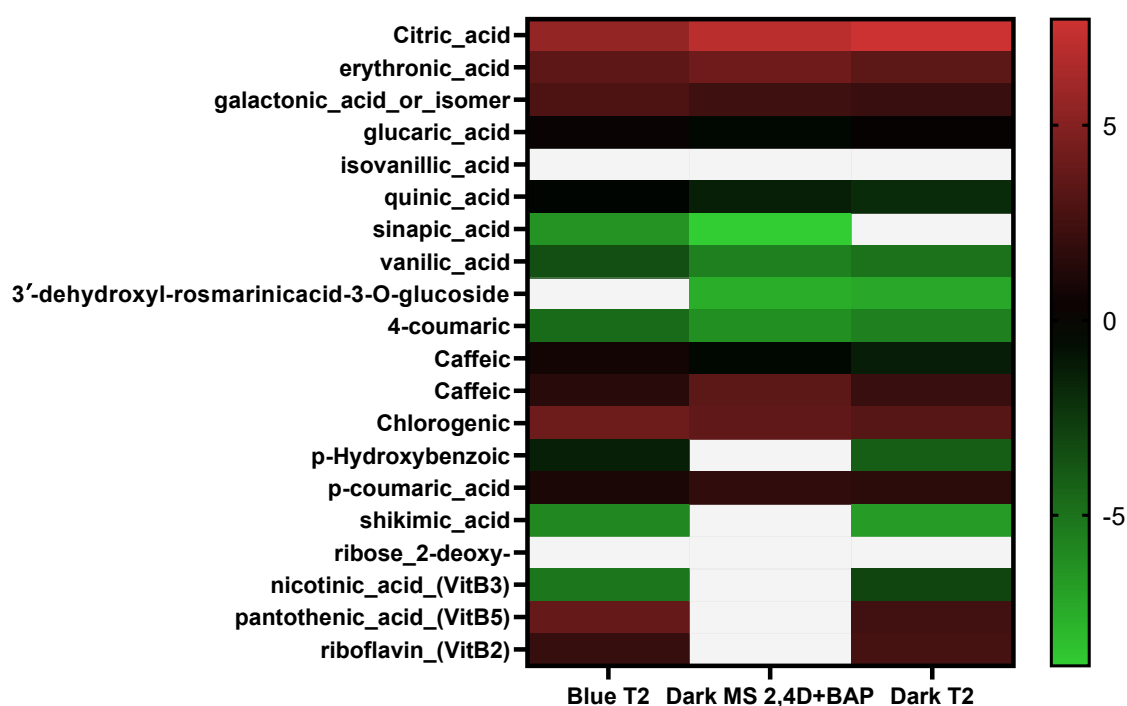


Figure 3.1.31. Heatmap of targeted LC-HRMS analysis of metabolites across the four different light and media conditions in *P. frutescens* suspension cultures. Data are expressed as $\log_2$ of the mean from three technical replicates, calculated from relative abundances normalized to the internal standard (Fold IS, Daidzein). Red and green colors refer to significantly up- and down-regulated metabolites, respectively.

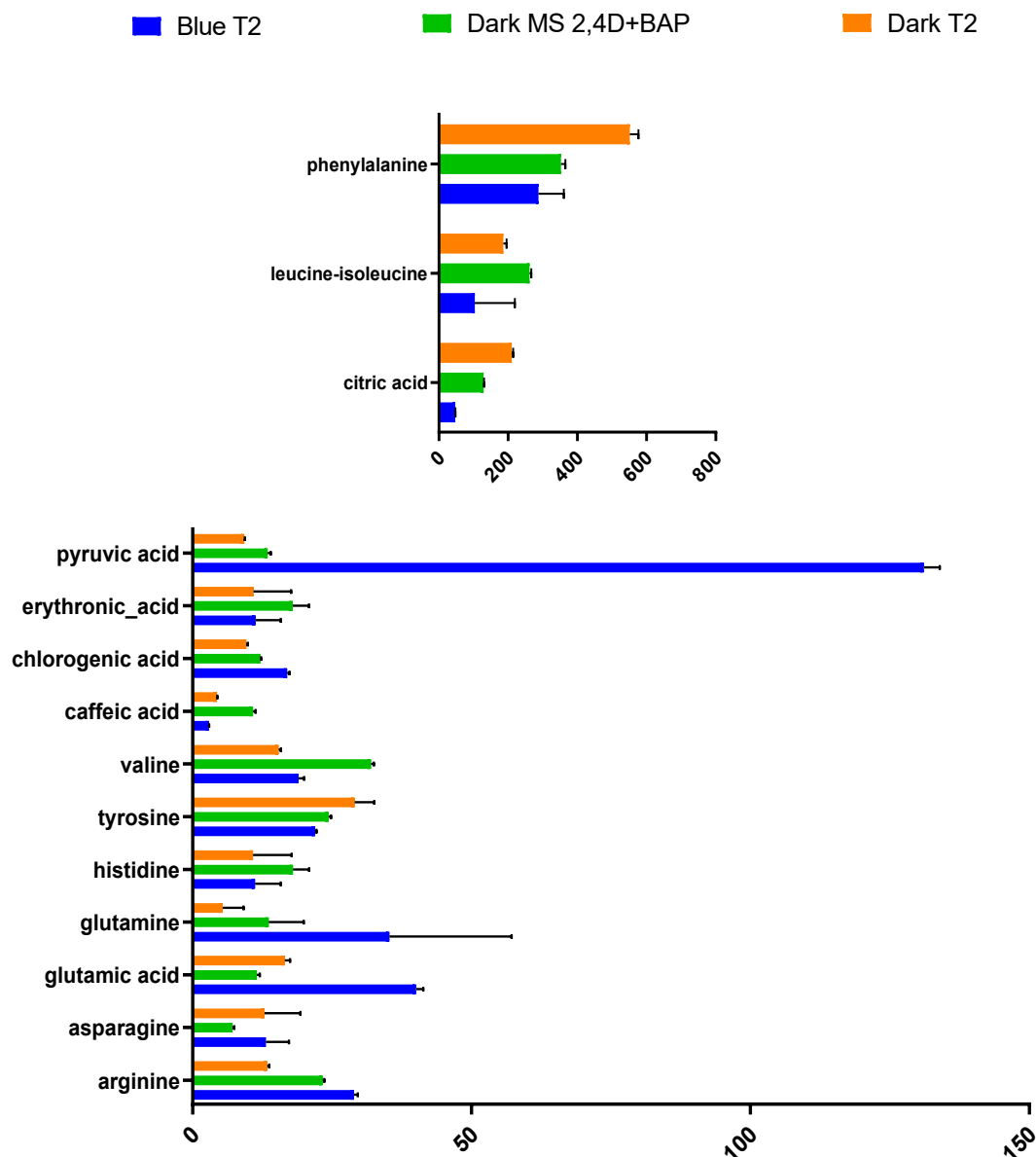


Figure 3.1.32. Histograms show the most abundant compounds among the metabolites detected through targeted metabolomics.

The targeted analysis conducted on the *P. frutescens* blue T2, dark T2 and dark MS 2,4-D + BAP cell suspensions revealed significant differences between the different experimental conditions.

In particular, the most abundant organic acid was found to be citric acid, with a higher concentration in dark T2 sample. Chlorogenic acid followed in abundance, particularly present in the suspension grown under blue light condition. Erythronic acid was also detected, especially in the dark MS 2,4-D

+ BAP sample, while caffeic acid 3-O-glucoside was the fourth most abundant compound, particularly in dark MS 2,4-D + BAP.

With regard to amino acids, the most abundant are phenylalanine, leucine-isoleucine, tryptophan, pyruvic acid, followed by tyrosine, valine, glutamic acid, glutamine, asparagine, arginine and spermidine. In particular phenylalanine, tryptophan and tyrosine are more abundant in dark T2 sample, leucine-isoleucine, valine and spermidine has the highest concentration in the dark MS 2,4D + BAP, while pyruvic acid, glutamic acid, glutamine, arginine and asparagine are more concentrated in the blue T2 condition.

As for vitamins, a significant presence of nicotinic acid (vitamin B3) is highlighted, especially in blue T2 condition. Pantothenic acid (vitamin B5) follows with an increase in the dark T2 sample.

3.1.8 Antioxidant activity and total polyphenols of plant cell cultures

The DPPH assay was conducted to assess the antioxidant capacity of methanolic extracts obtained from both callus and suspension culture under optimal dry matter conditions for *P. frutescens*. Suspension cultures exhibited the highest antioxidant activity and the greatest accumulation of polyphenols in both species compared to the callus cultures (Figure 3.1.33).

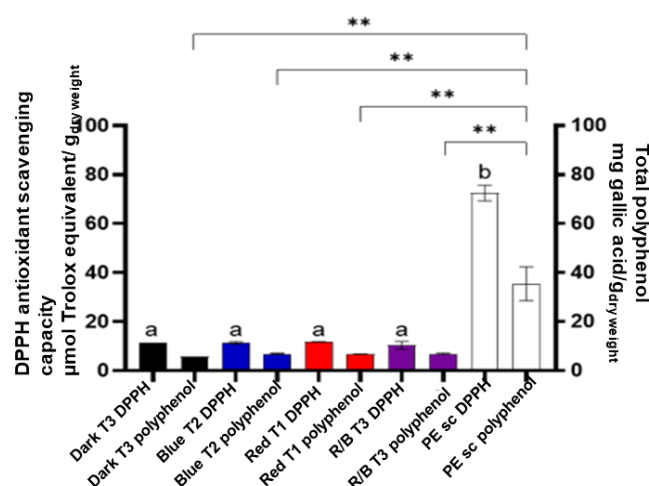


Figure 3.1.33. DPPH antioxidant capacity and total polyphenols on the best samples of *P. frutescens*. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001) for total polyphenols. Letters indicate statistically significant differences (p-value ≤ 0.001) related to DPPH.

3.1.9. Study on the replacement or substitution of synthetic plant growth regulators in the culture media

3.1.9.1 Casein hydrolysate and yeast extract as plant growth regulators substitutes in *P. frutescens* callus culture

Casein hydrolysate and yeast extract were evaluated as potential substitutes for synthetic plant growth regulators contained in the recipes of the media traditionally used for the culture of plant cell cultures (Hakkinen et al., 2020; Al-Khayri 2011). Casein hydrolysate supplies essential nutrients and growth factors, providing a natural alternative to synthetic regulators, while yeast extract, which contains plant growth regulators, such as auxins and cytokinins, can promote callus growth.

A first study assessed *Perilla frutescens* callus growth in media supplemented with varying concentration of casein hydrolysate and yeast extract under dark condition. Growth parameters were measured in terms of fresh weight (at day 14, 21 and 28 from sub-culture), dry weight (day 21) and dry matter (at 21 days). The measurements of dry weight and dry matter were assessed at 21 days as this time point corresponded to the peak of dry matter accumulation of *P. frutescens* under standard condition (see Table 3.1.3). Control data were also included, corresponding to callus growth in standard medium (containing 0.22mg/L 2,4-D) and in a medium without plant growth regulators, casein hydrolysate, and yeast extract (Table 3.1.15 and Figure 3.1.34).

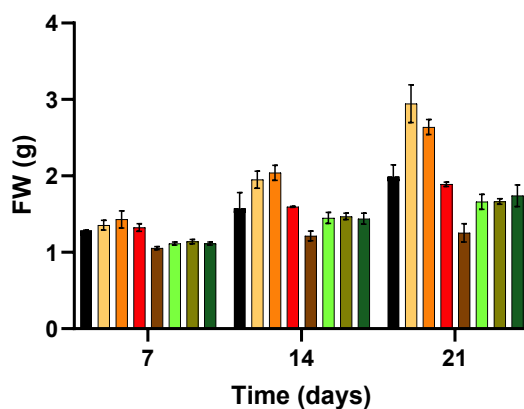
Time (days)	Fresh weight (g)											
	MS (g)			MS+2,4-D (g)			MS+ casein 0,5g/L			MS+ casein 1 g/L		
	Sample 1	Sample 2	Mean±S D	Sample 1	Sample 2	Mean±S D	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±S D
7	1,28	1,29	1,29±0,07	1,31	1,40	1,35±0,06	1,51	1,35	1,43±0,11	1,29	1,36	1,32±0,04
14	1,72	1,43	1,57±0,2	1,87	2,03	1,95±0,11	2,11	1,97	2,04±0,1	1,59	1,60	1,59±0,01
21	2,10	1,88	1,99±0,15	2,77	3,12	2,95±0,24	2,71	2,57	2,64±0,1	1,87	1,91	1,89±0,02
Time (days)	MS+ casein 2 g/L			MS+Yeast extract 1g/L			MS+ Yeast extract 2,5g/L			MS+ Yeast extract 5g/L		
	Sample 1	Sample 2	Mean±S D	Sample 1	Sample 2	Mean±S D	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±S D
	1	2	D	1	2	D	1	2	Mean±SD	1	2	D
7	1,04	1,07	1,05±0,03	1,10	1,13	1,12±0,02	1,12	1,16g	1,14±0,02	1,1	1,13g	1,12±0,02
14	1,17	1,26	1,21±0,06	1,50	1,40	1,45±0,07	1,50	1,44g	1,47±0,04	1,49	1,39g	1,44±0,07
21	1,17	1,34	1,25±0,12	1,73	1,59	1,66±0,09	1,69	1,64g	1,66±0,03	1,84	1,64g	1,74±0,14

	Dry weight (g)											
Time (days)	MS (g)			MS+2,4-D (g)			MS+ casein 0,5g/L			MS+ casein 1 g/L		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
21	0,13	0,15	0,14±0,01	0,13	0,15	0,14±0,01	0,16	0,15	0,16±0,01	0,11	0,11	0,11
Time (days)	MS+ casein 2 g/L			MS+Yeast extract 1g/L			MS+ Yeast extract 2,5g/L			MS+ Yeast extract 5g/L		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
21	0,07	0,08	0,08±0,01	0,13	0,11	0,12±0,01	0,11	0,11	0,11	0,16	0,11	0,14±0,03

	Dry matter (%)											
Time (days)	MS (g)			MS+2,4-D (g)			MS+ casein 0,5g/L			MS+ casein 1 g/L		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
21	4,7	4,8	4,75±0,07	4,6	4,8	4,75±0,14	6	5,9	5,87±0,07	5,8	5,7	5,75±0,07
Time (days)	MS+ casein 2 g/L			MS+Yeast extract 1g/L			MS+ Yeast extract 2,5g/L			MS+ Yeast extract 5g/L		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
21	5,85	5,9	5,9±0,07	7,82	7,2	7,51±0,42	6,7	6,9	6,8±0,14	8,9	7	7,9±1,3

Table 3.1.15. Evaluation of fresh weight (FW), dry weight (DW) and dry matter (DM) in duplicate. Mean and standard deviation were calculated for each parameter.

■ MS ■ Cas 0.5g/L ■ Cas 2g/L ■ MS YE2,5g/L
 ■ MS2,4D 0,22mg/L ■ MS YE1g/L ■ MS YE5g/L



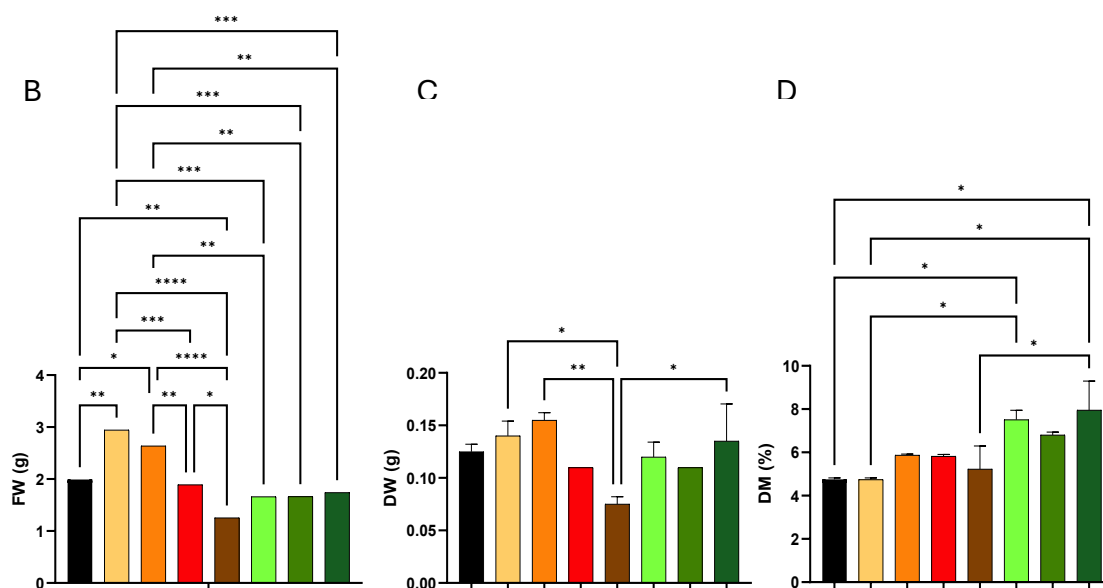
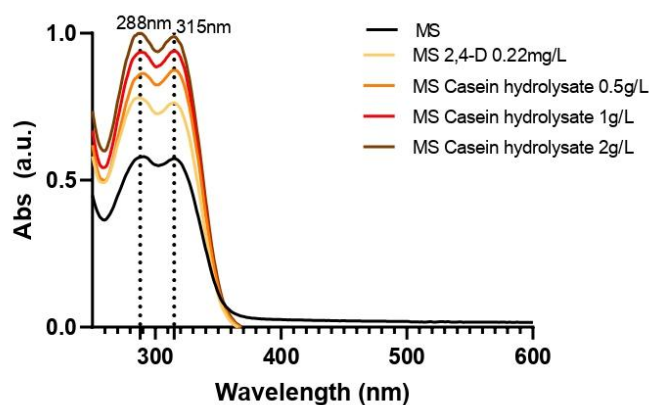


Figure 3.1.34. Effects of different concentrations of casein hydrolysate and yeast extract on the fresh weight (A) (with a focus at day 21 from subculture(B)), dry weight (C) and dry matter (D) of *P. frutescens* callus at 14, 21, and 28 days. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001). Abbreviation: Cas, casein hydrolysate; YE, yeast extract

The results indicated that among the tested concentrations of casein hydrolysate, 0,5g/L was the most effective, both for fresh weight, dry weight and dry matter. Notably, for dry weight and dry matter, an improvement was also observed compared to standard culture conditions. Although yeast extract resulted in comparatively lower fresh weight than casein hydrolysate and standard culture conditions, with no significant differences between the three concentrations tested at 21 days, an increase in dry weight and, in particular, in dry matter was observed at concentrations of 1g/L and 5g/L. However, the morphological features of the callus were not optimal due to lack of friability and browning. Consequently, casein hydrolysate was selected as the substitute with the best performances to be considered for further analysis focusing on the UV-visible spectral profile and cell viability at three different concentrations, compared to standard growth media and to media without any plant growth regulators or substitutes.

The UV-visible spectral analysis of extracts was conducted on day 21 from sub-culture, the time point with the peak accumulation of dry matter for *Perilla frutescens* callus culture grown on the standard conditions (see Table 3.1.3) (Figure 3.1.35). Although the medium with 2g/L of casein hydrolysate exhibited an improvement metabolic profile, its overall yield (in particular in term of dry weight) did not support satisfactory accumulation of secondary metabolites of interest. Based on these findings, the medium containing 0,5g/L of casein hydrolysate was selected for subsequent analyses.



	Abs <i>P. frutescens</i>				
Wavelength	MS (g)	MS+2,4-D (g)	MS+ casein 0,5g/L	MS+ casein 1g/L	MS+ casein 2g/L
288nm	1,626±0,2	2,190±0,055	2,420±0,12	2,621±0,03	2,802±0,01
315nm	1,609±0,3	2,14±0,03	2,45±0,12	2,634±0,01	2,77±0,012

Figure 3.1.35. UV-visible spectra of hydroalcoholic extract in standard medium, in medium without plant growth regulators and in media containing different concentration of casein hydrolysate (0,5g/L, 1g/L and 2g/L). The absorbance values in the graph and reported in the table refer to samples diluted 1:10.

Figure 3.1.36 presents the assessment of cell viability in *P. frutescens* callus culture grown with 0.5g/L casein hydrolysate, observed under a fluorescence microscope at 20X magnification. At time 0, cell viability reached 83%.

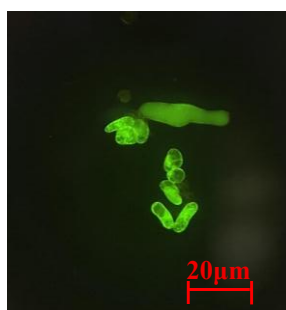
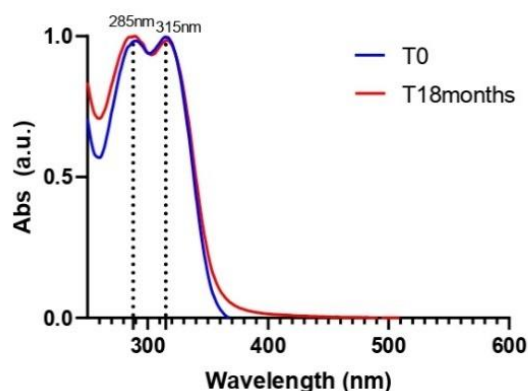


Figure 3.1.36. Assessment of cell viability in the sample grown with 0,5g/L casein hydrolysate at time 0, observed with fluorescence microscope at 20X magnification. Bar 20µm.

After 18 months, the callus culture grown on the only 0,5g/L casein hydrolysate exhibited stable growth with friability comparable to that observed under standard conditions. A new extraction showed no significant differences in secondary metabolite accumulation or profile compared to the initial callus culture, confirming its stability and the efficacy of the substitution over time (Figure 3.1.37)



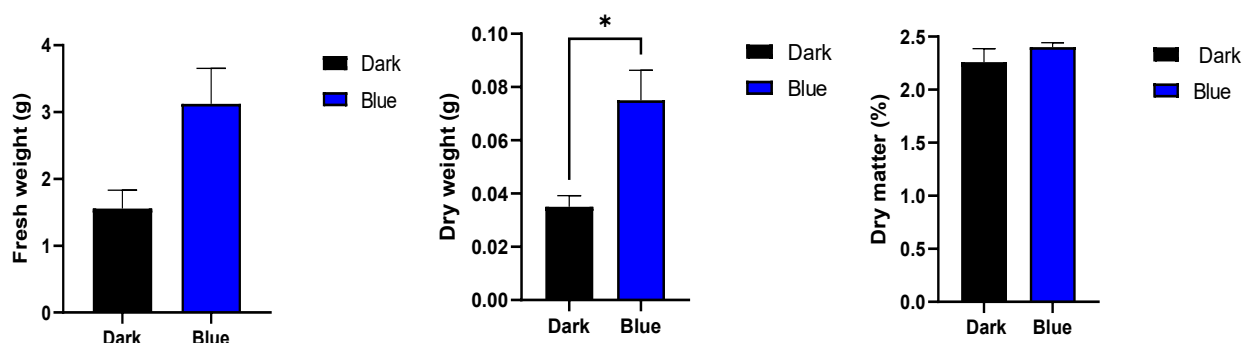
Abs <i>P. frutescens</i> callus culture		
Wavelength	T0	T18 months
285nm	2,37	2,45
318nm	2,45	2,42

Figure 3.1.37. Comparison of UV-visible spectra of *P. frutescens* calli grown in a modified medium containing 0,5g/L casein hydrolysate at time 0 and after 18 months. The absorbance values reported in the table refer to 1:10 diluted samples.

3.1.9.2 Casein hydrolysate as a plant growth regulator substitute in *P. frutescens* suspension culture

The friable callus obtained in the modified medium containing 0,5g/L casein hydrolysate, was transferred to the corresponding liquid medium (MS 3% sucrose with casein hydrolysate 0.5g/L). Additionally, to enhance growth and metabolite accumulation, as previously done for the callus culture, elicitation with blue light (best LED light condition for *P. frutescens*) was tested.

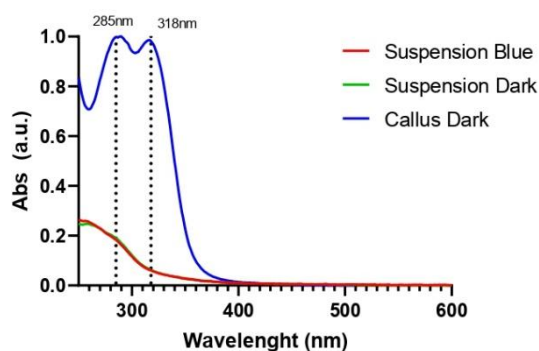
Two replicates of *P. frutescens* suspension culture were grown in liquid medium, with one set cultivated under blue LED light and the other in darkness, both without plant growth regulators but supplemented with 0.5g/L casein hydrolysate. After 21 days, which corresponds to the peak of dry matter accumulation for both *P. frutescens* callus and suspension culture, the cell biomass was harvested. Fresh weight, dry weight (after lyophilization process) and dry matter were recorded (Figure 3.1.38).



Time (days)	Fresh weight (g)			Dark weight (g)			Dry matter (%)		
	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD	Sample 1	Sample 2	Mean±SD
Blue	2,75	3,5	3,12±0,53	0,06	0,08	0,075±0,001	2,43	2,37	2,40±0,04
Dark	1,75	1,36	1,55±0,28	0,03	0,04	0,035±0,004	2,35	2,17	2,26±0,12

Figure 3.1.38. Evaluation of fresh weight, dry weight and dry matter of *P. frutescens* suspension culture grown on modified media with 0,5g/L casein hydrolysate, under blue LED or dark. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001).

UV-visible spectra of the extracts from the lyophilized samples derived from *P. frutescens* suspension culture grown in the modified liquid media without plant growth regulators and with 0.5g/L casein hydrolysate, either under dark conditions or Blue LED light, were compared (Figure 3.1.39)



Wavelength	Abs <i>P. frutescens</i> suspension culture		Abs <i>P. frutescens</i> callus culture
	Blue	Dark	Dark
285nm	0,451	0,468	2,452
318nm	0,148	0,153	2,413

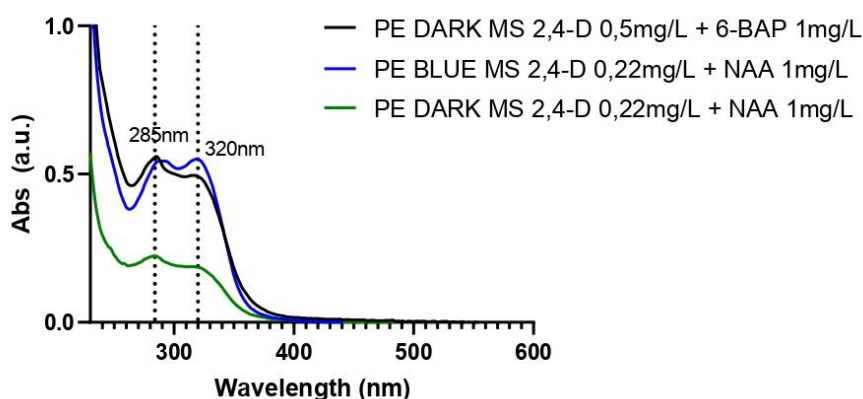
Figure 3.1.39. UV-visible comparison between *P. frutescens* suspension grown in plant growth regulators-free medium with 0.5g/L casein hydrolyzed caseinate, both in dark conditions and elicited with blue light, and callus grown in the same medium conditions but only in dark condition. Dilution 1:10 of extracts.

While the fresh weight and dry weight results had previously indicated an increase in biomass under elicitation, the UV-visible analysis revealed no significant differences in absorbance between the two conditions. Blue light elicitation did not provide any advantage in the accumulation of target molecules. Additionally, when compared to callus grown under the same conditions, but without LED elicitation, the callus culture showed a significantly greater accumulation of molecules of interest. This led to the exclusion of the liquid formulation containing only casein hydrolysate from subsequent studies.

3.1.9.3 LED Light as a plant growth regulator in *P. frutescens* suspension culture

Given the unsatisfactory results related to the substitution of synthetic plant growth regulators by casein hydrolysate in the suspension culture, the liquid medium of *P. frutescens* suspension culture was modified by replacing the synthetic regulators mostly endowed with a toxic potential and characterized by the lowest ADI, in order to define a safer medium composition in terms of future human consumption.

Consequently, a T2 medium, formulated without BAP and with 2,4-D 0,22mg/L + NAA 1mg/L was evaluated. Although the UV-visible spectral analysis of the hydroalcoholic extract in T2 dark conditions showed a lower absorbance compared to standard medium (Dark 2,4-D 0,5mg/L + 6-BAP 1mg/L), the addition of Blue LED light during growth (wavelength: 460nm, intensity: 50 $\mu\text{mol}/\text{m}^2/\text{s}$, timing: 24-hrs/24-hrs during the entire cultivation cycle, see Table 2.2.4) successfully restored absorbance levels to those obtained under standard culture conditions (Dark 2,4-D 0,5mg/L + 6-BAP 1mg/L) while ensuring a medium composition more suitable and safer for potential food applications and human consumption. (Figure 3.1.40)



	Abs <i>P. frutescens</i> suspension culture		
Wavelength	Dark T2 (2,4-D 0,22mg/L+NAA1mg/L)	Blue T2 (2,4-D 0,22mg/L+NAA1mg/L)	Dark 2,4-D 0,5mg/L+ 6-BAP 1mg/L
285nm	0,645	1,865	1,869
318nm	0,598	1,870	1,75

Figure 3.1.40. UV-visible spectra comparison of *P. frutescens* suspension culture in standard condition (Dark MS 2,4-D+BAP), in Blue light condition on MS 2,4-D+BAP medium and in Blue light condition on T2 medium. The absorbance values reported in the table refer to samples diluted at 1:10.

3.1.10 Stability tests

The stability of the lyophilized powders from *P. frutescens* suspension cultures samples (Blue 2,4-D 0,22mg/L+NAA1mg/L; Dark 2,4-D 0,5mg/L+ 6-BAP 1mg/L, Dark T2 (2,4-D 0,22mg/L+NAA1mg/L) was tested through the UV-visible analysis of their hydroalcoholic extracts.

An increase in absorbances was observed at the end of the treatment (45°C for one week). For *P. frutescens* suspension culture grown under blue LED on T2 medium and harvested at day 21 and *P. frutescens* suspension culture under standard condition (MS 2,4-D + BAP), the UV-visible spectra exhibited two main absorbance peaks around 285nm and 320nm. In contrast, lyophilized *P. frutescens* suspension culture grown under dark on T2 medium and harvested at day 21 showed a decrease in absorbances after the test.

The extracts from the three samples showed remarkable similarity in absorbance of the identified peaks before and after the stability test (Figure 3.1.35).

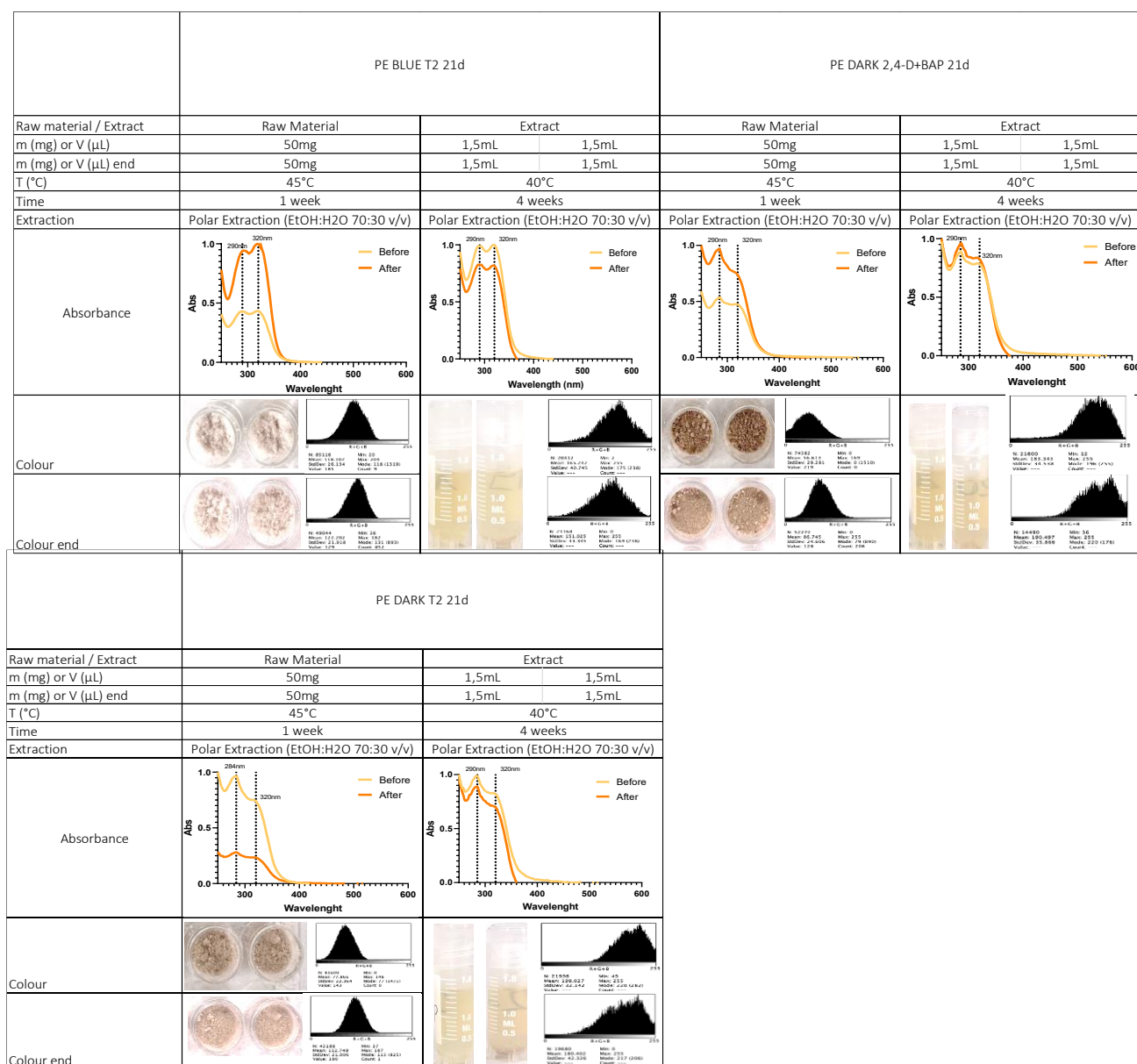


Figure 3.1.41. Summary of stability features of raw material (lyophilized powders) and hydroalcoholic extracts form *P. frutescens* suspension culture under different conditions.

