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# Vegetable by-products as a source of bioactive compounds for food applications

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## Abstract

Food processing wastes is an issue for the food industry sector as they need disposal treatment and management efforts to meet the SDG (Sustainable Development Goal) target 12.3. The economic interest is moving to functional ingredients and food application because vegetable by-products are a valuable source of a broad spectrum of phytochemicals with biological activity. The overall objective of this thesis is to explore different food application solutions to valorise promising vegetable industrial wastes, deriving mainly from brassica and coffee streams with a heavy impact on food waste, into a new multi-opportunity for both the food industry and consumers through the investigation of their potentiality as sources of bioactive and functional compounds.

This overall objective is composed by 3 aims: 1) to develop fortified pizzas in glucosinolates, carotenoids and phytosterols for the first time with high levels of incorporation (10 and 30%) of stalks or leaves from *Cheddar* and *Depurple* cauliflower; 2) to evaluate different vegetable by-products (black cabbage stem, tomatoes peels and seeds, spent coffee ground) as a potential source of functional ingredients (glucosinolates/isothiocyanates, lycopene and chlorogenic acids) in brewing beers, taking into account the impact on the volatile and amino acid profile; 3) to optimize the coffee silverskin by-product as basal rearing substrate for *Hermetia illucens* larvae with the enrichment of marine microalga (spirulina) to modulate the nutritional composition (fatty acids, carotenoids and amino acids) of larvae in order to use the larvae as high value ingredient in aquafeed.

Considering fortified pizza application, functional bakery products with different chemical and textural characteristics can be obtained from the incorporation with coloured cauliflowers discards. Orange stalks flour (10 and 30%) better retained the glucobrassicin (-4 and -19%, respectively). In general, β-carotene and glucobrassicin were highly impacted within the pizza preparation and cooking as they showed the higher decrement, with respect to lutein and total phytosterols. Glucobrassicin showed a range of fortification of 1.44-12.00 μmol, 4-670 μg for vitamin A, and 22.1-46.4 mg for total phytosterols per 100 g of dry weight pizza. In conclusion good levels of fortification of bioactive compounds were achieved.

Focusing on the first beer exploration with vegetable by-products, physicochemical parameters, untargeted volatile and free amino acid profile were affected by the brewing with vegetable by-products. Beers with different characteristics were obtained. Black cabbage and tomato beers displayed the lowest value of total essential amino acids and the highest value of total non-essential amino acids. Tomato peels produced a slight increment of lycopene (5.0±0.4 μg/mL). Spent ground coffee is valuable source for chlorogenic acid enriched beer (2.3±0.2 μg/mL). Along the untargeted volatile profile, satisfactory differentiation by PLS-DA score plots was obtained, evidencing that black cabbage beer was the most differentiated sample.

For the application for aquafeed needs, the black soldier fly larvae used in this study can be successfully grown on diets composed of coffee by-products enriched with microalgae. The dietary levels of microalgae (5, 10, 15, 20% of *Arthrospira platensis*) added to coffee silverskin diet did not affect the larval crude protein content and the amino acid profile, except for threonine, lysine, histidine, and tyrosine. Feeding with 15% of microalgae led to larvae with the highest PUFA content (1600.7 ± 62.7 mg/100g dry weight). A significant

increase of carotenoids was observed in larvae fed on coffee silverskin enriched with microalgae, reaching the maximum bioconversion on diet containing 20% of microalgae.
This thesis is a multidisciplinary approach to help research and food industries in designing and developing new food applications from wastes.

## Sommario

Gli scarti di lavorazione degli alimenti sono un problema per le industrie poiché necessitano di trattamenti di smaltimento e sforzi di gestione per raggiungere l'obbiettivo di sviluppo sostenibile (SDG) 12.3. Gli scarti vegetali sono una preziosa fonte di sostanze fitochimiche con attività biologiche, perciò, l'interesse economico si sta muovendo verso ingredienti funzionali e applicazioni alimentari. L'obbiettivo di questa tesi è di esplorare diverse applicazioni alimentari per valorizzare i sottoprodotti industriali vegetali, ricchi di composti bioattivi e funzionali, derivanti principalmente dalla lavorazione di brassicaceae e caffè, con nuove opportunità sia per l'industria alimentare che per i consumatori.

L'obiettivo generale ha 3 scopi: 1) sviluppare pizze fortificate con glucosinolati, carotenoidi e fitosteroli con alti livelli (10 e 30%) di aggiunta di gambi o foglie di cavolfiori *Cheddar* e *Depurple*; 2) valutare diversi scarti vegetali (gambo di cavolo nero, bucce e semi di pomodoro, fondi di caffè) ricchi in glucosinolati/isotiocianati, licopene e acido clorogenico, rispettivamente, come potenziali ingredienti funzionali per la produzione di birre, considerando l'impatto sul profilo dei volatili e degli amminoacidi; 3) ottimizzare il coffee silverskin come substrato di crescita per le larve di *Hermetia illucens* tramite l'arricchimento con spirulina per regolare la composizione nutrizionale (acidi grassi, carotenoidi e amminoacidi) delle larve al fine di utilizzarle come mangime di alto valore per l'acquacoltura.

Riguardo le pizze fortificate, si possono ottenere prodotti da forno funzionali con diverse caratteristiche chimiche dall'aggiunta di scarti da cavolfiori colorati. La farina di gambi arancioni (10 e 30%) ritiene meglio la glucobrassicina (4% e 19%, rispettivamente). In generale, il β-carotene e la glucobrassicina sono soggette a forte impatto nella preparazione e cottura della pizza mostrando un decremento maggiore rispetto a luteina e fitosteroli totali. La glucobrassicina ha mostrato un intervallo di fortificazione di 1.44-12.00 µmol, 4-670 µg per la vitamina A e 22.1-46.4 mg per i fitosteroli totali per 100 g di peso secco di pizza. In conclusione, buoni livelli di fortificazione di composti bioattivi sono stati raggiunti.

Riguardo la birra da sottoprodotti vegetali, questi ultimi hanno influenzato sia i parametri fisico-chimici, sia il profilo dei composti volatili e degli amminoacidi liberi. La birra al cavolo nero e quella al pomodoro hanno mostrato il valore più basso di amminoacidi essenziali e il più alto di amminoacidi non-essenziali. Le bucce di pomodoro hanno prodotto un lieve aumento di licopene (5.0±0.4 µg/mL). Il fondo di caffè è una preziosa fonte di acido clorogenico (2.3±0.2 µg/mL) nella birra. Riguardo il profilo dei volatili, una buona differenziazione dei campioni è stata ottenuta dai grafici PLS-DA, evidenziando la birra con cavolo nero come il campione più differenziato.

Per i mangimi in acquacoltura, le larve di mosca soldato nera usate in questo studio possono essere cresciute su diete composte da scarti del caffè arricchiti con microalghe. L'alimentazione con il 15% di microalghe ha prodotto larve con un contenuto di acidi grassi polinsaturi più elevato (1600.7 ± 62.7 mg/100g su peso secco). Un aumento significativo di carotenoidi è stato osservato nelle larve nutrite con il coffee silverskin arricchito di microalghe, raggiungendo la massima conversione con la dieta contenente il 20% di microalghe.

Questa tesi si basa sull'approccio multidisciplinare per aiutare la ricerca e le industrie agroalimentari nella progettazione e nello sviluppo di nuove applicazioni alimentari a partire da scarti.

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# Food waste and losses

## General information

Food loss is a reduction in the quantity or quality of food due to the decisions and the actions made by food suppliers along the supply chain, excluding retailers, food service providers, and consumers. It could be any food thrown away, burnt up, or otherwise discarded from the food supply chain from either harvest, slaughter, catch up to, but excluding the retail level, and not later used in any form of production either as feed or seed. Whereas food waste refers to a reduction in the quantity or quality of food resulting from decisions and actions by retailers, food service providers, and consumers. There are several ways through which food gets wasted. Fresh product that drifts from its standard idea as seen in its shape, size, and colour, is often removed and discarded from the supply chain during sorting processes. Foods having a near or beyond "best-before" date are often discarded by retailers and consumers. Thirdly, wholesome edible food could be unused and discarded from household kitchens and eating establishments. In addition, less food loss and waste would lead to more efficient land use and better water resource management with positive impacts on climate change and livelihoods (FAO 2019).

## Global situation

About one-third (1.3 billion tons per year) of food meant for consumption worldwide is either lost or wasted across the entire supply chain (Gustavsson et al. 2011). For over a decade, the problem of food waste and loss is referred to as "any food, and inedible parts of food, removed from the food supply chain to be recovered or disposed of" (Östergren et al. 2014), has been on the increase swiftly becoming a matter of international concern (Smith 2020). As of 2014, the amount of food loss and waste was estimated at about USD 936 billion, regardless of the social and environmental costs of the wastage that are paid by society as a whole (FAO 2014). FAO's Food Loss Index (FLI) estimates that globally, around 14 percent of all food produced is lost from the post-harvest stage up to, but excluding, the retail stage (FAO 2019).

## European situation

The European food-processing industry produces yearly huge volumes of aqueous wastes. Such waste comes from fruit and vegetable residues, molasses, bones, flesh and blood from meat and fish processing, stillage and other residues from wineries, distilleries and breweries, dairy products, and wastewaters cleaning operations (Kosseva 2009). In the EU, approximately 87.6 million tons of food is wasted on a year basics with associated costs estimated at 143 billion euro, and households waste contributes to more than half of the total food waste in the EU (47 million tonnes) with 70% of food waste arising at household, food service and retail (Stenmarck et al. 2016). While an estimated 20% of the total food produced in the EU is lost or wasted (De Schutter 2019), 33 million people cannot afford a quality meal every second day (EUROSTAT 2018).

## Food waste by sector

According to the European Commission, food waste has been placed into three groups: "Loss of food: foodstuffs that are lost at the production stage; Inevitable food waste: foodstuffs that are lost at the consumption stage (for example peels of some fruits); and Unnecessary food waste: foodstuffs that are not consumed but lost at the consumption stage" (European Commission 2014).

The food waste in the EU falls under four groups of the food supply chain: Manufacturing (treatment and processing of food products allocated for distribution), Retail/Wholesale (distribution and sale to individuals or organizations), Food Service Sector (ready-to-eat food preparation, catering, and restaurants), and Households (household consumption). Figure 1 divides this waste between the four stages described. Household waste contributes the most significant percentage of food waste: it is equal to 42% of the total (25% of the food expense per weight) and amounts to about 76 kg/year/person (60% of which could be avoided). The portion attributed to food processing (39%) and the portion from catering and restaurant services (14%) are also quite substantial (Gruszkowski 2011).

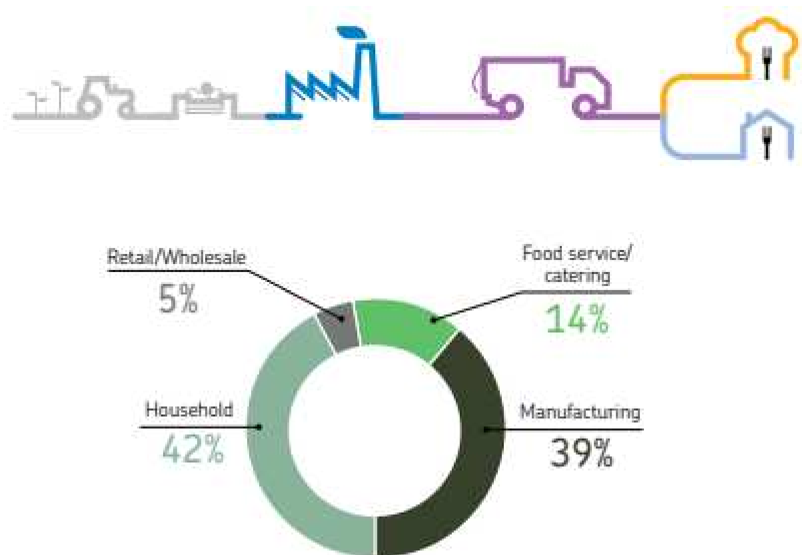



*Figure 1. Estimates of European food waste by sectors*

## Strategies to reduce food waste

The European Commission is highly concerned about the issue of food waste. Food waste reduction has a huge potential for lowering the resources used to make the food we eat. Food will be preserved for human consumption, money will be saved, and the environmental impact of the food production and consumption will be reduced. To combat food waste, all important parties from the governments to industries must collaborate to better identify, measure, understand, and find solutions to the problem. Because the food chain is a complex and dynamic system, there is no specific factor with a clear solution. From farmers, processors, manufacturers, and retailers to consumers themselves, all participants in the food chain must work together to develop answers. Lawmakers, researchers, food banks, and other non-governmental organizations (NGOs) all play a vital role (European Commission. 2016a).

Several techniques have been used by the EU and its Member States to prevent food loss and waste. When this is not possible, they choose to re-use, recycle, or repurpose food. The EU waste functional areas contain the guiding principles. Member States must either decrease food waste during production and distribution, reduce food waste in homes, encourage food donation, or monitor and assess the EU's food waste prevention initiatives. Furthermore, any food product could be used in the composition of animal feed or compost if possible (Council of the European Union 2016). In addition, depending on the sector of the food supply chain (primary production, processing and manufacturing, distribution, food services, and households), the European Commission has established a variety of legislative approaches that might be used to track food waste (Zambrzycki 2016).

The EU Platform on Food Losses and Food Waste is examining strategies to reduce food waste without jeopardizing food safety in close collaboration with business, consumers and other NGOs, research institutes, and EU country policy specialists, as well as exploring ideas for possible EU initiatives. Collaboration had been encouraged prior to the founding of the EU Platform by the EU nations Expert Group and a stakeholder Working Group on Food Losses and Waste (European Commission. 2016b).

The majority of food waste is produced by retailers and customers over-purchasing and then tossing perfectly edible goods. Technological improvements do not provide long-term answers to the problem of food waste. The mindsets and actions of a variety of stakeholders throughout the food supply chain must be addressed. Food waste is mostly driven by consumer behaviour and economic decisions in high-income countries, as well as policies and regulations affecting other industries. Authorities must realize that food waste may be rational from an objective point of view, coming from the 'optimizing behaviour' of farmers, processors, traders, and consumers, when addressing the behavioural causes. Policies must create conditions that allow diverse individual agents throughout the food supply chain to behave in such a way that food losses and waste are kept to a socially acceptable level. Another technique for decreasing food waste is the recovery and redistribution of safe and nutritious food (FAO 2017). A recent strategy is to recover bioactive compounds from food discards.

## Vegetable and fruit by-products

38% of food waste is produced during industrial processing vegetable by-products (Helkar et al., 2016). Agro-industrial wastes especially by-products can have a sustainable application. Food industry is oriented towards circular economy approach. Academy supports this approach by studying and developing useful applications of food industry by-products (Sicari, 2018). Waste proper management leads to several benefits in terms of health, environment, and economy (Mirabella et al., 2014). Brassica vegetables and coffee are interesting streams as they generate high amount of wastes since they are produced in high quantities and they are drivers of a wide pool of bioactive compounds.

## Study case: cauliflowers

Cauliflower is a variety of *Brassica oleracea* var. L. *botrytis*. There are many kinds of cauliflower and each one is characterized by different colour of inflorescence: white, green, purple, and orange, as Table 1 shows. In general, cauliflower is native of Mediterranean region, especially of Turkey. Nowadays, the world's largest producer of cauliflower is China with about 8.3 million tons (46% of world production), followed by India with 5 million tons (28%) and Italy with 455.000 tons (2.3%). In Europe, the main producers of cauliflower are Italy, Spain, France, and Poland (Faostat). Nowadays, scientific community is also giving importance to minority components as: polyphenols and carotenoids that are found in considerable quantities in purple and orange cauliflower, respectively.