3.2 Formulation of pre-industrialized prototype food products containing cells and bioactive molecules

3.2.1 Ink formulation

3.2.1.1. Study for defining the final recipes of the inks

This section will describe in detail the empirical process of obtaining the inks, illustrating the development phases through intermediate and final formulations, as well as printing tests.

Ink 1: biscuit-like layer of the "multilayer bar.", formulated to reproduce the consistency and appearance of a biscuit-like outer layer.

1% blueberry by-product extract in 5% citric acid was chosen to enrich the product with flavonoids (particularly anthocyanins) and to improve the shelf-life of the product (due the citric acid), and to enrich it with flavonoids (particularly anthocyanins) thanks to the mild extraction operated by citric acid and the high-value content of this byproduct. The blueberry extract was also considered in order to confer a dark color to the layers containing this ingredient, evocative of a biscuit. To obtain a biscuit-like visual appearance and consistence, 1,72g of apple peel containing 0,15g of pectin (Chen et al., 1988) was mixed to 5mL of 1% blueberry by-product extract in 5% citric acid. This amount of apple peel would ensure a final pectin concentration of 30g/L, useful for the gelation process (Vancauwenberghe et al., 2019). The fibrous component of the apple peel also was considered to increase viscosity, possibly improving the printing process. The sequence of trials performed to reach the optimal extrusion product is schematically described in Figure 3.2.1, which provides a visual representation of the methodological steps adopted. To promote gelation, 1mL of calcium lactate 0,25M was initially added in the first trial for a final concentration of 41,6mM according to Vancauwenberghe et al., 2017 and 2019 (Trial 1). However, due to the discontinuity of the product, it was necessary to integrate 1mL of high oleic oil, which improved flowability and printability, while providing a glossier finish to the final product (Trial 2). Subsequently, the elimination of calcium lactate in favor of the addition of 0,6g of sapin honey (Trail 3) was evaluated, both for honey antiseptic properties and for its natural tendency to crystallize, with the aim of improving the overall stability of the ink. A decrease in high oleic oil (from 11mL to 0,125mL) refined printability (Trial 4). Subsequently, a change in the type of honey was evaluated, in favor of multi-flower honey (thicker), leading to the optimal final result (Trial 5). In accordance with the requirements for baked goods, which must have a residual moisture content less than 60% for shelf life and stability (Smith et al 2004), the final recipe of Ink 1 (i.e. Trial 5) was manually deposited with a 2mL syringe (2mL BD

Emerald 1mm diameter) without needle and then subjected to a baking process using a Thermobalance (MA 50X2ICAWH Moisture Analyzer, Radwag, Poland) set at 45°C or at 60°C, or using an incubator with a thermostat dome set at 45°C (715/CT, Bioclass s.r.l., Italy) to verify which method was suitable to reach a residual moisture content of 44% (see section 3.3.2.1) for the results obtained in this sense).

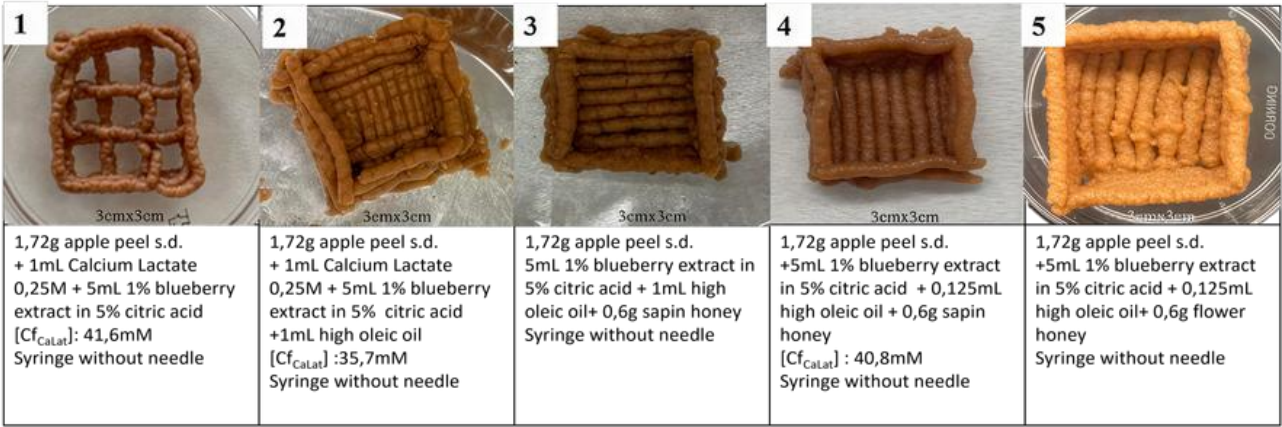


Figure 3.2.1. Development and optimization of Ink 1 formulation describing the different trials that were conducted, with both photos and recipes reported.

Ink 2: molten filling layer of the "multilayer bar.", conceived as an intermediate layer with characteristics similar to a white chocolate filling.

This ink was designed to enrich the product with a carbohydrate component. The sequence of the trials is schematically described in Figure 3.2.2. Initially, an ink made up of Solution 2 (prepared with 4,91mL of clear, concentrated apple juice diluted in 95,09mL of double-distilled water and 3g of amidated pectin, see section 2.3.4) a solution containing amidated pectin, as a base for gelation, was evaluated for its ability to gel with small quantities of calcium chloride, the concentration of which was progressively varied (test 1,2). Subsequently, the addition of powdered citric acid was considered to lower the pH to the mixture and promote gelation in the presence of a lower quantity of calcium chloride that could confer a bitter taste to the product (Trial 3). Subsequently, the addition of powdered calcium chloride was evaluated (Trial 4) to promote crosslinking among the pectin molecules without changing the volume of the formulation that could impair printing. A gradual reduction in the concentration of calcium chloride was then performed (Trial 5) to verify whether it was possible to obtain gelation even with lower calcium chloride quantities. This allowed to define the minimal calcium chloride concentration to obtain gelation (15mM). Subsequently, the replacement of calcium chloride with calcium lactate, a food ingredient endowed with a neutral flavor, was considered (Trial 6). The addition of locust bean gum, a chocolate substitute with viscosity-increasing properties, was evaluated to give to the ink a white chocolate-like consistency (Trial 7). In fact, locust bean gum (commonly used as a gluten substitute in baked goods and as a

healthy alternative to chocolate) acts as a thickener, stabilizer, and binder. Locust bean gum can also inhibit enzymatic activities involved in sugar metabolism. In addition, it has a higher fiber content, especially galactomannans, than other flours, which contributes to the stability, viscosity, and workability of the mixture (Vukušić Pavičić et al., 2021), while also adding dietary value.

A further modification involved replacing 1mL of solution 2 (out of a total of 6mL) with a 1% blueberry by-product extract in 5% citric acid. This allowed the introduction of citric acid no longer as a powder, but within the blueberry extract, with the additional advantage of improving the nutritional value of the formulation (Trial 8). Subsequently, the elimination of calcium lactate was evaluated. Even in its absence, thanks to the pH values of the mixture, a satisfactory extrusion was possible (Trial 9). Finally, to give added value to the final product, the addition of banana powder (0,19g) was taken into consideration (Trail 10). Indeed, banana powder is low in fat and has a good protein content (3-4%) with an interesting phenolic profile (gallic acid, catechins).

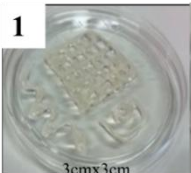
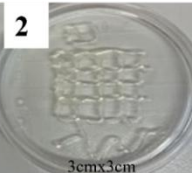
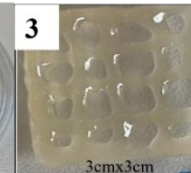
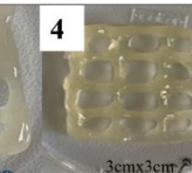
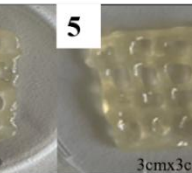
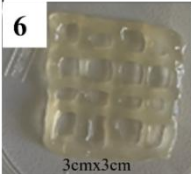
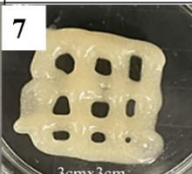
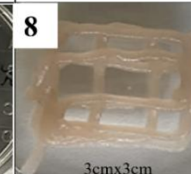
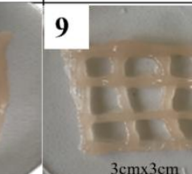
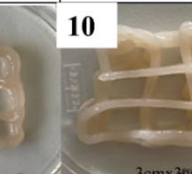
				
2mL Solution 2 + 0,75mL CaCl ₂ 20mM [Cf _{CaCl₂} :5,45mM] Syringe with 22G needle	2mL Solution 2 + 1,1mL CaCl ₂ 20mM [Cf _{CaCl₂} : 7,09] Syringe with 22G needle	2mL Solution 2 + 300mg of citric acid + 30μL CaCl ₂ 20mM [Cf _{CaCl₂} :2,6mM] Syringe with 22G needle	6mL Solution 2 + 900mg citric acid+ 18mg CaCl ₂ Cf _{CaCl₂} = 20mM Syringe with 22G needle	6mL Solution 2 + 900mg citric acid + 10mg CaCl ₂ Cf _{CaCl₂} = 15mM Syringe with 22G needle
				
6mL Solution 2 + 900mg citric acid + 29mg Calcium lactate [Cf _{CaLat} 15mM] Syringe with 22G needle	6mL Solution 2 28mg Calcium lactate 300mg locust bean gum, 900mg citric acid [Cf _{CaLat} :15,2mM] Syringe without needle	5mL Solution 2+ 1mL 1% blueberry extract in 5% citric acid + 200μL CaLat 1M 0,500g locust bean gum [Cf _{CaLat}] 32,2mM Syringe without needle	5mL Solution 2+1mL 1mL 1% blueberry extract in 5% citric acid +0,4g locust bean gum Syringe eppendorf	5mL Solution 2 + 1mL1% blueberry extract in 5% citric acid + 0,19g banana powder + 0,5g locust bean gum Syringe without needle

Figure 3.2.2. Development and optimization of Ink 2 formulation describing the different trials that were conducted, with both photos and recipes reported.

Ink 3: “gelée”-like filling layer in the "multilayer bar" and "honey pearl" products, as well as a formulation for cell inclusion, with properties suitable for their viability and integrity preservation.

Ink 3 was studied as a “gelée”-like filling for the inclusion of plant cells in both the final 3D printed products. The hydrogel was produced starting from three different liquid base solutions: diluted apple juice (Ink 3c), 1% strawberry by-product extract (Ink 3) and 1% blueberry by-product extract (Ink

3b) both in 0,5% citric acid. The use of 5% citric acid was excluded to avoid an excessively low pH that could compromise cell viability.

As a consequence, the study involved the development of three ink formulations in parallel, in order to identify the most suitable to preserve cell viability. The pectin concentration was kept at 15g/L or 30g/L.

Calcium chloride was added firstly as a powder and then, in the final trials, as a solution added 'drop to drop' to promote cross-linking among pectin molecules to obtain the gelation of the hydrogel under constant stirring, to ensure a homogeneous dispersion in the solution optimizing the gel formation and to empirically determine the optimal concentration needed in the formulation.

The sequence of trials is schematically described in Figure 3.2.3. Initially, 75mg of LMP (low-methoxylated pectin) were added to 5mL of 1% blueberry extract in 0,5% citric acid, along with calcium chloride powder (for a final concentration of 68 mM, and carob flour, the latter chosen to improve the viscosity of the formulation (Trial 1). Subsequently, the calcium concentration was gradually increased (Trial 2) until reaching 272 mM (Trial 3).

After this step, the removal of the locust bean gum, the increase in the amount of pectin and the addition of casein hydrolysate (Trial 4) were evaluated. Casein hydrolysate is a protein with colloidal properties (beneficial for 3D printing ink formulation) derived from dairy processing, offering added dietary value. Dairy waste, if not adequately treated, presents a high organic, nitrogen, and phosphorus load, posing significant environmental risks (Rivas et al., 2011; Prazeres et al., 2012). Recovering these by-products aligns with circular economy principles and helps mitigate this issue. Additionally, in the early development of plant cell culture technique, casein hydrolysate was widely used as a nutrient for plant cells. For these reasons, casein hydrolysate was then considered to facilitate the creation of a sustainable ink that could support plant cell survival in the absence of culture medium, undesirable in food applications.

Subsequently, a further increase in calcium was considered, with the aim of obtaining an even more stable structure (Trial 5).

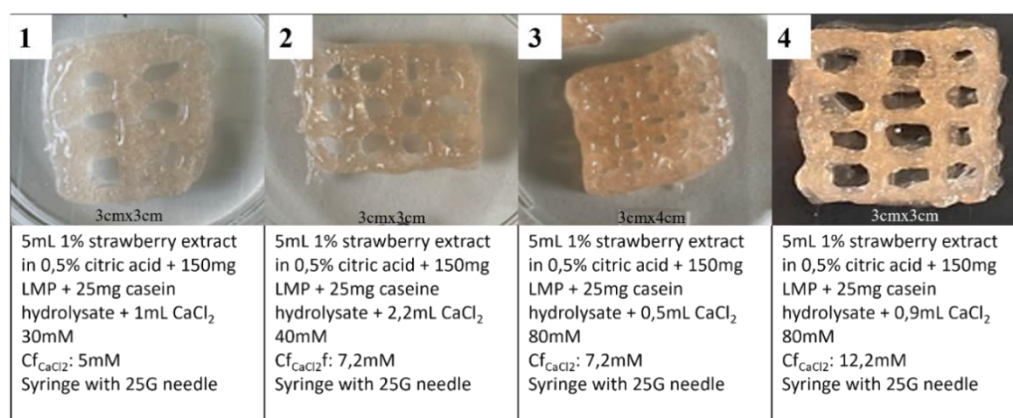


Figure 3.2.3. Development and optimization of Ink 3b formulation with blueberry extract and describing the different trials that were conducted, with both photos and recipes reported.

A formulation based on the 1% strawberry by-product extract in 0,5% citric acid were devised, which initial composition was kept similar to Trial 5 of the Ink 3 formulated with the blueberry extract but trying different calcium chloride concentration (as a solution). The sequence of trials is schematically described in Figure 3.2.4.

Initially, 1mL of a 30mM calcium chloride solution was used to reach a final concentration of 5mM in the ink (Trial 1). Subsequently, 2,2mL of a 40mM solution was used, for a final concentration of 7,2mM in the ink (Trial 2). To further optimize the formulation, the starting calcium chloride solution to be added was increased to 80mM. This allowed to reduce the amount of liquid solution to be added, still obtaining a final concentration of 7,2mM necessary for good gelation (Trial 3). Finally, starting from the 80mM solution, a final calcium chloride concentration of 12mM was tried in the ink, so as to improve its consistency and achieve the desired final texture (Trial 4).

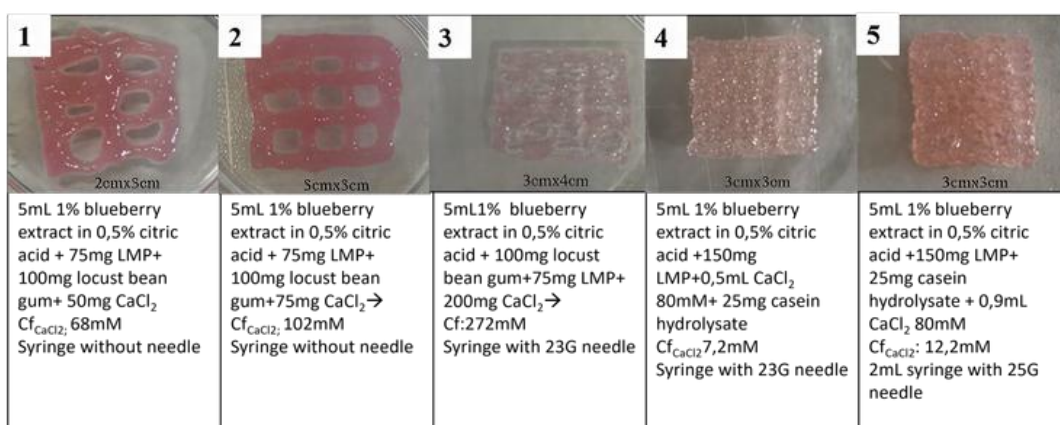


Figure 3.2.4. Development and optimization of Ink 3 formulation with strawberry extract and describing the different trials that were conducted, with both photos and recipes reported.

Another formulation of Ink 3 (Ink 3c) was studied for cell inclusion that was formulated starting from Solution 1 (Solution 1: prepared with 4,91mL of clear, concentrated apple juice diluted in 95,09mL of double-distilled water and 3g of low methoxylated pectin (LMP), see section 2.3.4) which already contains 3% LMP, and focused exclusively on varying the calcium chloride solution. The sequence of trials is schematically described in Figure 3.2.5. Initially, a 20mM calcium chloride stock solution was added, varying the volume added to obtain different final calcium chloride concentrations in the ink (Trials 1 and 2). Subsequently, a 30mM stock solution of calcium chloride was used, varying the volume to be added and, therefore, its final concentration in the ink (Trials 3, 4, 5 and 6). Subsequently, a 40mM stock solution was used, obtaining a final concentration of 9,23mM in the ink (Trial 7). Finally, the addition of a calcium chloride 80mM stock solution to reach a final concentration of 11.25mM in the ink (Trial 8) turned out to define the optimal recipe for the Ink 3c formulation.

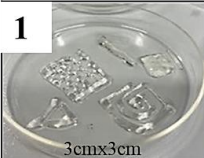
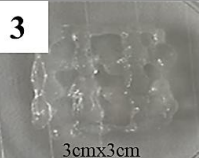
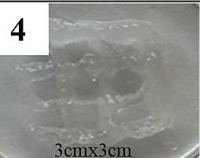
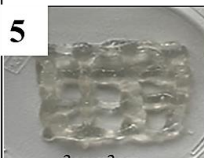
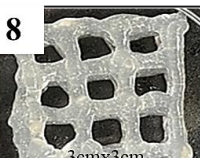
 2mL Solution 1 + 1,1mL CaCl₂ 20mM Cf_{CaCl₂}: 7,09 Syringe 22G needle	 2mL Solution 1 + 1,8mL CaCl₂ 20mM Cf_{CaCl₂}: 9,47mM Syringe 22G needle	 2mL Solution 1 1,35mL CaCl₂ 30mM → Cf_{CaCl₂}: 12mM Syringe 22G needle	 2mL Solution 1 1mL CaCl₂ 30mM → Cf_{CaCl₂}: 10mM Syringe 22G needle
 2mL Solution 1 + 0,8 mL 30mM Cf_{CaCl₂}: 7,3mM Syringe 25G needle	 2mL Solution 1 + 0,7 mL 30mM Cf_{CaCl₂}: 7,7mM Syringe 25G needle	 2mL Solution 1 + 600μL CaCl₂ 40mM [Cf_{CaCl₂}: 9,23mM] Syringe 25G needle	 5mL Solution 1, 900μL CaCl₂ 80mM. Cf_{CaCl₂}: 11,25 mM Syringe 25G needle

Figure 3.2.5. Development and optimization of Ink 3c formulation with apple juice and describing the different trials that were conducted, with both photos and recipes reported.

Ink 4: shell of the honey pearl, developed to reproduce a gummy-like outer layer

Ink 4 was developed to create the outer “shell” of a honey pearl which also would incorporate Inks 3 (with or without cells). Thanks both to the experience in the formulation of inks consolidated with Inks 1, 2 and 3 and to literature (in particular, the study by Andriotis et al., 2020) it was possible to define the optimal proportions among the use of pectin, honey and citric acid to obtain a 3D-printable object. As a first trial (Trial 1), 13 % w/v LMP and 2 % w/v sapin honey were used (Figure 3.2.6). However, in order to optimize the final product and enrich it in honey, this composition was modified by a mixture containing 10 % w/v LMP and 5 % w/v sapin honey. Furthermore, while the reference

study used 2% citric acid in double-distilled water to prepare the mixture, we used the 1% blueberry extract in 5% citric acid (Trial 2). The lower pH and the combination of pectin and honey turned out to offer the benefit of eliminating the need for a cross-linking phase with CaCl₂. As a manual trial before using a 3D-printer, Ink 4 was cast into a mold to simulate the final sphere. The molded sphere was then subjected to a heat treatment process to obtain solidification, at 50°C for 50 minutes (Trial 3).

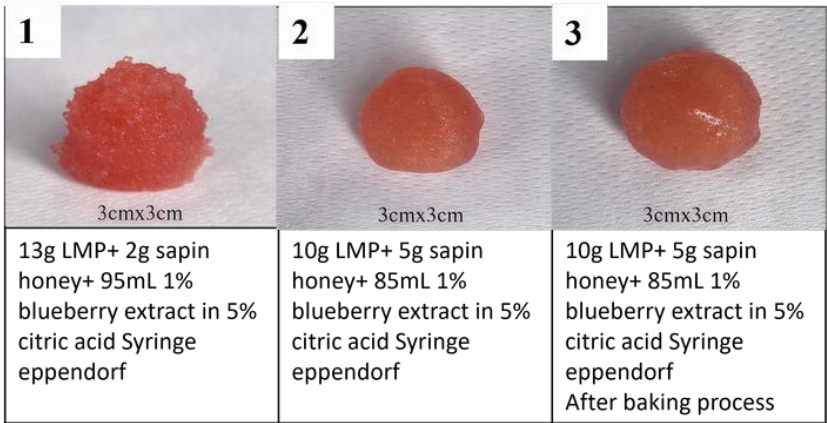


Figure 3.2.6. Development and optimization of Ink 4 formulation describing the different trials that were conducted, with both photos and recipes reported.

3.2.1.2 Evaluation of *P. frutescens* cell suspension culture viability in value-added by-product extracts and apple juice

Perilla frutescens suspension culture was counted and evaluated for its viability into either 1% strawberry or blueberry processing by-product extracts in 0,5% citric acid, or into clear apple juice concentrate diluted 5% in water (1x10⁶cells/mL), with viability in its standard medium (2,4-D 0,5mg/L and BAP 1mg/L) being the control. The results reported in Table 3.2.2. are a mean of two experiments. Viability was assessed for 192 hours (8 days), maintaining samples at 4°C. The viability of cells resuspended in blueberry by-product extract turned out to decrease to 0% between 2 hours and 24 hours (Data not shown). Therefore, blueberry by-product extract wasn’t considered to resuspend cells in the context of Ink 3b. The results obtained for the other two elements were shown in the following table (Table 3.2.2.), micrographs (Figure 3.2.7) and diagrams (Figure 3.2.8).

Time in hours	To in culture media	T1: 2h	T2: 24h	T3: 48h	T5: 120h	T6:144 h	T7:168 h	T8: 192h
Solution								
Apple juice (1x10 ⁶ cells /mL) Mean of experiment 1		74%	73%	64%	45%	46%	33%	39%
Apple juice (1x10 ⁶ cells /mL) Mean of experiment 2		80%	64%	70%	75%	72%	60%	36%
Mean of two experi-ments	84%	77%	69%	67%	60%	59%	47%	38%
Strawberry extract (1x10 ⁶ cells cells/mL) Mean of experiment 1		79%	79%	60%	35%	41%	38%	20%
Strawberry extract (1x10 ⁶ cells cells/mL) Mean of experiment 2		75%	72%	71%	55%	45%	29%	8%
Mean of two experi-ments	84%	77%	76%	66%	45%	43%	34%	16%
Standard medium (1x10 ⁶ cells /mL) Mean of two experi-ments	90%	87%	85%	82%	73%	72%	71%	69%

Table 3.2.2. Viability of *P. frutescens* cells resuspended in 1% strawberry by-product extract in 0,5% citric acid or clear apple juice concentrate (diluted with double-distilled water) or in its standard culture medium as a control.

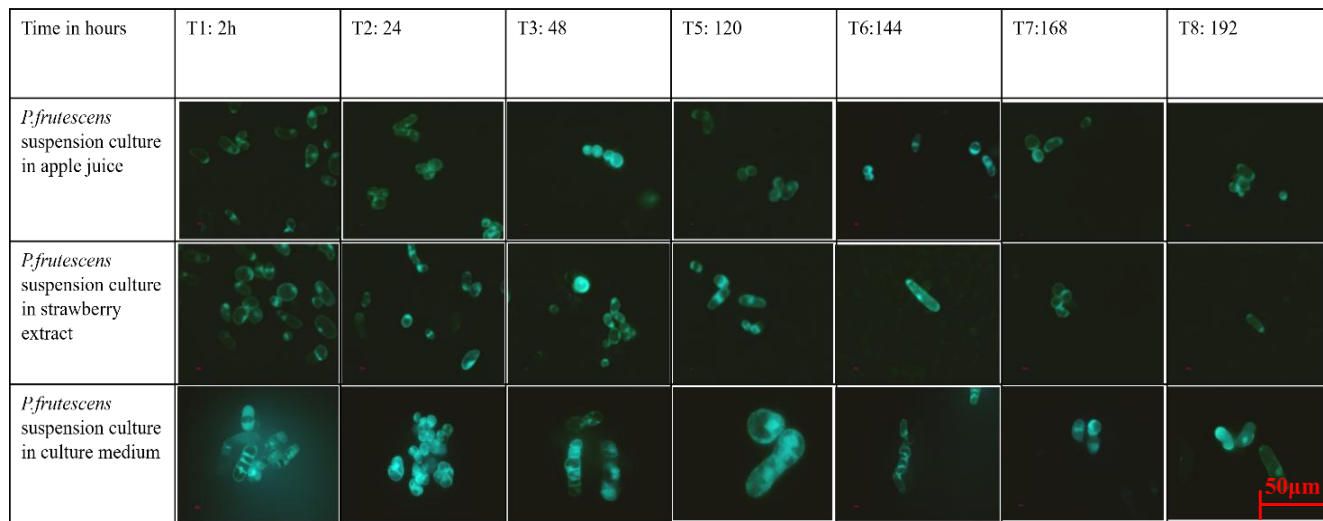


Figure 3.2.7. Fluorescence micrograph depicting the viability of *P. frutescens* cells resuspended in 1% strawberry by-product extract in 0,5% citric acid or clear apple juice concentrate (diluted with double-distilled water) or in its standard culture media. Images depict FDA-stained cells from T1 (2 hours) to T8 (192 hours, day 8). Bar 50µm.

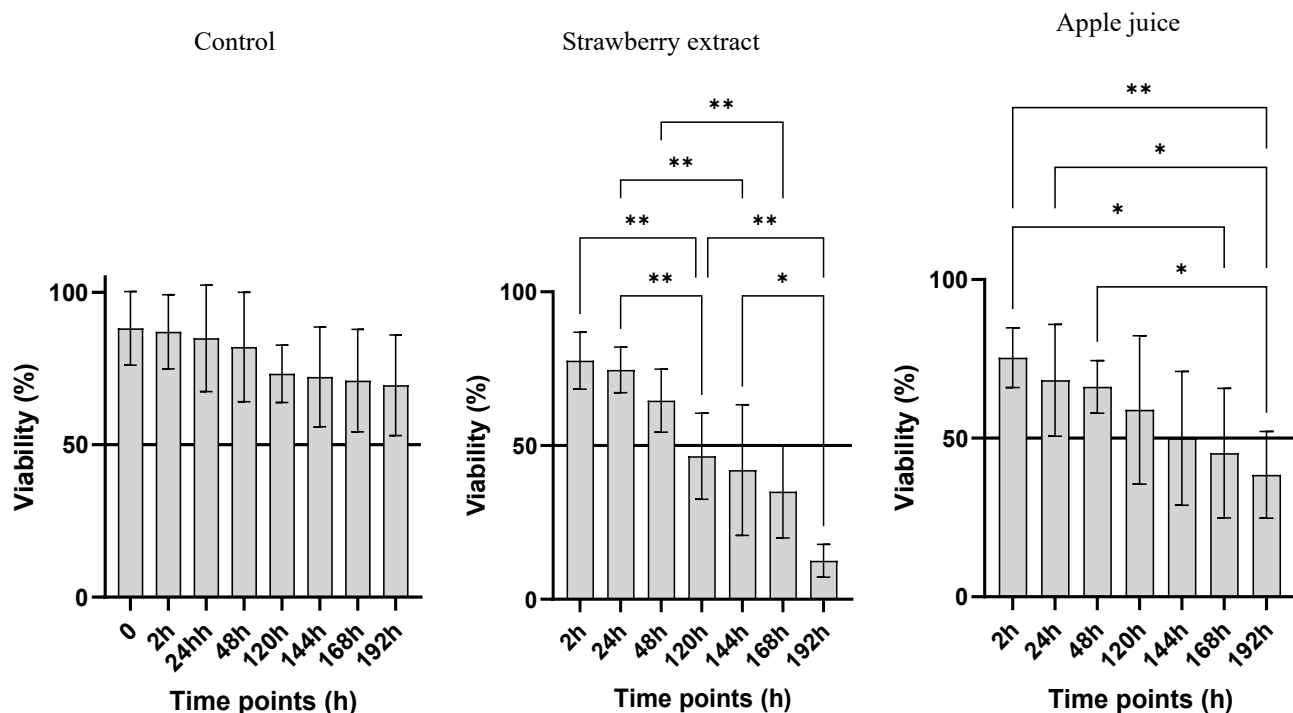


Figure 3.2.8. Viability of *P. frutescens* cells resuspended in 1% strawberry by-product extract in 0,5% citric acid or clear apple juice concentrate (diluted with double-distilled water) or in its standard culture media. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05 , **p-value ≤ 0.01 and ***p-value ≤ 0.001).

These results highlighted that both 1% strawberry by-product extract in 0,5% citric acid and clear apple juice concentrate (diluted into 5% v:v in water) can preserve at least 50% of live cells for approximately 5 days (in particular, at day 5, 60% viable cells are preserved in diluted apple juice and 45% viable cells in strawberry by-product extract) maintaining an aliquot of viable cells up to 192 h, that is higher in apple juice than in strawberry by-product extract (38% viable cells and 16% viable cells, respectively). As expected, in the standard culture medium, viability declines much slower (73% viable cells at day 5 days and 69% viable cells at 8 days) than in the two solutions. These results allowed to confirm at least two options as solutions to resuspend *P. frutescens* cells in the context of Ink 3 in the two versions of Ink3 and Ink 3c.

3.2.1.3 Evaluation of *P. frutescens* plant cell suspension culture viability in the by-product extracts- and high-value apple juice-based hydrogel (Ink3and 3c)

In parallel, experiments were performed to evaluate the viability of *P. frutescens* cells in Ink 3- and Ink 3c-based printed objects. However, in the case of Ink 3c that contained already diluted apple juice, which showed to support cell viability at a higher extant than strawberry by-product extract particularly at 192 hours, the possibility of omitting casein hydrolysate from the ink recipe was evaluated. In the series of experiments described in this paragraph, *P. frutescens* cells were

resuspended in standard culture medium (MS 2,4-D 0,5mg/L and BAP 1mg/L) at a concentration of 1×10^6 cells/mL and 1mL of strawberry extract or juice was replaced with 1 ml of cells in their standard culture medium, so that 1×10^6 cells could be added to the Ink 3 or Ink 3c formulation, respectively. Cell viability at different time points is shown in the following table (Table 3.2.3), and micrographs (Figure 3.2.9) and diagrams (Figure 3.2.10).

Solution	Time in hours	To in cul- ture media	T1: 2h	T2: 24h	T3: 48h	T5: 72h
Ink 3c (apple juice) w/o casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 1		85%	83%	79%	42%	25%
Ink 3c (apple juice) w/o casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 2		83%	83%	67%	34%	25%
Mean of two experiments		84%	83%	73%	38%	25%
Ink 3c (apple juice) w/ casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 1		85%	73%	73%	49%	28%
Ink 3 (apple juice) w/ casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 2		83%	70%	63%	54%	28%
Mean of two experiments		84%	72%	68%	52%	28%
Ink 3c (strawberry extract) w/ casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 1		85%	80%	72%	46%	30%
Ink 3c (strawberry extract) w/ casein hydrolysate 1×10^6 cells cells/ml Mean of experiment 2		83%	80%	76%	63%	44%
Mean of two experiments		84%	80%	74%	55%	37%

Table 3.2.3. Viability of *P. frutescens* cells in Ink 3c (apple juice) w/o or w/ casein hydrolysate and in Ink 3 (strawberry extract) w/ casein hydrolysate

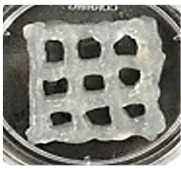
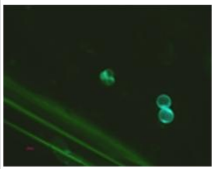
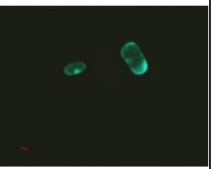
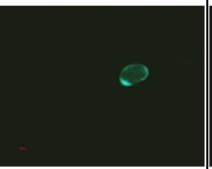
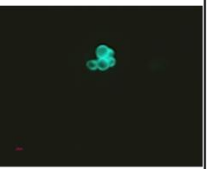
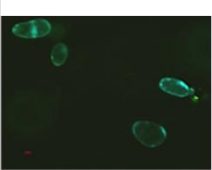
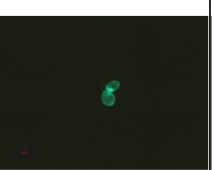
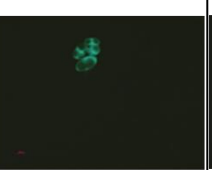
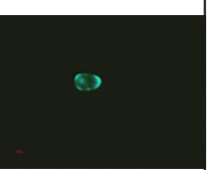
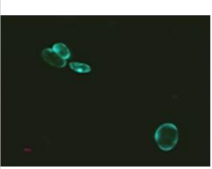
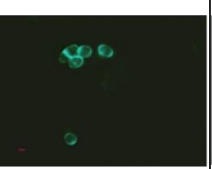
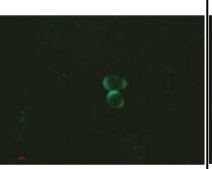
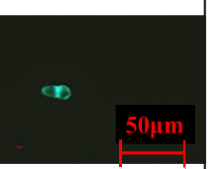
Time in h	To in culture medium	T1: 2h	T2: 24	T3: 48	T5: 72
Printed object with <i>P. frutescens</i> suspension culture in apple juice without casein hydrolysate					
Printed object with <i>P. frutescens</i> suspension culture in apple juice with casein hydrolysate					
Printed object with <i>P. frutescens</i> suspension culture in strawberry extract with casein hydrolysate					

Figure 3.2.9. Printing and fluorescence micrograph depicting the viability of *P. frutescens* in Ink 3c (apple juice) without or with casein hydrolysate and in Ink 3 (strawberry extract) with casein hydrolysate. Images depict FDA-stained cells from T1 (2 hours) to T8 (192 hours, day 8). Magnification 50X.