Cauliflower composition is similar in floret (30% vegetable's biomass) and by-products (leaves and stalks) glucosinolates, isothiocyanates, polyphenols, dietary fiber, proteins, tocopherols, carotenoids, and minerals (Campas-Baypoli et al. 2009; Dominguez-Perles et al. 2010).

Literature data suggest that cauliflower and broccoli leaves could constitute a functional food additive. The chemical composition and antioxidant potential of bioactive compounds found in broccoli leaves have to be analyzed to validate their functional properties.

*Table 1. Varieties of cauliflower*

| White | Green | Purple | Orange |
|---|---|---|---|
| Snow Cloud | Emeraude | Graffiti | Cheddar |
| Snowball | Vitaverde | Violetta | Orange Burst |
| Cloud | Green Macerata | Purple of Sicily | Sunset |
| Aviso | Monte Verde | Mulberry | |

## Study case: coffee

The global consumption of coffee exceeded 9.3 billion kg in 2016 (International Coffee Organization, 2017). 90% of the brewed coffee ends up in the waste called spent coffee ground, according to an estimation of Murthy and Naidu (2012). By 2050 the earth population will reach 9.6 billion people, producing enormous quantities of food waste such as spent coffee ground and coffee silverskin (Zabaniotou and Kamaterou 2019). Compost, landfill disposal and animal feed use of waste (including spent ground coffee) is no longer a sustainable way (Directive1999/31/EC,1999).

Spent coffee ground is composed of cellulose 8.6-13.3% by weight, hemicellulose 30-40%, proteins 6.7-13.6%, oil 10-20%, lignin 25-33%, polyphenols 2.5% and caffeine 0.02% (Stylianou et al. 2018; Murthy and Naidu 2012; Mussatto et al. 2011). These compounds can be exploited to produce bio-syngas, electricity generation, soil amendment, green composites, antioxidant additives/nutraceuticals, fruiting bodies and enzymes production, bioethanol, polyhydroxyalkanoate polymers, food ingredient in bakery products, absorbents and more (Kourmentza et al. 2018; Stylianou et al. 2018; Kovalcik, Obruca, and Marova 2018).

Coffee silverskin is a thin tegument, enveloping the coffee bean, constituted of dietary fiber, proteins, minerals, lipids, chlorogenic acids, melanoidins, vitamins, and caffeine (Borrelli et al. 2004; Santos et al. 2021). Coffee silverskin is the most abundant solid by-product generated during roasting of coffee processing. The total production of coffee worldwide went up to 168,68 million of bags in 2019/20 (ICO 2020).

Roasted coffee contains many bioactive compounds showing beneficial effects on human health. Cafestol and kahweol, present in the coffee unsaponifiable matter, have positive effects against cancer and diabetes. Tocopherols and polar phenolic compounds have well known antioxidant abilities. In particular, coffee is the major dietary source of chlorogenic acids (i.e., the quinate esters of hydroxycinnamic acids) that are considered responsible of the positive biological effects related to the coffee beverage consumption. Caffeine as well displayed therapeutic effects (Farah et al. 2019).

# Functional foods

## Definition

The origin of the term "functional food" is attributable to an expression arisen in Japan in the last 80th and introduced in "Systemic Analysis and Development of Food Functions" memorandums of Ministry of Education, Science and Culture, to describe *food enriched with ingredients able to produce health benefit effects*. The term "*functional food*" was proposed on the basis of the observation of the progressive increase in life expectancy, ascribed to particular physiological effects of the Japanese diet by successive studies. Then, the phrase "functional food" was employed by several other countries around the world, causing the beginning of a disputation of its exact definition. Today does not exist a univocal and legally recognized definition for all the different countries, but several definitions linked to different cultures, market requirements, and different approaches of various competent authorities towards nutrition sciences. Many terms are used as synonymous with "functional food", as "novel food", "special food", "nutraceuticals", "medical food", "pharmaceutical food" and "FOSHU" (meaning foods for a special purpose).

A notable table in a review of Kaur and Das (2011) collects and explains the different terms linked or interchanged with functional foods, giving an exhaustive explanation for each term.

Given that, it is necessary to take into consideration the different definitions of functional food employed in industrialized countries.

- **Japan**

In 1984-1986, the Ministry of Education, Science, and Culture, during a study entitled "Systematic Analysis and Development of Food Function", established two basic criteria for the food selection: in the first, food has to satisfy the nutritional needs of the organism, then, it has to please also the sensorial requests. Meanwhile, from studies on the diet's capability in preventing the development of diseases, evidence of the presence in foods of substances that can regulate the immune, the endocrine, the nervous, and the digestive systems interacting with the course of pathologies was extracted. Considered that, in 1988 the Japan Ministry

of Health and Welfare (MHW) promoted a meeting to delineate the characteristics of a new and emergent class of foods: the Functional Foods. During this event, functional foods were defined as specifically designed and processed food, with the aim to positively interact with biological mechanisms of the organism to modulate for example immune system, body rhythm and body weight. From this meeting indispensable characteristics of functional food were individuated:

1- It should have the same aspect as a conventional food
2- It has to be part of the staple diet
3- It should be labelled as food having properties to control biological mechanisms

Whit this definition, some functions previously permitted only for drugs were given to this kind of food too. In 1990-1991 the MHW decided to regulate functional foods into the Nutritional Improvement Law. In this text, the term "functional food" was substituted with the expression "foods for specified health use" synthesized with the "FOSHU" acronym. FOSHU was defined officially as a sub-group of foods for special dietary uses. The most important function in FOSHU is the biological activity of the organism, more than the nutritional and sensorial ones. In this period the criteria needed by food to be labelled FOSHU were delineated:

(a) The food has to maintain or improve the health state of the organism.
(b) Scientific evidence must prove the benefits provided by foods or by their constituents.
(c) The recommended daily amount of the food or its active components have to be definable based on medical and nutritional knowledge.
(d) The food should be safe to eat.
(e) The presence in the food of biologically active compounds must be defined in a qualitative and quantitative approach.
(f) The processes to the formulation of these foods, using appropriate technologies, should not involve losses in nutritive constituents.
(g) The product has to be taken as part of the conventional diet, not in an occasional way.
(h) The aspect of the food must be the same as a traditional product. For example, FOSHU cannot be produced as pills or capsules.
(i) The product should not be consumed in place of drugs or only for medical purposes.

- **UK and EU**

In 1995 the UK Ministry of Agriculture, Fisheries, and Food (MAFF) defined Functional Food as "food that has had a component incorporated into it to give a specific medical or physiological benefit, other than a purely nutritional effect". Nevertheless, benefits produced by the consumption of these kinds of food are well proved in various experimental studies, the UK government today still prohibits medical claims in the labelling. The EU position about the Functional Food question is expressed by the International Life Sciences Institute Europe (ILSI – Europe). This last institute, in the early years of the past decade, established in a published study that "a food can be regarded as functional if it is satisfactorily demonstrated

to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved state of health and well-being and/or reduction of risk of disease" (Roberfroid 2002). Moreover, substances that could be used for the production of functional food in the EU perspective are grouped in three groups by a paper Roberfroid (1997):

**GROUP 1**- An essential macronutrient having specific physiological effects, such as resistant starch or omega-3 fatty acids;

**GROUP 2**- An essential micronutrient if it confers a special benefit through the intake over and above recommended daily intake (RDI);

**GROUP 3**- A non (essential) nutrient giving specific physiological effects, such as some oligosaccharides and phytochemicals.

In the EU, considering studies of relevant international scientific authorities, a functional food must own some characteristics:

- It must have the aspect of normal food (not pills, tablets, or powders)
- It should be consumed as part of the traditional diet
- It cannot contain a synthesized component
- It should have a measurable effect on a target physiological function
- It should increase the health status, prevent the development of pathologies, and improve psychophysical and behavioural abilities
- It must be labelled with solid scientific-based claims

In an educational text from the European Commission, Directorate E – Biotechnologies, Agriculture, Food; Unit E.3 – Food, Health, Well-being, under the Directorate-General for Research, published in 2010, a working definition of Functional Food is reported, proposed by the EC Concerted Action on Functional Food Science in Europe (FUFOSE): "a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement". This definition is an integration of the previous one given by the ILSI – Europe. Following this definition, nowadays, in the EU, some foods considered to be functional are natural whole foods where new scientific information about their health qualities can be used to proclaim benefits. Many, if not most, fruits, vegetables, grains, fish, and dairy and meat products contain several natural components that deliver benefits beyond basic nutrition. Others think that only fortified, enriched, or enhanced foods with a component having a health benefit beyond basic nutrition should be considered functional (Bahorun, Aruoma, and Neergheen-Bhujun 2019).

- **USA**

A specific law about functional foods is not yet present in the USA. Functional foods are currently regulated the same as conventional foods by the US Food and Drug Administration (FDA). Despite this, most organizations believe that functional foods need a specific documentation. All functional foods would have a health claim. Lacking a formal definition, FDA for its public hearing use the IFT (Institute for Food Technologists) definition for functional foods: "food and food components that provide a health benefit beyond basic nutrition". In USA, functional foods definition relates to food components too, therefore also food supplements can be considered functional foods. All the statements on food labels related to health and nutrition are subjected to regulation or enforcement by FDA. Functional foods, by their nature, are implying a health claim or a structure/function claim, so FDA should ensure that functional foods provide the functions claimed. All things considered, nowadays there is not a definition for functional food term recognized and adopted all around the world. A full and reliable collection of the main definitions employed by various governments and institutions are summarized in a table in an exhaustive review paper of (Bigliardi and Galati 2013).

## European Regulation

The regulation on food is extensive and complicate, particularly for functional foods where health claims are involved (Moors 2012). There is no specific regulatory structure for functional foods in the EU. Although, it has gained in popularity, its legal status with respect to food laws is not yet fully established in many countries. It still required by law that these functional foods be regulated in the market depending on the nature of the products involved (Duttaroy 2019; Díaz, Fernández-ruiz, and Cámara 2019).

As regards the regulatory framework, the European regulation considers functional foods as an idea rather than a certain food classification and they must meet general food laws. Thus, functional foods having any nutritional and health claim related to vitamins, minerals or other substances in its labelling, presentation or advertising, have to meet the specific requirements established by the Regulation (EC) 1924/2006. Concerning the nutrition and health claims made on functional foods, Regulation (EC) No 1924/2006 ensures that any claim displayed on its labelling, presentation or advertising is clear, accurate and based on scientific evidence (Díaz, Fernández-ruiz, and Cámara 2019). This is the first specific set of legal rules allocated to health and nutrition claims to coordinate foods in the European Union (Moors, 2012). The major aim of this regulation is to ensure that any claim made on a food label in the EU is clear and supported by scientific evidence (Duttaroy 2019). Functional foods with novel ingredients or those which are made by novel processes fall under Regulation (EU) 2015/2283.

The European Food Safety Authority (EFSA) is the body in charge of the evaluation of health claim applications. The EFSA assesses the health claim dossiers and advises the European Commission, which finally makes the decision (Moors 2012).

## Classification

Nearly all categories of food can be processed to obtain functional food, and considering the product, the ways to obtain the functional properties could be classified in five categories (Siró et al. 2008; Lau et al. 2012; Bigliardi and Galati 2013):

- ***Fortified products:*** food fortified with additional nutrients (fruit juices fortified with vitamin C, E, folic acid, zinc, and calcium)
- ***Enriched products:*** food with additional new nutrients or components not normally found in a particular food (margarine with plant sterol ester, probiotics, prebiotics)
- ***Altered products:*** food from which a deleterious component has been removed, reduced, or replaced by another with beneficial effects (fibers as fat releasers in meat or ice cream products)
- ***Non-Altered Products:*** foods naturally containing increased content of nutrients or components (natural foods)
- ***Enhanced commodities:*** food in which one of the components have been naturally enhanced (eggs with increased omega-3 content achieved by altered chicken feed)

Another method to classify functional foods divides them into three classes, based on the aims of the different functional foods and their effects on the health status (Bigliardi & Galati, 2013):

- Functional foods that add good and improvements to your life (for example probiotics and prebiotics)
- Functional foods reduce risks connected to health problems (for example foods with the ability to control cholesterol levels)
- Functional foods that make your life easier (for example products without lactose or gluten)

Moreover, behind their classification following the production processes or the aim, the main types of functional foods present on the market can be divided by their nature. The main kinds of functional foods are briefly outlined below.

- **Probiotics**

Probiotics are living microorganisms, as lactic acid bacteria and bifidobacteria, that "if they are consumed in adequate numbers confer a health benefit on the host". They can colonize the intestine, regulating the balance of the bacterial flora. Moreover, probiotics can enhance the immune system, through effects on lymphatic tissue connected to the intestinal mucosa and exploiting the systematic turnover of lymphocytes of intestinal origin. Food formulations containing probiotics are employed for the prevention of gastrointestinal tract diseases, as diarrhoea, constipation, colitis, and cancer.

- **Prebiotics**

Prebiotics are non-digestible food ingredients, especially non-digestible carbohydrates, that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health. The principal effect is the significant variation of the colon microflora during the period in which is consumed the prebiotics-containing food. The main prebiotics are fructooligosaccharides

(FOS), inulin, isomalto-oligosaccharides (IMO), polydextrose, lactulose and resistant starches. Excessive consumption of prebiotics can lead to intolerance phenomena, with flatulence, bloating, and diarrhoea.

- **Symbiotic**

Symbiotics are food in which probiotics and prebiotics are present together. From the potential synergy between these two kinds of functional components, enhanced health benefits can be achieved.

- **Functional Drinks**

Functional drinks are non-alcoholic beverages fortified with vitamins A, C, and E or other functional ingredients. The main products in this class are ACE drinks, cholesterol-lowering drinks (with a combination of ω-3 and soy), drinks for the improvement of eye health (with lutein), or for the enhancement of bone health (through calcium and inulin). These beverages have the objectives to reduce cholesterol levels, stimulate antioxidant function, and avoid the inhibition of growth and the deformation of the bones.

- **Functional cereals**

Functional cereals are cereals (as for example oat, barley, rye) that contain water-soluble dietary fiber, such as β-glucan and arabinoxylan, oligosaccharides as like galacto- and fructo-oligosaccharides, and resistant starch (Díaz, Fernández-ruiz, and Cámara 2019). Functional cereal can exploit multiple beneficial effects in different ways. For example, non-digestible oligosaccharides and starches are prebiotic components, providing fermentable substrates for the growth of probiotic microorganisms. Moreover, some functional cereals components (starch) can be used as encapsulation materials for probiotics in order to improve their stability during storage and enhance their viability during their passage through the adverse conditions of the gastrointestinal tract. Dietary fiber of functional cereals can be used in dairy and bakery industries, supplying fats in the manufacture of low-fat ice creams and yogurts.

- **Bakery products**

In bakery products, the development of functional foods is not yet at the level of other sectors of food (dairy or confectionery). Bakery products however provide an ideal matrix by which functionality can be delivered to the consumer in an acceptable food. One functional bakery product developed is white bread with some nutritional elements' characteristic of brown bread, as like dietary fiber, group B vitamins, iron, zinc, inulin and, starch from wheat.

- **Spreads**

Different types of functional foods are present in this class of products, and the main health claim related to them is a lowering-cholesterol effect. Margarine added with phytosterol esters spreads containing camelina oil as a source of ω-3 fatty acids, low-cholesterol butter obtained by β-cyclodextrin addition are the principal items in this group.

- **Functional meat**

Functional meat is "meat modified by adding ingredients considered beneficial for health or by eliminating or reducing components that are considered harmful". Different strategies are employed to obtain functional meat, as the modification of fatty acid composition, the addition of antioxidants, dietary fiber, or probiotics.

To achieve this purpose, industries can act on raw or processed materials. Also, the control and the modification of the cattle feed could be a way to obtain functional meat.

- **<u>Functional eggs</u>**

Functional eggs can contain enhanced amounts of ω-3 fatty acids, vitamins D, E, B12, folic acid, and selenium. This kind of eggs can reduce the possible formation of blood clots and can control blood pressure. To the production of this functional food, chicken feeding modification is the main method.

## Market Trends

The trends in the market for functional foods are large and increasing continuously globally, intensely competitive and many new products are launched regularly. The functional products aim at health and mental well-being have led the food industry to increase the research and development of these foods. There are potentials to explore, nevertheless, matters relating to regulations and information has to be improved. Therefore, investments in the field of functional foods will prove beneficial only if specific legislation, an international collaborative approach and a direct and an effective strategy for communication between producer and consumer will be developed (Vicentini, Liberatore, and Mastrocola 2016).

There has been a significant improvement in the market trends. In 2019, the worldwide functional food market had an estimated size of around 178 billion U.S. dollars. Among other products, this covers vitamins and minerals, proteins, amino acids, probiotics, prebiotics, and dietary fiber (Figure 3) (Kamble and Deshmukh 2020). According to a report published in Statista (Wunsch 2021) on the functional food market, it was found that the functional food market size was valued at $177,770.0 million in 2019, and is estimated to reach $267,924.4 million by 2027, registering a CAGR of 6.7% from 2021 to 2027 as seen in Figure 2.

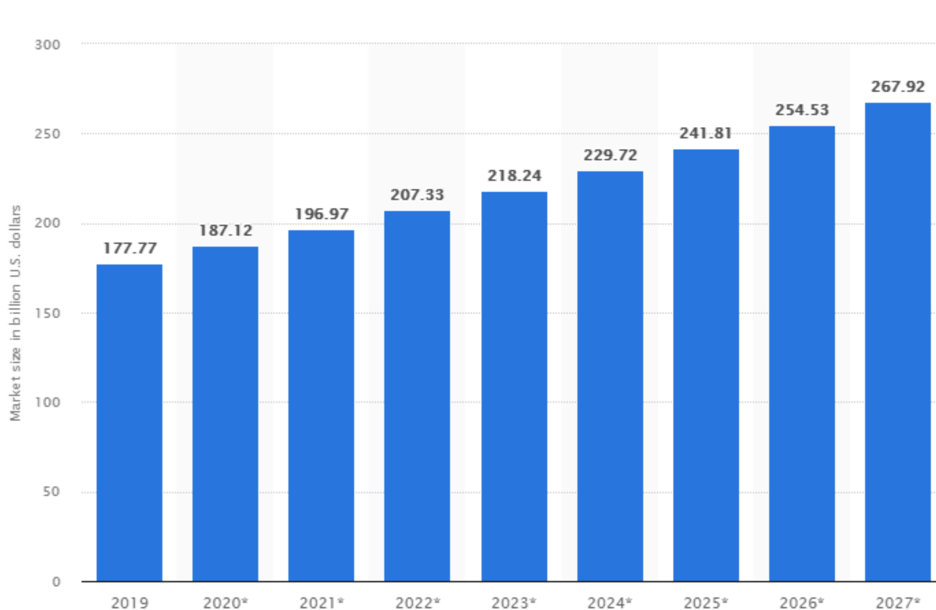
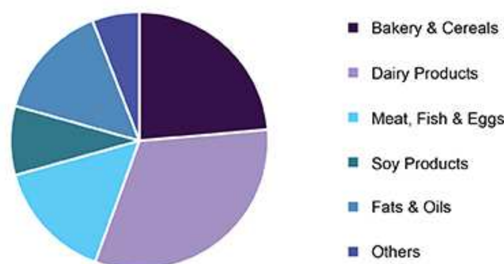



*Figure 2. Size of the functional food market worldwide from 2019 to 2027 (in billion U.S. dollars)*



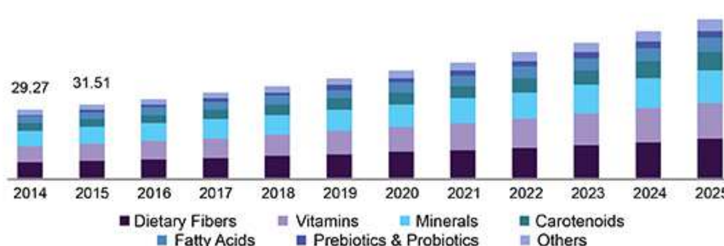




*Figure 3. Functional market size by product and by ingredient* (Grand View Research)

# Bioactive compounds

## General definition

A type of chemical found in small amounts in plants and certain foods (such as fruits, vegetables, nuts, oils, and whole grains). Bioactive compounds have actions in the body that may promote good health. They are being studied in the prevention of cancer, heart disease, and other diseases. They are divided in classes: vitamins, salts and minerals, dietary fiber, phytoestrogens, antioxidants. Examples of bioactive compounds include glucosinolates, carotenoids, phytosterols, polyphenols, tocopherols.

## Phytosterols

## Chemical description

Phytosterols (plant sterols) are lipophilic steroid alcohols naturally present in plants. Phytosterols are included in the family of triterpenes represented by tetracyclic structure and a side chain in the C-17 position. The majority of plant sterols are 4-desmethyl due to the lack of any methyl groups on the fourth position in the structure of the sterol ring (nomenclature of the ring structure and carbon numbering according to the IUPAC) (Figure 4). Their chemical structure is similar to the cholesterol one, with the only difference of side chains and ring structure saturation (Moreau et al. 2018).

*Figure 4. Phytosterols chemical structure (Moreau et al. 2018).*

Phytosterols are divided into two classes, plant sterols, with an unsaturated sterol ring (double bond at the position 5), and plant stanols, with a saturated ring (MacKay and Jones 2011). Sterols can be represented by "free" unbound OH sterol lipid class and by conjugates (Moreau et al. 2018).

## Functional effect

In plants, sterols represent by free form (free sterols, FSs) and are conjugated as steryl esters (SEs), steryl glycosides (SGs) and acyl steryl glycosides (ASGs) (Ferrer et al. 2017). Free phytosterols have in the plant cell membranes the role that cholesterol has in the animal cell membranes, stabilizing the phospholipid bilayers and fulfilling an important structural function (Moreau et al. 2002). SEs are responsible for membrane sterol homeostasis and the storage pool of sterols in plant tissues. SGs and ASGs are important structural elements in the lipid rafts of the plant cell plasma membrane, therefore, contributing to the function of plasma membrane enzymes. Conjugated sterols are constantly confirmed to be involved in plant stress response (Ferrer et al. 2017). Moreover, phytosterols contribute to the synthesis of essential bioactive compounds namely brassinosteroids, phytoecdysteroids, steroidal saponins and glycoalcaloids (Moreau et al. 2018).

Some of the main phytosterols employed as functional ingredients are β-sitosterol, campesterol, stigmasterol, brassicasterol, avenasterol. The two main phytostanols are campestanol and sitostanol. Phytosterols and phytostanols are used as functional ingredients for their protective effects against colon, prostate, and breast cancer, and, mainly, for their well-known total and low-density lipoprotein cholesterol-lowering activity (Gonzalez-Larena et al. 2012). Cardiovascular diseases are one of the principal causes of death in the world, hence plant sterols and stanols enriched food, mostly due to their anti-cholesterolaemic activity, are main actors in a world scale market of functional foods. Recent studies have demonstrated that phytosterols and phytostanols, both esterified and free, formulated in a manner that makes them "equally bioavailable" may give similar effects on the lowering of LDL-C levels (Moreau et al. 2002). Further studies are needed to compare the activity of different kinds of phytosterols and phytostanols.

Foods with a high natural content of phytosterols and phytostanols are oils like corn oil, canola oil, soybean oil, sunflower oil, olive oil, palm oil, cereals (corn, rye, wheat, barley, rice, etc.), nuts (peanuts and almonds), vegetables (broccoli, cauliflower, carrots, tomatoes, etc.), fruits and berries (avocado, passion fruit, orange, apple, banana, etc.). For a more exhaustive and deep vision, the review of Gylling et al. (2014) can be examined.

## Application for food enrichment

There is a high variability across plants in their phytosterol content in terms of individual phytosterols as well as conjugated to (Moreau et al. 2018). Behind the natural phytosterols and phytostanols containing food, a lot of plant sterols and stanols pharmaceutical preparations and enriched food are produced. The formulation of the phytosterol and its delivery vehicle is important for ensuring the fulfilment of the target functions. For example, free phytosterols can remain crystalline or trapped in the matrix. The difficulty in dissolving high amounts of free plant sterols in aqueous mixtures can result in detestable taste and unpleasant feeling of texture leading to a lack of palatability, in addition to limited effectiveness. These reasons brought to the discontinuation of Cytellin, a pharmaceutical preparation of free phytosterols (MacKay and Jones 2011*)*. Free phytosterols and phytostanols are only in a small extent soluble in oils too, this bringing to problems also in the enrichment of food with plant sterols extracts. The first solution to overcome these obstacles was the production of phytosterol fatty acid esters. Free phytosterols and phytostanols extract are esterified with fatty acids, mainly of vegetable oils, enhancing by tenfold their solubility in oils and fats. This process is employed since the first appearing of functional food with plant sterols, margarine enriched with phytostanols fatty acids esters introduced in Finland in 1995 (MacKay & Jones, 2011).

Phytosterols and phytostanols fatty acid esters in the place of free form present the advantage to be soluble in food matrix with significant fat content, enabling the production of different functional foods like margarine, spreads, cream cheese, salad dressing. Moreover, esterification with fatty acids from vegetable oil or with functional properties can improve the nutritional and functional effects of phytosterols, for example, procuring ω3 fatty acids too. However, target of the functional foods enriched with plant sterols and stanols are mainly people with dyslipidaemia and high cholesterol problems. This class of patients is suggested to follow a poor-fat diet, so there is the need to develop functional foods with very low percentages of lipids. Moreover, dyslipidaemia patients are usually treated with low caloric dietetic regimen, and phytosterols and phytostanols fatty acid esters furnish more calories than free forms. Low-fat functional foods are developed too, like functional drinks (juice and milk-based ones), baked products, ice creams, meats and soups, green teas, dark chocolate (Tasan et al. 2006; Botelho et al. 2014). Some methods used to produce low-fat functional foods with free plant sterols and stanols are based on homogenization and emulsion techniques using stabilizing agents such as lecithin (MacKay and Jones 2011).

## Some technological aspects

Plant sterols and stanols can be extracted from tall oil, a phytosterol-rich by-product of wood production. Phytosterols present in tall oil are refined and purified, then chemically hydrogenated to obtain phytostanols. In the end, esterification can be performed to obtain plant sterols and stanols fatty acid esters, by food-grade fatty acids from vegetable oils (often canola oil). From the refining process of soy oil and other vegetable oils, phytosterols extracts can be obtained too (Moreau et al. 2002; Tasan et al. 2006). Sterol enriched fractions can be extracted from mushrooms by-products too (Gil-Ramírez et al. 2013).

## Glucosinolates

### Chemical description

Glucosinolates (*β*-thioglucoside-*N*-hydroxysulfates, GSLs) are thioglycosides that differ in the structure of the aglycone side chain. GSLs occur naturally as salts of sulphate and are classified as aliphatic, aromatic, and indolic, depending on the amino acid from which they are derived (Figure 5) (Soledade et al. 2010). It has been reported 132 different natural glucosinolates  vary in the length of side chain and secondary modifications of amino acids such as oxidation, hydroxylation, sulfation, glycosylation, methoxylation, and desaturation (Agerbirk and Olsen, 2012).

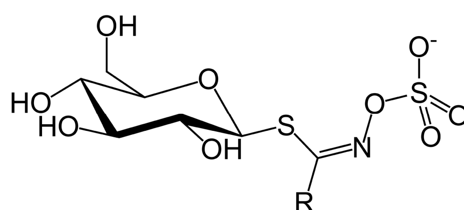



*Figure 5. General structure of glucosinolates*

### Functional effect

Glucosinolates (GSLs) are mainly represented in cruciferous plants such as the Brassicaceae family, including broccoli, cabbage, cauliflower, rapeseed, mustard, and horseradish (Prieto et al. 2019). GSLs and their derivatives are dietary plant secondary metabolites playing a role in plant defence. Additionally, GSLs are potentially involved in the survival mechanism of the Brassicaceae family (Martinez-Ballesta et al. 2015). Plant tissue damage leads to a combination of glucoraphanin with the enzyme myrosinase and subsequent formation of isothiocyanate, and sulforaphane. Sulforaphane is a naturally occurring health product with cell death protective features in neurodegenerative diseases and anticancer activity. According to Ladak et al. (2021), rich in sulforaphane, broccoli sprouts have been found to be “protective” in the fetal brain in preclinical rodent models of placental insufficiency, perinatal stroke, and fatal inflammation. Moreover, GSLs showed antibacterial, antioxidant, anti-inflammatory, anti-carcinogenic activities beneficial for human health (Baskar et al. 2012).

### Application for food enrichment

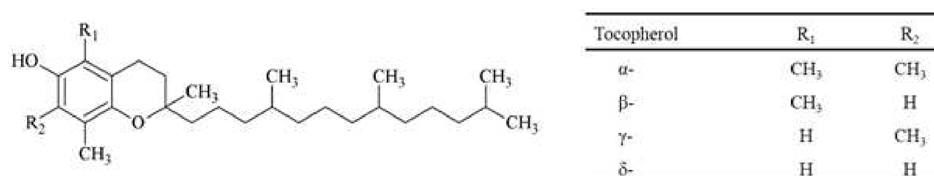
By-products of broccoli are sources of bioactive compounds such as nitrogen–sulphur compounds (glucosinolates and isothiocyanates (ITC)), phenolic (derivatives of chlorogenic and sinapic acid), and

important nutrients (vitamins and minerals). They represent a high interest in terms of source of health-promoting compounds, and essential ingredients for the evolution of functional food (Dominguez-Perlez et al. 2011). Broccoli by-products received attention in terms of the application of health-promoting compounds being characterized by a range of biological activities that could enhance the healthy attributions of beer for the development of new types of craft beers (Ramirez et al. 2020). Brassica-food has been widely and successfully used in the evolution of functional products such as functional soup reach in bioactive sulforaphane (Ramirez et al. 2020), a new beverage made with green tea and minimally processed broccoli by-products (Dominguez-Perles et al. 2011). Green tea enriched with broccoli extracts demonstrated enhanced phytochemical composition, physical activity, and antioxidant capacity. Beneficial results for human health should encourage the use of broccoli by-products as ingredients in functional food. The usage of broccoli by-products on the industrial scale as a component of new functional food may contribute to giving value to the crop wastes, therefore, reducing environmental impact (Dominguez-Perles et al. 2011).

## Tocopherols

### Chemical description

Tocopherols are fat-soluble class of compounds discovered in 1922 by the scientists Evans and Bishop-being isolated from green vegetables (Evan and Bishop, 1922). The general structure of tocopherols is shown in Figure 6. They are founded in 4 conformations: α-, β-, γ-, and δ-tocopherol that differ in the number and position of methyl groups in the aromatic ring (Della Penna and Pogson 2006).




| Tocopherol | $R_1$ | $R_2$ |
|---|---|---|
| α- | $CH_3$ | $CH_3$ |
| β- | $CH_3$ | H |
| γ- | H | $CH_3$ |
| δ- | H | H |

*Figure 6. Tocopherols chemical structures*

These amphipathic molecules with the polar chromanol ring and hydrophobic saturated side chain take part of vitamin E compounds. Among them, α-tocopherol is believed to present the most biological antioxidant activity, since it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Cruz and Casal 2013, Polat et al. 2013).