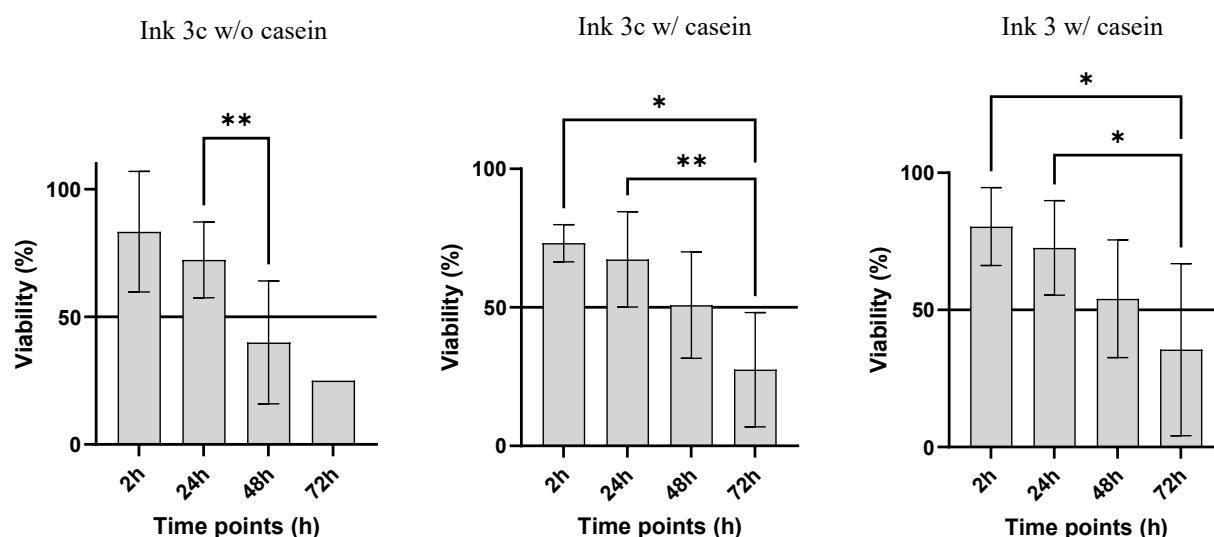


Figure 3.2.10. Viability of *P. frutescens* cells in Ink 3c (apple juice) w/o or w/ casein hydrolysate and in Ink 3 (strawberry extract) w/ casein hydrolysate. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value $\leq$ 0.05, **p-value $\leq$ 0.01 and ***p-value $\leq$ 0.001). Bar 50 μ m.

From this analysis emerged that, although cells resuspended in diluted concentrated apple juice had shown the highest viability in the previous series of experiments, the final Ink 3 formulation that maintained the highest overall viability with the least statistically significant variations over time was

the one containing strawberry by-products extract in citric acid, casein hydrolysate and pectin (Ink 3; no statistically significant differences were observed up to 72 hours, with a p-value <0.005).

Given that the primary objective was to develop a hydrogel compatible with cell viability and integrity, the formulation based on strawberry by-product extract was selected (Ink 3). The next experiment will assess this formulation (strawberry by-product, LMP and casein hydrolysate), but with the cells resuspended directly in the strawberry by-product extract to evaluate the feasibility of eliminating the culture medium.

3.2.1.4. Evaluation of *P. frutescens* plant cell suspension culture viability in the by-product extracts (Ink 3)

The series of experiment described in this paragraph , were conducted using Ink 3 (which contained 1% strawberry by-products extracts in 0,5% citric acid, casein hydrolysate and LMP, see section 2.3.4) and directly resuspending 1×10^6 or 2×10^6 *P. frutescens* cells in 1% strawberry by-products extract in citric acid 0,5%, while resuspension of 1×10^6 cells in 1mL of standard culture medium (MS 3% sucrose containing 0,5mg/L 2,4-D+1mg/L BAP), represented the internal control. Results are described in Table 3.2.4, and in micrographs (Figure 3.2.11) and diagrams (Figure 3.2.12).

Solution	Time in hours	To in culture media	T1: 2h	T2: 24h	T3: 48h	T5: 120h
Ink 3 + <i>P. frutescens</i> suspension culture in strawberry extract (2×10^6 cells/mL) Mean of experiment 1		81%	77%	65%	33%	31%
Ink 3 + <i>P. frutescens</i> suspension culture in strawberry extract (2×10^6 cells/mL) Mean of experiment 2		83%	83%	74%	47%	17%
Mean		82%	80%	70%	40%	24%
Ink 3 + <i>P. frutescens</i> suspension culture in strawberry extract (1×10^6 cells/mL) Mean of experiment 1		81%	79%	70%	43%	33%
Ink 3 + <i>P. frutescens</i> suspension culture in strawberry extract (1×10^6 cells/mL) Mean of experiment 2		83%	82%	77%	57%	40%
Mean		82%	81%	74%	49,5%	37%
Ink 3 + <i>P. frutescens</i> suspension culture in media		81%	78%	77%	30%	27%

(1 x 10 ⁶ cells/mL) Mean of experiment 1					
Ink 3 + <i>P. frutescens</i> suspension culture in media (1 x 10 ⁶ cells/mL) Mean of experiment 2	83%	83%	80%	50%	44%
Mean	82%	81%	79%	40%	36%

Table 3.2.4. Viability of *P. frutescens* cells in Ink 3. Cells were resuspended in 1% strawberry by-product extract in 0.5% citric acid at different concentrations and in their standard medium

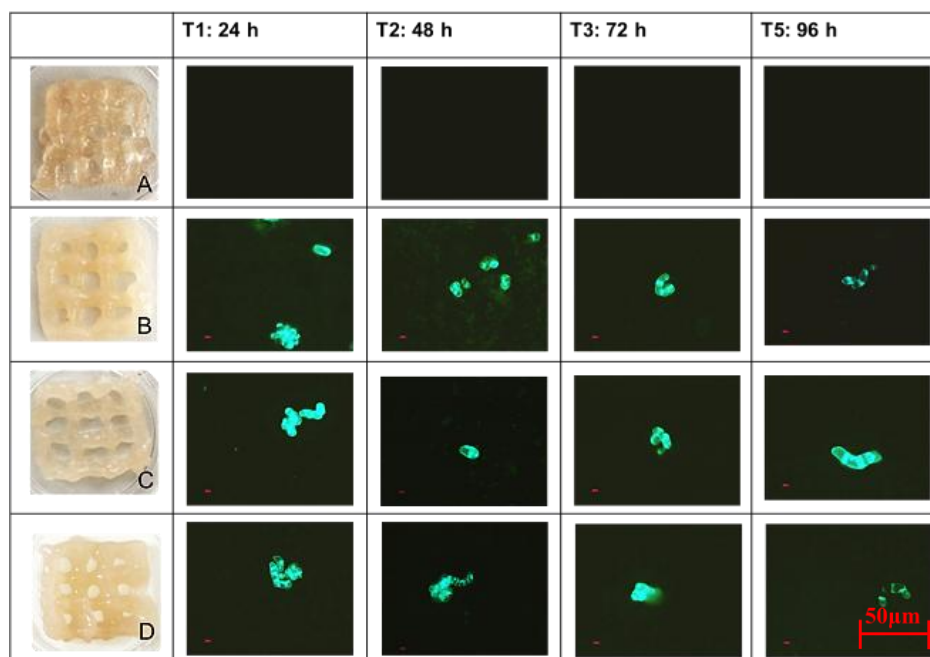


Figure 3.2.11. Printing and fluorescence micrograph depicting the viability of *P. frutescens* in Ink 3. Cells were resuspended in 1% strawberry by-product extract in 0.5% citric acid at different concentrations (B, C) and in their standard medium (D). Figure A represents negative control: ink3 without *P. frutescens* cell culture.

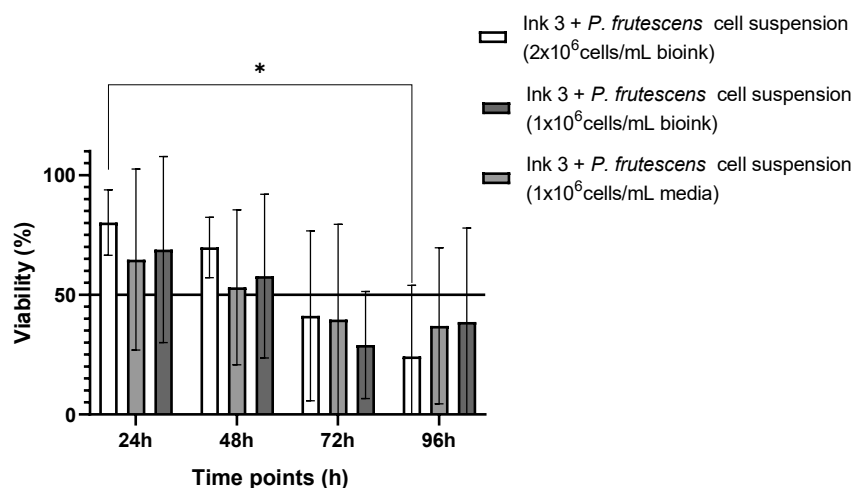
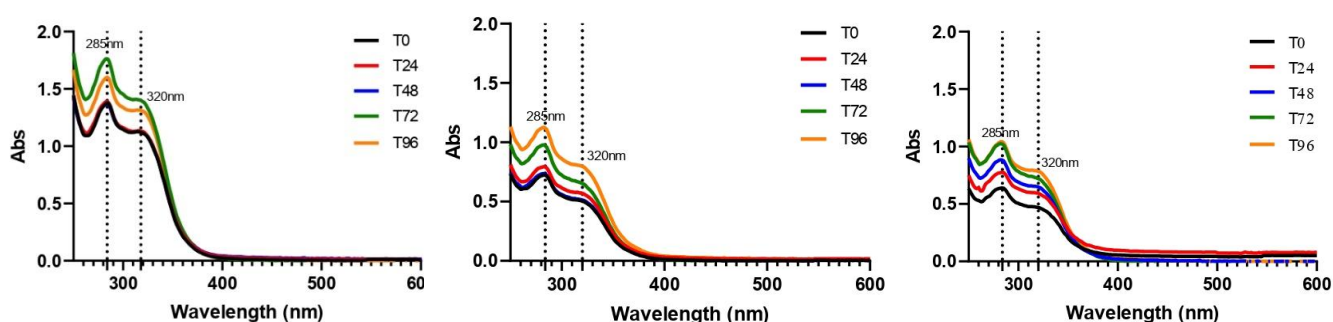


Figure 3.1.12. Viability of *P. frutescens* cells in Ink 3. Cells were resuspended in 1% strawberry by-product extract in 0.5% citric acid at different concentrations and in their standard medium. The analysis of variance was performed by one-way ANOVA with multiple comparisons and Tukey's post-hoc test. Asterisks indicate statistically significant differences (*p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001).

The difference in viability of cells is not statistically relevant among the three samples meaning that the presence of cell culture medium to the ink is not necessary (beside being non desirable). Up to 72h the cells resuspended in strawberry by-product extract and incorporated into the ink maintain a percentage of 49,5% viable cells. For this ink, the timing at which 50% of cell viability is inhibited still vital is between 48 and 72h. The UV-visible spectra of the hydroalcoholic extracts of the inks under the three conditions indicate a substantial stability of the compounds over time (Figure 3.2.13). The absence of significant differences in absorbance between an equal number of cells resuspended either in their standard medium or in strawberry by-product extract, suggests that the addition of medium is not necessary to maintain the integrity and the metabolic activity of *P. frutescens* cell in the Ink 3 in the interval of this observation. The increase in absorbance over time is probably attributable to the loss of water over time, implying very small variations in absorbance and indicating a substantial stability of the content of bioactive molecules of the flavonoid type in the printed Ink 3.



λ	Compounds	B0	B24	B48	B72	B96
288	Flavanones	1,377 $\pm$ 0,06	1,389 $\pm$ 0,02	1,369 $\pm$ 0,02	1,763 $\pm$ 0,16	1,6 $\pm$ 0,31
320	Hydroxycinnamic acids	1,128 $\pm$ 0,04	1,13 $\pm$ 0,019	1,12 $\pm$ 0,03	1,409 $\pm$ 0,13	1,31 $\pm$ 0,25
λ	Compounds	C0	C24	C48	C72	C96
288	Flavanones	0,724 $\pm$ 0,04	0,794 $\pm$ 0,003	0,736 $\pm$ 0,20	0,977 $\pm$ 0,014	1,125 $\pm$ 0,28
320	Hydroxycinnamic acids	0,514 $\pm$ 0,03	0,579 $\pm$ 0,002	0,525 $\pm$ 0,14	0,676 $\pm$ 0,018	0,8 $\pm$ 0,2
λ	Compounds	D0	D24	D48	D72	D96
288	Flavanones	0,755 $\pm$ 0,12	0,846 $\pm$ 0,002	0,884 $\pm$ 0,10	1,026 $\pm$ 0,085	1,042 $\pm$ 0,08
320	Hydroxycinnamic acids	0,599 $\pm$ 0,13	0,624 $\pm$ 0,003	0,657 $\pm$ 0,074	0,741 $\pm$ 0,068	0,794 $\pm$ 0,05

Figure 3.2.13. UV-visible spectrum of samples B, C, D from time T0 to time T96h.

3.3 Characterization of final printed products

3.3.1 Printing tests

The printing conditions for the various inks were established using the CellInk Bio X-Bioprinter (CELLINK, Gothenburg, Sweden) and a custom-made extrusion-based printer built and patented by the Project Partner G&A Engineering (<https://www.gaengineering.com/nutri3d>). The printing conditions for the various inks were established:

3.3.1.1. Ink 1

Fixed parameters:

-Room temperature.

-Dimensions: 20x20x3 mm and 30x30x3mm.

-Distance between extruder nozzle tip and printing stage/deposited top layer 0,01 mm.

Ink	Speed mm/sec	Nozzle diameter (G / μm)	Infill %	Pressure (KPa)	Note	Photo
Test 1.1						
1	10	20G	20	20 50 100	Insufficient pressure for extrusion	
		20G	20	200	Non-uniform extrusion, non-homogeneous sample	
Test 1.2						
1	10	20G	20	150	Non-uniform extrusion, non-homogeneous sample. Tip obstruction	
Test 1.3a						
1	20	18G	20	20 50 70	Pressure tested but insufficient for extrusion	
			20	100	Incomplete print, uneven sample	
Test 1.3b						

1	20	18G	20	150	Speed too high for deposition resulting in repeated filament breakage	
Test 1.3c						
1	10	18G	20	150	Inhomogeneous print due to tip clogging	
Test 1.4						
1	10	18G	15	170-180	Complete print and good resolution	

Table 3.3.1. Defining printing conditions for ink 1.

For ink 1, based on the conducted tests, the minimum extrusion pressure is 150 KPa and a nozzle size between 20 and 18 G is found to be suitable

3.3.1.2. Ink 2

Fixed parameters:

-Room temperature.

-Dimensions: 20x20x3cm.

-Distance between extruder nozzle tip and printing stage/deposited top layer 0,01 mm.

Ink	Speed mm/sec	Nozzle diameter (G / μ m)	Infill %	Pressure (KPa)	Note	Photo
Test 2.1						
2	10	20G	20	20 50 100	Insufficient pressure for extrusion	
2	10	20G	20	110	Non-uniform extrusion	
2	10	20G	20	150	Full structure print	
Test 2.2						

2	10	22G	20	20 50 100	Tested pressure insufficient for extrusion	
2	10	22G	20	130	Drag effect, the layers do not adhere	

Table 3.3.2. Defining printing conditions for ink 2

For ink 2, minimum extrusion pressure of 150 kPa is required to achieve well-defined structure with properly adhering layers. The optimal nozzle size is 20G. If a higher shear force is applied, such as when using a 22G nozzle, layer cohesion is compromised during deposition, resulting in a dragging effect and structural instability.

3.3.1.3. Ink 3

Fixed parameters:

-Room temperature.

-Dimensions: 20x20x3cm.

-Distance between extruder nozzle tip and printing stage/deposited top layer 0,01 mm.

Ink	Speed mm/sec	Nozzle diameter (G / μm)	Infill %	Pressure (KPa)	Note	Photo
Test 3.1						
3	10	22G (644 μm)	20	10 20 50 100 150 200	Unsuitable nozzle	
Test 3.2						
3	10	20G (812 μm)	20	10	Print obtained at minimum speed. Structure completed but with low resolution	
3 +	10	18G (812 μm)	20	70	Print obtained at minimum speed. Structure completed with improved resolution	

Table 3.3.3. Defining printing conditions for ink 3

Ink 3, despite being soft and low in viscosity, fails to extrude through a small diameter 22 G nozzle (644 μm), even at the maximum allowable pressure of 200 kPa for the current printer configuration)

However, it demonstrates good printability with a 20G nozzle at a speed of 10 mm/sec (the instrument's minimum setting), although the structure tends to collapse, making the ink more suitable for use as a filling material.

Nanocellulose gel networks, when incorporated into an aliquot of ink 3, exhibit self-assembly behavior in an aqueous medium. Additionally, printing tests also with ink 3 + containing cells resulted in a more defined structure under the following conditions: 10 mm/sec, 18G nozzle, 20% infill and 70 kPa extrusion pressure

3.3.1.4. Ink 4

Fixed parameters:

-Room temperature.

-Dimensions: 20x20x3cm and 30x30x3 mm

-Distance between extruder nozzle tip and printing stage/deposited top layer 0.01 mm.

Ink	Speed mm/sec	Nozzle diameter (G / μ m)	Infill %	Pressure (KPa)	Note	Photo
TEST4.1a						
4	10	20G	15	20 22 25	discontinuous	
TEST 4.1b						
4	10	18G (1020)	22	25	Complete print of the structure, but dragging of the thread, probably low cohesion between the layers	
TEST 4.1c						
4	10	18G	22	28 30	Complete print of the structure, but dragging of the thread, probably low cohesion between the layers	
TEST 4.1d						
4	10	18G	22	35	Full structure print.	

4	10	18 G	22	40	Pressure tested but too high. The syringe empties without giving structure to the form.	
TEST 4.1e						
4	10	18G	25	35	Full structure print (30x30x3mm)	

Table 3.3.4. Defining printing conditions for ink 4

For sample 4, the use of 20 G nozzle was excluded due to filament discontinuity and breakage. The optimal pressure range for extrusion is between 28 and 35Kpa

3.3.1.5. Best printing conditions obtained for the 4 inks

Table 3.3.5 summarizes the two best printing scenarios obtained from the tests illustrated above in detail and also after further optimization for each ink.

Sample	Speed	Tip	Infill %	Kpa	Shape
Ink 1	10	18 G	20	150	20x20x3mm
Ink 1	20	18 G	20	200	20x20x3mm
Ink 2	10	20	20	150	20x20x3mm
Ink 2	10	22	20	130	20x20x3mm
Ink 3	10	20	20	10	20x20x3mm
Ink 3 with nanocellulose or with plant cell culture	10	18	20	70	20x20x3mm
Ink 4	10	18	22	30	20x20x3mm

Table 3.3.5. Defining printing conditions for the four inks

3.3.1.6. Images of final printed products

The defined parameters were applied to the prototype printer (Nutri3D Printer, ELTHUB S.r.l, Oricola, Italy) developed within the framework of the project. The application of these settings allowed to obtain the final products, which were subsequently subjected to sensorial evaluation through panel tests to verify their qualitative characteristics.

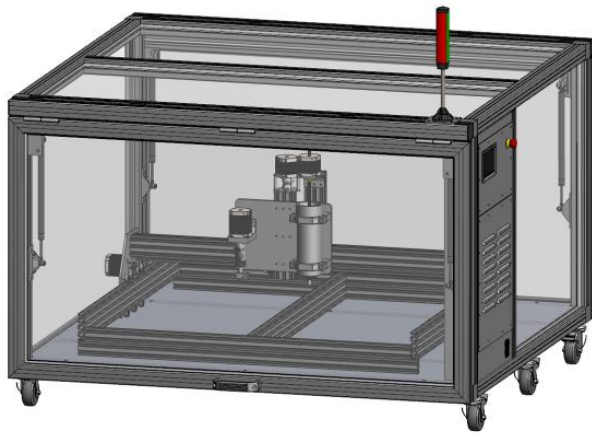


Figure 3.3.1. 3D Rendering of the Nutri3D Printer.



Figure 3.3.2. Multilayer bar composed of an external biscuit-like shell, an intermediate layer of dark or white chocolate-like filling, and an internal core of gelée filling for the inclusion of cells

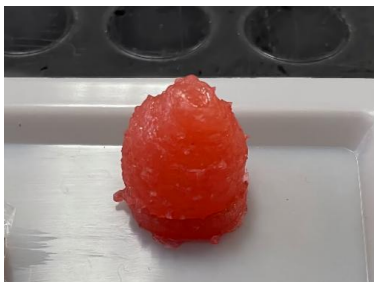


Figure 3.3.3. Honey sphere with an inner gelée filling that contains cells.

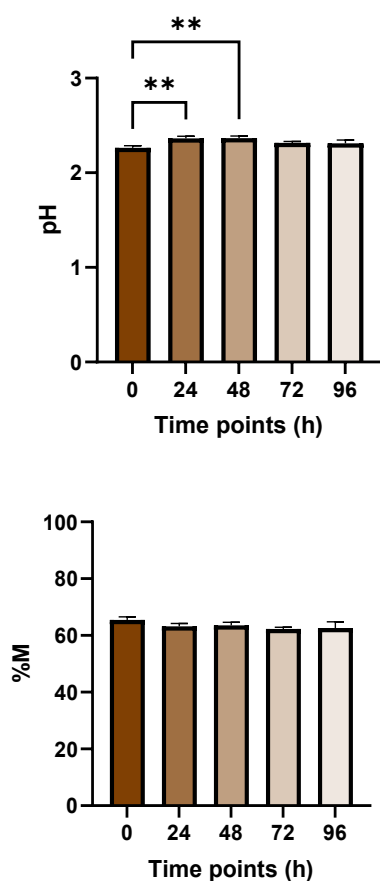
3.3.2 Evaluation of UV-visible spectra, pH and moisture content on individual inks

The four final recipes were evaluated for the UV-visible spectrum of hydroalcoholic extract and for chemical-physical characteristics mainly related to shelf life (total humidity and pH).

3.3.2.1 Ink 1

The data indicate an increase in pH, with significant differences (p value < 0.005) observed between time 0 and time 24h, as well as between time 0 and time 48h, according to Tukey's multiple comparison test (Figure 3.3.1). This basification may be attributable to the progressive degradation of citric acid over time, as well as the acid hydrolysis of polysaccharides present in apple fiber (Rehman et al., 2014). The analysis determined that the total moisture content of the sample is

65,50%. On the contrary, the moisture content of the ink after storage at 4°C did not exhibit statistically significant variation over the observation period (T0-T96; Tukey's multiple comparison test) (Figure 3.3.4).



Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	**	0,004	2,263	2,363
0 vs. 48	**	0,004	2,263	2,367
0 vs. 72	ns	0,14	2,263	2,317
0 vs. 96	ns	0,23	2,263	2,310
24 vs. 48	ns	>0,99	2,363	2,367
24 vs. 72	ns	0,23	2,363	2,317
24 vs. 96	ns	0,14	2,363	2,310
48 vs. 72	ns	0,18	2,367	2,317
48 vs. 96	ns	0,11	2,367	2,310
72 vs. 96	ns	>0,99	2,317	2,310

Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	ns	0,26	65,50	63,23
0 vs. 48	ns	0,39	65,50	63,54
0 vs. 72	ns	0,07	65,50	62,31
0 vs. 96	ns	0,11	65,50	62,61
24 vs. 48	ns	>0,99	63,23	63,54
24 vs. 72	ns	0,90	63,23	62,31
24 vs. 96	ns	0,97	63,23	62,61
48 vs. 72	ns	0,76	63,54	62,31
48 vs. 96	ns	0,89	63,54	62,61
72 vs. 96	ns	>0,99	62,31	62,61

Figure 3.3.4. Study of the variation of pH and humidity over time (0-96h) of Ink 1 by Tukey's multiple comparison test (Ti: 0h; Tf: 96h) for statistical significance.

Subsequently, an assessment of the potential degradation of high added value molecules (flavonoids) in the ink following the cooking process was performed. The UV-visible spectra were found to be superimposable across different temperatures in the thermobalance, as well as between thermobalance and the incubator (Figure 3.3.5.). Given the absence of significant differences, IR cooking at 60°C was selected as the optimal condition, as it allows for achieving the target moisture in a shorter time. As a result of water removal after cooking, the absorbance related to flavonoids (peak at 282nm) per unit of weight (30mg), tripled in the cooked product compared to the raw sample, thereby enhancing the added value of the final product.

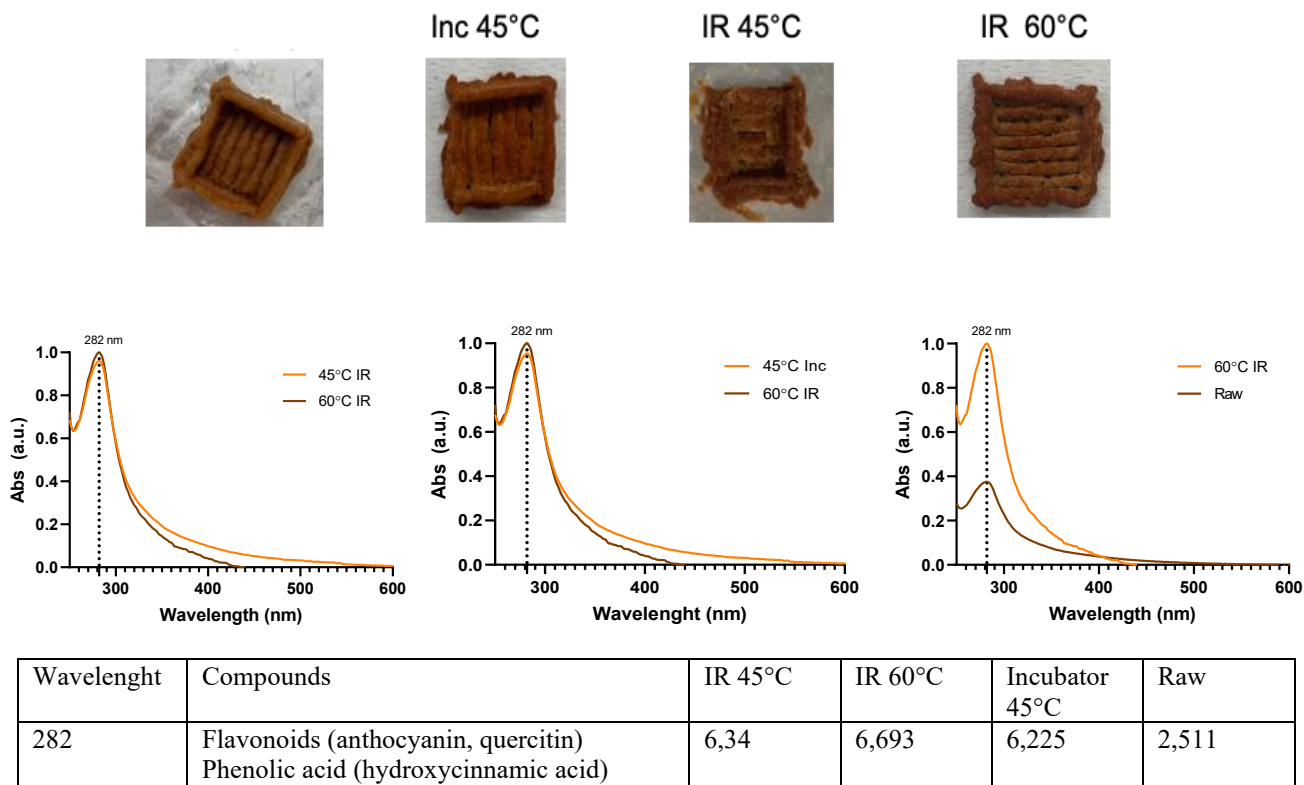
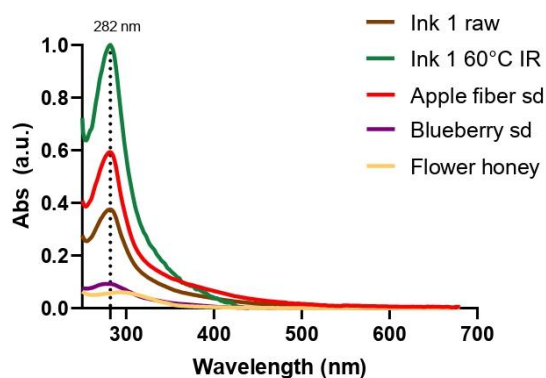


Figure 3.3.5. Comparison of UV-visible spectra of ink1 cooked in an infrared (IR) thermobalance at 45°C or at 60°C (a), IR thermobalance at 60°C and 45°C incubator (b) and raw and cooked product at 60°C in thermobalance (c) (IR: cooked in infrared thermobalance; inc.: cooked in incubator; raw: raw ink). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

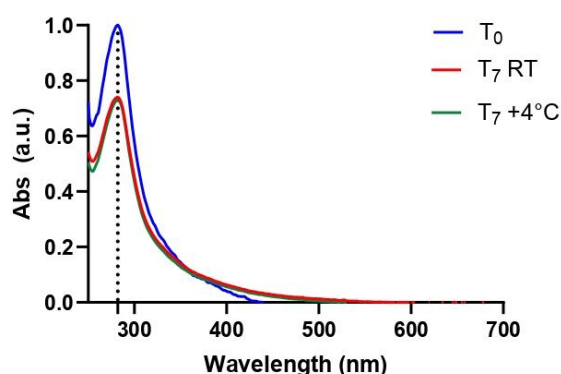
In both cooked or raw Ink 1 (30mg), flavonoids (absorbance peak at 282nm; related to flavonols and attributable to the blueberry by-product extract and apple fiber), are preserved through the cooking process and with respect to the spectra of the single components of the Ink, considering their respective concentration in the formulation (Figure 3.3.6.)



Wavelength	Compounds	Abs Ink 1 raw	Abs Ink 1 60°C	Abs Apple fiber sd	Abs Blueberry sd	Abs Honey
282	Flavonoids (anthocyanin, quercitin) Phenolic acid (hydroxycinnamic acid)	2,511	6,693	3,967	0,619	0,405 (293nm)

Figure 3.3.6. UV-visible spectra of MeOH:H₂O 70:30 v:v extracts of ink 1 and its ingredients (Blueberry SD: spray-dried blueberry by-product powder; Abs: absorbance). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

Figure 3.3.4. shows the results of the UV-visible spectrum of the hydroalcoholic extract from Ink 1 at time 0, after 7 days under vacuum at room temperature, and after 7 days under vacuum at +4°C. Regardless of the storage mode, the UV-visible spectrum shows the same decrease in absorbance over time, while maintaining the spectral profile unchanged.

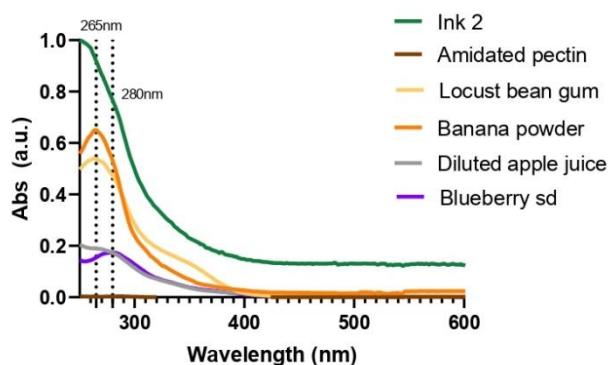


λ	Recipe1 60°C T0	Recipe1 T7 RT	Recipe1 T7 +4°C
282	6,693	4,956	4,883

Figure 3.3.7. UV-visible of Ink extract 1 at time 0 (T0), after 7 (T7) under vacuum at room temperature and after 7 days under vacuum at +4°C (Abs: absorbance). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

3.3.2.2 Ink2

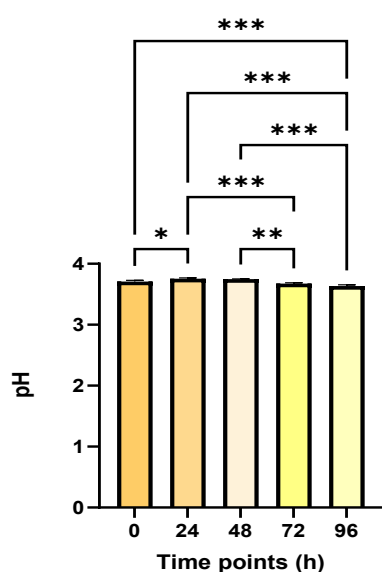
The UV-Vis spectrum of the MeOH:H₂O (70:30 v:v) extract from Ink 2 (Figure 3.3.8) exhibit a distinct absorption peak at 265 nm, consistent with characteristic UV-visible signatures of gallic acid reported in literature. These findings align with compositional data of locust bean gum (Goulas et al., 2019) and banana powder (Afzal et al., 2022), both of which recognized sources of gallic acid as predominant phenolic components.



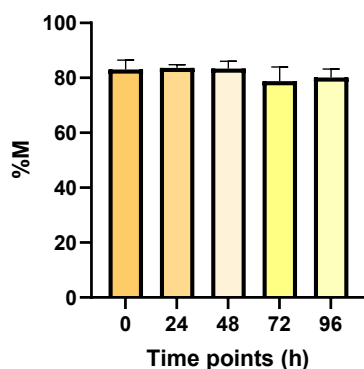
λ (nm)	Compounds	Ink 2	Banana powder	Amidated pectin	Locust bean gum	Diluted apple juice	Blueberry sd
265	Phenolic acid (gallic acid)	0,656	0,464	-	0,384	-	
280	Flavonoids (anthocyanins)	-	-	-	-	0,123	0,123

Figure 3.3.8. UV-visible spectrum of MeOH:H₂O 70:30 v:v extract of Ink 2 in relation with those of its components (Abs: absorbance). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

The most pronounced pH variations were observed within the first 24 hours post preparation, characterized by a slight but significant acidification trend. However, these fluctuations remained minimal, with changes confined to the first or second decimal place (e.g., pH 3,757 at 24h vs pH 3,633 at 72h), indicating a good stability in the ink's pH profile at 4°C (Figure 3.3.6). These minimal variations highlight the intrinsic buffering capacity of the formulation and the absence of significant chemical degradation pathways. Furthermore, this behavior is consistent with the Ink 2 moisture stability (95.94-97.46 %) (Figure 3.3.9), collectively confirming its physicochemical reliability under the defined short-term storage conditions.



Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	*	0,03	3,710	3,757
0 vs. 48	ns	0,10	3,710	3,747
0 vs. 72	ns	0,10	3,710	3,673
0 vs. 96	***	<0,001	3,710	3,633
24 vs. 48	ns	0,93	3,757	3,747
24 vs. 72	***	<0,001	3,757	3,673
24 vs. 96	***	<0,001	3,757	3,633
48 vs. 72	**	0,001	3,747	3,673
48 vs. 96	***	<0,001	3,747	3,633
72 vs. 96	ns	0,07	3,673	3,633

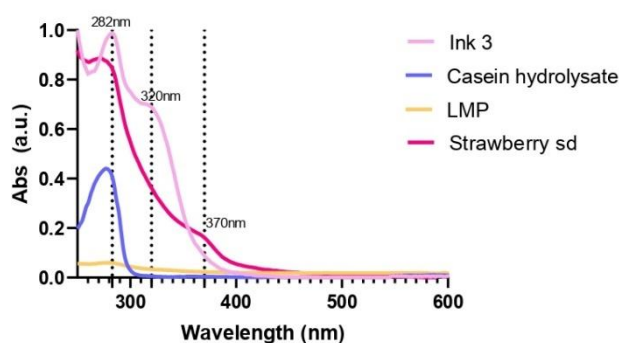


Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	ns	>0,99	83,03	83,67
0 vs. 48	ns	>0,99	83,03	83,38
0 vs. 72	ns	0,56	83,03	78,77
0 vs. 96	ns	0,87	83,03	80,13
24 vs. 48	ns	>0,99	83,67	83,38
24 vs. 72	ns	0,44	83,67	78,77
24 vs. 96	ns	0,78	83,67	80,13
48 vs. 72	ns	0,50	83,38	78,77
48 vs. 96	ns	0,83	83,38	80,13
72 vs. 96	ns	>0,99	78,77	80,13

Figure 3.3.9. Study of the variation of pH and humidity over time (0-96h) of Ink 2 at 4°C by Tukey's multiple comparison test (Ti: 0h; Tf: 96h).

3.3.3.3 Ink 3

The UV-visible spectrum of Ink 3 extracted in MeOH:H₂O 70:30 (v:v) reveals a peak of absorbance at 278nm, attributable to pelargonidin 3-glucoside (Lopes da Silva et al., 2007), an anthocyanin predominantly found in strawberries. Casein hydrolysate contributes to the overall absorbance at 280nm. This is consistent with the characteristic absorption of most proteins at this wavelength, primarily due to their tryptophan content (Stefanescu et al., 2017). The higher absorbance observed in the UV-visible spectrum of the spray-dried strawberry by-product powder compared to the complete Ink 3 (Figure 3.3.10) can be attributable to its dilution in the final recipe due to the presence of the other ingredients in the formulation.



λ (nm)	Compounds	Ink 3	Pectin	Casein hydrolysate	Strawberry sd
280	Flavonoids (pelargonidin 3-glucoside), Amino acids (tryptophan)	0,382	-	0,317	0,634
320nm	Flavonoids	0,257	-	-	-
370nm	Flavonols	0,027	-	-	0,118

Figure 3.3.10. UV-visible spectrum of MeOH:H₂O 70:30 v:v extract of Ink 3 in relation with those of its components (Abs: absorbance). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra

The pH of Ink 3 remains stable over time, although slight but significant variations are observed within the first 24 hours post-preparation. From 0h to 48h, a gradual increase in pH is observed, followed by a slight acidification, which may be influenced over time by the presence of calcium chloride in the ink, as demonstrated in a study of Suriati et al., 2022 where calcium chloride causes an increase in acidity during storage. This pH variation, however, remains within the first or second decimal place, highlighting the overall stability of this parameter in Ink 3 (Figure 3.3.11).

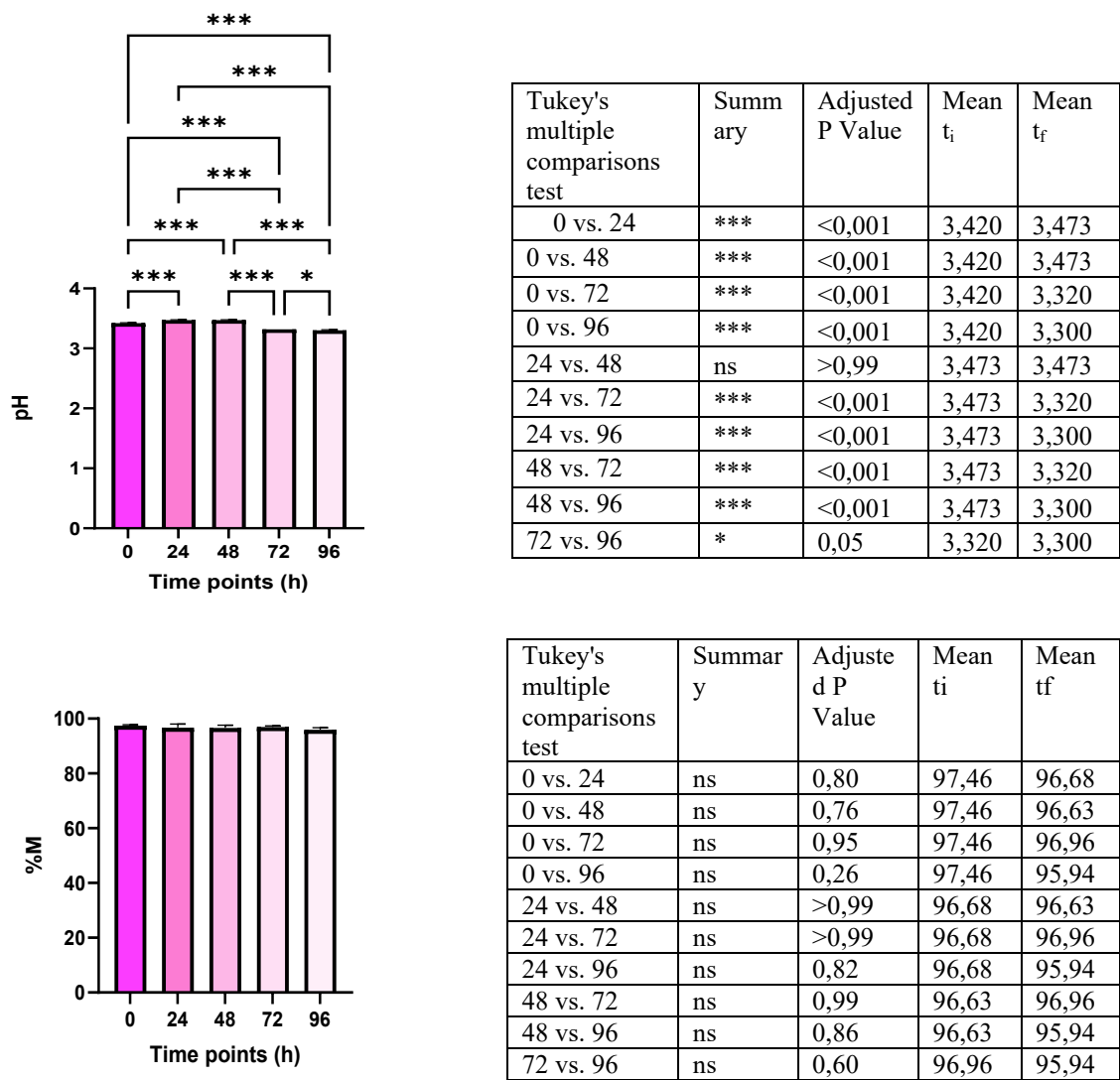
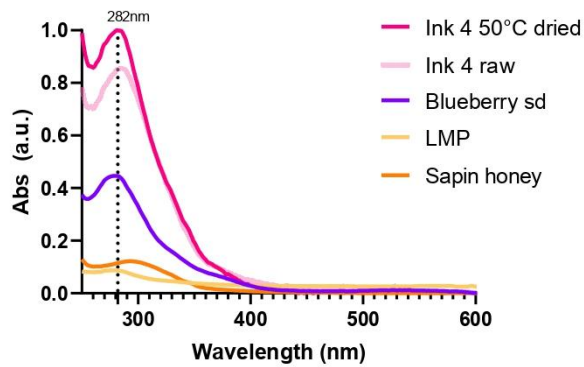


Figure 3.3.11. Study of the variation of pH and percentual humidity over time (0-96h) of Ink 3 at 4°C by Tukey's multiple comparison test for statistical significance. (Ti: 0h; Tf: 96h).

3.3.3.4 Ink 4

The UV-visible spectrum obtained from the MeOH:H₂O 70:30 (v/v) extraction of Ink4 presents a slight increase in absorbance (peak at 282nm; attributable to flavonoids, including anthocyanins, in

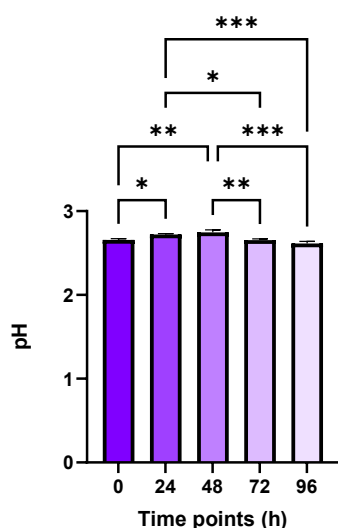
blueberry and vanillin in the honey; Cheung et al., 2019) when the final drying step is applied (Figure 3.3.12).



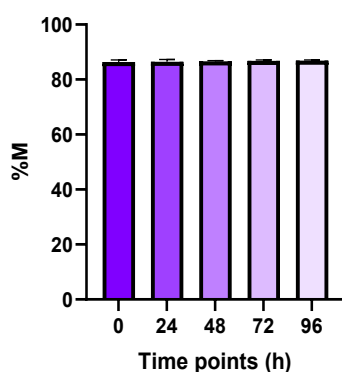
λ	Compounds	Ink 4 dried	Ink 4 raw	Pectin (LMP)	Honey (sapiin)	Blueberry sd
282nm	Flavonoids (anthocyanins and vanillin)	2,080	1,772	0,180	0,238	0,927

Figure 3.3.12. UV-visible spectrum of MeOH:H₂O 70:30 v:v extract of Ink 4 in relation with those of its components (Abs: absorbance). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

A statistically significant acidification of the pH was observed primarily between 24 and 96 hours, and between 48 and 96hours (Figure 3.3.10). However, these variations remain within the first or second decimal value, indicating the overall stability of this parameter in Ink 4. The observed moisture variations were not statistically significant (86,39% at 0h- 86,86%) (Figure 3.3.13)



Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	*	0,02	2,657	2,720
0 vs. 48	**	0,002	2,657	2,747
0 vs. 72	ns	>0,99	2,657	2,653
0 vs. 96	ns	0,14	2,657	2,613
24 vs. 48	ns	0,52	2,720	2,747
24 vs. 72	*	0,02	2,720	2,653
24 vs. 96	***	<0,001	2,720	2,613
48 vs. 72	**	0,002	2,747	2,653
48 vs. 96	***	<0,001	2,747	2,613
72 vs. 96	ns	0,18	2,653	2,613



Tukey's multiple comparisons test	Summary	Adjusted P Value	Mean t_i	Mean t_f
0 vs. 24	ns	>0,99	86,39	86,50
0 vs. 48	ns	0,98	86,39	86,64
0 vs. 72	ns	0,87	86,39	86,81
0 vs. 96	ns	0,82	86,39	86,86
24 vs. 48	ns	>0,99	86,50	86,64
24 vs. 72	ns	0,95	86,50	86,81
24 vs. 96	ns	0,92	86,50	86,86
48 vs. 72	ns	>0,99	86,64	86,81
48 vs. 96	ns	0,98	86,64	86,86
72 vs. 96	ns	>0,99	86,81	86,86

Figure 3.3.13. Study of the variation of pH and percentual humidity over time (0-96h) of Ink 4 at 4°C, by Tukey's multiple comparison test for statistical significance. (Ti: 0h; Tf: 96h).

3.3.3 UV-visible profile stability of the multilayer bar and honey pearl under different storage conditions

The multilayer bar and the complete honey pearl were also evaluated for the preservation of the UV-visible profile under different composition/storage conditions:

3.3.3.1 Multilayer bar

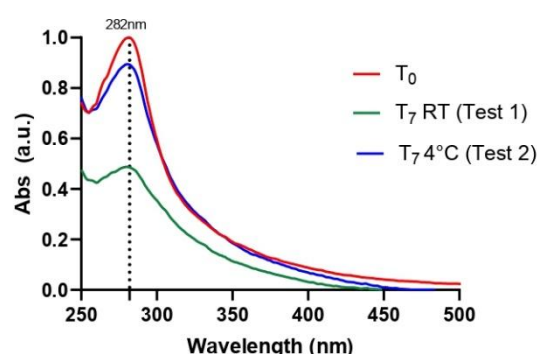
- Test 1: bar with layers of Ink 1 (dried; 45% humidity), + Ink 2 and Ink 3; kept under vacuum and at room temperature.
- Test 2: bar with layers of Ink 1 (dried; 45% humidity), + Ink 2 and Ink 3; kept under vacuum and at 4°C.

- Test 3: bar with layers of Ink 1, Ink 2 and Ink 3; dried (45% humidity) and kept under vacuum and at room temperature;
- Test 4: bar with layers of Ink 1, Ink 2 and Ink 3; dried (45% humidity) and kept under vacuum and at 4°C.
- Test 5: bar with layers of dried (45% humidity) Ink 1, Ink 2 and Ink 3 (with 1×10^6 cells/mL *Perilla frutescens* suspension cells); kept under vacuum at room temperature.
- Test 6: bar with layers of dried (45% humidity) Ink 1, Ink 2 and Ink 3 (with 1×10^6 cells/mL *Perilla frutescens* suspension cells); kept under vacuum at 4°C.

The UV-visible spectra were acquired following the extraction of the 6 formulations/conditions using MeOH:H₂O 70:30 (v:v). The absorbance values are indicative of the concentration of specific components within the ink.

Tests 1 and 2

A notable stability was observed when the sample was stored at 4°C (Test 2), as evidenced by the minimal change in the UV-visible spectrum compared to T0. In contrast, the spectrum of the sample within Test 1 (stored at room temperature), reveals a decrease in absorbance in correspondence of the 282nm peak (Figure 3.3.14).

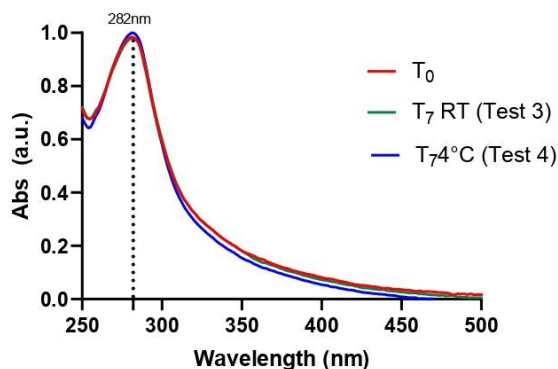


λ (nm)	T0	T7 RT (Test 1)	T7 +4°C (Test 2)
282	2,242	1,088	2,000

Figure 3.3.14. UV-visible spectrum of the multilayer bar, stored for 7 days at room temperature (Test 1) or at 4°C (Test 2), respectively. Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

Tests 3 and 4

A significant stability was observed both after storage at room temperature (Test 3) and at 4°C (Test 4), in comparison to T0. This suggests that processing the entire formulation, rather than just Ink 1 as initially thought, more effectively preserves the metabolite profile (Figure 3.3.15).

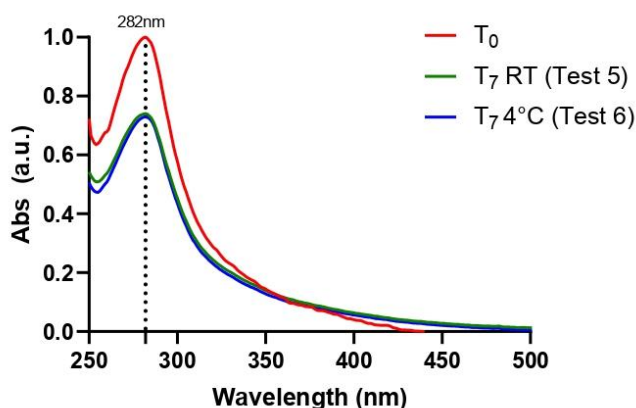


λ (nm)	T0	T7 RT	T7 +4°C
282	3,535	3,524	3,595

Figure 3.3.15. UV-visible spectrum of the multilayer bar prepared as per Test 3 and 4 (i.e., stored for 7 days at room temperature or 4°C, respectively). Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

Tests 5 and 6

A decrease in absorbance at 282nm was noted for both storage conditions compared to T0. However, due to the contribution of plant cells, the absorbance at 282nm (which corresponds to metabolite classes such as hydroxycinnamic acids and flavanones) is increased from 2,242 to 4,635 (Figure 3.3.16).



λ (nm)	T0	T7 RT	T7 +4°C
282	4,635	3,029	3,130

Figure 3.3.16. UV-visible spectrum of the multilayer bar, upon Test 5 and 6 (i.e., stored for 7 days at room temperature or 4°C), respectively. Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.

3.3.3.2 Honey pearls

- Test 1: dried honey pearl, under vacuum and at room temperature.
- Test 2: dried honey pearl, under vacuum and at 4°C.
- Test 3: dried honey pearl filled with Ink 3, under vacuum and at room temperature.
- Test 4: dried honey pearl filled with Ink 3, under vacuum and at 4°C.
- Test 5: dried honey pearl filled with Ink 3 with cells (1×10^6 cells/mL *Perilla frutescens* suspension cells), under vacuum at room temperature.
- Test 6: dried honey pearl filled with Ink 3 with cells (1×10^6 cells/mL *Perilla frutescens* suspension cells), under vacuum and at 4°C.

Tests 1 and 2

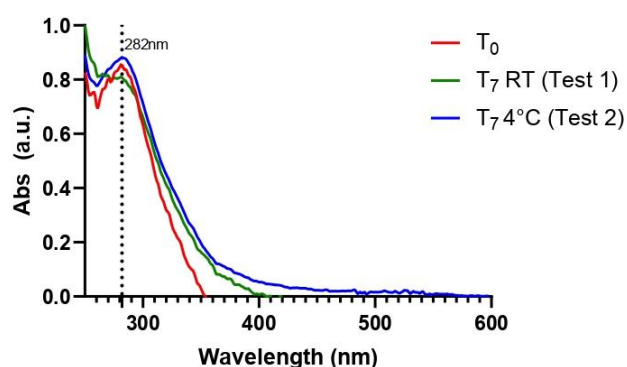
A substantial spectral stability was observed at both room temperature and +4°C, when compared to the initial spectrum at T₀ (Figure 3.3.17).

Tests 3 and 4

Upon Test 3 and 4 (honey sphere filled with ink 3 without cells), storage at +4°C resulted in slightly higher stability of flavonoids, as indicated by the peak at 282nm, compared to the sample stored at room temperature (Figure 3.3.18).

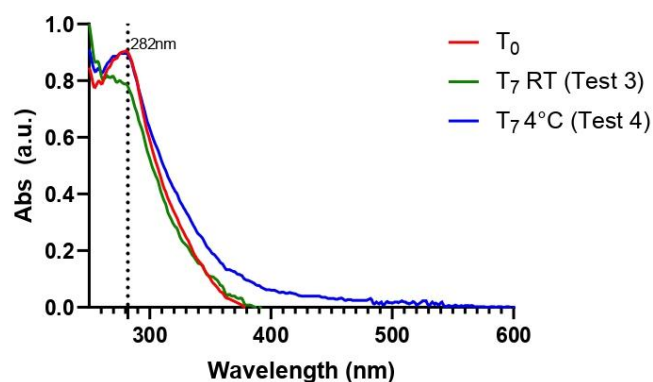
Tests 5 and 6

Upon Test 5 and 6 (honey sphere filled with Ink 3 with 1×10^6 cells/mL of *Perilla frutescens* suspension) a slight decrease in UV-visible spectrum, particularly at the flavonoid peak, was observed, with a more pronounced effect at room temperature (Figure 3.3.19). The contribution of plant cells to absorbance at 282nm, which corresponds to metabolite classes such as hydroxycinnamic acids and flavanones, is less significant compared to the multilayer bar. This is attributable to the lower content in Ink 3 (approximately five times less than in the multilayer bar).



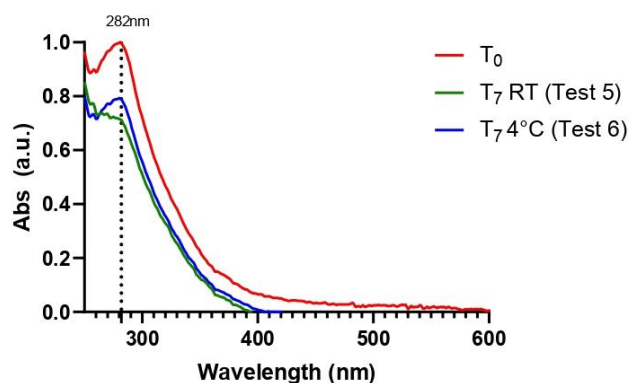
λ (nm)	T0	T7 RT	T7 4°C
282	0,353	0,334	0,364

Figure 3.3.17. UV-visible spectrum of the honey pearl upon Test 1 and 2 (i.e., stored for 7 days at room temperature or 4°C), respectively. Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra.



λ (nm)	T0	T7 RT	T7 4°C
282	0,436	0,380	0,433

Figure 3.3.18: UV-visible spectrum of the honey pearl upon Test 3 and 4(i.e., stored for 7 days at room temperature or 4°C), respectively. Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra



λ (nm)	T0	T7 RT	T7 4°C
282	0,533	0,386	0,421

Figure 3.3.19. UV-visible spectrum of the honey pearl upon Test 5 and 6 (i.e., stored for 7 days at room temperature or 4°C), respectively. Real (calculated) O.D. values are reported in the table, while arbitrary units (a.u.) in the spectra

3.3.4 Shelf life

3.3.4.1 Determination of the Shelf-life of 3D Products

The data presented in Table 3.3.6. indicate that, at time zero, the microbial load of the four analyzed samples, expressed in colony forming units (CFU), was as follow:

$6,1 \times 10^3$ (A), $6,6 \times 10^3$ (B), $6,9 \times 10^3$ (C), $8,3 \times 10^3$ (D).

Time	Total aerobic mesophilic microflora [log10 CFU/g]			
	Determination on Plate Count Agar (PCA) at 30°C			
	Bars		Honey pearls	
	A (with cells)	B (no cells)	C (with cells)	D (no cells)
0d	3,79± 0.14	3,82±0.90	3,84±0.90	3,92±0.31
7d	3,83± 0.08	3,84 ±0.89	3,86 ±0.17	3,99±0.290
16d	4,09± 0,37	4,02± 0,35	4,89±0.20	4,91±0.25

Table 3.3.6. Results obtained from microbiological tests conducted on the printed products. The samples were stored at refrigeration temperature (4°C). The results are the average of three determinations. Results are expressed as log10 CFU/g

The plate counts reveal two distinct morphologies, corresponding to a yeast (present in sample A, B, C, D) and a mold (C, D) (Figure 3.3.20).

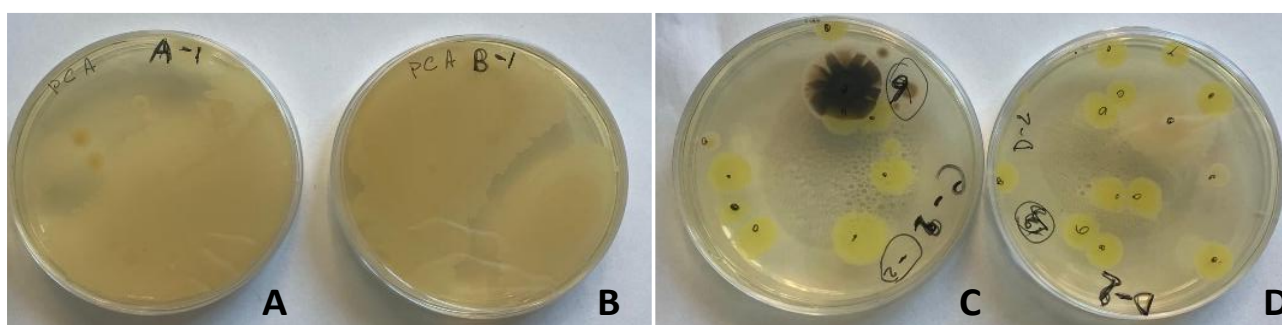


Figure 3.3.20. Determination of the total mesophilic microflora of samples A, B, C, D stored at refrigeration temperature (4°C) (Times: 0, 7th, 16th day; agar culture medium: Plate Count Agar, PCA; incubation T 30° C, 72 hours).

The plates of sample A, containing the plant cells, also display two distinct inhibition zones (Figure 3.3.21), suggesting an inhibitory effect on microbial growth exerted by the plant cells.



Figure 3.3.21. Detail of the determination of total mesophilic microflora on sample A stored at refrigeration temperature (4°C) in which two clear inhibition halos are evident (Times: 0, 7th, 16th day).

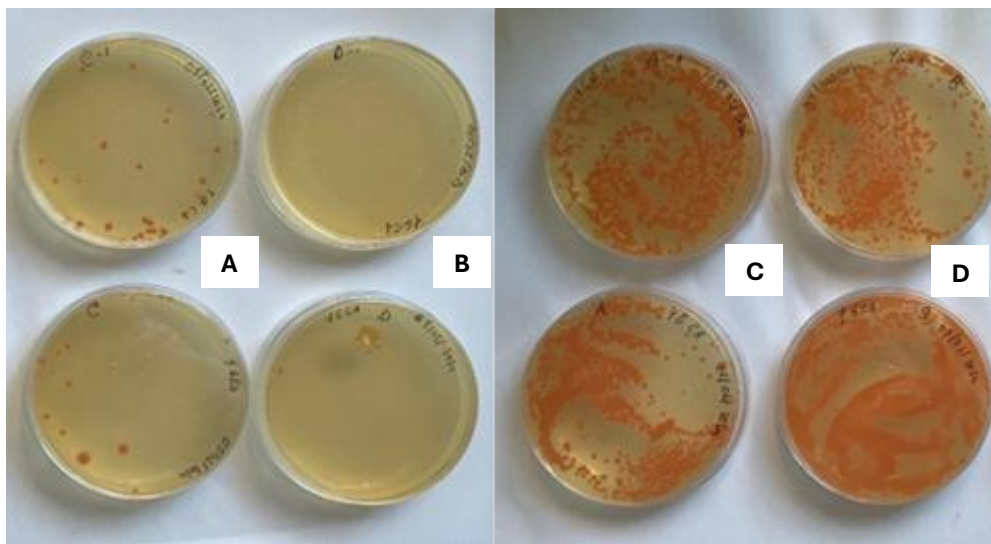


Figure 3.3.22. Isolations conducted on selective medium for yeasts and fungi, at 25°C developed from samples (A, B, C, D) stored at 4°C.

Plating the samples on a selective medium for fungi and yeasts (Yeast Glucose Chloramphenicol Agar) revealed salmon-colored colonies, morphologically identifiable as yeasts (predominantly in sample A and B) and colonies morphologically classified as molds (observed in samples C and D).

The analysis of microbial growth kinetics over the observation period, aimed at estimating the shelf-life, showed microbial growth values ranging from 1×10^4 (sample B) and 8.1×10^4 (sample D) on day 16. These microbial alterations became more pronounced on day 20, when the samples exhibited signs of *spoilage*, structural degradation and liquefaction (Figure 3.3.23).

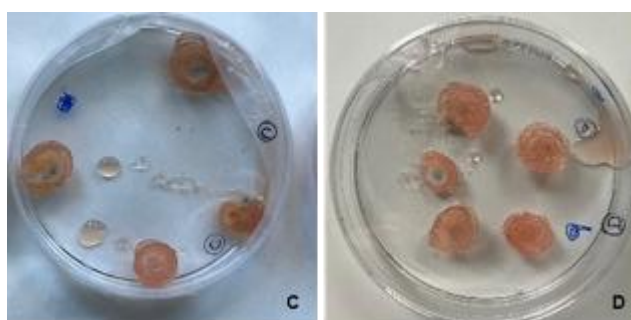


Figure 3.3.23. Samples C and D, on the 20th day of observation. Mold development, structural degradation, and liquefaction are evident.

3.3.4.2. Determination of the chemical-physical parameters pH, aw, water content and hardness

As shown in Table 3.3.7, all samples exhibit pH values below 3, classifying them as products potentially susceptible to contamination by lactic acid bacteria, mold or yeast, particularly

considering the composition of the ingredients. Regulatory guidelines, however, indicate a pH threshold of 4.5 as the safety level to ensure product stability and microbiological safety.

Furthermore, samples A and B display a_w values that are generally unfavorable for microbial growth, likely due to the thermal stabilization process applied during their preparation. In contrast, the honey pearl has a_w values higher than those of the bar, potentially increasing its susceptibility to microbial spoilage, despite also undergoing thermal stabilization. Therefore, given the substantially similar pH levels between samples A-B and C-D, a_w emerges as the primary discriminating factor in estimating the microbiological shelf-life of these two products groups.

The *texture* of the four samples was assessed based on hardness measurement for products stored at 4°C (Table 3.3.7.).

Time 0 (Starting point)	Determination of chemical-physical parameters			
	Multilayer bar		Honey pearl	
	A	B	C	D
Fresh weight (g)	2.054± 0.10	2.020±0.10	0.8829± 0.09	0.6088± 0.07
Moisture % (gH ₂ O/100g matrix)	42,6%± 0.09	37,9%±0.12	64,0%±0.11	66.3%±0.17
Dry matter (g/100 g fresh weight)	57,4%± 0.06	62,1%± 0.20	36,0%±0.15	33,7%±0.21
Water activity (a_w)	0.58± 0.02	0.56 ±0.01	0.94. ±0.02	0.95±0.01
pH	2.51± 0,31	2.49± 0,30	2.70±0.10	2.72±0.11
Hardness (Newton)	0.23± 0,02	0.25± 0,01	0.31±0.06	0.28±

Table 3.3.7 Determination of chemical-physical parameters linked to the microbiological and structural stability of the products

Table 3.3.8 shows the data relating to the monitoring of the pH of the products in the observation interval (0, 7 days, 16 days) to estimate the shelf life.

pH	Monitoring of the pH of the products in the three analytical observation times			
	Multilayer bar		Honey pearls	
	A	B	C	D
t0	2.51± 0,31	2.49± 0,30	2.70±0.10	2.72±0.11
t7	2.53± 0,17	2.55± 0,21	2.74±0.09	2.77±0.10
t16	2.98± 0,21	2.99± 0,17	3.01±0.19	3.03±0.13

Table 3.3.8. Monitoring of the pH of the products in the three analytical observation times

The pH remained consistently stable around the indicated values, indicating that the products are microbiologically safe concerning the proliferation of pathogenic bacteria. The structural modifications in the samples, along with their dehydration, quantified as weight reduction, were monitored at room temperature (22 °C). Samples C and D maintained microbiological stability up to the 16th day. However, in contrast to their counterparts stored under refrigerated conditions (Table 3.3.9), samples exhibited a significant weight reduction ranging from 80% to 83.1%. This dehydration process was accompanied by vitrification/crystallization phenomena, leading to a complete alteration of the texture/structure.

Weight (g)	Monitoring of the weight of products kept at room temperature (to, 7th day, 16th day)							
	Multilayers bar				Honey pearls			
	A	*%	B	*%	C	*%	D	*%
To	2.017± 0,10		2.109± 0,09		0.996±0.10		0.867±0.06	
T7	1.596± 0,12	20.8	1.644± 0.08	22,0	0.219±0.09	78	0.248±0.10	71.3
T16	0.856± 0,21	57.5	0.877± 0,13	58.4	0.198±0.11	80	0.146±0.06	83.1

Table 3.3.9. Monitoring of percent weight loss due to dehydration and texture of samples between 0 and 16 days at room temperature

3.3.5 Analytical characterization of inks

3.3.5.1 Analytical characterization of inks by FTIR-ATR

Results of optical microscope photographs (10X objective), Raman and FTIR-ATR spectra of the inks are reported in the following figure (Figure 3.3.24).

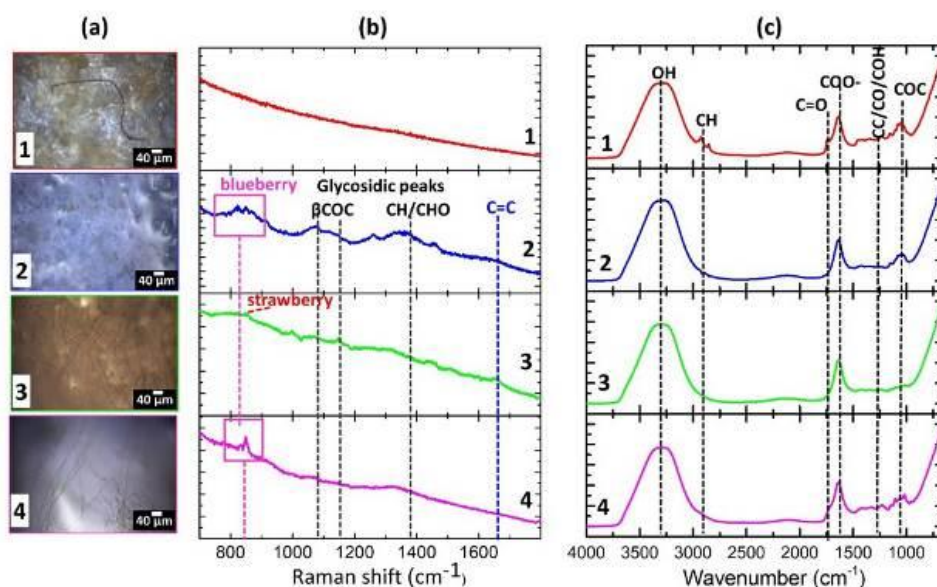


Figure 3.3.24 (a) Optical microscope photographs with 10x objective; (b) Raman and (c) FTIR-ATR spectra of the inks.⁴

The Raman spectral profile of the four inks exhibit variation based on the nature of the ingredients utilized. Ink 1, due to the presence of oily components and its consistency, hinders the acquisition of the spectrum. In contrast, Inks 2, 3 and 4 display characteristic signals around 800 cm^{-1} , attributable to red fruit extracts, with additional peaks between 1000 and 1500 cm^{-1} corresponding to glycosidic components and a band near 1600 cm^{-1} , indicating the presence of C=C double bonds. Notably, the glycosidic signals are marked in Ink 2, linked to the sugar component of banana powder and carob flour.