### Functional effect

Tocopherol (vitamin E) is an antioxidant, which neutralizes free radicals donating hydrogen from its chromanol ring. α-Tocopherol represents the dominant form of vitamin E since the transfer protein in the liver binds mainly α-tocopherol, hence preventing its degradation. Tocopherols are important components in the membrane structure therefore responsible for its integrity. Tocopherols prevent red blood cells haemolysis by oxidizing agents. Moreover, tocopherols protect polyunsaturated fatty acids from

peroxidation. The wide range of tocopherol functions connects directly or indirectly to its antioxidant properties (Ahsan et al. 2014). Vitamin E has been shown to be protective of cancer, cardiovascular, eye, bone, and neurological diseases. Additionally, tocopherols have been known for anti-inflammatory, immune-stimulatory, and nephroprotective features (Peh et al. 2016).

### Application for food enrichment

In our days, it is commonly established that diets rich in vegetables and fruits are healthy mainly due to the high amount of antioxidant compounds. Hence, food enriched with antioxidants of natural origin is receiving wide interest for health and technological reasons. Different vegetable oils are important, widely used lipid food and their enrichment with antioxidant compounds with a natural origin such as tocopherols may be useful (Alberdi-Cedeno et al. 2020). Tocopherols are mainly used as food additives to reduce the oxidation of fatty acids and vitamins (Delgado et al. 2020). Wagner and Elmadfa (2000) registered the oxidative stability of olive oil and linseed oil under heating enriched previously with tocopherols. Alizadeh et al. (2019) demonstrated that mayonnaise enriched with tocopherols has longer shelf life due to reduction of oxidation and inhibition of formation volatile compounds such as hexanal and heptanal. Based on those studies, the application of tocopherols as an alternative to synthetic antioxidants of food is highly recommended (Delgado et al. 2020). In addition, the extracts of food by-products such as watermelon and pumpkin peel and seeds, contain tocopherols that can be further used in the production of fruit bars, cakes, muffins enriched in vitamin E. Moreover, the utilization of food by-products decreases and environmental pollution (Comunian et al. 2021).

## Chlorogenic acid

### Chemical description

Chlorogenic acid (CGA) (Figure 7) is a group of specialized metabolites produced by several species and an essential component of coffee. Chlorogenic acid is the most abundant polyphenol compound in the human diet. CGA is an ester built from cinnamic and quinic acid and is known as 5-O-caffeoylquinic acid (5-CQA) or 3-CQA. The most common form of CGA is 5-caffeoylquinic acid (5-CQA) (Meng et al. 2013).

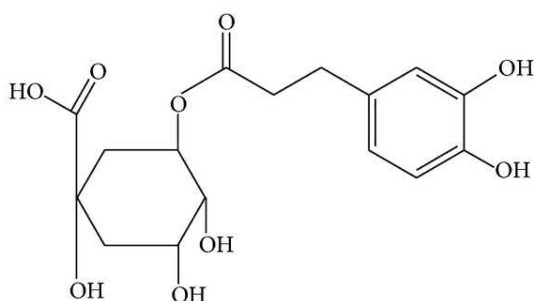



*Figure 7. General structure of chlorogenic acid (Meng et al. 2013).*

## Functional effect

Chlorogenic acid is receiving attention as a potential cardiovascular-preserving effect. Coffee, Lonicerae Japonicae Flos, and yerba mate are the main source of chlorogenic acid. The use of chlorogenic acid can contribute to the reduction of risk of atherosclerosis, heart failure, hypertension, myocardial infarction, and additional factors related to cardiovascular diseases as diabetes and obesity. It was indicated that chlorogenic acid showed circulatory guarding functions through oxidative stress elimination, infiltration of leukocytes, platelet aggregation, leucocyte-platelet interconnection, vascular remodeling, apoptosis, and, moreover, regulation of lipid and glucose metabolism (Li et al. 2020). In addition, the oxidative stress elimination property of chlorogenic acid may contribute to the prevention of several neurodegenerative diseases such as Alzheimer and Parkinson, which have been connected with chronic oxidative stress as well as with proinflammatory mechanisms leading to the neuronal damage (Nabavi et al 2017).

## Application for food enrichment

Several studies indicated that chlorogenic acid showed antimicrobial properties and can be used for the preservation of food products. Additionally, it has been discovered no inhibition effect of chlorogenic acid of probiotic bacteria growth, which are beneficial to human health. Moreover, chlorogenic acid is responsible for the inhibition of anthocyanins degradation, which is favourable for human health phytochemicals found in foodstuff. Kopjar et al. (2012) explored the prevention of anthocyanins degradation in blackberry juice by enrichment of sugars and chlorogenic acid. Chlorogenic acid also demonstrated the activity of probiotics since enhancing the growth of beneficial bacteria in the microbiota of the human intestine. Food products enriched with chlorogenic acid significantly increase the amount of Bifidobacterium spp. as well as *Clostridium coccoides-Eubacterium rectale* group, showing potentially advantageous effects to human health (Mills et al. 2015). The usage of chlorogenic acid as a food enrichment is a new area of research therefore more studies are needed to investigate the proper concentration of CGA as a prebiotic and preservative component (Santana-Galvez. 2017).

# Carotenoids

## Chemical description

Carotenoids are the most widespread pigments in the colour range from yellow to red. Currently, 1204 carotenoids have been identified in 722 organisms, as reported in the Carotenoid Database (http://carotenoiddb.jp/). They are biosynthesized *ex novo* by plants, bacteria, algae, fungi, and some arthropods, but they were also found in other animals that introduced them through diet (Rodriguez-Concepcion et al. 2018). The basic structure of carotenoids consists of isoprene units covalently linked, which are responsible for light interaction. They are classified into two main groups: “carotenes” (e.g., lycopene, α-carotene, β-carotene) and “xanthophylls” (e.g., lutein, zeaxanthin, β-cryptoxanthin), the latter being characterized by the presence of oxygen (Saini, Nile, and Park 2015). In nature, these compounds can be also associated with other molecules such as sugars, fatty acids, or proteins (Melèndez-Martìnez 2016).

## Functional effect

During the last decades, the beneficial effects of dietary carotenoids in the human organism have been widely investigated through epidemiological studies, lab tests, etc. (Meléndez-Martínez et al. 2020). From a nutritional perspective, carotenoids are potent antioxidants and some of them (e.g., β-carotene, α-carotene, β-cryptoxanthin) can be also converted to vitamin A, namely retinol, which is an essential micronutrient involved in growth, vision, and immune system functions (Andrew 2021).

Furthermore, some authors revealed that the optimal intake of these compounds has been related to reduced risk of developing cancers, cardiovascular diseases, Age-related Macular Degeneration (AMD), and other disorders (Van Hoang et al. 2018; Eisenhauer et al. 2017).

## Application for food enrichment

Vitamin A deficiency is common especially in poor countries where cereals and tubers account for staple food. Therefore, a variety of crops (e.g., rice, maize, cassava, wheat, sorghum, potato) were engineered in order to increase carotenoid accumulation (Beyene et al. 2018; Che et al. 2016). On the other hand, since carotenoids are pigments, they can be used as colorants in food industry, being allowed by Regulation (EC) No. 1333/2008 on food additives. In this regard, synthetic colorants have been commonly employed for food application because of their higher stability, but now consumers are more and more interested in "clean label", and thus food companies have to find natural alternatives (Asioli et al. 2017). Currently, the research is focused on finding sustainable techniques to extract these bioactive compounds from fruit and vegetables by-products, and on their stabilization for the formulation of functional foods such as cookies (de Toledo et al. 2017), bread (Waghmare and Arya 2014), cereal bar (V. S. Carvalho and Conti-Silva 2018) and chicken sausage (Zaini, Sintang, and Pindi 2020).

# Health claims

"Health claim" is defined by the Codex Alimentarius as "any representation that states, suggests, or implies that a relationship exists between a food or food constituents and health" (FAO and WHO 2004). In European Union, Regulation 1924/2006 establishes that health claims should be only authorized for use after scientific assessment carried out by European Food Safety Authority. The list of authorized health claims is reported within the Commission Regulation (EU) No 432/2012, and those referred to the bioactive compounds investigated in this study (i.e., phytosterols, carotenoids, tocopherols) are summarized in Table 2.

As regards phytosterols, several meta-analyses have been conducted to evaluate their optimal daily intake, and a recent study revealed that an intake of 0.6-3.3 g/day of phytosterols can help to reduce LDL cholesterol by 6-12% (Ras, Geleijnse, and Trautwein 2014). For vitamins, the recommended daily intakes (in adults) are reported in the Regulation (EU) No 1169/2011 on food information to consumers, and especially vitamin A and E have values corresponding to 800 µg and 12 mg, respectively.

Conversely, no health claim has been established yet for glucosinolates, although many epidemiological studies highlighted the inverse association between the intake of glucosinolates-rich foods and the risk of different types of cancer or other disorders (Marino et al. 2021). For example, it was observed that the consumption of 30 g of broccoli per day (containing approximately 121 mg of glucosinolates) helps to attenuate chronic inflammation in overweight subjects (López-Chillón et al. 2019).

In the same way, chlorogenic acids have not yet received an authorized health claim. Nevertheless, the potential anticarcinogenic capacity of these phenolic compounds was demonstrated by several studies (Kjølsrud Bøhn, Blomhoff, and Paur 2014; Pauwels and Volterrani 2021), and a moderate coffee consumption (e.g., 2-4 cups/day, containing up to 98% of chlorogenic acids) was associated with reduced all-cause mortality (Kim, Je, and Giovannucci 2019).

*Table 2. List of authorized health claims (extracted from Commission Regulation (EU) No 432/2012).*

| **Substance** | **Claim** |
|---|---|
| Plant sterols and plant stanols | "Plant sterols/stanols contribute to the maintenance of normal blood cholesterol levels" |
| ***Condition of use**: In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of at least 0,8 g of plant sterols/stanols.* | |
| Vitamin A | "Vitamin A contributes to normal iron metabolism"<br>"Vitamin A contributes to the maintenance of normal mucous membranes"<br>"Vitamin A contributes to the maintenance of normal skin"<br>"Vitamin A contributes to the maintenance of normal vision"<br>"Vitamin A contributes to the normal function of the immune system"<br>"Vitamin A has a role in the process of cell specialisation" |
| ***Condition of use**: The claim may be used only for food which is at least a source of vitamin A as referred to in the claim SOURCE OF [NAME OF VITAMIN/S] AND/OR [NAME OF MINERAL/S] as listed in the Annex to Regulation (EC) No 1924/2006.* | |
| Vitamin E | "Vitamin E contributes to the protection of cells from oxidative stress" |
| ***Condition of use**: The claim may be used only for food which is at least a source of vitamin E as referred to in the claim SOURCE OF [NAME OF VITAMIN/S] AND/OR [NAME OF MINERAL/S] as listed in the Annex to Regulation (EC) No 1924/2006.* | |

## Aim of the thesis

Food processing wastes is an issue for the food industry sector as they need disposal treatment and management efforts to meet the SDG (Sustainable Development Goal) target 12.3 to halve food waste by 2030 and reducing food losses The economic interest is moving from use of wastes to produce fertilisers, biofuels, animal feed to functional ingredients and food application because vegetable by-products is valuable source of a broad spectrum of phytochemicals with biological activity. This strategy embraces the circular economy approach by utilizing both products and by-products along the production chain.

Within this framework, the **overall objective** of this thesis is to explore different food application solutions to valorise promising vegetable industrial wastes, deriving mainly from brassica and coffee streams with a heavy impact on food waste, into a new multi-opportunity for both the food industry and consumers through the investigation of their potentiality as sources of bioactive and functional compounds.

This overall objective is composed by **3 aims**, namely:

To develop fortified pizzas in glucosinolates, carotenoids and phytosterols for the first time with high levels of incorporation (10 and 30%) of stalks or leaves from *Cheddar* and *Depurple* cauliflower and evaluation of textural properties.

To evaluate different vegetable by-products (black cabbage stems, tomatoes peels and seeds, spent coffee ground) as a potential source of functional ingredients (glucosinolates/isothiocyanates, lycopene and chlorogenic acids) in brewing beers, taking into account the impact on the volatile and amino acid profile.

To optimize the coffee silverskin by-product as basal rearing substrate for *Hermetia illucens* larvae with the enrichment of marine microalga (spirulina, *Arthrospira platensis*) to modulate the nutritional composition (fatty acids, carotenoids, and amino acids) of larva in order to use the larvae as high value ingredient in aquafeed.

## Impact of the thesis

The reduction of wastes: in the circular economy vision, wastes and by-products are managed as secondary raw materials and not simply discarded. They are exploited to prolong as much as possible the production processes, in order to reduce the pressure on the environment (reduction of soil exploitation, water consumption, nutrients requirements, energy demand and GHG demand). Vegetable discards will be avoided, and they will be used as starting point for the development of innovative products, following the "3R principles (repair, reuse, and recycle) belonging to the Circular Action Plan 2020 (one of the pillar of the European Green Deal).

Innovation: adding value to by-products implies a degree of innovation that makes a by-product that could be considered as waste, to be used as raw material subjected to further processing into edible food items.

# Chapter 1

# Assessment of bioactive compounds content and texture profile of pizza made with coloured cauliflower by-products

## Abstract

Coloured cauliflowers by-products are rich in bioactive compounds such as carotenoids, glucosinolates and phytosterols that can be found in stalks and leaves. Freeze-dried flours from violet stalks, orange stalks and leaves from both cauliflowers were prepared after hot boiling of raw materials. Two levels (10 and 30%) of enrichment of pizza with the prepared flours were assessed in terms of bioactive compounds recovery (glucosinolates, carotenoids and phytosterols) and texture profile. The results showed that hot boiling pre-treatment, pre-cooking and cooking pizza affected the recovery of bioactive compounds in pizza. Considering the retention, calculated on the predicted value, the lowest % were found for leaves flour pizza at 10% of enrichment for β-carotene, lutein and glucobrassicin. The highest ones in violet stalks flour at 10% for β-carotene, lutein, total phytosterols. Orange stalks flour (10 and 30%) better retained the glucobrassicin (-4 and -19%, respectively) than other samples (mean value = - 65%). In general, β-carotene and glucobrassicin were highly impacted within the pizza preparation and cooking as they showed the higher decrement, with respect to lutein and total phytosterols. Good levels of fortification of bioactive compounds were achieved. Glucobrassicin showed a range of fortification of 1.44-12.00 µmol, 4-670 µg for vitamin A, and 22.1-46.4 mg for total phytosterols per 100 g of dry weight pizza. The most interesting formulation of pizza was POF30 as it was the richest in phytosterol, the second richest for β-carotene and glucobrassicin content. POF30 and all 10%-fortified pizzas showed good values of springiness and hardness when compared to the conventional formulation of pizza. These results confirmed that cauliflower by-products enhance the nutritional value of bakery products and can be turned into an opportunity for the industries.

## Introduction

The SDG (Sustainable Development Goal) target 12.3 is halving food waste by 2030 and reducing food losses. Food waste is also associated with significant economic costs, estimated to amount to around € 143 billion in the EU. This includes costs to producers, who leave produce un-harvested; processors, who discard edible products that do not adhere to market size and aesthetic standards; retailers, households. In addition, there are also additional financial costs for collecting, managing, and treating food waste. 38 % of food waste is produced during industrial processing vegetable by-products (Bharat Helkar and Sahoo 2016).