The spectra of Inks 2 and 4 show the typical peaks of blueberry extract at 820 cm^{-1} , whereas the peak at 850 cm^{-1} in Ink 3 suggests the presence of strawberry extract, along with a peak at 1600 cm^{-1} attributable to the C=C double bonds of casein hydrolysate.

The FTIR-ATR analysis further elucidated the chemical composition of the inks, as shown in Figure 3.3.21c. All spectra display a broad peak around 3200 cm^{-1} , corresponding to –OH groups in absorbed water and stretching of –OH groups in polysaccharides and pectin. Only the spectrum of Ink 1 reveals peaks near 2930 cm^{-1} , associated with the vibrations of –CH groups. This can be attributable to the presence of lignin and hemicellulose in the apple fiber, along with the hydrophobic effect of the oil. The band at around 1750 cm^{-1} corresponding to the stretching of C=O groups in carboxylic esters, while the peak near 1650 cm^{-1} is linked to the vibrations of free carboxyl groups in carboxylate compounds. The wavenumbers below 1500 cm^{-1} reveals a series of peaks attributable to the vibrations of the C-C, C-O, and C-OH groups, while the peaks around 1200 cm^{-1} are indicative of C-O-C glycosidic bonds.

3.3.5.2 Deswelling Behavior and Water Release Over Time

In Figure 3.3.22 the trend of the swelling degree (SD%) and the water retention (WR) is shown during time.

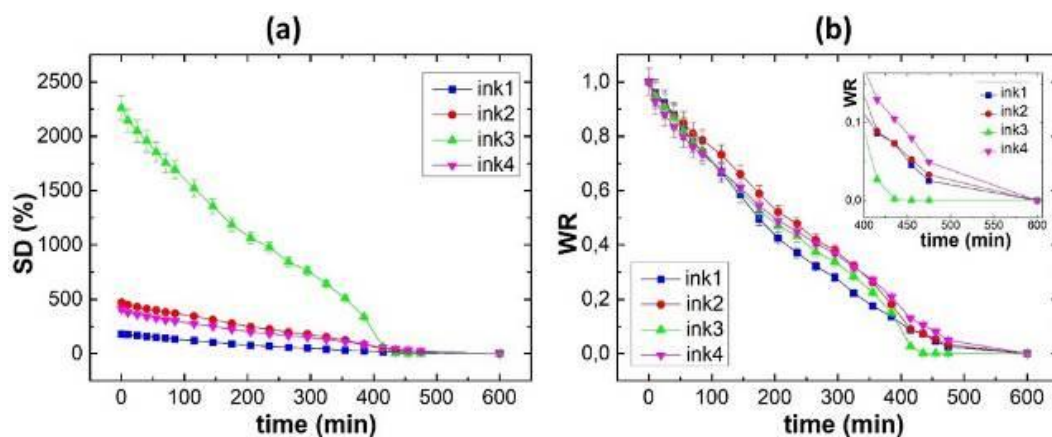


Figure 3.3.25. Swelling degree (SD%; a) and water retention (WR; b) trends as a function of drying time. The error was calculated as the standard deviation on three sets of experiments.

The degree of *swelling* measured at time zero ($t=0$) is approximately 190, 500, 2200 and 480 for inks 1, 2, 3 and 4, respectively. The variation in the amount of water absorbed is likely attributed to the distinct chemical composition of the ink mixtures. Ink 1, due to the presence of hydrophobic substances such as oil, lignin and cellulose, exhibits the lowest swelling capacity, thus retaining the least amount of water. In contrast, Ink 2 and Ink 4, which have a high content of sugary substances, show similar swelling percentages, resulting in comparable SD% value. Ink 3, with its significantly higher degree of swelling, can be explained by the presence of calcium chloride ions, which enhance the water retention capability by increasing the number of anchoring sites for water molecules within the ink three-dimensional structure.

As shown in the figure, all four inks reach dehydration after approximately 400 minutes, revealing a notable capacity to retain water molecules over time.

3.3.5.3. Functional groups involved in drying mechanisms via FTIR analysis over time

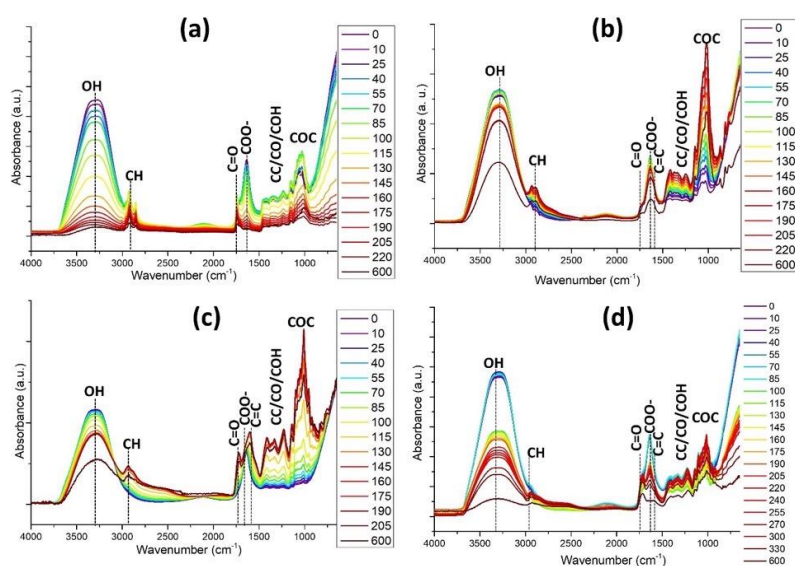


Figure 3.3.26. Time-lapse FTIR-ATR spectra acquired for ink (a) 1, (b) 2, (c) 3 and (d) 4.

The spectral profiles vary based on the chemical composition of the inks, suggesting different interactions between the inks and water molecules.

The signals at approximately 3500, 2900, 1750 and 1650 cm^{-1} are respectively attributable to the OH, CH, C=O(ester) and COO groups. The series of peaks at wavenumbers lower than 1500 cm^{-1} are attributable to vibrations of the C-C/C-O/C-OH groups, while the signals between 1200 and 1000 cm^{-1} can be ascribed to the C-O-C glycosidic bonds. As the drying time increases, an additional signal appears in the spectra of inks 2, 3 and 4 around 1600 cm^{-1} attributable to the vibrations of the C=C double bonds.

3.3.5.4 Molecular interactions between ink components

The analysis of FTIR spectra over time provides essential insights into the molecular interactions among ink components. The synergistic interaction between water molecules, any ionic additives and organic molecules, such as pectin, polysaccharides, peptides, modulates the hygroscopic behavior of the inks, depending on their specific chemical composition.

Ink 1: the progressive reduction in the intensity of the OH band until complete dehydration is likely attributable to the presence of hydrophobic components, such as oil and apple fiber, which facilitate water removal. The observed increase in the intensity of the C=O ester groups, alongside the concomitant decrease in the COO⁻ peak, suggests that cross-linking occurs between the polymer chains present in the ink. Given the structural rigidity achieved by Ink 1 at the end of the drying

process, it is reasonable to assume that the cross-linking primarily involves interaction between the glycosidic units and carboxylate groups within the apple fiber.

Ink 2: the decrease in OH band intensity is associated with water evaporation. The ratio between the intensity of C=O and COO⁻ signals remain relatively stable, however, a marked increase in the relative intensity of the C-O-C signals is observed. This suggests that cross-linking within the polysaccharide chains occurs primarily through the formation of inter-chain glycosidic bonds.

Ink 3: The presence of calcium chloride plays a crucial role in the water-retention properties of Ink 3. The OH band intensity decreases over time, while the C-O-C signals increase. Additionally, a significant increase in the relative intensity between the C=O and COO⁻ peaks is detected after the first 100 minutes of drying. This suggests that cross-linking between polymer molecules within the ink occurs via both esterification and inter-chain glycosidic bonds formation. In agreement with the observed swelling behavior, the substantial increase in the C=C intensity indicates a pronounced expulsion of water molecules following extensive inter-chain cross-linking.

Ink 4: the inclusion of honey results in a behavior similar to that of Ink 1. The formation of the C=C bonds is likely attributable to beta-elimination mechanisms occurring at the glycosidic units within the pectin chains.

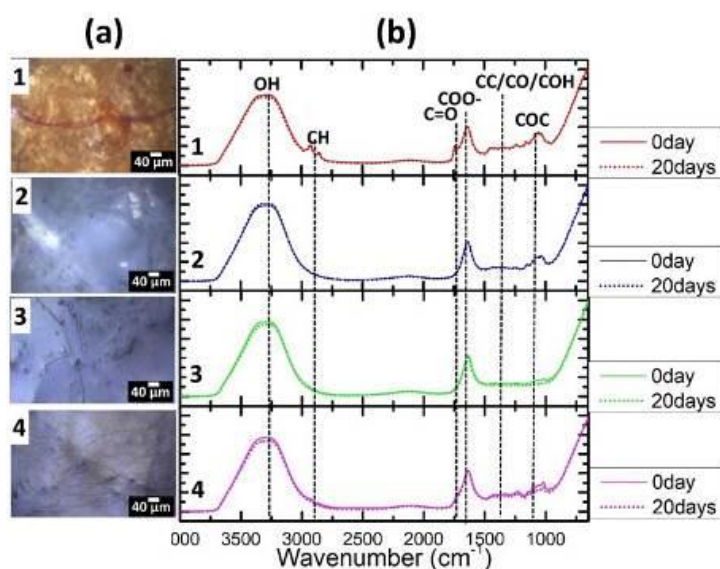


Figure 3.3.27. (a) optical microscope photographs of samples stored 20 days at 4°C; (b) comparison of the FTIR-ATR spectra acquired on freshly produced inks (solid line) and on inks stored for 20 days at 4°C (dashed line).

The FTIR-ATR spectra acquired on inks stored for 20 days at 4°C are perfectly superimposable with those of freshly produced food matrices (Figure 3.3.27).

3.3.5.5 Effect of the presence of plant cells on the hygroscopic behavior of Ink 3

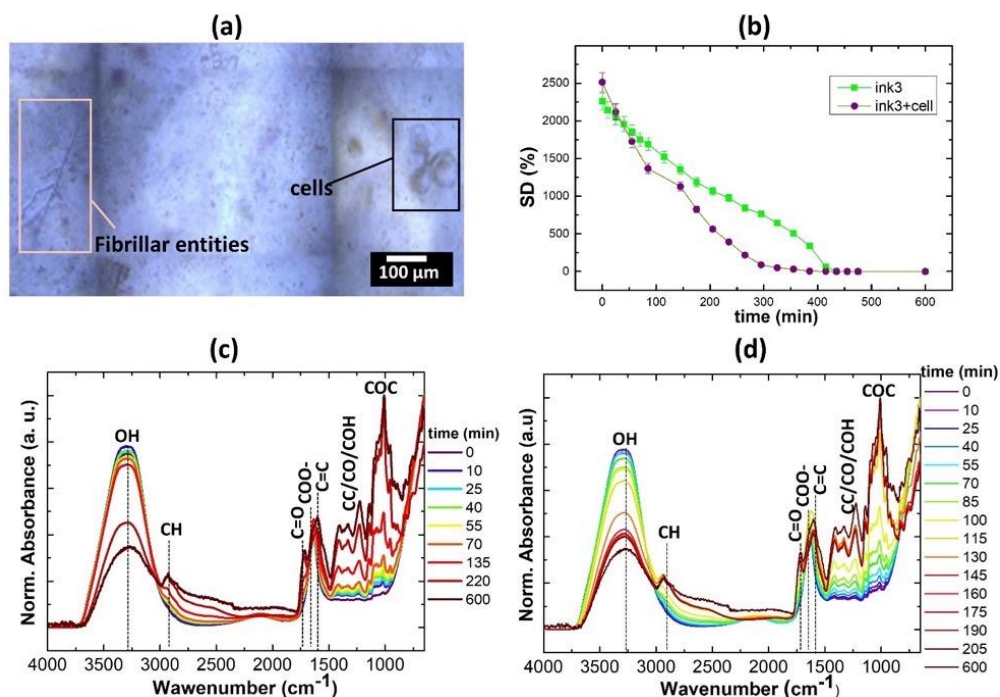


Figure 3.3.28 (a) optical microscope photo of Ink 3 with cells, (b) trend of swelling degree over time for ink 3 with and without cells, FTIR-ATR over time for ink 3 (c) with and (d) without cells.

The incorporation of cells within the ink modulates the water retention behavior of the hydrogel.

The swelling degree trend over time indicates that the ink containing the cells exhibits a lower water retention capacity compared to the reference ink without cells. However, since this retention capacity decreased progressively over time, Ink 3 with embedded cells demonstrates a more stable and predictable behavior. In contrast, hydrogel without cells has shown an abrupt loss of retention capacity around 350 minutes. Despite the differing hygroscopic properties of Ink 3 with and without cells, the comparison of FTIR-ATR spectra, reported in Figure x c-d, for the hydrogel with and without cells, respectively, suggests that the dehydration mechanisms remain unchanged in both cases.

3.3.6. Texture Analysis

The texture analysis led to the following results, reported in Table 3.3.10.

Sample	Stiction	Plateau force
1	10830,869	5008,170
2	3240,870	3013,234
3	746,139	733,503

3+	578.16	684.12
4	263,274	6281,105

Table 3.3.10. Extrusion force values at the start of sliding and at the plateau reached during extrusion at constant speed.

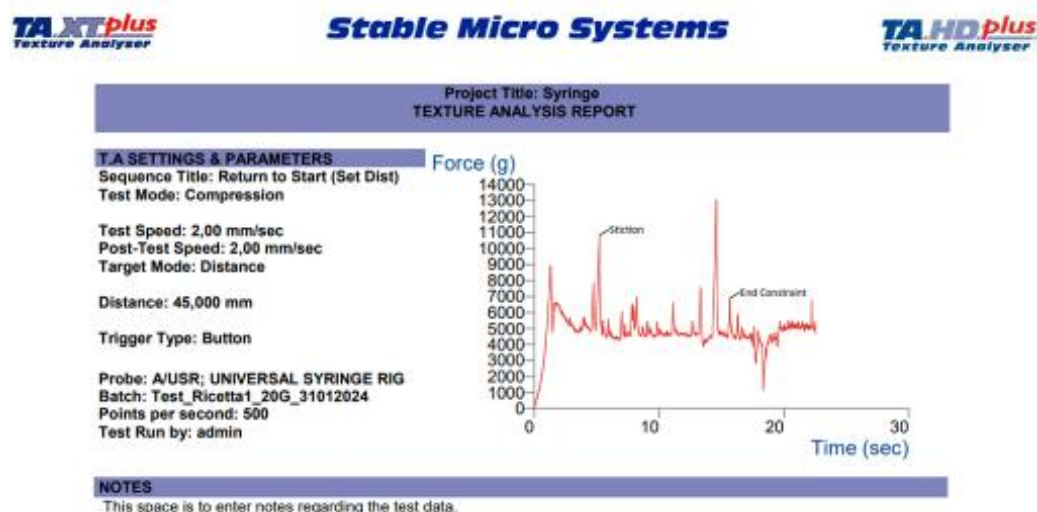


Figure 3.3.29. Texture analysis report for Ink1

Ink 1: the force required to initiate extrusion is approximately 10830g (106 Newton), corresponding to the sample hardness. During the plateau phase, the average force decreases to 5008g. However, considerable variability in the measured value is observed, likely attributable to the irregular grain size of the sample.

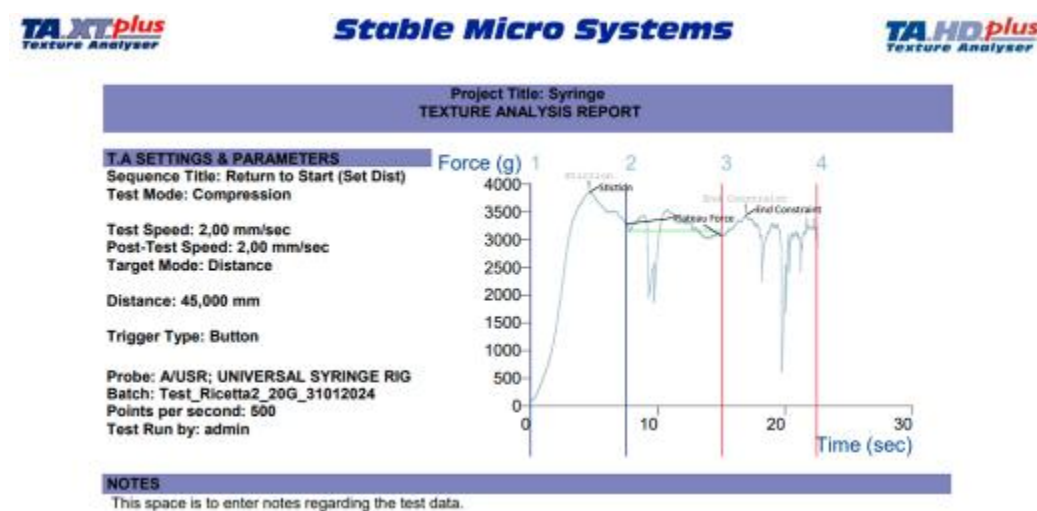


Figure 3.3.30. Texture analysis report for Ink2

Ink 2: Extrusion occurs at an average force of 3240g, with a plateau of 3013g. The sample exhibits high homogeneity. However, the presence of two minimum force values at 10 and 20 seconds is likely attributable to the presence of air in the ink.

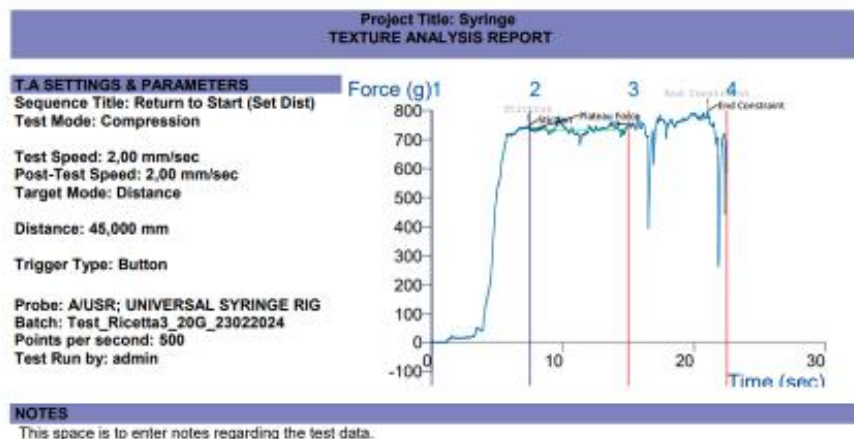


Figure 3.3.31. Texture analysis report for Ink3

Ink 3: the force required for extrusion of the gelatinous sample is approximately 746g, with a plateau at 733g. The extrusion force remains very low, and the sample demonstrates high homogeneity.

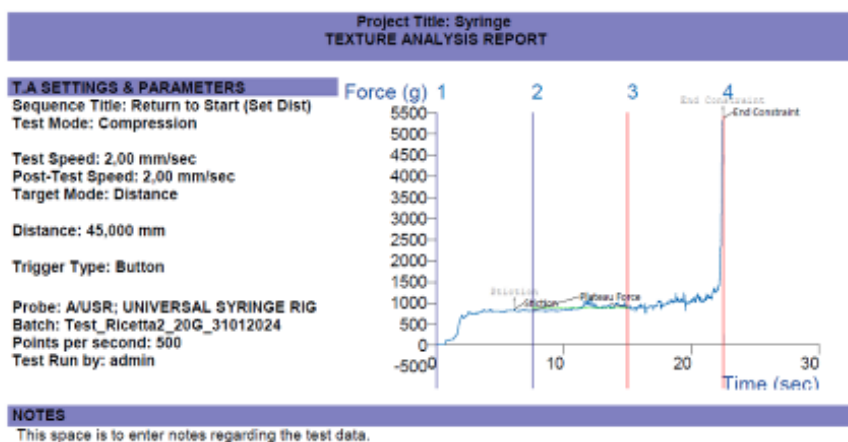


Figure 3.3.32. Texture analysis report for Ink3+

Ink 3+ with cells: The extrusion behavior is closely that of Ink, with an extrusion force of 578g and a plateau of 684g.

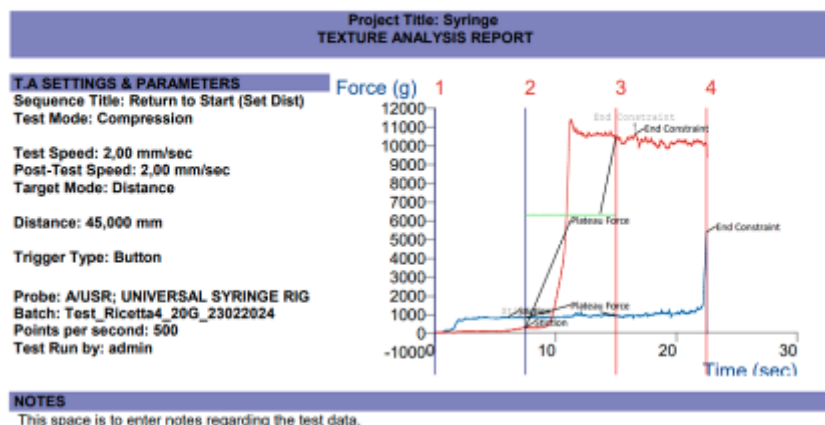


Figure 3.3.33. Texture analysis report for Ink4

Ink 4: Extrusion initiates at 263g, while the plateau phase reaches approximately 2681g. This substantial difference may be attributed to the presence of air produced during sample preparation.

3.4 Characterization of the biological activities of the food matrices after *in vitro* digestion

3.4.1. *In vitro* digestion

As described in section 2.6.1, the samples were named C2, C3, and C4: replicas of samples obtained with Inks 1 + 2 + 3; P3, P5, P6: replicas of samples obtained with Inks 1 + 2 + 3 with Ink 3 also containing a cell suspension of *Perilla frutescens* 1×10^6 cells/mL.

Following *in vitro* digestion, the eluates obtained by centrifugation were collected, aliquoted and stored at -80°C for subsequent analyses. Before freezing, the degrees Brix were read in each sample, thus obtaining the percentage of solutes present (Table 3.4.1.). The value of the samples was quite homogeneous, while the blank was much lower (about 45%).

Sample	Brix degree (%)
C2	4,5
C3	5,8
C4	5,2
P3	5,7
P5	5,8
P6	5,2
Blk	2,4

Table 3.4.1. Brix degrees of digested foods (Blk: blank where the food matrix was replaced with water).

3.4.2. Characterization of potential activities present in food matrices after *in vitro* digestion.

3.4.2.1. Measurement of antioxidant activity

As shown in Figure 3.4.1, digested and filtered samples P3, P5 and P6 show a significantly higher antioxidant activity than samples C2, C3, C4, suggesting that the presence of *Perilla frutescens* cells inside the matrix can confer to the food a greater antioxidant protective activity.

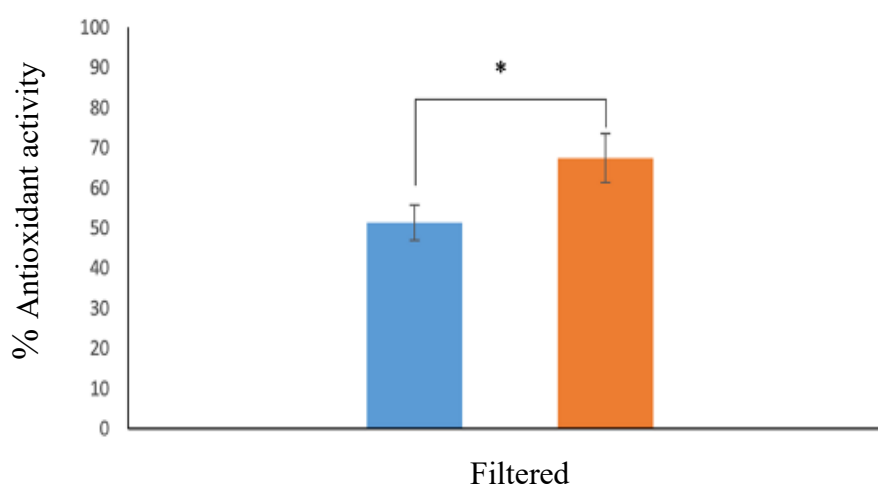


Figure 3.4.1. Measurement of antioxidant activity expressed as mean $\pm$ standard deviation in samples C2, C3, C4 (in blue) and P3, P5, P6 (in orange) after digestion and filtration in Centricon. The digested blank control (Blk) was subtracted from each sample of type C or P, to eliminate possible interferences. * $p < 0.00098$ (unpaired t-test).

3.4.2.2. Evaluation of prebiotic activity

The study of the potential prebiotic activity of the products analyzed in the project was conducted through the evaluation of the effect exerted by the products obtained from *in vitro* digestion on the growth of probiotic bacteria,

The results obtained, reported in Table 3.4.2, show that the probiotic *Lacticaseibacillus paracasei* probiotic strain was able to grow using both types of samples (P and C, as described in Table 3.4.1) as a carbon source, reaching OD600 values between 1.85 and 2.22, comparable, although lower, to the values obtained in the presence of glucose or fructooligosaccharides (FOS).

	Samples C2, P5	Samples C4, P6
Growth medium ¹	OD600	OD600
MRSnoG	0.98	0.92
MRSnoG + Glu 2%	3.98	3.99
MRSnoG + FOS 2%	3.97	3.99
MRSnoG + Blk	0.82	0.79
MRSnoG + C	1.85	2.20
MRSnoG + P	2.46	2.22

Table 3.4.2. Growth values, expressed as optical density at 600 nm, of the probiotic strain *L. paracasei* CNCMI-1572 after 24 h of incubation in the indicated growth media. 1MRSnoG: complete medium without carbon source; Glu: glucose; FOS: fructooligosaccharides; Blk: blank; C: Control; P: Perilla. Control samples tested: C2 and C4; Perilla samples tested: P5 and P6.

3.4.3. Effects of digests on the Caco-2 intestinal cell model

Since the intestine represents the first site of impact between the food contained in the diet and the organism, the biological effects of digests were evaluated using an *in vitro* intestinal system represented by the human intestinal cell line Caco-2: an experimental system widely validated for *in vitro* studies of bioavailability and intestinal biological activity (see section 2.6.4).

As shown in Figure 3.4.2, the TEER values of epithelial cells treated with digests remain high, demonstrating that digests do not exert harmful effects on the intestinal barrier. In cells incubated with C2 and P5 digests, the TEER values are higher than the control cells, these trends are probably due to electrical phenomena independent of the permeability of the epithelial monolayer, such as trans-epithelial movement of electric charges due to ionic transport in the different solutions.

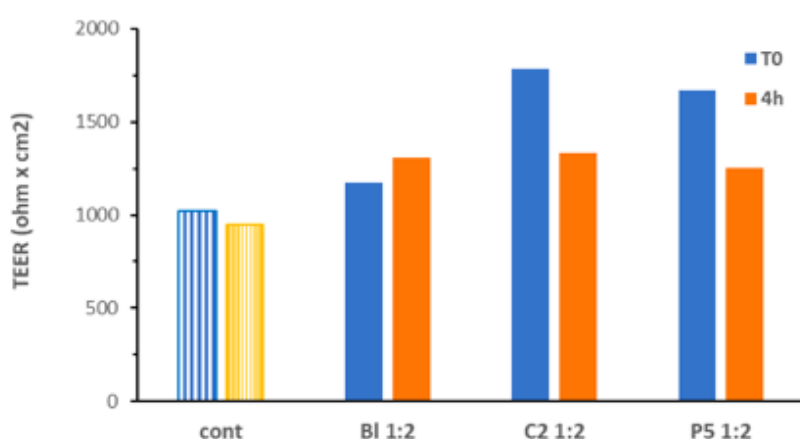


Figure 3.4.2. TEER (Trans-Epithelial Electrical Resistance) values at the beginning (T0) and at the end of the 4 h incubation in the presence of digests diluted 1:2. cont: Caco-2 cells maintained in saline solution not treated with digest; Bl: blank; C2: digest from sample C2; P5: digest from sample P5 which contained *Perilla frutescens* cells.

The Phenol Red passage data confirmed the results obtained from the TEER measurements (data not shown). In fact, in all the conditions tested, the Phenol Red passage rate (calculated as Apparent

Permeability, Papp) was very low and always lower by at least one order of magnitude than the Papp threshold value of 10⁻⁶ cm/sec. This value is considered a reference above which the monolayer is no longer perfectly intact. The results obtained therefore confirm that even using relatively high concentrations of eluates the epithelial monolayer does not suffer damage to its integrity.

3.5 Sensory Analysis of 3D Products

3.5.1. Panel test

3.5.1.1. Bars

Sensory Parameter	Commercial Bar	3D Multilayer bar
Visual homogeneity	5,56	4,44
<i>Visual defects</i>	0,00	0,00
Intensity of berry aroma	8,89	1,11
Intensity of apple aroma	1,48	8,52
<i>Off-odors</i>	0,00	0,00
Sweetness	10,00	0,00
Acidity	0,00	10,00
Intensity of apple flavor	1,85	8,15
Flavor persistence	4,81	5,19
Chewiness	6,67	3,33
Crumbliness	0,00	10,00
Ease of chewing	0,37	9,63
Stickiness	9,63	0,37
<i>Off-flavors</i>	0,00	0,00

Table 3.5.1. The average results with the relative standard errors (calculated as the ratio between the standard deviation and the square root of n, number of trials = 30).

For all attributes, apart from visual defects, foreign smells and tastes (the so-called off notes), values close to 5 indicate that the products are very similar for the attribute considered. In fact, in this case, 50% of the judges consider an attribute more accentuated in a product, while the remaining 50% consider it less accentuated. On the other hand, the more the value differs from 5, the greater the number of judges who agree on assigning the same value 0 or 10 to an attribute and, therefore, the more marked the diversity of the samples for that attribute.

As can be seen from Table 3.5.1., the two samples are clearly different for all attributes except for visual homogeneity and flavor persistence, which were found to be non-discriminating. Both products have no off notes.

In particular, the 3D printed bar compared to the commercial one has:

- lower intensity of the smell of wild berries,
- greater intensity of apple odor and flavor,
- less sweetness,
- greater acidity,
- less rubberiness,
- greater friability,
- easier chewing,
- less stickiness.

3.5.1.2. Honey pearls

Sensory Parameter	Commercial honey pearl	Honey pearl (3D)	Honey pearl + cells (3D)
Visual homogeneity	7,67±0,74 a	4,73±0,72 b	4,33±0,79 b
<i>Visual defects</i>	0,00	0,00	0,00
Overall aroma intensity	7,87±0,74 a	4,30±0,75 b	3,23±0,72 b
<i>Off-odors</i>	0,00	0,00	0,00
Sweetness	9,67±0,33 a	1,80±0,52 b	1,43±0,37 b
Acidity	0,93±0,52 a	8,33±0,44 b	8,23±0,48 b
Intensity del complexity flavor	8,00±0,74 a	3,33±0,74 b	4,03±0,65 b
Intensity of berry flavor	8,33±0,69 a	2,77±0,64 b	3,03±0,62 b
Flavor persistence	7,63±0,78 a	3,73±0,74 b	4,77±0,69 b
Compactness	9,67±0,33 a	3,30±0,52 b	0,80±0,38 c
Ease of chewing	0,63±0,44 a	7,47±0,42 b	9,27±0,37 c
Stickiness	10,00±0,00 a	2,30±0,4 b	0,37±0,19 c
Juiciness	1,20±0,53 a	7,17±0,59 b	8,53±0,53 b
<i>Off-flavor</i>	0,00	0,00	0,00

Table 3.5.2. the average results with the relative standard errors (calculated as the ratio between the standard deviation and the square root of n, number of trials = 30).

All products have no off notes. For all attributes, the commercial product is significantly different from the other two, which are the same apart from texture-related attributes such as firmness, ease of chewing and stickiness. The two pearls obtained by 3D-printing, with and without cells, are very similar, except for the texture. The addition of the cells involves, all other attributes being equal, a reduction in compactness and stickiness and an increase in the ease of chewing.

The developed 3D pearls obtained lower value than those of the commercial product for all attributes except for acidity (as also found in the bars), ease of chewing and juiciness. The commercial pearl

has a more intense smell, flavor and persistence of flavor, but it is also sweeter, more compact and stickier, less juicy and less easily chewable.

3.5.2. Consumer test

The study investigated consumer willingness to purchase innovative and sustainable fruit-based foods produced using plant cell cultivation in controlled environments.

3.5.2.1 Demographic profile of consumers interviewed

As indicated in Table 3.5.3, the surveyed individuals reside in central-southern Italy (93%), mirroring the age and gender distribution of the Italian population. Regarding employment, half of the participants are public employees. Most respondents have a higher level of education than the Italian average. This is probably due to a shift in the technique used to select consumers. The high level of education could explain why 67% of the interviewees answered that they live without worries, which does not fully agree with the official Italian statistical data on family incomes in the Center-South (ISTAT, 2024). To assess the impact of education on participants' responses, we first analyzed the questionnaire results for the entire survey sample and then for the three education-based groups: A = middle and high school diploma (111 participants); B = bachelor's or master's degree (193 participants); C = PhD or postgraduate specialization (105 participants).