A strategy to the waste valorisation is to use by-products from fruit and vegetable processing as additional sources of nutrients and functional ingredients (O'Shea, Arendt, and Gallagher 2014) without increasing production costs (Drabińska et al. 2018a). An interesting vegetable rich in a wide spectrum of bioactive compounds, produced worldwide is brassica vegetable in particular broccoli and cauliflower. According to FAOSTAT, the largest global producers of broccoli and cauliflower are China, India and the United States (average 1994 – 2017). Within the European Union, the major growers of broccoli and cauliflower are Spain, Italy, and France. Cauliflower composition is similar in floret (30% vegetable's biomass) and by-products (stem and leaf): glucosinolates, isothiocyanates, polyphenols, dietary fiber, proteins, tocopherols, carotenoids, and minerals (Campas-Baypoli et al. 2009; Domínguez-Perles et al. 2010).

The functional food market based on Brassicaceae vegetables is relatively recent. However, it contains interesting potential to offer new food products and formats with beneficial effects on health. Broccoli, cabbage, and kale are predominant in the search for new functional products based on Brassica vegetables, due to their phytochemical composition and the extensive knowledge collected in the scientific literature until this moment. Only several authors have described the nutritional composition and antioxidant activity of broccoli by-products (Ramirez et al., 2020) and of cauliflower ones (Ramirez et al. 2020).

Based on SCOPUS database (17/11/2021), when selecting the most relevant studies on broccoli and cauliflower by-products with functional food applications (Shi et al. 2020; Angiolillo et al. 2019; Krupa-Kozak et al. 2019; Drabińska et al. 2018b; Alvarez Jubete et al. 2014; Domínguez-Perles et al. 2010; Llorach et al. 2003; Llorach, Tomas-Barberan, and Ferreres 2005; Amofa-Diatuo et al. 2017; Abul-Fadl 2012; Caliceti et al. 2019; Montone et al. 2018; Soengas et al. 2012; Stojceska et al. 2008; Krupa-Kozak et al. 2021; Sedlar et al. 2021; Dominguez-Perles et al. 2011; Gliszczyńska-Świgło et al. 2006; Castillejo, Martínez-Hernández, and Artés-Hernández 2021; Eriksson et al. 2021; Sanz-Puig et al. 2017; Huynh et al. 2018; Zykwinska et al. 2009; Ng et al. 2011; H. Zhang et al. 2020; Larrosa et al. 2002) it emerged that the research focus mostly on polyphenols and antioxidant activities (57% of the selected articles), followed by proteins (20%), less in glucosinolates and isothiocyanates (17%), some in minerals (6%), especially in leaf and poorly in stem and stalks. Pre-treatment operations involved drying, freeze-drying, blanching and ethanolic and water extracts to improve antioxidant, nutritional and functional, physico-chemical and structural, antimicrobial effect properties of foods such as soups, beverages, breads, snakes, gluten free bakery products but animal foods such pork meat (sausages or patties) and tuna oil. Thus, there is a lack of knowledge on cauliflower by-products more than broccoli by-products, especially on stem and stalks and in

new cauliflower varieties appeared recently on the market such as spontaneous mutation of the white counterpart (*Brassica oleracea* L. var. botrytis). Moreover, glucosinolates and their bioactive counterparts (isothiocyanates) deriving from myrosinase hydrolysis (reference), carotenoids in which range cauliflower is richer, and phytosterols are less investigated as fortified food applications. Among vegetables, cauliflower and broccoli have been found to be the richest in phytosterols with a contribution of around 310-400 mg/kg (fresh weight) for cauliflower (Tolve et al. 2020) and having hypoglycaemic and hypolipidemic activities (EFSA health claim), no data was found regarding cauliflower by-products.

Coloured cauliflower by-products could be a non-expensive source of health benefits from glucosinolates, phytosterols and carotenoids. In this framework, we developed a food product spread worldwide (pizza) fortified for the first time with high levels of incorporation (10 and 30%) of stalks or leaves from *Cheddar* and *Depurple* cauliflower. So then, we investigated the thermal effect during the pizza production on bioactive compound less o more resistant to heat (carotenoids, glucosinolates and phytosterols).

## Materials and methods

### Chemicals and reagents

Glucosinolates standards (100%, glucoraphanin, glucobrassicin and sinigrin potassium salts) were purchased by PhytoLab GmbH & Co (Vestenbergsgreuth, Germany), carotenoids standards (> 95% purity; lutein and β-carotene), 19-hydroxycholesterol standard, Sylon BTZ, solvents HPLC grade (>95% purity, acetone, acetonitrile, dichloromethane, ethanol, methanol, *n*-hexane, diethyl ether, isopropanol, acetic acid, water), ammonium acetate (HPLC grade, >99%) were purchased by Merck (Darmstadt, Germany). Potassium hydroxide (85%) and anhydrous sodium sulphate were purchased by ITW Company (Darmstadt, Germany). MilliQ water was purified with Millipure System (Millford, USA).

### Preparation of cauliflower by-product powder

Cauliflower's by-products *(Brassica oleracea* L. var. botrytis) were provided from a local company (Agrinovana S.r.l Petritoli, Fermo, Italy). Stalks of *Cheddar* variety (orange cauliflower), stalks of *Depurple* variety (violet cauliflower) and stems and leaves from *Cheddar* and *Depurple* cauliflower were provided. Damaged tissue was removed from each by-product tissue, water washed and cut into small pieces of 10 ± 5 g of around 4 cm in diameter and 4 cm in length. The selected raw by-product typologies (stalks of Orange cauliflower = raw O; stalks of Violet cauliflower = raw V; stems and Leaves from both varieties = raw L) were split in two lots: the first for direct freeze-drying process to keep the raw samples, grounded to a fine flour (flour = F; flour of stalks of Orange cauliflower = raw OF; flour stalks of Violet cauliflower = raw VF; flour stems and Leaves from both varieties = raw LF); the latter for a strong blanching step in hot water for 5 min to inactivate enzymes hydrolysing biologically active compounds (such as myrosinase and polyphenol oxidase) and to investigate the effect of blanching on bioactive compounds. Blanched cauliflower by-products were freeze-dried and ground to produce flours to use in pizza preparation (flour of stalks of Orange

cauliflower = OF; flour stalks of Violet cauliflower = VF; flour stems and leaves from both varieties = LF). The flours were at -18°C under vacuum for further use.

## Preparation of pizza

Two enrichment levels (10 and 30%) with special flours from by-products of coloured cauliflowers were performed by replacing an equivalent amount of wheat flour with respect to the control sample (Table 3). 10 and 30 % are two strong levels of incorporation in pizza which were chosen to have a significative impact on the valorisation of cauliflower by-products. Samples of pizza were prepared (Figure 8) in a professional bakery laboratory (Pomorilla.it, Ascoli Piceno, Italy) to standardize the production till a half-processed product (pre-cooked and packed pizzas, stored at -18°C) to be sold to catering services. The preparation of fortified pizza included the preparation of biga dough by mixing the ingredients with a professional spiral mixer with fixed bowl (SPI 60 F E, Esmach Ali Group, Vicenza, Italy) as starting and once final dough was obtained, it was sized and round in dough portions of 70 g, allowed to leaven in a leaving chamber (Cella 1900x2300, 2021/085, Atrepan, Verona, Italy) at 35°C for 60 min, pizzas were draft, allowed to leaven a second time. Uncooked pizzas were precooked (300°C, 2.30 min) in an electric rotary rack oven (Esmach-Bongard, Esmach Ali Group, Vicenza, Italy) and stored at -18°C for 3 months, then samples were cooked setting the pizza dedicated program (220°C, 8 min) in a modern oven for consumer use (Bosch, HSG636ES1) purchased in a local distributor (MediaWorld Italy).

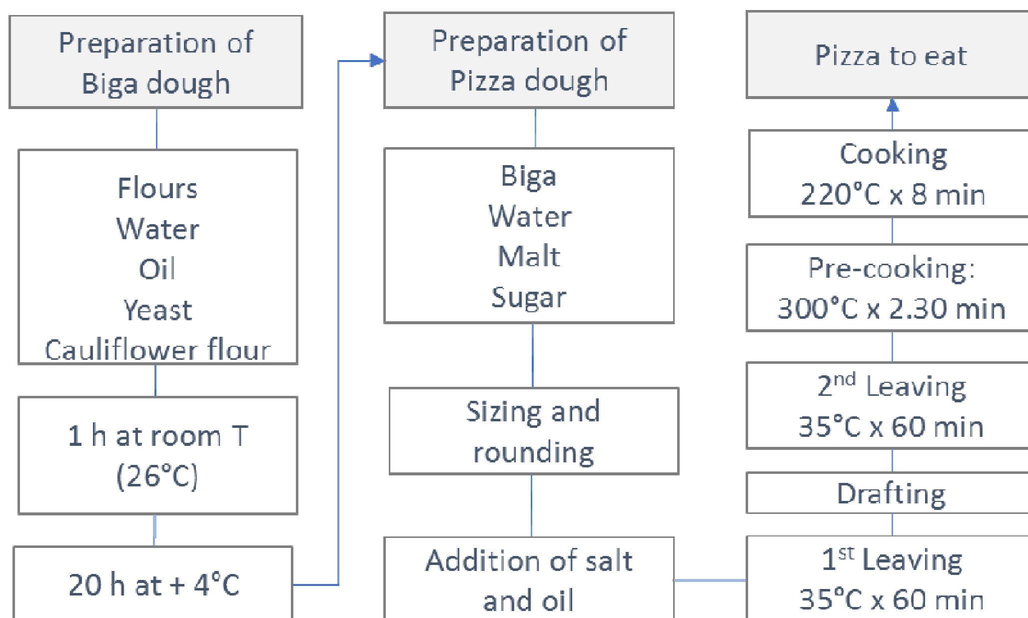



*Figure 8. Flow chart of pizza preparation.*

*Table 3. Percentage composition of control and fortified pizzas. Two enrichment levels (10 and 30%) with special flours from by-products of coloured cauliflower were performed by replacing an equivalent amount of wheat flour with respect to the control sample. (P=pizza; F=flour; L=leaf; O=orange cauliflower; V=violet cauliflower). Data are reported as fresh weight (FW) and dry weight (DW). Water was added in biga and final dough preparation.*

| | Control | PLF10 | PLF30 | POF10 | POF30 | PVF10 | PVF30 | Control | PLF10<br>POF10<br>PVF10 | PLF30<br>POF30<br>PVF30 |
|---|---|---|---|---|---|---|---|---|---|---|
| | % FW | | | | | | | % DW | | |
| Flour Manitaly | 28.9 | 26.0 | 19.6 | 26.0 | 14.8 | 26.0 | 17.2 | 44.5 | 40.0 | 31.3 |
| Flour Beta | 14.6 | 13.1 | 9.8 | 13.1 | 7.4 | 13.1 | 8.6 | 22.4 | 20.1 | 15.6 |
| Flour "0" | 14.6 | 13.1 | 9.8 | 13.1 | 7.4 | 13.1 | 8.6 | 22.4 | 20.1 | 15.6 |
| By-product flour | | 5.8 | 16.8 | 5.8 | 12.7 | 5.8 | 14.7 | | 8.9 | 26.7 |
| Malt | 0.6 | 0.6 | 0.5 | 0.6 | 0.4 | 0.6 | 0.5 | 0.9 | 0.9 | 0.9 |
| Sugar | 1.1 | 1.1 | 1.1 | 1.1 | 0.8 | 1.1 | 0.9 | 1.7 | 1.7 | 1.7 |
| Salt | 1.5 | 1.5 | 1.4 | 1.5 | 1.1 | 1.5 | 1.2 | 2.3 | 2.3 | 2.3 |
| Sunflower oil | 3.9 | 3.9 | 3.7 | 3.9 | 2.8 | 3.9 | 3.3 | 6.0 | 6.0 | 6.0 |
| Water in uncooked pizza | 34.9 | 34.9 | 37.3 | 34.9 | 52.5 | 34.9 | 45.1 | | | |
| Water in cooked pizza | 9.0 | 11.6 | 16.9 | 15.9 | 19.3 | 12.4 | 22.0 | | | |

## Glucosinolates determination

Glucosinolates were extracted in accordance with Baenas et al. (2019). Vegetable flours (200 mg) and pizza powders (1 g) were extracted (30 min, 70°C), with 2 mL methanol/water (70:30, v/v), vortexed each 5 min. Samples were cooled in ice and centrifuged (6000 rpm, 2 min). The supernatant was taken to dryness with a rotary evaporator (35°C), reconstituted in water (0.5 mL), centrifugated (13500 rpm, 12 min) filtered through a 0.2 μm (Sartorius Regenerated Cellulose Membrane) and injected (5 μL) in a Acquity Ultra Pressure Liquid Chromatographic H-class system (Waters Corporation, Milford, US), equipped with Photodiode Array Detector (PDA). The separation was carried out following Lin et al. (2014) on an analytical column UPLC CSH C18 (2.1 mm x 100 mm, 1.7 μm). The mobile phase was composed of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). The linear gradient was from 4 to 15% B (v/v) at 10 min, from 15 to 70% at 20 min, isocratic for 5 min, and decreased to 4% B (v/v) at 30 min. Flow rate was 0.3 mL/min, column oven was set at 35 °C and sample loading was carried out at 20 °C. PDA analysis was performed at 225 nm wavelength upon a spectrum scanning in the 210 - 500 nm range. Glucosinolates were identified by comparison of retention time and absorbance spectrum with pure standards. Their quantification was performed by external calibration. Stock and working standards of glucoraphanin, glucobrassicin and sinigrin were prepared by dissolving the salts in deionised water. Good correlation coefficients ($R^2$) of 0.999 were obtained in all cases. The instrumental limit of detection (LOD) and quantification (LOQ), calculated at signal-to-noise ratio of 3:1 and 10:1 respectively, were as followed: sinigrin, 42 and 140; glucobrassicin, 23 and 75, glucoraphanin, 18 and 59 ng/mL.

## Carotenoids determination

Carotenoids were extracted and analysed in accordance with Nartea et al. (2021). Briefly, freeze dried vegetable (100 mg) or freeze-dried cooked pizza was added of acetone (5 mL, 4 °C), kept at 4 ± 1 °C (15 min), vortexed (5 min) and centrifuged (1370 rpm, 10 min, 4 °C), repeating the acetone extraction a second time. The supernatant was filtered (0.45 µm, Sartorius Regenerated Cellulose Membrane), dried, resuspended in 0.5 mL acetone for pizza samples and 0.25 mL for tissue vegetable and injected in a Acquity Ultra Pressure Liquid Chromatographic H-class system (Waters Corporation, Milford, US), equipped with Photodiode Array Detector (PDA) and an Acquity column UPLC BEH C18 (2.1 mm x 100 mm, 1.7 µm). The mobile phase was composed by phase A consisting of acetonitrile (75 %), dichloromethane (10 %) and methanol (15 %), and phase B consisting of acetate ammonium in water (0.05 M). Gradient started at 75:25 (A:B) till 10 min, 98:2 (A:B) from 10 to 11 min, 98:2 (A:B) till 20 min. Flow rate was 0.4 mL/min, column oven was set at 35 °C and sample loading was carried out at 20 °C. PDA analysis was performed at 450 nm wavelength upon a spectrum scanning in the 210 - 500 nm range. Carotenoids were identified by comparison of retention time and absorbance spectrum with pure standards. Their quantification was performed by external calibration. Good correlation coefficients ($R^2$) of 0.999 were obtained in all cases. The instrumental limit of detection (LOD) and quantification (LOQ), calculated at signal-to-noise ratio of 3:1 and 10:1 respectively, were as followed: lutein, 5 and 16 and β-carotene, 5 and 18 ng/mL. Retinol activity equivalent (RAE) was calculated considering 1 µg retinol for each 6 µg of β-carotene or 12 µg of other provitamin A carotenoids (EFSA NDA Panel 2015).

## Phytosterols determination

Acid hydrolysis and alkaline saponification were performed to extract the total amount of phytosterols (esterified and acylated) in pizza and cauliflower flours as suggested by Toivo et al. (2001) for cereal-based matrices and adapted with new modification. By-product flours (0.5 g) and 2.0 g of pizza were added of internal standard solution (19-hydroxycholesterol, 50 µg in 2.5 mL ethanol) absolute ethanol (1 mL), shaken, added again hydrochloric acid (6M, 5mL), shaken and samples were placed in a water bath (80°C, 60 min) and shaken every 10 min. Then, samples were cool down, added of absolute ethanol (5 mL) and *n*-hexane:diethyl ether (20 mL, 1:1, v/v), vortexed again (10 min), centrifuged (4500 rpm, 10 min). The organic phase was collected and taken to dryness with a rotary evaporator (40°C). The saponification (80°C, 10 min) was performed by adding pyrogallol/ethanol solution (8 mL 3%, w/v), aqueous KOH (0.5 mL, 1.3% (w/v) to each dried sample, placed in the water bath and shaken every 2 min. The samples were cooled down and extracted with *n*-hexane (20 mL) and deionized water (12 mL), vortexed (10 min), centrifuged (4500 rpm for 10 min). The upper organic phase (15 mL) was evaporated to dryness at 40°C and added with Sylon BTZ allowing the derivation reaction (1h at room temperature). The sample was taken to dry and dissolved in 250µL of n-hexane. All samples were injected (1µL) in a GC/EI-MS (ThermoScientific, Waltham, MA, USA) system, equipped with a split/splitless injector, and a single quadrupole analytical column MDN-5 (30 m×0.25 mm ID, 0.1µm dF. Da). The oven temperature was set at 200°C, held for 1 min, increased to 280°C

(2°C/min) and held 1 min, using helium flow at 1.2 mL/min. The injector in splitless mode was set at 320°C, the ionization source (70 eV) was set at 250°C and the auxiliary line at 280°C. The acquisition was performed in total ion current (TIC) in a mass range of 70–650m/z and with a detector gain of 1.0. Trimethylsilane-phytosterols were identified by comparison with pure standards of each phytosterol (retention time and mass spectra). Quantification was performed by internal calibration. The instrumental limit of detection (LOD) and quantification (LOQ) were 0.6 and 1.8 ng/mL.

### Rheological properties of pizza samples

Rheological properties of pizza samples were evaluated in terms of viscoelastic properties according to Nartea et al. (2021) using a Universal Testing Machine (Zwick GmbH and Co, Ulm, Germany). Creep and recovery tests were conducted under small-deformation conditions on cooked pizza samples (90 $mm^2$ area, 15mm height). A creep load target (5 N) was reached during the loading step by imposing high crosshead speed (600 N/min) and kept constant for 300 s (15min) under load-controlled mode. Then the target load was removed, and the displacement (mm) was informed for 300 s (5 min) during the unloading step, allowing the equilibrium recovery in the residual structure. A preload of 0.05 N was used to keep in touch with the samples before loading and during unloading steps. Six replicates of displacement were acquired for each pizza formulation. Creep behavior was modeled by a tailored Burger's function using Robust algorithm and Profit software ver. 7 (QuantumSoft, Zurich, Switzerland). The goodness of model fitting was testified by a good square relative mean error (<2). Model parameters were estimated together with their 95% confidence intervals by performing 500 Monte Carlo simulations simultaneously with 95% of confidence. Model parameters: J0 (kPa−1) and Ji (Pa−1) are the instantaneous and retarded reversible responses, respectively. J1, J2, and J3 (kPa−1) are the retarded reversible viscoelastic responses; RTi (s) are the characteristic relaxation times governing the retarded compliance under the viscoelastic regime. JN (kPa−1) is the steady-state irreversible compliance; ERA is the elastic recovery ability at the cell scale, used as an indirect measure of the loss of cell membrane and cell wall integrity. Jmax and Jeq are the maximum creep compliance and the equilibrium compliance at the end of the recovery step.

### Texture profile analysis of cooked pizza

Concerning the uniaxial compression tests, cooked pizza samples of 90 $mm^2$ area and 15mm height were placed on the stationary steel plate of the Universal Testing Machine equipped with a 2.5 kN load cell, with the convex side of the sample facing up and compressed with an upper steel plate. A preload of 5 N was reached with a crosshead speed of 5 mm/s and 10 s of resting time to relax preload stress; then, compression tests were conducted under position-controlled mode with a crosshead speed of 10 mm/s. Two consecutive compression cycles each with 50% of deformation (expressed as a percent of displacement for the initial thickness of the sample) were performed and load (N) was registered versus time. From each load–time curve registered during the two compression cycles of the TPA, textural parameters (hardness, cohesiveness, resilience, springiness, gumminess, chewiness, and adhesiveness) were extracted, which are considered to be

well correlated with sensory evaluation (Gámbaro et al., 2002; Rubel, Pérez, Manrique, & Genovese, 2015). Six replicates were performed for each pizza formulation.

### Statistical analysis

Data are reported as mean values ± standard deviation (SD) of three replicates. Data were analysed by one-way ANOVA and Tukey's mean comparison test at a significance level of $p<0.05$ as well as principal component analysis (PCA) using R software version 3.5.0.

## Results

### Bioactive compounds of cauliflower by-products: composition and pre-treatment thermal effect

The composition of Bioactive compounds of raw cauliflower by-products is reported in Table 4. The investigated compounds are both lipophilic and hydrophilic with different thermal stabilities, thus the impact of the thermal pre-treatment (5 min of boiling) of the orange and violet cauliflower by-products was evaluated. Coloured cauliflowers (*Brassica oleracea* L. var. botrytis), such as *Cheddar* (orange) and *Depurple* (violet), appeared as spontaneous mutation, altering the carotenoid or anthocyanin accumulation, respectively (Nartea, Fanesi, et al. 2021). Our outcomes highlighted such peculiarities also in their by-products even if carotenoids and anthocyanins mainly accumulate in the florets. Raw stalks of orange cauliflower (Raw OF) were 3.7-fold richer in β-carotene rather than the stalks from *Depurple* cauliflower (Raw VF). Raw cauliflower by-products showed a different bioactive compound composition. For instance, leaves were characterized by the highest value of carotenoids and the lowest amount of phytosterols. In raw OF, very low amount of glucobrassicin was recorded, while the raw VF displayed the highest value. Stalks contained more phytosterols than leaves.

In all samples, the effect of thermal pre-treatment (5-min boiling) on raw materials produced flours of cauliflower wastes with higher amount bioactive compounds (glucobrassicin, carotenoids and phytosterols). The glucobrassicin content was increased till 100% in orange cauliflower (OF), 88% violet one (VF) and 85% in leaves (LF). The β-carotene raised up till 94% (LF), 92% (OF), 78% (VF) while the lutein remained constant in OF, VF and increased only in LF (23%). The β-sitosterol, the dominant phytosterol, reached the highest increment in OF (39%), followed by VF (22%) and LF (12%).

*Table 4. Bioactive compounds (mg/kg DM) in raw cauliflower by-products and in flour of 5-min boiled orange stalks (OF), violet stalks (VF) and leaves (LF). Data are reported as mean of three replicates ± standard deviation.*

| | glucobrassicin | | | β-carotene | | | lutein | | | campesterol | | | stigmasterol | | | β-sitosterol | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| raw OF | 0.71 | ± | 0.01 | 5.43 | ± | 0.07 | 0.44 | ± | 0.01 | 298.44 | ± | 17.26 | 19.78 | ± | 1.07 | 1216.58 | ± | 31.35 |
| raw VF | 59.26 | ± | 1.05 | 1.48 | ± | 0.02 | 0.46 | ± | 0.02 | 262.04 | ± | 30.07 | 28.91 | ± | 2.09 | 951.49 | ± | 34.65 |
| raw LF | 43.74 | ± | 1.04 | 20.03 | ± | 0.39 | 79.70 | ± | 3.09 | 116.90 | ± | 11.25 | 11.88 | ± | 1.32 | 584.92 | ± | 15.12 |
| | | | | | | | | | | | | | | | | | | |
| OF | 161.21 | ± | 3.39 | 68.37 | ± | 1.14 | 0.43 | ± | 0.02 | 508.13 | ± | 38.17 | 16.71 | ± | 0.99 | 2005.42 | ± | 58.87 |
| VF | 476.62 | ± | 4.36 | 6.81 | ± | 0.26 | 0.45 | ± | 0.01 | 323.64 | ± | 14.68 | 19.09 | ± | 1.09 | 1224.68 | ± | 31.66 |
| LF | 283.97 | ± | 7.61 | 357.26 | ± | 6.15 | 103.47 | ± | 5.09 | 131.78 | ± | 11.17 | 4.61 | ± | 0.33 | 665.21 | ± | 21.67 |

## Bioactive compounds in fortified pizzas

Glucobrassicin, carotenoids, phytosterols amounts in samples pizza are reported in Table 5. Glucobrassicin ranged from 6.5 to 11.6 in fortified pizzas with 10% of by-product, while from 24.0 to 41.3 in pizza with 30% of incorporation. The glucobrassicin enrichment with orange cauliflower stalks of 10 and 30% (POF10, POF30) gave the best results in terms of mg/kg retained in pizza (when comparing values against predicted values) while the highest value (around 50 mg/kg DM) was reported in pizza made with 30% of violet cauliflower stalks. Leaf flour (LF) produced the pizza (PLF10) with the lowest content of glucobrassicin (6.5 mg/kg DM).

Considering the carotenoids, the richest sample in β-carotene and lutein was PLF30, followed by POF30 (for β-carotene) and PLF10 (β-carotene and lutein), PVF10 was the poorest sample in carotenoids. The results are in line with the values of the starting material submitted to hot boiling pre-treatment. The effect of enrichment of pizza with coloured cauliflower increased the carotenoid concentration of pizza control, that displayed low amount of solely lutein.

Among samples with a 10-level of by-product incorporation, PVF10 showed the higher amount of total phytosterols (241.0 mg/kg DM) increasing 0.5-fold more their value against control pizza, but no statistical difference was found among samples with 10% of special flour. Differently, 30% of enrichment gave statistical different samples, with POF30 as the most highest in total phytosterol, increasing 1.9-fold more the control sample.

*Table 5. Level of bioactive compounds in control and fortified cooked pizza (P) with OF, VF and LF at 10 and 30% of incorporation. Data are reported as mean of three replicates ± standard deviation. Different letters in the same row mean statistical difference (ANOVA, p<0.05).*

| | Control | POF10 | POF30 | PVF10 | PVF30 | PLF10 | PLF30 |
|---|---|---|---|---|---|---|---|
| | | | | *mg/kg of pizza (dry weight)* | | | |
| Glucobrassicin | <LOD | 11.6±0.1$^{b}$ | 41.3±0.9$^{e}$ | 15.7±0.1$^{c}$ | 53.8±0.7$^{f}$ | 6.5±0.2$^{a}$ | 24.0±0.8$^{d}$ |
| β-carotene | <LOQ | 2.3±0.0$^{b}$ | 7.0±0.2$^{d}$ | 0.2±0.0$^{a}$ | 1.0±0.7$^{ab}$ | 3.8±0.2$^{c}$ | 40.9±1.2$^{e}$ |
| Lutein | 0.3±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.2±0.0$^{a}$ | 0.8±0.0$^{a}$ | 5.3±0.3$^{b}$ | 30.4±1.5$^{c}$ |
| Ergosterol | 4.4±0.3$^{bc}$ | 4.0±0.10$^{b}$ | 7.4±0.8$^{d}$ | 4.3±0.2$^{bc}$ | 5.0±0.1$^{c}$ | 1.8±0.0$^{a}$ | 2.5±0.2$^{a}$ |
| Campesterol | 21.8±1.7$^{a}$ | 29.6±2.7$^{ab}$ | 76.6±5.6$^{e}$ | 40.1±4.0$^{c}$ | 56.4±4.6$^{d}$ | 38.9±1.9$^{bc}$ | 58.7±3.3$^{d}$ |
| Campestanol | 4.6±0.30$^{b}$ | 7.5±0.6$^{c}$ | 4.3±0.30$^{b}$ | 4.4±0.30$^{b}$ | 4.9±0.3$^{b}$ | 2.4±0.2$^{a}$ | 3.3±0.5$^{a}$ |
| Stigmasterol | 6.0±0.5$^{a}$ | 5.9±0.4$^{a}$ | 9.5±0.5$^{c}$ | 7.2±0.4 $^{ab}$ | 8.6±0.6$^{bc}$ | 6.8±0.8$^{a}$ | 8.9±1.0$^{bc}$ |
| β-sitosterol | 97.6±4.4$^{a}$ | 104.2±5.7$^{b}$ | 331.9±9.3$^{d}$ | 161.5±4.2 $^{b}$ | 248.3±8.1$^{c}$ | 156.5±6.4$^{b}$ | 302.6±22.7$^{d}$ |
| $\Delta^{7}$-avenasterol | 17.0±1.3$^{a}$ | 28.5±2.1$^{b}$ | 25.4±1.7$^{b}$ | 14.9±0.7$^{a}$ | 17.8±0.9$^{a}$ | 14.4±0.7$^{a}$ | 15.4±1.0$^{a}$ |
| Stigmastadienol | 6.9±0.5$^{ab}$ | 6.0±0.4$^{a}$ | 9.3±0.8$^{c}$ | 8.6±0.5 $^{c}$ | 7.0±0.4$^{ab}$ | 8.5±0.4$^{bc}$ | 12.0±0.9$^{d}$ |
| | | | | *mg/kg of pizza (wet weight)* | | | |
| Glucobrassicin | <LOD | 9.8±0.1$^{b}$ | 32.3±0.6$^{e}$ | 13.8±0.1$^{c}$ | 42.0±0.5$^{f}$ | 5.7±0.1$^{a}$ | 19.9±0.5$^{d}$ |
| β-carotene | <LOQ | 1.9±0.0$^{b}$ | 5.6±0.2$^{d}$ | 0.2±0.0$^{a}$ | 0.8±0.0$^{ab}$ | 3.4±0.2$^{c}$ | 34.0±0.8$^{e}$ |
| Lutein | 0.3±0.0 $^{a}$ | 0.3±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.2±0.0 $^{a}$ | 0.7±0.0 $^{b}$ | 4.7±0.2 $^{c}$ | 25.3±1.0 $^{d}$ |
| Vitamin A *(RAE, µg retinol/200g)** | 41.7±0.1$^{a}$ | 68.8±0.6$^{b}$ | 193.4±5.1$^{c}$ | 10.2±0.1$^{a}$ | 36.9±0.5$^{ab}$ | 191.3±2.4$^{c}$ | 1554.3±27.9$^{d}$ |
| Ergosterol | 4.0±0.3$^{b}$ | 3.4±0.1$^{b}$ | 6.0±0.5$^{c}$ | 3.8±0.2$^{b}$ | 3.8±0.0$^{b}$ | 1.6±0.0$^{a}$ | 2.1±0.1$^{a}$ |
| Campesterol | 19.8±1.2$^{a}$ | 24.8±1.8$^{a}$ | 61.8±3.7$^{d}$ | 35.1±2.9$^{b}$ | 44.0±2.9$^{c}$ | 34.4±1.4$^{b}$ | 48.7±2.2$^{c}$ |
| Campestanol | 4.2±0.3$^{c}$ | 6.3±0.4$^{d}$ | 3.5±0.2$^{bc}$ | 3.8±0.2$^{c}$ | 3.8±0.2$^{c}$ | 2.1±0.2$^{a}$ | 2.7±0.3$^{ab}$ |
| Stigmasterol | 5.5±0.3$^{ab}$ | 5.0±0.2$^{a}$ | 7.6±0.3$^{d}$ | 6.3±0.2$^{ad}$ | 6.7±0.4$^{bd}$ | 6.0±0.6$^{abc}$ | 7.4±0.7$^{cd}$ |
| β-sitosterol | 88.8±3.3$^{a}$ | 117.9±3.9$^{b}$ | 267.8±6.1$^{d}$ | 141.4±3.0$^{b}$ | 193.7±5.1$^{c}$ | 138.4±4.6$^{b}$ | 251.4±15.4$^{d}$ |
| $\Delta^{7}$-avenasterol | 15.5±0.9$^{a}$ | 24.0±1.5$^{c}$ | 20.5±1.1$^{b}$ | 13.1±0.5$^{a}$ | 13.9±0.6$^{a}$ | 12.8±0.5$^{a}$ | 12.8±0.1$^{a}$ |
| Stigmastadienol | 6.27±0.4$^{ab}$ | 5.1±0.3$^{a}$ | 7.5±0.5$^{b}$ | 7.6±0.4$^{b}$ | 5.4±0.2$^{a}$ | 7.5±0.3$^{b}$ | 10.0±0.6$^{c}$ |

*LOQ, Limit of quantification;* n.d. not detected; *Retinol activity equivalent (RAE) was calculated considering 1 µg retinol for each 6 µg of β-carotene or 12 µg of other provitamin A carotenoids. The recommended serving size of pizza is 200g.