		%
Origin	Northern Italy	7
	Central Italy	39
	Southern Italy and isles	54
Gender	Female	56
	Male	44
Age	19 – 24 years	6
	25 – 34 years	16
	35 – 44 years	19
	45 – 54 years	35
	55 – 64 years	19
Education	Middle school diploma	3
	High school diploma	24
	Bachelor's degree	12
	Master's degree	35
	PhD or specialization	26
Employment	Private employee	18
	Public employee	52
	Entrepreneur	1
	Freelancer	13
	Retiree	3
	Students	9
	Unemployed	3
Family income	I live comfortably and can also afford luxuries	11
	I live peacefully without any worries	67
	I barely pay the expenses without being able to afford luxuries	14

	My income is not even enough for the essentials	1
	I prefer not to answer	8

Table 3.5.3. Demographic profile of the surveyed sample: number and percentage of respondents (n= 409).

3.5.2.2. Diet type and purchasing habits

As regards the type of diet, participants mainly follow Mediterranean and Omnivorous diets (47,5% and 42,8% respectively) with about 2% adapting it to their intolerances. As shown in Figure 3.5.1, participants mainly consume breakfast food and fresh fruit, but also fresh vegetables. Probably, breakfast foods consist mainly of beverages as there is no frequent consumption of sweets and snacks, nor cereals and fiber; the consumption of jams, marmalades and honey is even predominantly rare. On the other hand, the consumption of both customized foods (for sport or illness) and healthy substitutes such as locust bean gum is very rare.

The consumer sample pays attention to the label (97%), in particular to the expiry date (53%) and information on ingredients and nutritional values (25%). Very little consideration (3%) is given to the promotional image or brand.

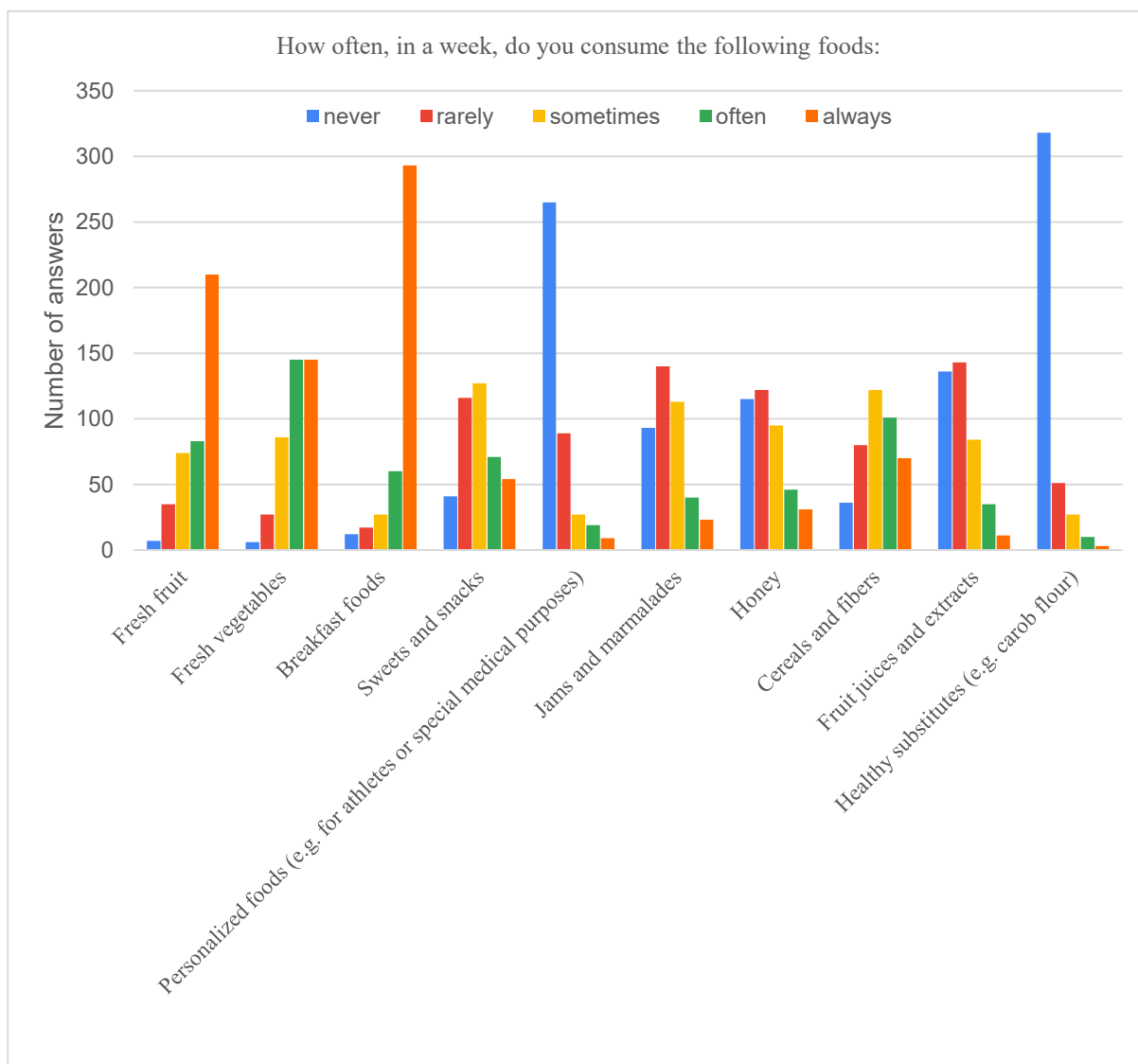


Figure 3.5.1. Frequency of consumption of food products by sampled consumers.

3.5.2.3. Factors and opinions influencing the choice of a new food product

Using a scale ranging from "not important at all" (corresponding to a score of 1) to "absolutely essential" (corresponding to a score of 5), we asked how important certain factors are in the decision-making process when comparing two similar products. The results (Figure 3.5.2) show that quality is absolutely essential, reporting an average score of 4.6 ± 0.7 (significantly different from all others). For a high percentage of consumers, quality/price ratio, ingredients, origin, and health benefits are equally essential, scoring around 4.1-4.2. All the parameters are important, even if "brand" and "valorization of by-products" differ significantly from the other factors, with total average scores around 3.0.

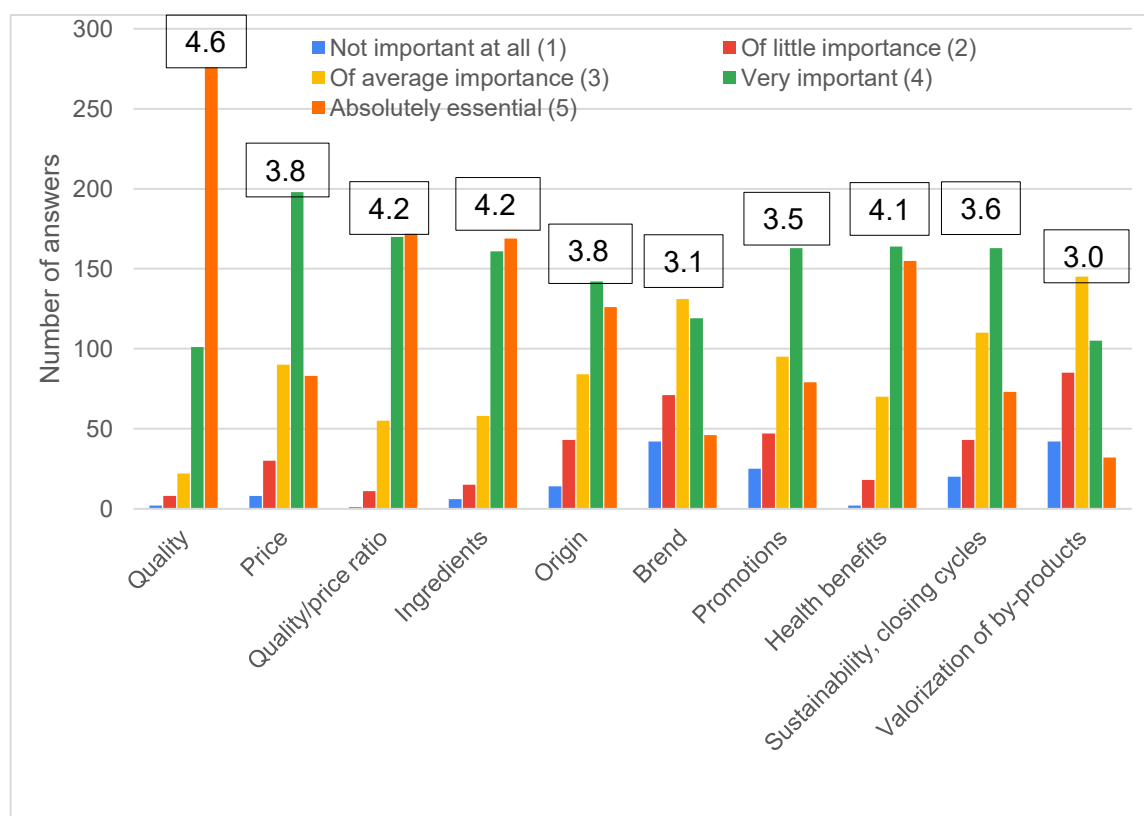


Figure 3.5.2. Importance of some factors in the decision-making process when comparing two similar products. Scale used: 1 = Not important at all, 2 = Of little importance, 3 = Of average importance, 4 = Very important, 5 = Absolutely essential.

The next question asked how important the opinions of friends, the mass media, the scientific world, or the internet are in choosing a new food product. With regard to the possible influences on the choice of new foods (figure 3.5.3), the opinion that most listened to is that of the scientific world. The opinions of the internet/social networks and the mass media obtain low scores.

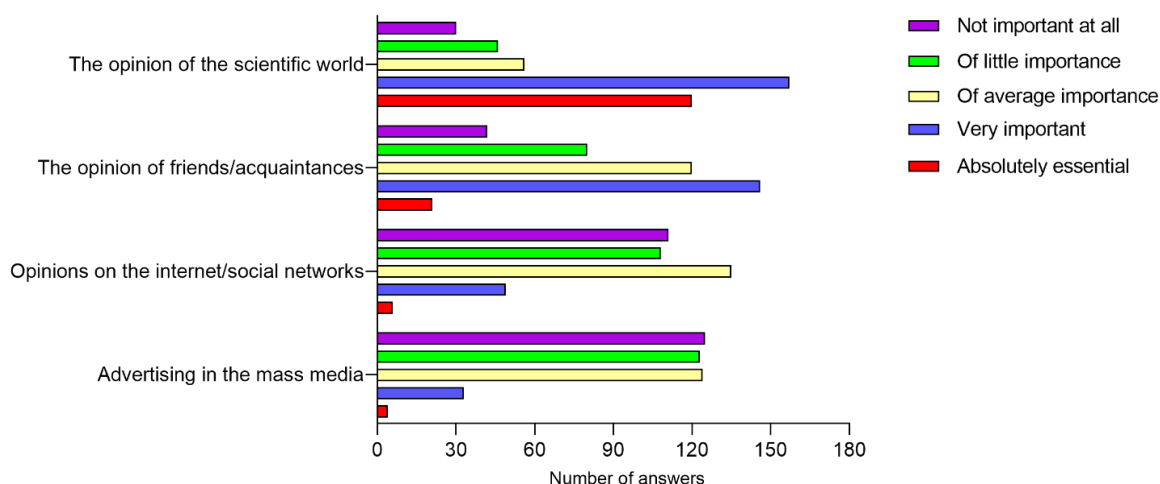


Figure 3.5.3. Importance of the opinions of certain subjects (scientific world, friends, social networks, mass media) in food choices.

3.5.2.4. Opinions on factors related to innovative and sustainability foods and propensity to taste them

Next question explored the perceived importance of certain characteristics in general food production and, more specifically, in innovative and sustainable food production. The scale was from 1 (not important at all) to 5 (absolutely essential). For simplicity, only the average scores obtained for each characteristic will be reported.

As shown in Figure 3.5.4, all the ten suggested characteristics received scores higher than 3, corresponding to “very important”. Communication with the customer scored the lowest (average value of 3.2 ± 1.1), while Quality and Authenticity scored the highest. These two characteristics are recognized as essential factors in food production.

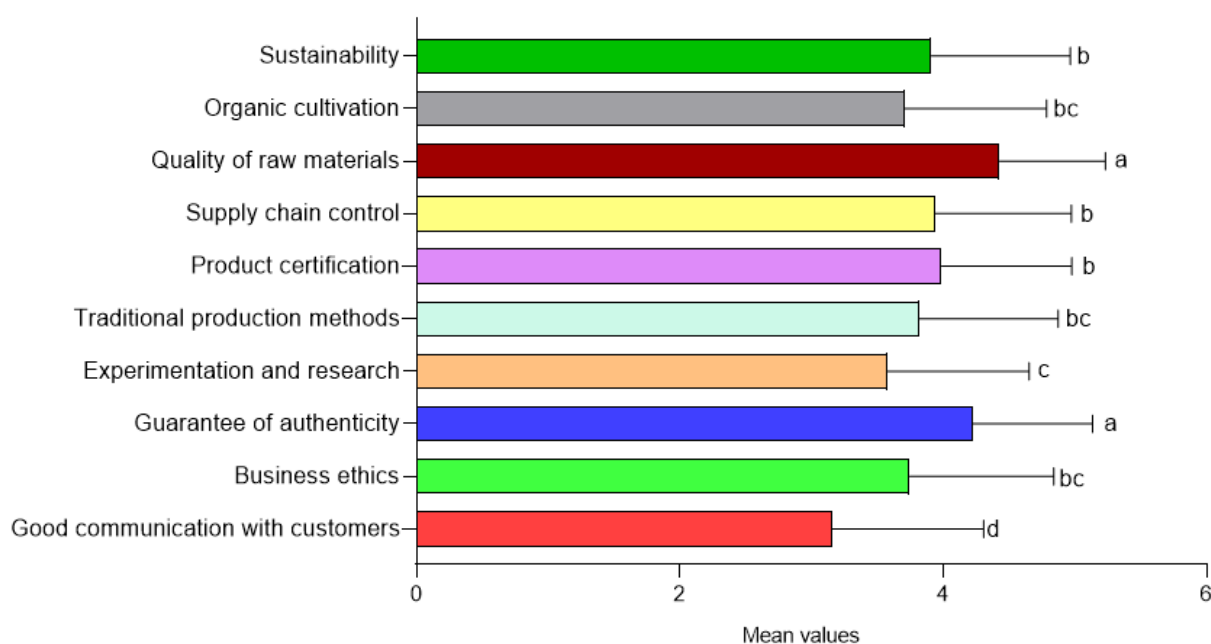


Figure 3.5.4. Scores relate the importance of some factors in food production. Scale used: 1 = not important at all; 2 = of little importance; 3 = of average importance; 4 = very important; 5 = absolutely important. Dunn's test was conducted. Equal letters correspond to non-statistically different values ($p < 0.05$).

The results indicated that almost 60% of respondents expressed a clear willingness to purchase such products, while over 26% were in favor but less certain. In contrast, only 14% responded negatively.

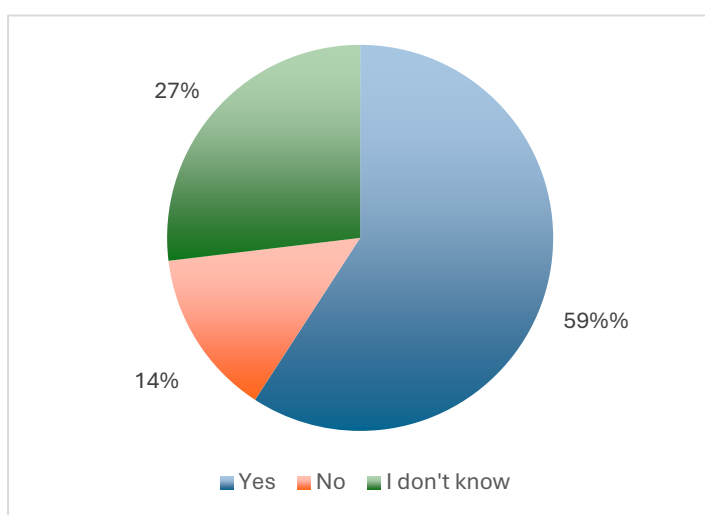


Figure 3.5.5. Pie chart showing the willingness to purchase innovative and sustainable fruit-based foods produced through the application of plant cell cultivation grown in controlled environments, which reproduce or improve natural environments.

Furthermore, 65% of consumers reported a preference for exploring new and different foods beyond their usual choices, and an equal percentage stated they are willing to try unfamiliar foods.

Responses to the statement “I try new foods (with ingredients) only if I think they can be good for your health” followed a Gaussian distribution. In contrast, for the statement, “I am interested in food produced with innovative production processes (e.g. 3D printing)” only 10% of respondents expressed strong agreement, whereas 26% completely disagreed.

Regarding multilayers bars, only 27% of respondents reported consuming them, with 21% doing so occasionally, 5% often, and 1% consuming them almost daily or always

In the case of honey-based products, 50% of those declared they regularly consume such products.

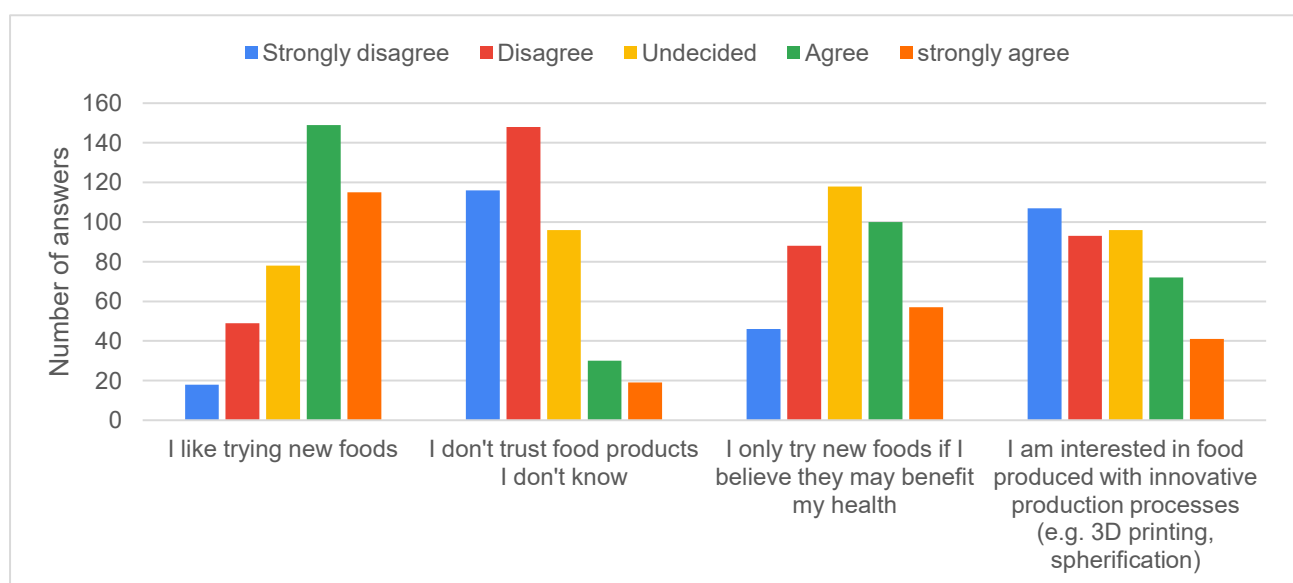


Figure 3.5.6. Consumer opinion to food innovations

3.5.2.5. Products appeal, evaluation of sensory parameters, and purchase intent

Participants were asked to evaluate the visual appeal of two products solely based on their appearance (visual perception), without any information regarding ingredients or production methods. A 5-point scale was employed, where 1 = not at all, 2 = slightly, 3 = indifferent, 4 = moderate, and 5 = yes. The overall appeal score for the bar was 1.9, positioning it just below “slightly.” In subsequent evaluation, respondents were asked to reassess the image and rate specific attributes of the product. As shown in Figure 3.5.7., the bar’s shape received the highest number of “indifferent” responses (i.e., 3), with a mean rating of 2.5. The pearl was perceived as slightly more appealing than the bar, achieving an average score of 2.3 (Figure 3.5.9). However, when analyzing appeal based on individual attributes, mean scores increased but remained below the “indifferent” threshold.

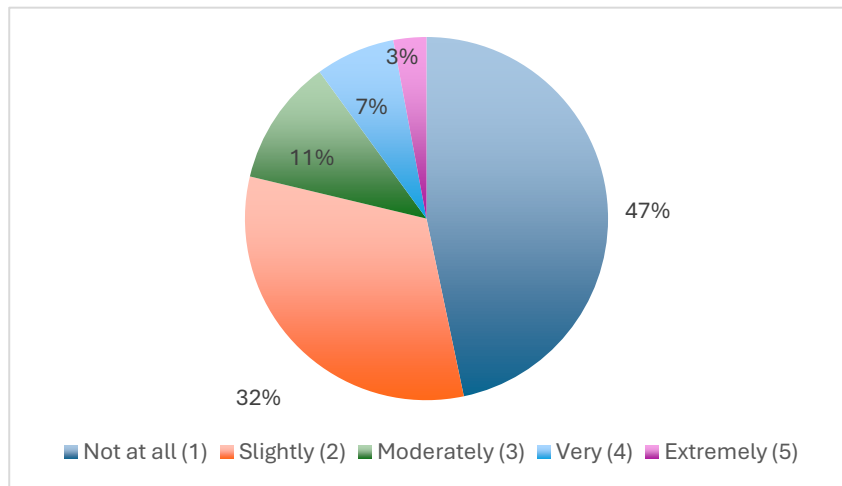


Figure 3.5.7. Judging the palatability of a bar by visual perception alone

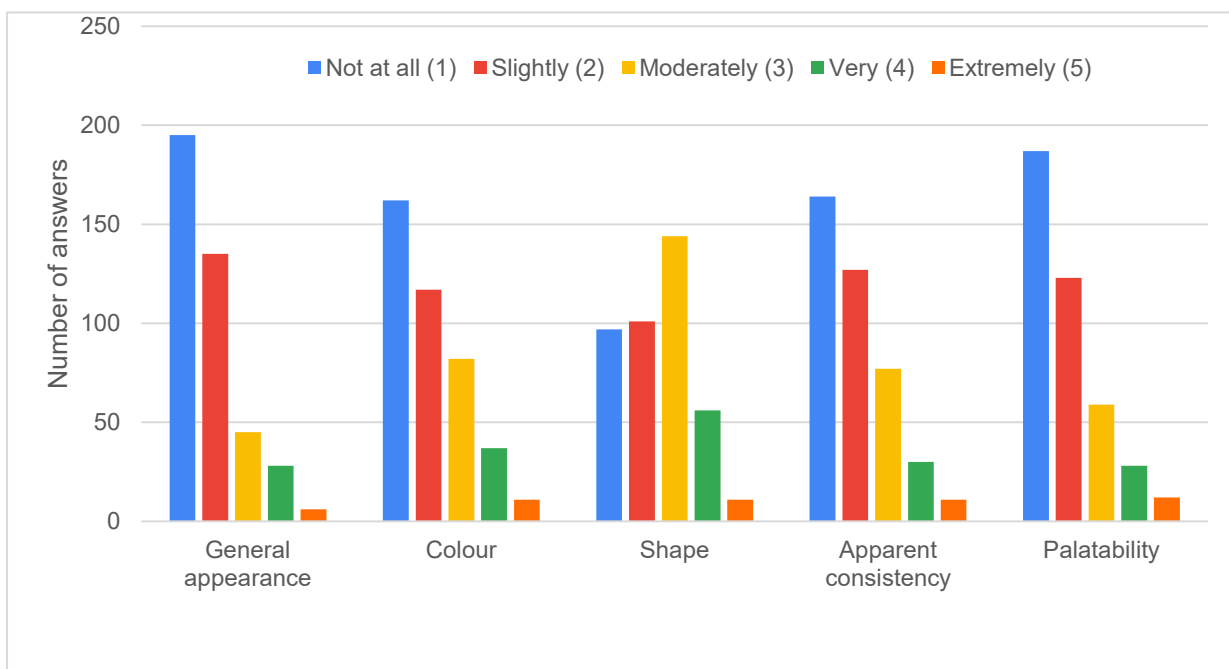


Figure 3.5.8. Judgment on some parameters of a bar by visual perception alone

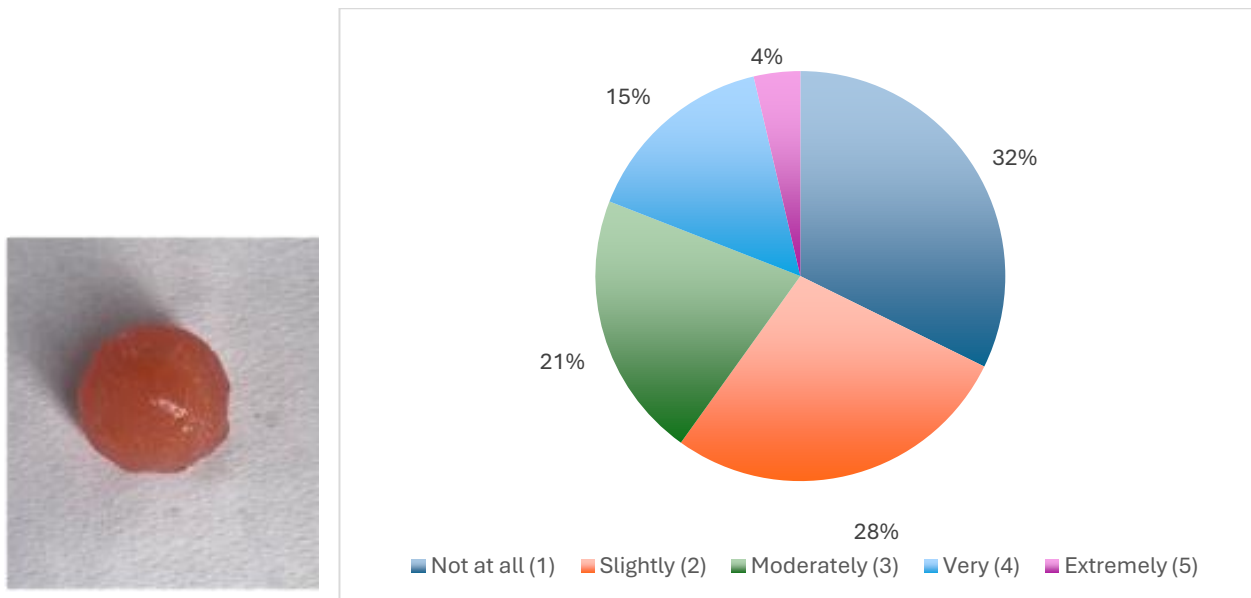


Figure 3.5.9. Judging the desirability of a pearl by visual perception alone

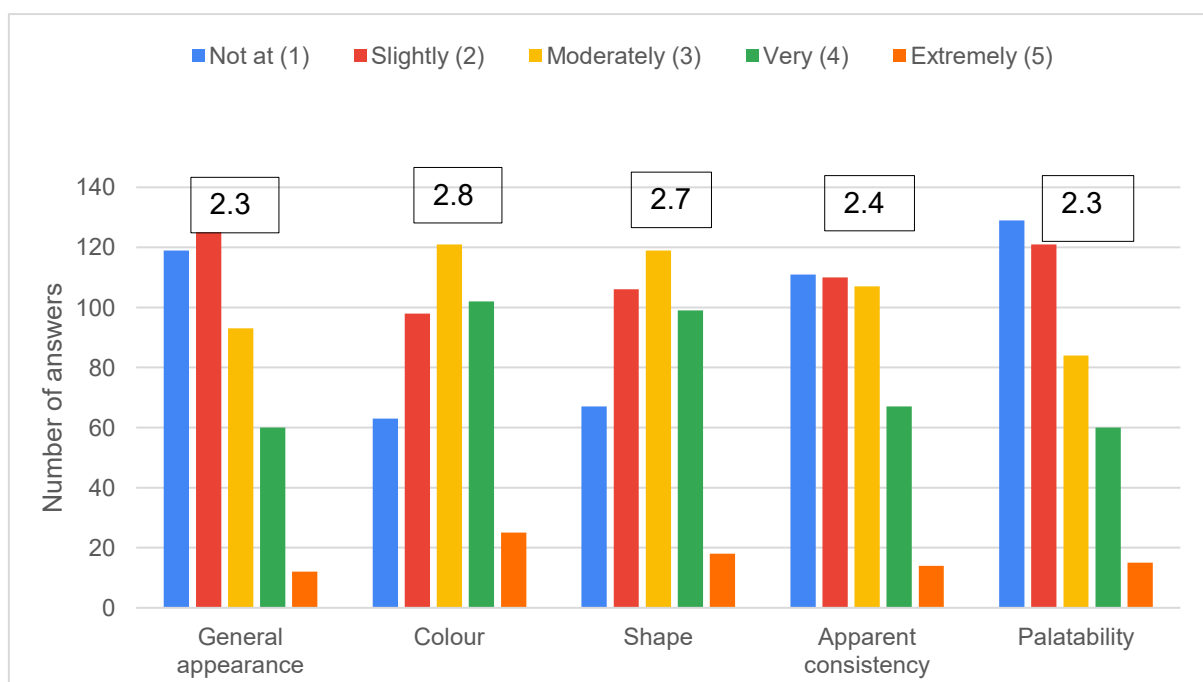


Figure 3.5.10. Judgment on some parameters of a pearl by visual perception alone

Respondents were subsequently asked to re-evaluate the appeal of the products after being informed that both items were produced using 3D printing technology, incorporating honey and by-products from berries and apples, which are rich in antioxidants and fiber. [The specific question was: Both foods shown in the previous images are created using 3D printing technology with honey, berry, and apple by-products which are rich in antioxidants and fiber. Now that you are aware of this, please share your opinion once again on whether you find the products in the photo appetizing]

As illustrated in Figure 3.5.11, the bar received a higher proportion of “negative” ratings (1 = not at all, 2 = slightly) compared to other response categories. Conversely, the pearl product obtained a slightly higher mean rating (2.9) compared to the bar (2.6), indicating a marginally more favorable perception. The analysis of average ratings reveals that the perceived appeal of both products increased after participants were informed about the production process and the incorporating of antioxidant- and fiber-rich by-products.

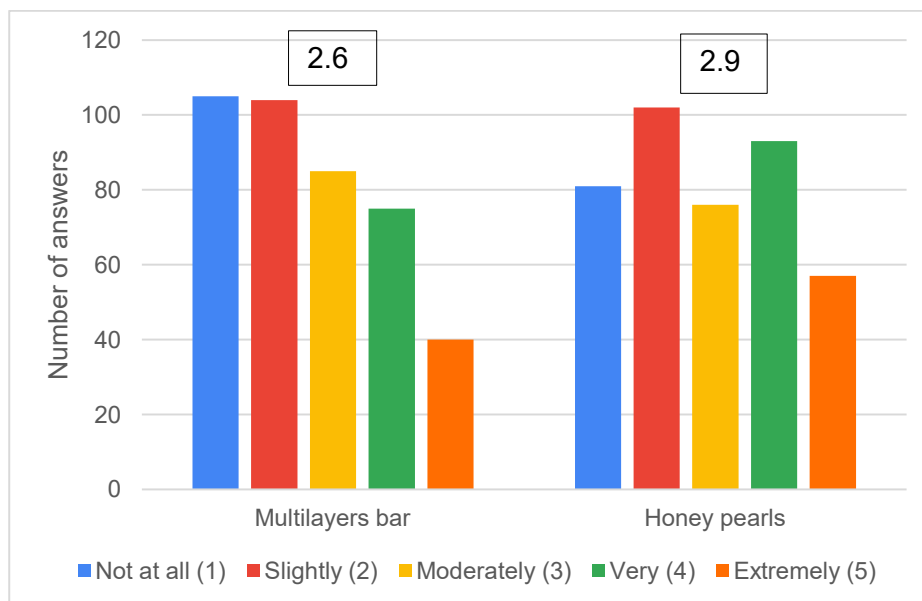


Figure 3.5.11. New review on innovative products after learning about the 3D process and the introduction of by-product ingredients rich in antioxidants and fiber.

Specifically, the appeal scores for the multilayer bar increased from 1.9 to 2.6, while the pearl product’s score rose from 2.3 to 2.9. In a subsequent question, respondents were asked to explain the reasoning behind their revised evaluations. As illustrated in Figure 3.5.12, the most frequently cited concerns were “seems unnatural” (22,9%) and “preference for traditional alternative” (24,2%). Health concerns were minimal, with only 2of respondents expressing apprehension. Conversely, 51% provided positive motivation, primarily citing curiosity (23,2%) and the valorization of by-products (16.4%). Additionally, 7,8% highlighted the potential health benefits as a key factor influencing their reassessment.

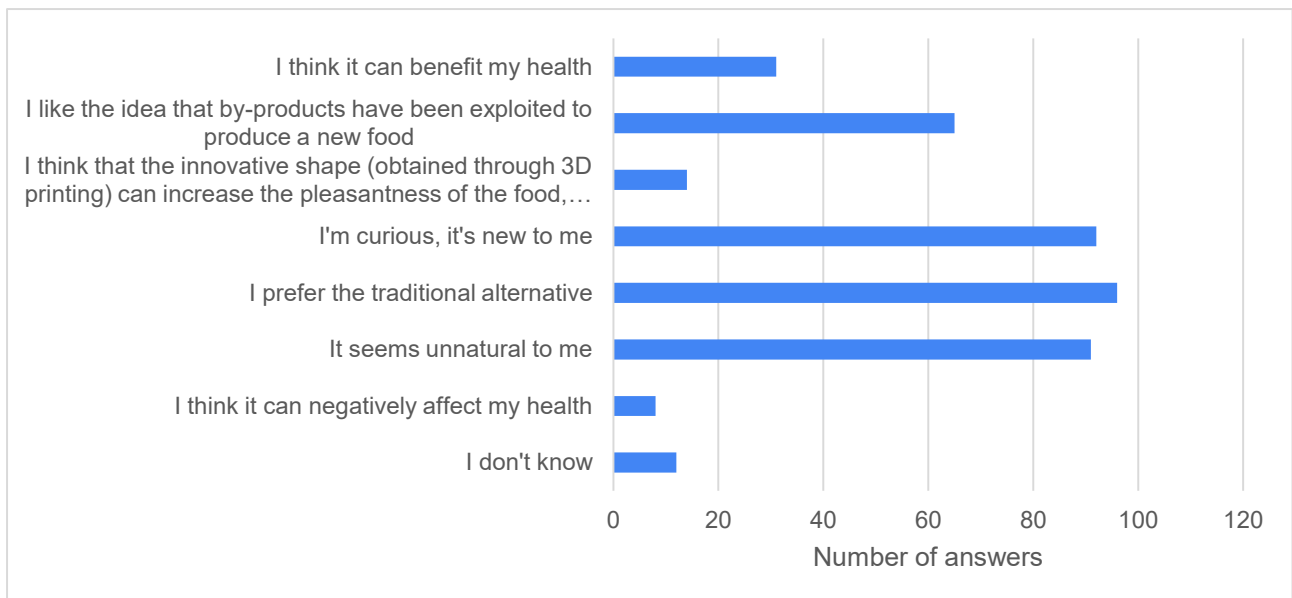


Figure 3.5.12. Reasons for the change in judgment after learning more about the products under consideration

With regard to purchase intent for the products depicted in the images (multilayer bar and pearl), the most polarizing statement was: “I am not willing to buy a product I moderately like or don’t like,” with an average score of 3, corresponding to “neither agree nor disagree.”