## Retention of bioactive compounds

The overview on the bioactive compounds in fortified pizzas with coloured cauliflower tissue of by-products is reported in Figure 9, by plotting the % of increment or decrement per each bioactive compound in function of the predicted value based on starting material (boiled cauliflower flours) to be expected on pizzas against the recorded experimental values. In general, different chemical classes of functional ingredients, from lipophilic to hydrophilic, from more thermostable to more thermolabile, from more or less bounded to the matrix, showed a decreasing trend in their retention in cooked ready pizzas. Only some compounds such as lutein displayed a little higher retention. The lowest retention % were found for leaf flour pizza at 10% of enrichment for β-carotene, lutein and glucobrassicin. The highest ones in violet stalks flour at 10% for β-carotene, lutein, total phytosterols. Orange stalks flour (10 and 30%) better retained the glucobrassicin (-4 and -19%, respectively) than other samples (mean value = - 65%). In all samples, the recorded values of glucobrassicin were lower than the predicted ones, with a decrement ranging from around 20% till 70%. In general, β-carotene and glucobrassicin were highly impacted within the pizza preparation and cooking as they showed the higher decrement, with respect to lutein and total phytosterols. It is important to consider that the retention value reported could derive from thermal degradation, enhanced or decreased extractability after cooking, the matrix effect and synergic effect of compounds.

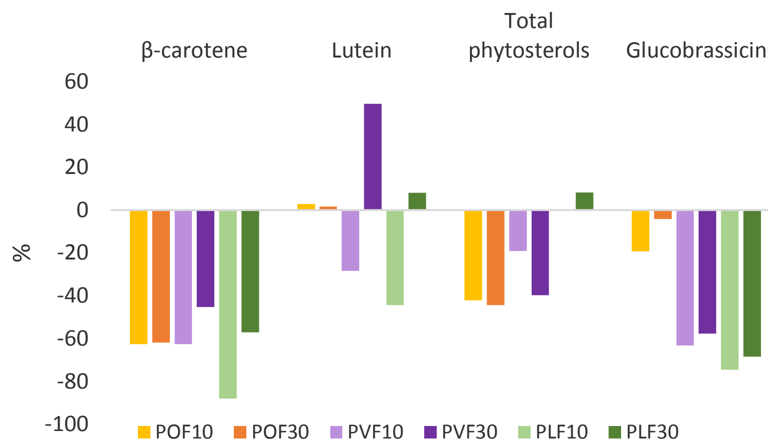



*Figure 9. % of increment or decrement (retention) of bioactive compounds calculated on the values recorded in pizza and the predicted values based on the flours as starting material. Fortified pizza (P) with OF, VF and LF at 10 and 30% of incorporation. *Predicted values = (bioactive compound (OF or VF or LF) * level of incorporation)/100%.*

## Rheological and textural properties

The quantitative effects of functional flours on technological and textural properties of functional cooked pizzas were investigated.

Figure 10 shows creep curves of pizzas describing the instantaneous, retarded, and steady-state compliance responses, which were fitted with Burger's model. The rheological properties were extracted from the Burger's creep curves and used as supplementary variables in the PCA (Principal Component Analysis) plotted (Figure 11) with textural independent variables to highlight their interplay in describing the pizza

samples. It emerged that the retarded elasticity (namely the springiness parameter) of the pizzas is opposite to all texture parameters (hardness, cohesiveness, chewiness, and gumminess) describing indirectly the ease to manage the bolus in the mouth. Springiness is positively related to viscoelastic (Ji) and plastic creep compliance (JN) as well as negatively related to the instantaneous compliance (J0) and work-to-deform W(Fmax) as measured at the end of the two compression cycles. The instantaneous elasticity (as described by the resilience) is negatively related to the instantaneous creep compliance (J0).

Samples with low content of special flour (10%) displayed similar features to pizza control (P-control). In particular samples at 10% of fortification were characterized by higher levels for springiness, viscoelastic compliance (Ji), and lower levels for hardness, chewiness, cohesiveness, and gumminess as well as lower levels for work-to-deform W(Fmax) more than samples with 30% of special flour incorporation, i.e., PVF30 and PLF30.

POF30 shows intermediate properties between samples with low and high content of functional flours.

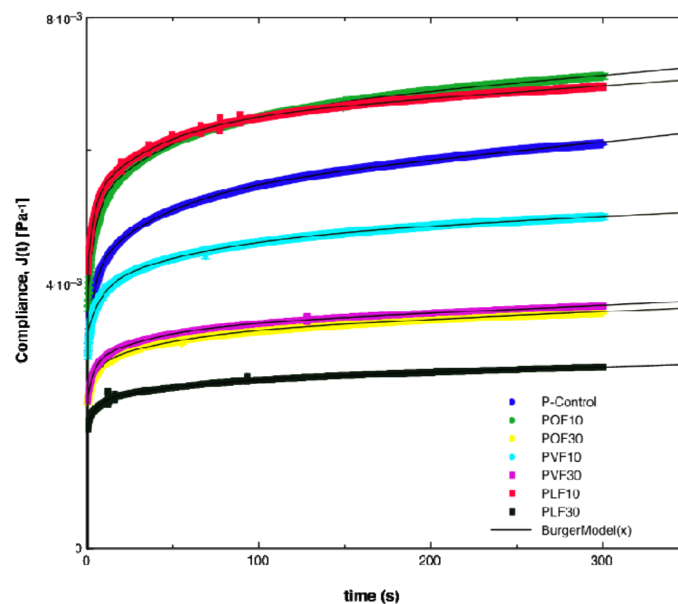



*Figure 10. Elastic, viscoelastic, and plastic behavior of conventional (P-Control) and functional pizzas.*

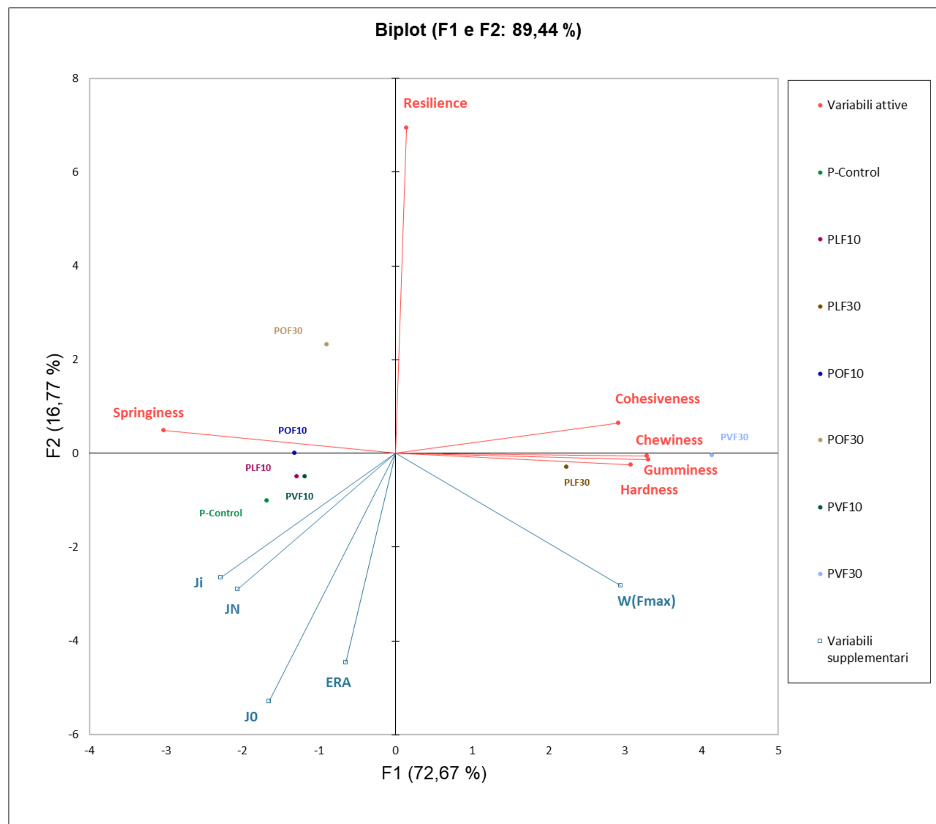



*Figure 11. Principal component analysis (PCA) performed using texture parameters as independent input variables and creep compliance parameters as supplementary variables for conventional and functional pizzas.*

## Discussion

To the best of authors' knowledge, in this work, for the first time we have designed and developed a fortified pizza in glucobrassicin, carotenoids and phytosterols with coloured cauliflower discards. The characterization of raw material and the effect of pre-treatment (5-min boiling) on raw material to produce the cauliflower freeze-dried flours was evaluated. A bakery product such as pizza was investigated as substrate of enrichment for glucosinolates (glucobrassicin), β-carotene (pro-vitamin A) and phytosterols (health claim).

In all samples, the effect of thermal pre-treatment on raw materials produced flours of cauliflower wastes with higher amounts of bioactive compounds with an increment from 12% to 100% (glucobrassicin, carotenoids and phytosterols).

For glucobrassicin, our results could be explained by the inactivation of myrosinase enzyme which breaks the glucosinolates in isothiocyanates and by tissue softening resulting in a higher extractability. The inactivation of myrosinase is a key point during the food processing and it directly impacts the recovery of glucosinolates (Barba et al. 2016). However, we cannot exclude that the water boiling can cause a leaching and/or a degradation. The degradation of plant tissue could have caused the release of bound glucobrassicin from cell walls of cauliflower stalks and leaves (Ciska et al. 2015). In raw vegetable discards we looked for sinigrin, glucoraphanin and glucobrassicin and we have found only glucobrassicin both in raw and pre-

treated samples, but in lower amounts with respect to white cauliflower, where the total glucosinolates content varied from 91-109 mg/100 g fresh weight (Possenti et al. 2016).
For carotenoids, the results agreed with previous findings in cauliflower florets (Nartea, Fanesi, et al. 2021; Gliszczyńska-Świgło et al. 2006; Miglio et al. 2008) where the effect of cooking on carotenoids depends on the level of tissue softening, which is considered higher in boiling water, provoking the release of matrix bound compounds. In boiled *Cheddar* cauliflower, the total carotenoids increased with cooking time until they were 13-fold than in raw cauliflower. In the *Depurple* variety, contents increased by half with respect to the orange variety (Nartea, Fanesi, et al. 2021).
For phytosterols, boiled stalks and leaves of cauliflower contained 80-720 mg/100g dry weight. Among vegetables, cauliflower and broccoli have been found to be the richest in phytosterols with a contribution of around 310-400 mg/100 g (fresh weight) for cauliflower (Tolve et al. 2020). Boiling enhanced the sterols extractability in coloured cauliflowers in accord with Nartea, Falcone, et al. (2021).
Once characterized the flours, the response of the bioactive compounds after the fortification was monitored at high temperature (300°C) in the pizza matrix. For instance, glucosinolates enrichment was applied in soup, juices, and bakery products (Dominguez-Perles et al. 2011; Alvarez Jubete et al. 2014; Drabińska et al. 2018). Among investigated bakery products (i.e., crackers, cakes, breads) the high temperature does not go upper than 180°C (Krupa-Kozak et al. 2021; Drabińska et al. 2018b; Franco et al. 2016), while we have evaluated the thermal impact at temperatures 220-300°C. The preparation of pizza (70 g) has involved a pre-cooking (300°C, 2.3 min) and a cooking step (220°C, 8 min). Most of studies described glucosinolates response to microwaving, steaming, stir-frying cooking (Sun et al. 2021), while the novelty of this study consisted in unravelling the glucobrassicin behaviour to oven baking, with hot air as heat transfer medium. In all samples, glucobrassicin amounts were lower than the predicted ones, from -20% to -70%. In general, β-carotene and glucobrassicin were highly reduced within the pizza preparation and cooking with respect to lutein and total phytosterols. Thus, hot air negatively affected the β-carotene concentration in pizza while hot boiling water of cauliflower discards positively affected the levels of β-carotene. The retention values can be a result of the combination of thermal degradation, extractability, and synergic effect of compounds. For instance, glucosinolates can be thermally degraded leading to the formation of volatile compounds such as nitriles and other compounds (Hanschen et al. 2012; Wu et al. 2021). Drabińska et al. (2018) hypothetically attributed the presence of synergic interactions between bioactive compounds and food ingredients in mini gluten free sponge cakes fortified with glucosinolates. Since the levels of inclusion in pizza (10% and 30% of flours) gave non proportional ratios in the retention values, it can be assumed that a combination of extractability, thermal degradation and synergism of compounds explains the outcomes. Giambanelli et al. (2015) emphasized that the ratio of glucosinolates to food components is crucial to determine thermal degradation of glucosinolates and that the interactions with the food matrix in bakery products require further investigation.
Moving to the health benefits issue, no health claim has been established yet for glucosinolates, although many epidemiological studies highlighted the inverse association between the intake of glucosinolates-rich

foods and the risk of different types of cancer or other disorders (Marino et al. 2021). 121 mg of glucosinolates per day helps to attenuate chronic inflammation in overweight subjects (López-Chillón et al. 2019). Along EFSA authorisation, “plant sterols/stanols contribute to the maintenance of normal blood cholesterol levels" can be claimed if the food provides at least 0,8 g of plant sterols/stanols intake per day.
Within this frame, the nutritional potential of fortified pizza is interesting as the fortification with different cauliflower by-products produced pizzas with 1.44-12.00 µmol of glucobrassicin, 4-670 µg vitamin A, and 22.1-46.4 mg of total phytosterols per 100 g of dry weight pizza. Similar values in total glucosinolates (9.03-15.05 µmol/100g fresh weight) were reported by Drabińska et al. (2018) in cakes enriched with 2.5-7.5% of broccoli leaf powder. 75 µmol/100 g of crackers added with 1% of *E. sativa* defatted seed meal, ensured a release of about 20 µmol/100 g isothiocyanates during chewing and 50 µmol glucosinolates in the gastrointestinal tract for the slow hydrolysis by gut microbiota in isothiocyanates, the bioactive metabolites of glucosinolates displaying health effect (Franco et al. 2016).
As regards rheological and textural properties, the fortification with cauliflower by-products affected the deformation behavior of pizza compared to P-control, depending not only on the fortification level (10, 30%) but also on the type of special flour which contain different amount of fiber (Abul-Fadl, 2012). As a first attempt to fortify pizza with cauliflower by-products, the comparison of data is difficult, but the higher hardness, gumminess, and cohesiveness, usually associated with negative sensorial properties (Sagar, Pareek, Sharma, Yahia, & Lobo, 2018), could be attributed to the partial substitution of viscoelastic wheat flour with cauliflower fibrous flours. The variation in texture properties can be explained by a worse gluten formation, not present in cauliflower, and an increment of fiber content in pizza as suggested for chickpea flour-enriched pizzas (Pasqualone et al., 2019).