The statement “I am willing to buy innovative products because they intrigue me,” yielded an average score of 2.9. In contrast, “Valorizing by-products is important for sustainable production,” received a higher mean score of 3.4, suggesting a gap in consumer awareness, not necessarily regarding sustainability as a concept, but rather the specific strategies for its implementation. This trend is further supported by responses to the statement, “I am willing to pay more to consume a healthier, more sustainable product,” which obtained an average score of 3.7, just below 4 (somewhat agree).

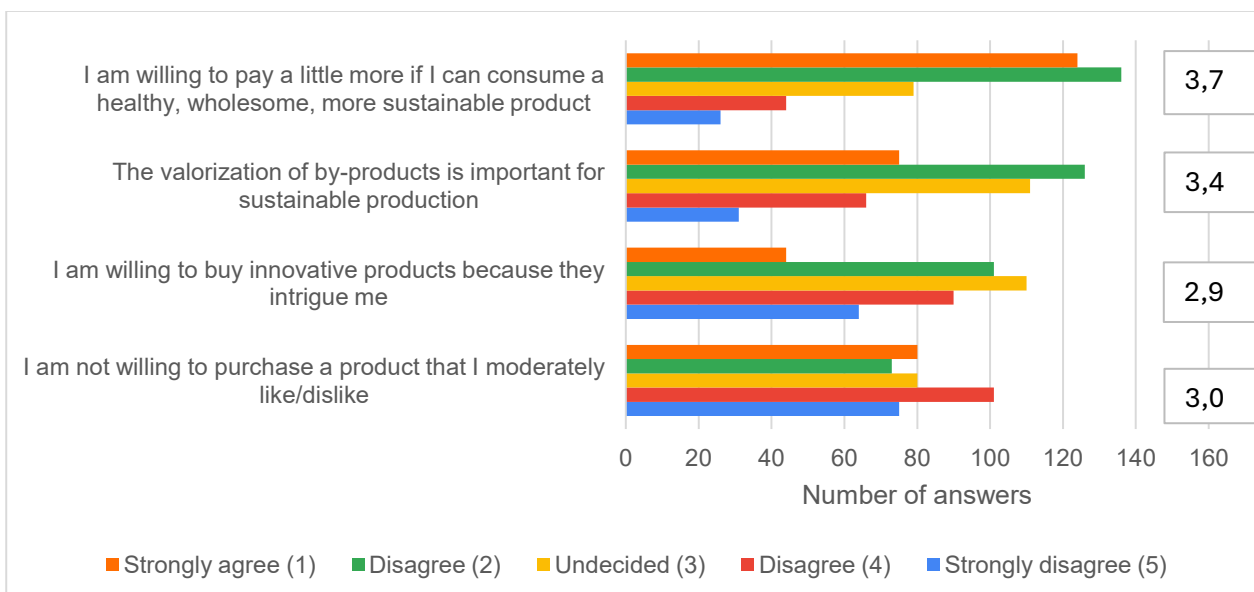


Figure 3.5.13. Propensity to purchase the products observed in the photo

4. DISCUSSION

The sustained growth of the global population, projected to reach 10 billion by 2050, is placing mounting pressure on agri-food systems. This demographic trend is expected to drive a substantial increase in food demand, with forecasts from the Food and Agriculture Organization (FAO) indicating a required production rise of approximately 35–56% by mid-century. Ensuring a reliable and nutritionally adequate supply of plant-based foods under these conditions represents a critical challenge for future food security. Simultaneously, the availability of arable land per capita is decreasing due to urbanization, erosion, salinization and desertification. These challenges are further exacerbated by climate change and the growing incidence of plant diseases, both of which negatively impact agricultural yields. These pressures are accelerating the demand for innovative and sustainable food production strategies.

A more modern and sophisticated kind of agriculture, known as "plant cellular agriculture", aims to produce plant-based food and other materials at the cellular level, preserving whole plants and natural resources through the set-up of plant cell *in vitro* cultures. Plant cell agriculture could be considered as an alternative production platform to address the challenges of certain commodities, in terms of land use, climate change and sustainability. The use of *in vitro* plant cell cultures can be a reliable approach to producing high-value plant-derived biomass, in a fully controlled environment, independently of environmental or seasonal factors or field-borne pests and possibly oriented to food purposes. The plant cell cultures technology involves isolation, growth and multiplication of plant cells in nutrient media and could serve as a sustainable platform for the production of biomass enriched with bioactive molecules. It offers key advantages such as standardized and reproducible results, scalability through bioreactor systems and reliability of plant-derived production process while preserving rare or endangered plant species. Moreover, it allows precise metabolic control for increased production of specialized metabolites valuable for human health, among which antioxidants.

The present study aimed to investigate an innovative application of plant cell cultures focused on the design of novel plant-based food systems including agro-industrial by-products as well, to develop functional food matrices with nutritional properties. Specifically, the research aimed to create food products with diverse consistencies, enriched with value-added specialized metabolites retrieved from agro-industrial by-products, and capable of including intact and possibly vital plant cells with their whole phytocomplex. This research was conducted as a part of the PON MiSE 'MaNUfacTuRIng 3D di alimenti vegetali di nuova generazione per la nutrizione sana (Nutri 3D)'. This project focused on developing next-generation plant-based innovative food by additive

manufacturing, opening the way to customizable food prototypes that meet emerging consumer demands for possibly sustainable and health-oriented diets. Through additive manufacturing, these prototypes may be tailored in terms of shape, color, flavor, and nutritional profile, offering a versatile solution for personalized nutrition. By integrating the plant cell culture technology with agro-industrial by-products, the research project aligns with the principles of circular economy and advances the potential for creating nutrient-dense, plant-based superfoods that address both environmental sustainability and dietary health.

Identification of edible plant species for cell cultures onset

The beginning of the study was devoted to the identification of promising genotypes within edible plant species. A comprehensive bibliographic review was conducted to assess the correlation between *in vivo* and *in vitro* accumulation of bioactive compounds characterized by documented health effects (i.e., antioxidant capacity, radical-scavenging activity, hypolipidemic, antimicrobial properties) and already used in the food industry as natural food colorants, or preservatives. The study considered also the adaptability of these species to *in vitro* culture and their responsiveness to elicitation aimed at enhancing biomass and secondary metabolite production in the culture. Based on this multi-criteria analysis, four species were selected for further investigation.

Among the analyzed species, *Ocimum basilicum purpurascens* (var. red rubin), *Perilla frutescens* (var. shiso purple), *Beta vulgaris* (var. rubra), and *Amarantus tricolor* (var. aztec) were chosen. *Perilla frutescens* and *Ocimum basilicum* were chosen due to their high polyphenol content and antioxidant activity, while *Beta vulgaris* and *Amaranthus tricolor* were of particular interest for their capacity to produce betalains, bioactive pigments with well-documented health benefits as hypolipidemic metabolites and growing relevance in the food industry as natural antioxidants, and as an alternative to synthetic dyes. To enhance the probability of accumulating bioactive molecules of interest in callus cultures, the choice of the age of the plant tissue for induction was restricted to the juvenile stage of microgreens (within 15 days post-sowing or at the first true leaf stage) for their higher concentration ($\mu\text{g/g}$) of the relevant bioactive molecules compared to adult or mature tissues and for the higher probability of callus formation from cells with lower differentiation levels. Additionally, since the microgreens stage for the selected species is fully edible, this helps minimize the risk of accumulating antinutritional compounds in the derived culture (Balik et al., 2025). Friable callus cultures were obtained independently of the explants (i.e., hypocotyl, cotyledons, or the first true leaf) revealing a general promptness of the chosen stadium for cell culture onset. The plant growth regulators selected to define the composition of the optimal culture medium was guided by literature, experimental data, and compliance with food regulatory requirements, particularly regarding maximum residue levels (MRL) in edible products as defined by the European Commission Pesticides Database (https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en).

Specifically, the acceptable daily intake (ADI) for NAA is 0,1mg/kg of body weight/day and the acute reference dose (ARfD) is 0,1mg/kg of body weight, while for 2,4-D, the ADI is 0,02mg/kg of body weight/day, the ARfD is 0,3mg/kg of body weight. Furthermore, MRL have been established in various food crops (0,06mg/kg for NAA and 0,1mg/kg for 2,4-D in blueberries, for example), providing a further safety demonstration for their use in the development of edible *in vitro* plant biomass. As a consequence, 2,4-D (0,2mg/L) and NAA (0,2mg/L, 1,0mg/L, 2,0mg/L) alone or in combination were preferred in this study.

Since callus friability is a critical parameter for the subsequent transition to liquid cultures, the best-performing medium formulation for each species, among those tested, was selected, prioritizing both rapid growth and friability.

Callus culture optimization on different media and in the presence or absence of physical elicitation with Light Emitting Diodes (LED)

One of the main objectives of this study was the optimization of secondary metabolite production and biomass accumulation in the plant cell cultures object of the study in order to produce a high value-added plant-derived biomass. In this context, an abiotic and non-chemical elicitation technique was employed, specifically the use of LED light stimulation, selected for its energy efficiency and minimal heat production, and key aspects for *in vitro* applications (Sena et al., 2024). Physical elicitation using LED light was preferred over chemical elicitation to reduce possible issues risks associated with human consumption. Nadeem et al., 2019 have highlighted that specific light wavelengths may selectively modulate biosynthetic pathways in *O. basilicum* callus cultures: blue light stimulates the phenylpropanoid pathway, enhancing the accumulation of rosmarinic acid and eugenol, while red light promotes the biosynthesis of flavonoids and anthocyanins, such as peonidin, mediated by the activation of phytochromes. We decided to apply blue, red and a combination of red and blue LED light at a specific intensity and wavelength to both *O. basilicum* and *P. frutescens*. In addition, in our experiment design, light elicitation was combined with variable plant growth regulators composition of the culture media: auxins concentrations were modulated by applying 2,4-D (0,22mg/L) and NAA (1mg/L or 2mg/L), individually or in combination.

A systematic analysis of growth kinetics, based on weekly measurement over a 28-day cultivation cycle, allowed the identification of four optimal culture conditions for each species in function of the combination of physical elicitation, growth regulators and harvesting time to maximize dry matter accumulation. The experimental results revealed that, in *P. frutescens* friable callus culture, maximum biomass accumulation in terms of dry matter under dark condition and red-blue light exposure (660nm + 460nm) was obtained in combination with a culture medium supplemented exclusively with NAA (2mg/L) and a harvesting time of 28 days. Conversely, when the culture was exposed to red light (660nm), the highest biomass accumulation was observed at 21 days in a medium containing

both auxins NAA (2mg/L) and 2,4-D (0,22mg/L). Exposure to blue light (460nm), combined with a medium containing NAA (1mg/L) and 2,4-D (0,22mg/L), favored the accumulation of dry matter, also within 21 days.

Two different friable callus cultures were obtained for *O. basilicum*, one showing a white pigmentation and one being heavily pigmented in violet. In the white *O. basilicum* friable callus culture, the condition observed under darkness replicated that of *P. frutescens*, while under red light, the optimal condition was obtained in a medium containing exclusively 2,4-D (0,22mg/L) and a reduced harvest time of 14 days. Both blue light and the red-blue combination required the presence of both auxins, 2,4-D (0,22mg/L) and NAA (1mg/L), with the highest performance recorded at 21 days.

For the pigmented (violet) callus culture of *O. basilicum*, being a material capable of accumulating anthocyanins in the absence of elicitation, the focus was exclusively on optimizing the culture medium and harvesting time. Dry matter content did not exhibit statistically significant differences in function of the different growth regulators. Consequently, all the four culture conditions were maintained for further analysis, with the aim of identifying the most effective condition based on spectrophotometric evaluation through UV-visible analysis.

In the pigmented *B. vulgaris* friable callus culture, that naturally accumulated betacyanins and betaxanthins on LS medium with 2,4-D 0,22mg/L, the medium composition was not modified and cultivation proceeded in the dark, without light elicitation also in order to avoid browning that occurred under light. Maximum dry matter accumulation was achieved at 14 days.

Screening of specialized metabolite classes

For the qualitative screening of specialized metabolite classes (polyphenols or betacyanins/betaxanthins), UV-visible spectrophotometric analysis was employed, following tailored extraction strategies. At the point of maximum dry matter accumulation, UV-visible analysis revealed that for *O. basilicum* white friable callus culture the highest absorbance values were recorded under blue LED combined with a MS medium containing NAA (1mg/L) and 2,4-D (0,22mg/L), followed by red-blue LED with the same medium. In *P. frutescens*, at 280nm, absorbance values (O.D.) were similar between those generated by cells grown under blue LED on a MS medium containing NAA (1mg/L) and 2,4-D (0,22mg/L), and those generated by the culture grown on MS medium supplemented exclusively with NAA (2mg/L). However, blue LED recipe administration induced the highest absorbance at 313nm. Since amino acids and flavanones have characteristic absorbance at 283nm (Bijlsma et al., 2022), while citric acid and hydroxycinnamic acids, including caffeic and rosmarinic acids, are linked to a peak around 320nm, different LED recipes were able to differentially improve accumulation of secondary metabolites. Additionally, flavones were identified based on their absorbance at 396nm, although the peak exhibit lower absorbance compared to the others.

The pigmented callus of *O. basilicum* showed the characteristic absorbance peaks of flavanones (290nm) and hydroxycinnamic acids (323nm) already observed in the white callus, but also a distinct peak at 518nm, indicative of anthocyanins, was detected. The highest absorbance at 290nm and 323nm was obtained in MS media containing both auxins (NAA 1mg/L or 2mg/L + 2,4-D 0,22mg/L), while the peak at 518nm was most pronounced in MS medium supplemented with NAA (1mg/L) + 2,4-D (0,22mg/L) or supplemented with only NAA (2mg/L). Based on these results, the MS medium supplemented with NAA (1mg/L) and 2,4-D (0,22mg/L) was preferred, as it demonstrated comparable absorbance value to the other media while presenting a more suitable composition for potential future human consumption use. *B. vulgaris* friable pigmented callus culture, grown in LS medium supplemented only with 2,4-D (0,22mg/L), showed a UV-visible spectrum characterized by distinct absorbance peaks at 280nm and 535nm, corresponding to betacyanins, in particular those containing Cyclo-Dopa residues (Sadowska-Bartosz et al 2021). Additionally, a peak at 477nm was detected, indicative of betaxanthins (Kugler et al 2007). Overall, with this study we were able to define species-specific optimal conditions by LED-growth regulators-harvesting time to effectively maximize biomass production and metabolite accumulation. *A. tricolor*, despite being able to produce a highly friable callus, did not generate sufficient biomass (data not shown) and, therefore, was not considered for further analysis. This species had been, indeed, initially considered in parallel with *B. vulgaris* to ensure cell culture-based betacyanin production from at least one of the two. Given the successful establishment of *B. vulgaris* cultures and their betacyanin production, the exclusion of *A. tricolor* did not compromise the objectives of this study.

Suspension cultures

To explore the potential for industrial applications and biomass production, starting from friable calli, liquid cultures were established using specific plant growth regulators formulations aimed at producing homogeneous cell suspensions. Standardizing growth parameters in suspension culture is indeed crucial for optimizing the production process, ensuring high homogeneity, vitality and productivity.

For *P. frutescens* and *O. basilicum*, the liquid media were optimized by introducing 6-benzylaminopurine (6-BAP), a cytokinin that had not been employed during the initial callus induction since it was specifically intended to promote homogeneity in cell suspension cultures. Indeed, the addition of 6-BAP promoted cellular homogeneity and the development of suspension cultures characterized by isodiametric cells with reduced size, in agreement with Woo et al., 2021.

In the case of the callus culture of *O. basilicum* violet, the initiation of a stable and homogeneous suspension culture was particularly challenging due to the non-uniform dispersed growth and the formation of compact cellular aggregates. Consequently, it was decided to maintain the culture under the current conditions. In future studies, it will be possible to consider the application of enzymatic

treatments, such as the use of pectinases and cellulases, in order to favor the disintegration of cellular aggregates and promote a more uniform suspension. The presence of small aggregates, in fact, could improve the production of secondary metabolites (Kolewe et al., 2011).

In contrast, for *B. vulgaris*, the same concentration of 2,4-D (0,22mg/L), used during callogenesis and subculture, was maintained in the liquid medium and judged capable of maintaining cells in suspension homogeneously. The only modification that was introduced was an increase in the sucrose concentration from 30g/L to 50g/L, which was necessary to prevent browning and to enhance cellular proliferation. *A. tricolor*, despite being able to produce highly friable calli, did not generate sufficient suspension biomass and, therefore, was not considered for further analysis. This species was, indeed, initially considered in parallel with *B. vulgaris* to ensure cell culture-based betacyanin production from at least one of the two. Given the successful establishment of *B. vulgaris* cultures and their betacyanin production, the exclusion of *A. tricolor* did not compromise the objectives of this study. Once identified the optimal growth conditions (in terms of growth regulators and/or LED lighting) giving the best dry matter accumulation of the suspension cultures deriving from the different species, a comparative analysis of UV-visible spectra was conducted versus polar extracts derived from the starting material (microgreens) and calli.

In vitro cultures, whether calli or suspension cultures, exhibited higher absorbance values compared to microgreens. Specifically, UV-visible analysis revealed that *O. basilicum* suspension cultures and *P. frutescens* callus cultures grown on NAA (1mg/L) and 2,4-D (0,22mg/L) and under Blue LED show higher polyphenol content than the original material. In *B. vulgaris*, calli exhibit a different spectrum compared to microgreens, with higher levels for betanin and betacyanin content (280nm), but not for betaxanthins (477nm and 535nm). Suspension cultures, on the other hand, displayed a similar spectrum but with higher absorbance values than callus cultures, along with an additional peak at 320nm, indicative of hydroxycinnamic acids, likely due to the enhanced accumulation of phenolic compounds stimulated by the sucrose-enriched liquid medium (Ali et al., 2016; Kriznik et al 2010; Sakuta et al., 1987)

The assessment of the antioxidant activity and of the polyphenol accumulation in *P. frutescens* and cultures under the four most effective growth conditions for dry matter accumulation in elicited calli (for *P. frutescens*, MS + NAA 2mg/L Dark 28days; MS + 2,4-D 0,22mg/L + NAA 2mg/L Red LED 21days; MS + 2,4-D 0,22mg/L+ NAA 1mg/L Blue LED 21days; MS + NAA 2mg/L Red/Blue LED 28days; while for *O. basilicum* MS + NAA 2mg/L Dark 28 days; MS + 2,4-D 0,22mg/L Red LED 14days; MS + 2,4-D 0,22mg/L+ NAA 1mg/L Blue LED 21days; MS + 2,4-D 0,22mg/L+ NAA 1mg/L Red/Blue LED 21days) confirmed that suspension cultures exhibit the highest antioxidant capacity and polyphenol accumulation in both species. This finding highlights the significant advantage of these systems (suspension cell cultures), as they demonstrate enhanced metabolic activity and greater

potential for compound production, supporting their suitability for further applications in biomass or bioactive compound generation.

Selection of *P. frutescens* as a candidate for the proof-of-concept of plant-cell food

Among the species analyzed, *Perilla frutescens* emerged as the most promising model system for subsequent applied phases of this project. This determination was based on its efficiency in accumulating both polar secondary metabolites and biomass (expressed as dry weight), as evidenced by a comparative analysis of UV-visible spectra obtained under optimized callus culture conditions (see section 3.1.5.5). The growth kinetic features of *Perilla frutescens*, in terms of high fresh and dry biomass, accumulation of specialized metabolites of interest within a shorter harvest time (21 days), stability over time both as a callus and as a suspension cell culture, led to its selection as the material of choice for subsequent activities.

Metabolomic analysis of hydroalcoholic extracts of *Perilla frutescens*

Metabolomic analysis of polar extracts of *Perilla frutescens* callus culture revealed a broad spectrum of bioactive metabolites of significant nutritional and pharmacological interest. Among the identified phenolic compounds, citric acid stands out for its abundance, highlighted by targeted analysis of both callus and suspension, particularly in dark growth conditions. This organic acid is a well-known co-factor in the absorption of minerals such as calcium and iron. Preclinical studies have highlighted its antioxidant and anti-inflammatory properties at brain and liver level, suggesting its potential use in neurodegenerative and inflammatory fields (Abdel-Salam et al., 2014). Among phenolic compounds, caffeic acid and its derivatives, detected both in callus and in the cell suspension in dark conditions, show a marked antioxidant, anti-inflammatory, antimicrobial and neuroprotective activity, with possible implications in the prevention of chronic, neurodegenerative and cardiovascular diseases. Caffeic 3-O-glucoside presents similar levels between callus and suspension, except in the suspension growth in culture medium with 2,4-D + 6-BAP, where an increase of up to five times is observed compared to callus. Rosmarinic acid, also identified, although only in callus, contributes to the protection of the central nervous system and to the regulation of lipid and carbohydrate metabolism thanks to its well-documented antioxidant and anti-inflammatory properties (Hitl et al., 2021).

Erythronic acid, a metabolite related to vitamin C, contributes to the antioxidant potential thanks to its free radical scavenging activity (Tao et al., 2017). It is expressed in greater quantities in suspensions, particularly in Dark MS with 2,4-D and 6-BAP.

Among esterified phenols, ethyl caffeate, which accumulates in the callus in dark conditions, has shown a marked immunostimulatory activity, improving immune responses in particular resistance to bacterial infections (Widodo et al., 2020). Glucaric acid, present in blue T2 cell suspension culture, is relevant for its liver detoxifying, hormone regulatory and tumor prevention properties (Ayyadurai et al., 2023). p-Coumaric acid, identified in the suspension culture, is known for its antioxidant,

antimicrobial, antiviral, anti-inflammatory, antithrombotic and anticancer activities, in addition to its potential role in the management of metabolic disorders such as obesity, diabetes, hyperlipidemia and gout (Pei et al., 2016). Shisoflavanone A, a flavonoid identified in callus under dark conditions, has promising anti-inflammatory properties (Nakajima et al 2015). Spermidine, an endogenous polyamine more abundant in callus under red-blue conditions, has recently emerged for its role in inducing autophagy and preventing cellular aging, with cardiovascular protective effects and potential nutraceutical applications (Chen et al., 2025). Esculetin, a coumarin present in both callus and suspension grown under blue conditions, has shown high antioxidant activity and is promising in the management of chronic inflammatory diseases such as rheumatoid arthritis and diabetes mellitus (Garg et al., 2022).

Among the essential amino acids, phenylalanine (L-Phe) is particularly abundant in both matrices under dark conditions with significantly higher concentrations in the suspension grown in Dark T2 conditions. Precursor of tyrosine, dopamine, noradrenaline and adrenaline, plays a key role in the control of appetite and energy metabolism, promoting the secretion of anorexigen hormones (GLP-1, CCK, PYY) and inhibiting ghrelin (Alamshah et al., 2017). Glutamine, predominant in the dark callus and in the blue suspension, is essential for intestinal homeostasis, acting as an energy substrate for enterocytes and modulating the immune response (Cruzat et al., 2018). Tryptophan, more abundant under dark condition, is involved in the synthesis of serotonin and melatonin, influencing mental health, sleep-wake rhythm and cardiovascular function (Friedman 2018). Tyrosine, more represented in the callus in R/B conditions, is known to improve cognitive and physical performance in stressful situations (Attipoe et al 2015). L-pipecolic acid, a lysine derivative, has recently gained interest for its action on intestinal transit (Li et al., 2023).

Perilla frutescens blue suspension culture is also rich in vitamin B5 (pantothenic acid), absent in callus culture, and essential for the synthesis of coenzyme A, energy metabolism, liver detoxification and modulation of the lipid profile. Riboflavin (B2), found in the callus in dark conditions, is crucial in mitochondrial processes and iron absorption, with protective effects on anemia. Vitamin B3 (niacin), detected in blue callus culture condition, influences lipid metabolism, vasodilation and cognitive function, with potential impact in the prevention of cardiovascular and neurodegenerative diseases.

The comparative analysis of the metabolic profiles of callus and cell suspension on Blue T2 medium reveals a partial overlap, in particular in the classes of amino acids, organic/phenolic acids and vitamins. However, significant differences emerge in terms of relative abundance and metabolic specificity between the two matrices. Both cultures contain citric acid and chlorogenic acid, with the latter being particularly abundant in suspension culture, where the content is approximately double that of callus. On the contrary, galactonic acid is more concentrated in callus. Callus shows greater

phenolic diversity, with the specific presence of ethyl caffeate, while suspension is characterized by the production of p-coumaric acid, not detected in callus. Both cultures show an accumulation of phenylalanine, with significantly higher concentrations in the suspension. Similarly, leucine, isoleucine, glutamine and glutamic acid are consistently more abundant in suspension. Arginine and tyrosine follow this trend, with a notable increase in suspensions, especially for arginine. Tryptophan is markedly enriched in the suspension, reaching levels much higher than in the callus, while valine also shows greater abundance. Notably, pyruvic acid is exclusively detected in the suspension, suggesting enhanced energy metabolism. As for vitamins, both callus and suspension contain comparable amounts of vitamin B2 (riboflavin), but vitamin B3 (niacin) is up to ten times more concentrated in the suspension. Additionally, vitamin B5 (pantothenic acid) is found exclusively in the suspension.

Replacement or partial substitution of synthetic plant growth regulators: callus culture

The utilization of plant cells and tissue cultures for the production of food ingredients necessitates that all components of the culture medium are food-grade. Synthetic plant growth regulators, some of which are used as pesticides in agriculture, are regulated by the EU, and specific acceptable daily intake (ADI) are recommended for them (ADI for NAA is 0,1 mg/kg of body weight/day, for 2,4-D, 0,02 mg/kg of body weight/day, for 6-BAP is 0,01 mg/kg of body weight/day). To comply with food safety standards and enhance the quality of the final product, exploring natural alternatives to conventional phytohormones is beneficial; such alternatives include casein hydrolysate, yeast extract, zeatin, and coconut water. Our study initially evaluated the substitution of the auxin 2,4-D with casein hydrolysate (0,5; 1 and 2 g/L) and yeast extract (1; 2,5, and 5 g/L) in *P. frutescens* callus culture medium. Casein hydrolysate, a milk-derived protein, was selected for its affinity with plant cells, promoting cell viability and making it a valuable ingredient for biotechnological applications. In the dairy industry, significant amount of casein-rich waste is produced during milk processing and equipment cleaning operations. Incorporating casein into plant substrates not only helps reduce environmental impact but also enhance the nutritional and sensory qualities of plant cells intended for human consumption. In our experiment, casein hydrolysate, particularly at a concentration of 0,5g/L, proved most effective in promoting callus growth, improving fresh weight, dry matter content, and the UV-visible profile, with performance surpassing that of standard cultures containing 2,4-D, without compromising cell viability. Conversely, yeast extract, while promoting an increase in dry weight biomass, resulted in non-optimal callus morphologies, lack of friability and browning, making it less suitable for subsequent applications.

Replacement or partial substitution of synthetic plant growth regulators: suspension culture

P. frutescens suspension cultures had been grown in a liquid medium containing both 6-BAP (1mg/L), and 2,4-D (0,5mg/L) to obtain suspension culture homogeneity, as previously explained.

However, since 6-BAP has the most restrictive ADI for human consumption (0,01 mg/kg body weight/day), it is not advisable to use it for food-related applications. In contrast, *B. vulgaris* suspension cultures grew easily with the help of only 2,4-D (0,22mg/L). Given the promising results of casein hydrolysate in media for callus cultures, the same formulation was tested in a liquid medium, specifically starting with *P. frutescens* callus previously cultured in this modified medium (without plant growth regulator and with 0,5g/L of casein hydrolysate). Additionally, blue LED treatment was applied to optimize growth and metabolite production, since we had previously shown it to effectively increase dry matter and bioactive compounds in *P. frutescens* callus cultures. However, UV-visible analysis revealed no significant differences in absorbance between suspension cultures with and without Blue LED treatment (in presence of casein hydrolysate and without plant growth regulators), suggesting that the Blue LED did not provide a significant metabolic advantage. Additionally, both metabolite profiles and dry matter accumulation of these suspension cultures was lower than in the starting callus culture (grown with casein hydrolysate in dark condition), leading to the exclusion of casein hydrolysate-only liquid formulation for further studies.

Therefore, it was decided to modify the composition of the standard medium used for *P. frutescens* suspension cultures (MS + 2,4-D 0,5mg/L + 6-BAP 1mg/L), adopting strategies aimed at reducing synthetic growth regulators, rather than completely eliminate them, with the same objective of improving their compatibility with food applications. Alternative media compositions avoiding the use of growth regulators with a restrictive ADI (specifically, 6-BAP 1mg/L) were evaluated in combination with specific wavelength LED light (blue: 460nm;50 $\mu\text{mol}/\text{m}^2/\text{s}$) application to eventually compensate the substitution. Based on the effectiveness in dry matter accumulation found in calli, the blue LED administration upon growth on MS supplemented with 2,4-D (0,22 mg/L) and NAA (1 mg/L) medium, was considered, given the absence of 6-BAP, and the reduced dose of 2,4-D (half compared to the standard formulation for *P. frutescens* suspension culture). This configuration showed a good balance between growth, accumulation of metabolites of interest, and reduced presence of synthetic regulators, in view of possible food applications. The comparison of UV-visible spectra between the polar extract under standard conditions (i.e., in the presence of 6-BAP) and optimized conditions revealed a spectral profile similar to that of the standard condition, but within a safer environment for food applications. Therefore, for the use of *Perilla frutescens* cell suspension cultures in hydrogels intended for the formulation of 3D food inks, modifying the culture medium under blue LED light emerged as a more effective strategy than completely replacing phytohormones with casein hydrolysate or yeast extract.

The presence of trace amounts of plant growth regulators indeed may not be harmful in itself, as demonstrated by a study led by the VTT research group, pioneering the evaluation of plant cell cultures for food purposes. In particular, one of their publications (Hakkinen et al., 2020) analyzed

the toxicity of birch (*Betula pendula*) and Arctic bramble (*Rubus arcticus*) cell cultures, grown in a culture medium containing 2 mg/L of 2,4-D and 0,5 mg/L of KIN for birch, and 0,1 mg/L of KIN and 1,0 mg/L of NAA for Arctic bramble, conditions comparable to ours (0,22 mg/L of 2,4-D and 1 mg/L of NAA). While for NAA the acceptable daily intake (ADI) is 0,1mg/kg of body weight/day, for 2,4-D the situation is more "critical," with an ADI of 0,02mg/kg of body weight/day. For this reason, these cell cultures were subjected to a toxicity assessment using the freshwater crustacean *Daphnia magna*. To assess toxicity, the EC50 value was calculated, which represents the concentration of the compound under examination at which 50% of the *Daphnia* neonates die and/or remain immobilized during a 24 and 48-hour incubation. In this way, the increase in the toxicity of the compound is reflected in a decrease in the EC50 value. The EC50 values of the extracts from birch and Arctic bramble cell cultures were 760mg/L and 730mg/L, respectively, after 48 hours of incubation, indicating a very weak toxic effect against *Daphnia magna*, compared to the 0,6 mg/L of K₂Cr₂O₇ used as a toxicity control. Moreover, the toxicity of the extracts did not exceed that of edible fruits such as blueberries. In the case of 2,4-D, the 24-hour EC50 value was 300mg/L, indicating relatively low toxicity and still below levels considered dangerous for humans. Regarding oral toxicity, the oral LD50 of 2,4-D in rats, mice, and guinea pigs ranges from 320 to 1000 µg/g. Therefore, the amount of free 2,4-D found at the collection point in a 100 g portion of dry weight birch cell culture would clearly remain below the reported DL50 levels. In conclusion, the amount of 2,4-D present in the cell cultures would not theoretically reach health-dangerous doses, even considering oral intake. These results suggest that, at small doses, such as those we use, the plant growth regulators used in cell cultures, such as 2,4-D, are not toxic to *Daphnia magna*, with concentrations well below the oral LD50 for mammals, making also the plant cell candidates chosen for this work suitable for safe food consumption.

Formulation of prototype food products containing plant cells and bioactive molecules from agri-food by-products

Technical solutions were analyzed and evaluated to support the pre-industrialization of prototypes for food products containing cells and bioactive molecules. The study focused on the development of (bio)inks designed for potential future use in 3D food printing technologies. Therefore, we focused on selecting basic ingredients that are suitable for these purposes. Three-D printable inks (and bio-inks) must possess the necessary chemical-physical and rheological characteristics for extrusion (speed, resolution, substrate, temperature). In addition, hydrogels conceived for being bio-inks must preserve the viability, nutritional properties, functional and biological characteristics of the embedded plant cells. Since the aim of the work was to produce inks for sustainable and healthy food applications, the ink ingredients had to be chosen not only for their nutritional benefits, but also for compliance with circular and sustainable agro-industrial production. To devise edible polymers with

printing-compatible properties that are generally recognized as safe (GRAS) by the Food and Drug Administration, we focused on hydrogels, that can be produced from natural molecules with colloidal properties in suitable conditions. Among these molecules, there are primarily polysaccharides, polypeptides, and food-grade lipids. To ensure stability and compatibility between primary and secondary ingredients, additives and secondary products (e.g., citric acid, CaCl_2) were identified for use in the printing process. The basic ingredients were identified from those provided by the Nutri 3D project partners and from other sources based on identified useful chemical-physical characteristics suitable for 3D food printable inks. The selection of ingredients was based on a rational approach, focusing on their chemical, physical, and nutritional properties. For instance, the apple juice concentrate from Rigoni di Asiago Srl, with a high °Brix value of 70, indicated a significant content of soluble solids, typical of concentrated products. This results in higher density and improved shelf life by reducing the risk of microbial growth. The product is formulated to enhance the concentration of natural sugars like fructose, glucose, and sucrose, while the addition of sorbitol, a sugar substitute, improves the taste profile without increasing the glycemic index. The pH of 3,28 and acidity level of 0,04% malic acid ensure microbiological stability and a balanced sweet-acidic flavor. From an organoleptic standpoint, the juice has a clear amber color, characteristic aroma, and a sweet-acidic taste, making it appeal both health-wise and in terms of flavor. Although the use of the extract in a diluted form with water, necessary to achieve an osmolarity suitable for plant cell resuspension may attenuate some of its functional properties, the results show that the pH remains acidic, ensuring good stability. However, the reduction of Brix degrees (due to the dilution) could compromise the shelf-life. To address this, additional components, such as citric acid, were incorporated in the final product to help extend shelf life. The sensory notes continue to be perceptible, contributing to the overall aromatic profile of the product.

Another key ingredient used was high-oleic sunflower oil, (Rigoni di Asiago Srl). This refined oil is extracted from organic sunflower seeds (*Helianthus annuus*) with a high oleic acid content. The fatty acid composition predominantly features oleic acid (80-90,7%), followed by linoleic acid (2,1-20%). The maximum oleic acidity is 0,40%, which contributes to its stability and nutritional value.

Pectins are a hetero polysaccharide polymer containing α -(1–4)-d-galacturonic acid units, naturally present in plant as important elements of primary cell wall and middle lamella in plants and fruits. Pectins exhibit colloidal properties that enable gel formation at room temperature under specific pH conditions, a property widely exploited in various industries (e.g. in food and pharmacy). Pectins are classified according to the degree of esterification (DE) in high methoxylated (HM) with a DE above 50% or low methoxylated (LM) with lower DE. Gelation behavior differs accordingly: HM-pectins forms gels in acid environments (pH 2,8-3,5) in the presence of co-solutes like sugars. LM-pectin, on the other hands, gel in presence of divalent cations (typically Ca^{2+}) across a broader range, following

the egg-box model. For our application, we utilized low-methoxyl, non-amidated pectins (LMP) known for their degree of methylation low ester content (32-40%) highly reactive to calcium, standardized with sucrose, manufactured from citrus peel with natural color variation from off-white to golden. These pectins offer uniform consistency and distribution in fruit products, excellent control over syneresis, and are suitable for organic products. In addition to LM pectin, amidated pectin represents another type of pectin of particular interest due to its functional properties/for its improved gelling characteristics. Amidation represents one of the most relevant chemical modifications to improve the Ca^{2+} -dependent gelation capacity of pectin. Specifically, we used amidated pectin (AM), features a low ester content (30-38%) and amidation (7-14%), standardized with calcium citrate to reduce the need for calcium salts, which can impart a bitter taste compared to non-amidated pectin, amidated pectin requires a lower amount of Ca^{2+} for gel formation, shows greater resistance and stability, thanks to the formation of hydrogen bonds between the amide groups, which favor the cross-linking of the polymeric network, especially in low pH environments.

Honey, specifically flower and sapin honey from Mielbio RdA (Rigoni di Asiago Srl), was selected for its preservation of beneficial nutrients, antioxidants, enzymes, and organic acids, as well as its natural antibacterial and antibiotic properties. This honey is processed at room temperature to maintain its characteristic flavor and nutritional profile.

Additionally, fruit by-products from industrial processing of strawberries and blueberries and apple peel fiber (Rigoni di Asiago Srl) for the manufacturing of fruit jellies were utilized as high-value recovery materials, promoting sustainability by repurposing “waste”.

Lastly, locust bean gum (Rigoni di Asiago Srl) serves as a stabilizer, thickener, and binder. Derived from the endosperm of *Ceratonia siliqua*, a Mediterranean fruit tree, it contains galactomannans (D-galactose and D-mannose) with a molecular weight of 50-3000 KDa and a mannose-to-galactose ratio of 4:1. This ingredient contributes to the structural integrity and rheological properties of the inks.

The research also included other ingredients, not supplied by the project Partners, such as casein hydrolysate, useful for imparting gel-forming, emulsifying, and foaming properties to the final food products. Using casein as an ingredient in plant substrates not only reduces environmental impact but also enriches the nutritional and sensory properties of plant cells intended for consumption. The properties of casein hydrolysate were carefully monitored to assess its potential in hydrogel formulation, with particular attention to its role in creating stable and biocompatible gel structures. Additionally, other ingredients were selected to enhance the functionality of the inks: citric acid was chosen to extract value-added compounds from fruit processing by-products and to regulate pH in ink formation, extending the shelf life of the final products. In the food sector, citric acid (E330) is used as an acidity regulator and flavor compound, calcium chloride acts as an excellent cross-linking agent for pectin solutions, improving the macroscopic properties of the gel at low pectin

concentrations following the egg-box model, where positively charged Ca^{2+} interacts with dissociated acid groups in polymer chains (Ahmed 2023). The gelation process for low-methoxyl pectin (LMP) based food inks relies on cross-linking interactions between Ca^{2+} ions and free carboxylic groups. The addition of calcium chloride (CaCl_2) was essential and carefully regulated based on stoichiometric ratios to ensure appropriate gel fluidity and partial cross-linking during printing (Vancauwenberghe et al., 2017). This approach allowed the gels to maintain structural integrity while enabling extrusion-based deposition. Calcium lactate can be used as an emulsifier in foods and as a cross-linking agent in LMP. The addition of calcium lactate to LMP solutions increased the viscosity and stability of emulsion (Fan et al., 2016).

Ink recipes

This project focused on 3D printing pectin-based inks with varying properties, using extrusion techniques. A rheological analysis of calcium/pectin-based food inks was first conducted to emphasize the critical role of calcium in forming printable pectin gels. Following this, inks were formulated using LMP and AMP and enriched with agro-industrial by-products to improve sweetness, antioxidant properties and texture variability. These formulations were thoroughly characterized to optimize their performance. To incorporate a diverse range of ingredients, base recipes were developed with specific attributes, such as minimal sugar content (from clear, concentrated apple juice) and controlled pH levels (using citric acid) to improve shelf life and to create the conditions to trigger gelation with a lower content of sugars. Gelation studies were then conducted under ambient temperature conditions to maintain the stability of key metabolites and plant cells. Preliminary tests were conducted to evaluate the printability of these inks, leading to the refinement of procedures that progressively incorporated all the selected elements. The pH stability and their ability to maintain structural integrity under multiple layers (initially tested via manual deposition using a syringe) were also assessed. These evaluations contributed to identifying optimal conditions for producing multi-functional food inks suitable for advanced 3D printing applications. The inks and final products were formulated based on a rational approach, prioritizing stability (evaluating pH stability and total moisture variation) and the preservation of key specialized metabolites, such as flavonoids. The formulations were developed with specific applications in mind, each tailored to meet the functional and nutritional needs of the final product:

1. A multilayer bar composed of an external biscuit-like shell, an intermediate layer of dark or white chocolate-like filling, and an internal core of gelée filling for the inclusion of cells
2. A honey sphere with an inner gelée filling that contains cells.

1. Multilayer bar composed by:

Ink 1: formulated to reproduce the consistency and appearance of a biscuit-like.

Ink 2: conceived as an intermediate layer with characteristics similar to a white chocolate filling.

Ink 3: designed for the inclusion of plant cells in a jelly-like structure, with properties suitable for their viability preservation.

2. Honey sphere composed by:

Ink 3: (as above) designed for the inclusion of plant cells in a jelly-like structure, with properties suitable for their viability preservation.

Ink 4: developed to reproduce a gummy-like outer layer.

During the development of the formulations for food 3D printing, each ink was specifically optimized to fulfil a defined function within the final multilayered product. 3D printing was not merely employed as a technical tool, but as a means to enhance both the sensorial experience and the nutritional functionality of the product. Layering was strategically used to "positively deceive" the palate, making the product healthier and more sustainable (integrating agri-food by-products and plant cells), while also increasing its appeal and consumer acceptance. By differentiating texture, flavors and nutritional profile across the layer, this approach fully leveraged the potential of 3D printing. It is based on recent scientific evidence demonstrating that manipulating the internal structure of a 3D printed food can significantly influence the perception of satiety, chewing and, in general, eating behavior. In our case, the multilayer bar was designed to offer a multisensory experience (appeal the palate) through the combination of a biscuit-like outer shell, a soft chocolate-like middle layer, and a gelée inner core enriched with viable plant cells. Similarly, the honey pearl exploits the contrast between a gummy outer shell and the gelée inner core, with embedded plant cells, offering a multi-textured, nutritionally enriched eating experience. Within this framework, four food inks were developed and optimized, each playing a distinct role in the final product. The formulation process followed an iterative strategy, where initial recipes based on agri-food by-products, juices, and pectin, were gradually refined. Modification in ingredients and concentration, as well as the adjustment to printing parameters, were essential to achieving the desired rheological and functional properties. The optimization process was driven by the need to ensure consistency, printability, biocompatibility and sensory appeal. Ultimately, this led to the creation of four stable, functional, and innovative inks, demonstrating how 3D food printing can be harnessed to produce complex, multi-textured, and health-promoting food systems.

For example, in Ink 1, designed for the outer shell of the bar, the inclusion of apple fiber powder and high oleic oil improved the rheological properties, structural stability, and nutritional profile by enhancing fiber and lipid. Honey was incorporated to further stabilize the matrix, leveraging its natural crystallization tendency. Ink 2, intended as the creamy filling, required multiple modifications to achieve the desired consistency. The inclusion of locust bean gum significantly enhanced the ink's stability and cohesion, while ingredients as concentrated apple juice and banana powder enriched the

formulation with bioactive compounds, including proteins and phenolic compounds. Ink 3, specifically designed for the inclusion of viable plant cells, presented the challenge of maintaining the vitality of the cells within a gelled matrix. Finally, Ink 4, intended to form the shell of the honey pearls, was adjusted to obtain a gummy-like texture. Increasing honey not only improved the sensory characteristics but also allowed for gelation without the need of calcium chloride. The final structure, based on low-methoxyl pectin (LMP) and honey resulted in a perfectly printable structure, which maintained its shape and consistency even after a thermal treatment at 50°C.

Embedding of cells into Ink 3

Ink 3 was developed in three slightly different formulations (namely Ink 3, 3b and 3c) with the aim of incorporating viable plant cells into a hydrogel matrix. This work is part of a broader vision of synergistic valorization between plant biotechnology, with particular reference to cell suspension cultures, and the principles of the circular economy, exploiting agri-food by-products as functional matrices. The initial formulation included three liquid solutions, strawberry (basis of Ink 3 version) and blueberry (basis of Ink 3b version) by-product extracts and clear, diluted apple juice (basis of the Ink 3c version), all combined with low-methoxylation pectin (LMP) and calcium chloride, to promote gelation. The main challenge was to ensure the formation of a stable gel without compromising the viability of the plant cells. The extracts were selected based on their acidity, favorable both to the stability of the hydrogel matrix and to the preservation of the cellular system. Particular attention was placed on apple juice's osmolarity, which, due to the high °Brix value, required dilution to ensure compatibility with plant cells. Preliminary cell viability tests, conducted at 4°C, showed that cells resuspended in strawberry by-products extract in citric acid and diluted apple juice maintained approximately 50% of the cells viable for up to 120 hours, with survivals observed even up to 192 hours. In a subsequent phase, complete formulations containing LMP pectin, casein hydrolysate, used as a natural substitute for growth regulators in plant cultures, and calcium chloride were tested. Comparative tests included the variation of the resuspension liquid (culture medium and strawberry extract) and the concentration of plant biomass. The results highlighted:

- Cell compatibility: the selected ingredients supported the viability of plant cells, maintaining approximately 50% of live cells between 48 and 72 hours, even in the absence of culture medium;
- Printability: the chemical-physical properties of the formulations allowed effective extrusion printing, even in the presence of incorporated cells;
- Nutraceutical value: the nutritional quality of the ink was directly related to the amounts of cells included and remained stable for at least 96 hours, confirming the ink's potential as a vehicle for bioactive ingredients.

Overall, Ink 3 proved to be a promising solution for advanced food 3D printing applications, thanks to its ability to combine biocompatibility, technological functionality and sustainability, through the

use of high added value agro-industrial by-products. The results obtained confirmed the full compatibility between plant cells and matrices derived from these by-products, demonstrating that the latter can act not only as sources of antioxidants within the hydrogel matrix, but also as valid resuspension liquids, alternatives to traditional culture media.

Printing test

The structural integrity of the four ink recipes (including Ink 3 with and without plant cells), were evaluated under multilayer deposition, initially using manual syringe-based extrusion methods. These assessments ensured the compatibility of each formulation with the printing process while preserving nutritional and functional properties. Trials with the CellInk Bio X 3D bio-printer allowed to define the printing parameters: speed, nozzle size, pressure, and infill. Two optimized printing scenarios with texture profiles were identified for each ink. In extrusion-based printing, the final product quality depends on material properties (e.g., viscosity), process parameters (e.g., nozzle diameter, speed), and post-processing treatments (e.g., baking). For soft materials, viscosity must balance ease of extrusion and layer adhesion. The smallest nozzle was chosen to ensure smooth extrusion. The printing trials were performed at room temperature ($25 \pm 3^{\circ}\text{C}$) to preserve nutritional integrity. Texture analysis was performed with a portable texture analyzer, measuring force, distance, and time to assess the inks' structural properties, crucial for optimizing product design and printing. The results demonstrate that For Ink 1 (the biscuit-like base), combinations with a speed of 10–20 mm/s, an 18G nozzle and a pressure between 150 and 200 kPa were effective. Ink 2, like Ink 1, required a speed of 10 mm/s, a 22G tip and a pressure similar to Ink 1 (130-150 kPa). Ink 3, containing live cells, required a much lower pressure (10 kPa), confirming its need for more controlled printing. Ink 4, intended for the creation of the gummy shell of the honey pearl, showed good printability with an infill of 22% and a low pressure (30 kPa). These results demonstrated the versatility and adaptability of the formulations, providing fundamental practical indications for the reproducibility of the printed products and for the optimization of the multilayer food design.

Analytical characterization of inks by FTIR-ATR

The chemical composition of the developed inks was investigated using a combination of micro-Raman and FTIR-ATR spectroscopy. Raman spectroscopy, a non-destructive light scattering technique, provides molecular-level information on structure, phases, and interactions by detecting the inelastic scattering of laser light as it interacts with chemical bonds. In micro-Raman systems, the integration with a microscope allows for precise analysis of micrometric regions of the sample.

FTIR-ATR spectroscopy, on the other hand, identifies molecular structures based on the absorption of infrared light at frequencies corresponding to bond vibrations. The ATR mode enhances measurement accuracy by allowing shallow penetration of infrared light, thus minimizing interference from complex or heterogeneous samples. This dual spectroscopic approach enabled

detailed characterization of the inks, even in the presence of dense or multi-phase matrices. The behavior of water release from the inks was also monitored over time, as influenced by ink composition and ink formation mechanisms. Key parameters evaluated included swelling degree (SD%) and water retention (WR), both critical for assessing ink stability. As inks lose water, degradation of the polymer network may occur, impacting structural integrity. Among the tested formulations, Inks 1, 2, and 4 exhibited gradual dehydration with comparable WR trends. In contrast, Ink 3 showed a drop in water retention between 400 and 450 minutes. This divergence is attributed to its specific chemical composition: as dehydration proceeds, calcium chloride concentration increases within the hydrogel network. Acting as a cross-linker, the calcium ions promote network stiffening and compaction, ultimately triggering the rapid expulsion of residual water.

Analysis of component interactions and gelling mechanisms

The interactions between ink components and their gelation mechanisms unveiled by the FTIR and Raman spectroscopy analyses, can be discussed based on the chemical composition of the resulting inks.

Ink 1. This formulation was designed to mimic the color and texture of cookie dough while maintaining a balanced nutritional profile. The synergistic interaction between the glycosidic units in honey, the polymers from apple fiber, and oil molecules realistically results in a compact and dense matrix. Water is retained within the polymer network through hydrogen bonding and electrostatic interactions with polar groups.

Ink 2. Gelation is primarily driven by pectin, a polysaccharide rich in galacturonic acid units. In aqueous solution, pectin chains interact via hydrogen bonding and electrostatic forces to form a stable three-dimensional network that entraps water. The presence of amidated pectin, citric acid, and sugars from locust bean gum and banana powder promotes gelation even in the absence of calcium chloride, by modulating pH and sugar concentration.

Ink 3. Gelation occurs through calcium-pectin crosslinking. Calcium chloride releases Ca^{2+} ions that form ionic bonds with the carboxyl groups of pectin, organizing the polysaccharide chains into a stable, structured network. Citric acid adjusts the pH, while casein and sugars from strawberry extract contribute to the cohesion and viscosity of the final hydrogel.

Ink 4. The network is formed through interactions among sapin honey, bilberry extract, and pectin. Hydrogen bonds form between pectin and water, as well as with glycosidic units in the extracts. Citric acid modulates pH to favor gelation, while sapin honey increases viscosity, resulting in a pasty, stable ink.

FTIR analysis over time

The temporal monitoring of FTIR spectra offered valuable insights into the dehydration dynamics of the inks, providing information on molecular interactions, phase transitions from gel to solid, and

potential chemical modifications occurring during water loss. The consistent overlap of FTIR spectra across time points, along with the absence of increased signal intensity in carboxylate and ketone regions, and the lack of additional peaks associated with oxidation by-products of lipids, proteins, or polysaccharides, strongly indicates that no significant structural or compositional changes occur under the experimental conditions applied. The presence of living plant cells within the hydrogel seemed to have a key role in modulating water dynamics. These cells remain turgid due to osmotic pressure, driven by water and solute content in the cytoplasm and vacuole, both of which are critical for sustaining metabolic activity and membrane exchanges. Their ability to absorb or release water in response to environmental stimuli directly influences the hydrogel's hydration status. Moreover, plant cells can physically interact with the pectin matrix and calcium ions derived from calcium chloride within the ink. These interactions may serve as anchoring points within the hydrogel, contributing to the stability of its three-dimensional network. Such cell-matrix interactions enhance the hydrogel's structural integrity and modulate its water-holding capacity. Notably, the presence of a C=C stretching signal in the IR spectra confirms that beta-elimination (a dehydration mechanism) remains a dominant dehydration mechanism, even in cell-containing systems. Overall, the inclusion of plant cells appears to improve the predictability and performance of the hydrogel matrix, particularly in Ink 3, by stabilizing water retention. This characteristic is especially relevant for its potential application in functional food products. Importantly, under the food-relevant storage conditions tested in this study (i.e., exposure to air at 4 °C), no evidence of oxidative degradation or significant compositional shifts was observed, confirming the chemical stability of the food-grade inks over time.

Determination of the shelf-life of 3D products

The microbiological evaluation conducted in this study proved to be fundamental to analyze the hygienic-sanitary stability of the developed prototypes, in relation to both their composition and storage conditions. In particular, two key parameters were monitored: the total microbial load at 30°C, indicative of the presence of mesophilic aerobic microorganisms, and the yeast and mold load at 25°C, useful for detecting any fungal contamination. The adoption of the ISO 4833 protocol guaranteed the reliability of the counting of the total mesophilic microflora; a parameter widely used to verify the effectiveness of hygiene practices and identify possible post-process contamination. The results obtained indicate that all the analyzed samples fall within the limits of microbiological acceptability, confirming the effectiveness of the process conditions and stabilization techniques used. However, differences in stability are observed between the two types of products. The multilayer bars, both with and without plant cells, proved to be particularly stable from a microbiological and chemical-physical point of view for at least 16 days in refrigeration, without evident signs of deterioration. This behavior can be attributed not only to the formulation but also to the heat treatment undergone, which reduced the water activity (a_w) to values not favorable to

microbial growth. Furthermore, maintaining a pH lower than 4.5 in all samples helped to inhibit the growth of pathogenic microflora, further increasing microbiological safety. On the other hand, honey pearls showed a greater susceptibility to microbiological alterations, with the first signs of deterioration visible around the 16th day, despite also having been subjected to heat stabilization. This behavior can be explained by the higher a_w values, characteristics of a sugar-rich and intrinsically more humid matrix, which could create an environment more favorable to microbial proliferation. At room temperature, the bars maintained good microbiological stability, but were subject to structural changes, probably related to moisture loss or internal migration of components. The pearls, on the other hand, did not show microbiological deterioration, but underwent vitrification phenomena, which altered consistency and texture, partially compromising their organoleptic quality. These results highlight how microbiological stability is not the only parameter to consider in the evaluation of shelf-life: structural and sensory aspects also play a crucial role in product preservation. Ultimately, the analysis demonstrated that multilayer bars are more stable over time, both for their formulation and for the technological treatment adopted, while honey pearls require more controlled storage conditions, particularly in terms of temperature and humidity, to preserve their microbiological and sensory integrity.

Characterization of the biological activities of the food matrices after *in vitro* digestion

The eluates obtained through the *in vitro* digestion protocol showed a significant antioxidant power, particularly marked in the bar containing *Perilla frutescens* cells. This suggests that the inclusion of these plant cells can amplify the functional properties of the final product. With regard to prebiotic activity, both digested samples (i.e., with and without cells), demonstrated the ability to support the growth of the probiotic strain *Lacticaseibacillus paracasei* CNCMI-1572, thus highlighting an interesting prebiotic potential, independently from the presence of cells. In parallel, tests conducted on *in vitro* intestinal models demonstrated that the eluates do not exert toxic effects on the intestinal barrier, a fundamental element to evaluate their safety. This result paves the way for the use of these formulations in bioavailability studies, confirming their compatibility with the simulated biological systems. In conclusion, the developed product, and in particular the bar enriched with *Perilla frutescens* cells, stands out for its concrete biological advantages: on the one hand, it contributes to intestinal health thanks to the prebiotic effect and the stimulation of beneficial flora; on the other hand, it offers effective antioxidant protection, potentially useful in counteracting oxidative stress. The confirmation of its safety and functionality *in vitro* reinforces its suitability as an innovative and healthy food, suitable for future nutritional and clinical studies.

Sensory Analysis of 3D Products: Consumer test

Consumer acceptance represents a critical factor in the successful commercialization of novel food products. To assess this aspect, a combined approach involving both a targeted panel and a broader

consumer survey was adopted. For the consumer survey, an online format was strategically selected to maximize participation. This approach enabled the collection of a statistically significant number of responses, which would have been logistically unfeasible using in-person methods due to limited participant availability. The data analysis revealed that 65% of respondents declared a willingness to try unfamiliar or novel foods. However, this openness appears closely associated with perceived health benefits. A more polarizing response emerged when participants were asked about their interest in foods produced using unconventional technologies such as 3D printing. Only 10% of respondents strongly agreed with the statement expressing interest, while 26% strongly disagreed. One core aspect of the study involved evaluating the perceived desirability of the proposed products, specifically a 3D-printed multilayer bar and a honey pearl. Participants assessed these products based solely on visual appearance, without any contextual information about their composition or production. The fruit bar received an average desirability score slightly below the threshold typically associated with "unappetising," suggesting an initial barrier in visual appeal. After receiving detailed information on the ingredients and production processes (use of 3D printing, incorporating honey and by-products from berries and apples, both known for their antioxidant and dietary fiber content), consumer perception improved, with both products showing increased palatability indices. These results highlight the importance of transparency and the promotion of functional components in enhancing consumer acceptance of innovative food items. Targeted communication strategies, emphasizing both the safety and health-promoting attributes of novel technologies, could play a pivotal role in improving consumer receptivity, particularly regarding purchasing intent and perceived sustainability, which are explored in the following section. Other key drivers include familiarity with ingredients, transparency of the production process, and clear communication of nutritional and environmental benefits. In this context, consumer education emerges as a crucial factor for promoting openness to novel food technologies and encouraging more sustainable consumption behaviors.

Sensory Analysis of 3D Products: Panel test

The sensory analysis of the innovative food products aimed to objectively characterize their sensory profiles through panel test. Trained assessors were tasked with evaluating key descriptive attributes to define the organoleptic identity of each product. For the multilayer bar, sensory profiling revealed that, excluding visual defects and the presence of off-flavors or odors (which were absent in all samples), scores close to 5 indicate that the attributes were perceived similarly across samples. A score significantly diverging from 5, conversely, reflects strong agreement among panelists in identifying a notable difference between the two bars. The 3D-printed and commercial bars were significantly different across nearly all evaluated attributes, except for visual homogeneity and flavor persistence, which were non-discriminating. Notably, both bars were free of any off-notes. Compared to the commercial bar, the 3D-printed version exhibited: lower intensity of berry aroma, higher

intensity of apple aroma and flavor, lower sweetness, higher acidity, lower gumminess, greater friability, easier mastication, reduced stickiness. The lower perceived sweetness may be partially explained by the higher acidity, which can suppress sweet perception. Furthermore, the improved texture descriptors, particularly ease of mastication and reduced stickiness, could offer objective advantages in terms of consumer appeal. For the pearl products, all samples (3D-printed pearl, 3D-printed pearl with added plant cells, and commercial reference) were free of off-notes. The commercial product was significantly different from both 3D-printed variants for all attributes except texture-related descriptors (compactness, ease of mastication, and stickiness). The two 3D-printed pearls were largely similar, but the inclusion of plant cells led to: lower compactness, lower stickiness, and improved ease of mastication. The commercial pearl showed higher intensities for aroma, flavor, and flavor persistence, alongside greater sweetness, compactness, and stickiness. In contrast, both 3D-printed pearls were perceived as more acidic, juicier, and easier to chew, echoing trends already observed in the multilayer bar.

5. CONCLUSIONS

This research is framed within the broader context of contemporary global challenges, marked by increasing demographic pressure, climate change, and the progressive depletion of available natural resources. In addressing this critical issue, the study explored innovative strategies that combine plant biotechnologies, principles of circular bioeconomy, and advanced food production technologies, with the aim of contributing to the development of a more sustainable and resilient agri-food system. The focus of the research project lies in the integration of *in vitro* plant cell cultures and agri-food industry by-products to formulate food-grade bio-inks suitable for 3D printing applications. These bioinks were developed and optimized to exhibit appropriate physicochemical and rheological properties, enabling the preservation of plant cells integrity, viability and bioactive compound profile. This qualifies them suitable in the design of personalized and functional food products. A pivotal component of the study was the set up and utilization of *Perilla frutescens* cell cultures, selected for their ability to accumulate polyphenols with proven health benefits. The cultivation protocol was optimized through physical elicitation methods, specifically LED light treatments, adopted as an alternative to chemical elicitors for their improved safety and sustainability. This approach led to a significant enhancement in biomass production and metabolite accumulation, as evidence by UV-visible spectroscopic analysis, while ensuring compliance with safety requirements for human consumption in terms of a controlled presence of plant growth regulators with suitable Acceptable Daily Intake (ADI). Metabolomic analysis of *P. frutescens* grown under different conditions revealed a remarkable variability in the accumulation of secondary metabolites of nutritional and pharmacological interest. Metabolomic analysis of polar extracts of *Perilla frutescens* revealed a rich diversity of bioactive metabolites with potential nutraceutical and pharmacological interest. Differences between callus and cell suspension, in terms of compound profile and abundance, are interesting, with suspensions generally richer in essential amino acids, organic acids and vitamins, particularly in dark or blue light culture conditions. Some metabolites, such as citric acid, chlorogenic acid, caffeic acid, p-coumaric acid, B vitamins and compounds such as spermidine and esculetin, suggest a potential use of *P. frutescens* as a source of molecules with antioxidant, anti-inflammatory, neuroprotective and metabolism regulatory action, with promising applications in the nutraceutical, pharmaceutical and food sectors.

Indeed, particular attention was given to the formulation of culture media. For callus culture, a plant growth regulators-free medium supplemented with casein hydrolysate demonstrated to perform exactly as a canonical medium with plant growth regulators, demonstrating the possibility of a complete substitution for the sake of safety without sacrificing the quality of the cell culture in terms of value-added compounds. Despite this approach did not work for suspension cultures, for this latter

culture format, a medium containing minimal concentration of plant growth regulators with acceptable ADI and consistent with the most recent scientific evidence on edibility and regulatory compliance, with the addition of a LED light treatment, demonstrated to be a suitable substitute for the optimal media containing canonical plant growth regulators. The cellular biomass, exhibiting a promising metabolic profile, valuable nutritional properties and suitability for food use, was subsequently incorporated into the formulation of functional food prototypes.

3D printing fits into this context, by potentially expanding the available products through the use of unconventional matrices, such as plant cells, as well as the valorization of waste materials.

3D food printing has proven to be a highly versatile technology, capable of fine-tuning the structural, sensory (such as shape, texture, flavor) and nutritional characteristics of food products. This innovative approach makes it possible to design multilayered items with high added value, both in terms of functionality and consumer appeal. Within this framework, four distinct ink formulations were developed, each offering complementary properties, contributing to the overall sensory profile and acceptability of the final product. The final functional 3D-printed food prototypes were the multilayer bar, consisting of a cookie-like crumbly outer shell, a soft intermediate layer, and a gelatinous core containing viable plant cells and the honey-based pearls, characterized by a complex texture comprising a gummy outer shell layer and a gel-like core embedding viable plant cells. The suitability of the formulations was validated through viability assay: hydrogels based on low methoxylated pectin enriched with strawberry by-product extracts preserved cell viability at approximately 50% even three days post-printing, a significant finding for the retention of nutraceutical functionality over time.

The strategic incorporation of agri-food by-products and high-value-added juices contributed not only to the structural and rheological performance of bioinks (in terms of gelation at room temperature, essential for the maintenance of the integrity and viability of cells), to sustain cell metabolic activity, but also to the valorization of materials otherwise destined for waste, aligning with a sustainable and circular model of food production.

3D printing trials enabled the refinement of process parameters critical to the quality and reproducibility of the final products, highlighting the flexibility of the developed formulation. Shelf-life evaluations further confirmed the structural and compositional stability of the multilayer bars over time. Conversely, honey pearls exhibit greater sensitivity to storage conditions, requiring careful control of temperature and humidity to maintain product integrity.

In vitro tests have demonstrated the functional efficacy of the bar formulation enriched with *P. frutescens* cells, highlighting its significant prebiotic and antioxidant potential. These findings support the suitability of the product for future nutritional applications and clinical investigations. The high polyphenol content and bioactive profile of the plant cells contributed to the functional value

of the final product. Moreover, the retention of cellular viability over time and under storage conditions further validates the stability and bio-efficacy of the developed formulation. Besides the technical aspect, the work also considered the response of consumers: the survey conducted highlighted an increase in interest in the products when accompanied by clear and transparent communication about their composition and benefits.

In particular, the presence of plant cells improved the sensory perception of the products, suggesting that food education can play a decisive role in the acceptance of new technologies applied to food.

Overall, this project represents a concrete step towards the commercial application of food 3D printing, with a transformative potential impact on the sector. The union of biotechnology, circular economy, and advanced printing technologies opens interesting scenarios to sustainably and innovatively meet the growing global demand for healthy, functional, and personalized foods. The approach also holds potential for applications in specific contexts such as space food, clinical nutrition and personalized dietary regimens for the population with particular nutritional needs (e.g., the elderly, athletes, or patients with metabolic disorders)

Future prospects include the scalability of processes in bioreactors, the increase of cellular viability in printable matrices, and the optimization of culture conditions to maximize the yield of bioactive compounds.

For the success of these kinds of food products, it will be crucial to consolidate scientific studies to confirm the safety and the sustainability of the underlying manufacturing strategies. Engaging policymakers, regulatory agencies, and stakeholders from the agri-food sector will be fundamental to enabling a regulatory framework that supports innovation while safeguarding consumer health and confidence. Under Regulation (EU) 2015/2283, food derived from plant cells and tissue culture are classified as novel foods, as they were not significantly consumed in EU before May 15, 1997. As such, they require pre-market authorization, including a safety assessment by the European Food Safety Authority (EFSA), which has provided specific guidelines on the data and information required for the characterization and approval of these foods. Public awareness efforts should follow, in order to promote the acceptance and commercial success of these new foods.

In summary, the present work demonstrates how real opportunities to rethink food production in a more sustainable, resilient, and health-oriented perspective can be considered. This integrated biotechnological platform provided by plant cell cultures, additive technologies (3D printing), and circular economy offers a forward-looking model for the next generation of food systems, where customization, sustainability and functionality converge to address both current and future global food challenges.

Supplementary materials

Stability Protocol (PAT-Merck, 19.07.2022)

Sample type:

- Plant extract with lead compound
- Plant extract without lead compound
- We don't know

Which extraction method did you use?

- Hydrophilic
- Lypophilic
- Sub-and supercritical CO₂

Color:

Odor:

Tests to assess extract stability

1) Stability studies of the raw material (powder or oil) at 45°C (1 week), (If you have an HPLC, you could analyze the lead compound and if not, please observe a change of color or odor).

If instabilities/degradation are observed under these **mild** conditions, it could be a **K.O. criteria**

What do you observe? →

2) Stability studies in the extraction solvent at 40°C for 4 weeks (If you have an HPLC, you could analyze the lead compound and if not, please observe a change of color, odor or a pH variation > 1) → it would be ideal if your lead compound is stable or if you do not note any color change or if you do not smell a weird odor or if the pH remains stable.

If degradation/instabilities are observed under these **mild** conditions, it could be a **K.O. criteria**

What do you observe? →

3) Stability in the extraction solvent at 70°C for 2 hours (If you have an HPLC, you could analyze the lead compound and if not, please observe a change of color, odor or a pH variation > 1) → it would be ideal if your lead compound is stable, if you do not observe any color change, if you do not smell a weird odor and if the pH remains stable, but it is **no K.O. criteria** if degradation/instabilities are observed under these **challenging** conditions!) (**optional**)

What do you observe? →

4) Photostability of the extract in the extraction solvent (beside a window 3-4 days). If you have an HPLC, you could analyze the lead compound and if not, please observe a change of color. A darkening of the solution is an indication of a photoinstability but it is **no K.O. criteria** (a negative phototoxicity test later AND the use of a dark glass container could be a solution) (**optional**)

What do you observe? →

Storage conditions (RT, fridge, in the dark, under inert conditions...):

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