## Conclusions

The results of this study testify that colored cauliflower by-products are a good source of bioactive compounds with different health benefits from chemopreventive (glucosinolates/isothiocyanates), vitaminic (β-carotene) and cholesterol lowering effect (phytosterols). Different discard tissues of cauliflowers produced fortified pizzas with different levels of bioactive compounds. This research provides indication for developing functional bakery products for food industries from vegetable by-products, looking also at new gluten-free products.

# Chapter 2

# Exploration on vegetable by-products as a source of bioactive compounds in beer production and their impact on free amino acids and volatile organic compound profiles


## Abstract

Vegetable by-products were explored for the first time as ingredient in pale beer brewing to valorise the bioactive compounds residues in plant tissues. Batches of 100 L of beer were produced with black cabbage stalks, tomato peels and seeds, water extracts from spent coffee ground (deriving from the preparation of espresso coffee) and coffee powder and compared with a control beer sample. The effect of the enrichment with lycopene, glucosinolates, chlorogenic acid was evaluated as well as the impact of free amino acid and volatile organic compound profile. Vegetable by-products affected physicochemical parameters of beer, as well as free amino acid profile and volatile profile. Spent coffee ground and coffee powder gave the beer with higher pH value and % of acidity. Special ingredients affected the colour with respect to ECB value of control beer and the highest value was recorded for coffee beer. Black cabbage and tomato beers displayed the lowest value of total essential amino acids and the highest values of total non-essential amino acids with respect to the other beer samples. Tomato peels produced a slight increment of lycopene (5.0±0.4 µg/mL). Spent ground coffee is valuable source for chlorogenic acid enriched beer (2.3±0.2 µg/mL). Glucosinolates were not detected in black cabbage beer. The content of total free amino acid in beer samples ranged from 370 to 680 mg/L and the variance was well explained with PCA. Along the untargeted volatile profile, satisfactory differentiation by PLS-DA score plots was obtained, evidencing that black cabbage beer was the most differentiated sample. The results can help research and food industries in designing and developing food application such as beer enriched with functional ingredients to meet market and sustainability needs.

## Introduction

Beer is one of the most consumed beverage and the market demand is characterized by a wide range of beers (Rošul et al. 2019) with new craft beers with an increasing trend in production and consumption in countries like Italy, Spain, France or Mexico. Higher quality and variety and healthier products are demanded by consumers (Carvalho et al. 2018). Functional beers are not so well studied as a driver of health benefits. Beer is mainly constituted by water, malted cereals, yeasts, and hops and the production of functional non-conventional is based on the substitution of an ingredient or the addition of a special ingredient at different step of the brewing system (i.e., boiling, wort extraction, fermentation, maturation). The most applied special ingredients are fruits and plant extracts proving enhanced sensorial characteristics but also higher antioxidant activity and phenol content (Paiva, Mutz, and Conte-Junior 2021).

Functional beers are designed to reduce alcohol, sugars, gluten, and carbohydrates by also to improve health functionalities by adding special ingredients drivers of bioactive compounds at different stages during beer production (Habschied et al. 2020). Fruits such as grapes increase carotenoids, polyphenols, and antioxidant activities (Nardini and Garaguso 2020; Habschied et al. 2020). Vegetables as well provide chemoprotective compounds such as sulforaphane from broccoli (Abellán et al. 2021), hepaprotective and hypolipidemic functions from artichoke (Schuina et al. 2019), β-carotene from sweet potato (Humia et al. 2020). The downside is the raw material cost of the special ingredients from herbal, species, fungi, vegetables, fruit, grape. To overcome the cost burden and meet the sustainability criteria, functional beers were suggested to be produced from vegetable by-products such as olive leaves for oleuropein and 3-hydroxytirosol (Guglielmotti, Passaghe, and Buiatti 2020), banana discards and coffee pulp (Sriwichai, Detchewa, and Prasajak 2020) for phenols and flavonoids.

The 54% of total food loss and waste occurs during production and postharvest and 46% during processing, distribution, and consumption (Mirabella, Castellani, and Sala 2014). Deeply, industrial processing vegetable by-products accounts for 38 % of food waste (Bharat Helkar and Sahoo 2016). Agro-industrial wastes especially by-products can have a sustainable application by recovering bioactive compounds to be use as functional ingredients in foods, contributing to the cost reduction of waste management, especially for smaller producers (Doria et al. 2021). Beer production includes the formation of different by-products in great quantities such as wastewater, spent grains, spent hops, and yeast. Global beer consumption is growing steadily and has recently reached 187.37 billion litres per year (Amienyo and Azapagic 2016). Thus, when designing functional beers also the environmental impact needs to be taken into account. A strategy could consist in the exploitation of bioactive compounds from vegetables by-products to be used in beers. Research supports this approach by studying and developing useful applications of food industry by-products (Sicari 2018), but few studies addressed beer production with vegetable by-products. Waste proper management leads to several benefits in terms of health, environment, and economy (Mirabella et al., 2014).

In this scenario, the aim of the study was the exploration of different vegetable by-products (black cabbage stems, tomatoes peels and seeds, spent coffee ground) as a potential functional ingredients in brewing beers since there are rich in well-known bioactive compounds such as glucosinolates/isothiocyanates, lycopene and

chlorogenic acids, respectively, displaying antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, antioxidant and anticancer activities (Yang et al. 2016; Imran et al. 2020; Naveed et al. 2018). The volatile and amino acid profile was investigated as well as to fill the gap between functional and sensorial characteristic in brewing.

## Materials and methods

Glucosinolates standards (> 95%, glucoraphanin, glucobrassicin and sinigrin potassium salts) were purchased by PhytoLab GmbH & Co (Germany), chlorogenic acid, caffeine, lycopene standard (>95 % purity), L-amino acids analytical standards (alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, hydroxyproline, glutamic acid, arginine, asparagine, lysine, glutamine, histidine, tyrosine and DL-norvaline as internal standard, 98.5 % purity), derivatization agent (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide, MTBSTFA, >99 % purity), solvents HPLC grade, hydrochloric acid (HCl), 37%, boron trifluoride-methanol solution ($BF_3$-MeOH, 14 % in methanol) were purchased by Merck (Darmstadt, Germany). Anhydrous sodium sulphate was purchased by ITW Company (Darmstadt, Germany). MilliQ water was purified with Millipure System (Millford, SC, USA).

### Plant material

Black cabbage by-products (*Brassica oleracea* L. var. *acephala*) were provided from a local company (Agrinovana S.r.l Petritoli, Fermo, Italy). Fresh leaves and stems as discards of vegetable production were removed of damaged tissue, water washed and cut with a mixer into small pieces ready for the use in beer production. Plant material was collected in the same day of beer production.

Tomato by-products were collected after producing tomato puree (San Marzano variety), provided by the local company (Agrinovana S.r.l Petritoli, Fermo, Italy). The material was stored at -4°C for one day before being brewed.

Coffee powder and spent coffee ground (*Coffea arabica* and *C. canephora* var. robusta from Guatemala) were supplied by a local industrial coffee roaster (Caffè del Faro, Robin S.r.l., Montegranaro, Italy). Spent coffee ground was collected after espresso coffee preparation by using a coffee machine VA388 Black Eagle Gravitech (Victoria Arduino, Italy). The grinding was performed by using a grinder machine K30 Mahlkönig (Hemro Manufacturing, Germany GmbH). Ground coffee (7 g) was inserted in a 1-cup filter holder, water temperature was 92 °C and pressure, 9 atm. The coffee powder and the correspondent spent coffee ground were collected. A hot water extract (250 g/L, 5 min of boiling) was prepared starting from the spent coffee ground and coffee powder and vacuum filtered on paper filter on a Büchner funnel. The extract was prepared the same day of beer production.

### Beer brewing

Pale beer samples were produced using 100 L Polsinelli brewing system (Polsinelli Enologia srl, Italy). Samples were produced in triplicate (3 batches of 100 L). The base recipe consists of 92.6% Pale Ale, 3.7 %

Carapils and 3.7% Caravienna as malts, Cascade hop (1.1 g/L) and Safale US-05 yeast (0.46 g/L), purchased by Mr Malt® (Udine, Italy) and scheme of brewing is point out in Figure 12. The mashing step started at 62°C (hold 20 min), increased till 68°C in 5 min and hold for 40 min. The mashing out step (78°C) was reached in 10 min and hold for 10 min. Wort was boiled for 60 min with Cascade hop and added of the special ingredient to characterize each different beer sample. Tomato (10%) and black cabbage (10%) by-products were added at the begging of the boiling, while coffee (0.75 g/L) and spent ground coffee (7.5 g/L) water extracts were added 5 min before the boiling end. After the whirlpool step, wort was filtered and cooled down till 18°C, inoculated with yeast. The fermentation lasted 10 days till the density dropped from around 1.060 till 1.010 g/L. Beer was bottled (750 mL dark glass bottles), added of sugar (2.5 g/L), closed with crown caps, and kept at room temperature for maturation for 6 months.
Preliminary tests were performed to select the amount of vegetable by-products as special ingredient in beer based on the compromise of sensorial appeal and tailored bioactive compound concentration in the beer. To avoid microbiological contamination, the by-products were introduced in the beer at 100°C during the boiling phase (for 60 or 5 min). The critical point of our production was the filtration of the boiled wort as we used fresh plant material. We used filter bags dipped in the boiling wort when introducing tomato and black cabbage by-products. In this way we avoid critical manipulation of beer during or after fermentation.

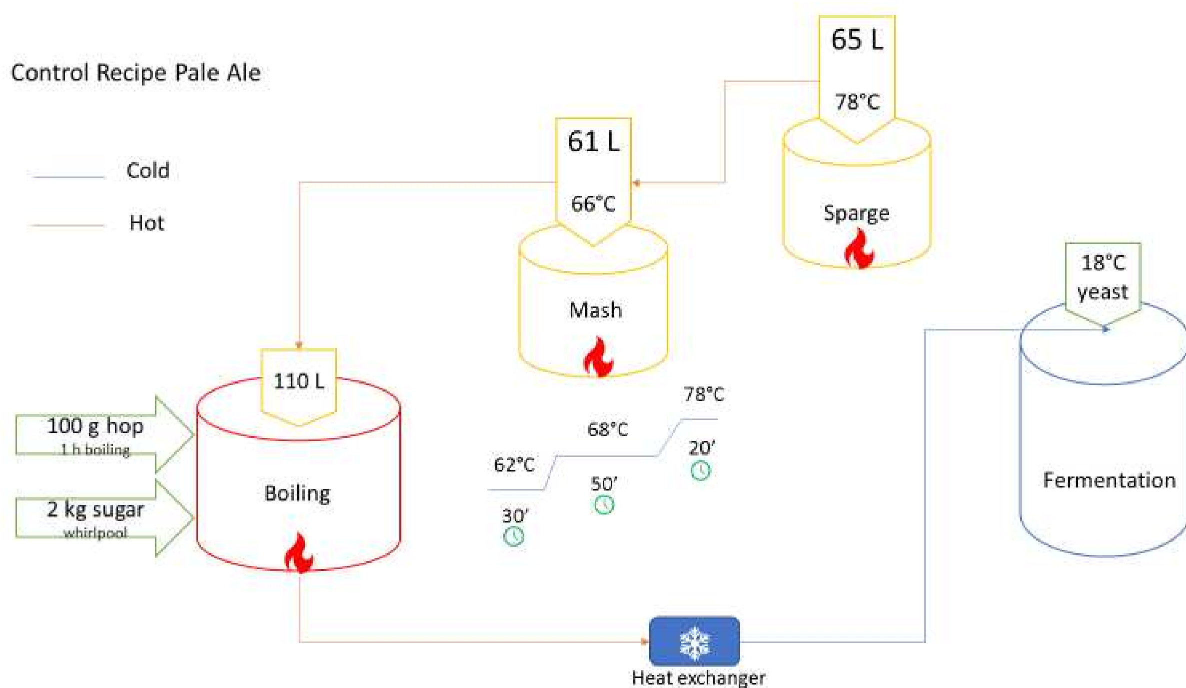



*Figure 12. Brewing scheme*

## Physico-chemical parameters of beer

Total soluble solids, total acidity, pH, alcoholic grade (% v/v), colour (EBC), and bitterness (IBUs) were determined as physico-chemical parameters in control and enriched beers. After 6 months of maturation, the samples from three bottles were analysed in duplicate. The total soluble solids, expressed as °Brix, and pH

were measured by using a digital refractometer at 20°C, and a pH meter, respectively. Total acidity was measured by titration with 0.1 N NaOH up to pH 8.1, using 1 mL of beer in 25 mL of distilled H2O, and results were expressed as % lactic acid (g lactic acid equivalent per 100 mL). Alcoholic grade was calculated by using the density data of the beers, which were obtained with a hydrometer before and after fermentation. Colour EBC and Bitterness (IBUs) were determined according with the Official Methods of the Analytical Division of European Brewery Convention, EBC Method 9.6 and 9.8, respectively, using a spectrophotometer (Varian 5000 UV-Vis NIR) (Abellán et al. 2021).

### Determination of free amino acids

Beer samples were decarbonated by shaking and precipitated of proteins (10 mL of beer +15 mL of methanol, keep 20 h in freezer at -18°C), filtrated through a Whatman No.4 filter paper. Then, 10 µL of the extract was taken to dryness, added of DL-norvaline as internal standard (50 µL of a 50 mg/mL solution in 0.1 M HCl) and taken to dryness under nitrogen flow. To ensure no water residual, dichloromethane (50 µL) was added to dried tube and dried again. The sample was derivatized with acetonitrile (50 µL) and MTBSTFA reagent (50 µL) at 100°C for 60 min, as reported by Jiménez-Martín et al. (2012).

All samples were injected (1 µL) in a GC/EI-MS (Thermo Scientific, USA) system, equipped with a split/splitless injector, single quadrupole and a fused silica capillary column MDN-5 (30 m, 0.25 mm, 0.25 µm, Supelco, Bellefonte, PA). The chromatographic conditions were in accordance with Fico et al. (2018). Oven temperature was set at 100 °C, hold for 2 min, increased to 280 °C (4 °C/min) and hold 15 min, using helium flow at 1,0 mL/ min. The injector in splitless mode was set at 250 °C, the ionization source (70 eV, positive mode) was set at 250 °C and transfer line, 280 °C. The acquisition was performed in Total Ion Current (TIC) in a mass range of 70-650 m/z and with a detector gain of 1.0. Amino acids were identified by the comparison with analytical standards following their retention time with and characteristic m/z ions. The quantitation was carried out in the selected ion monitoring (SIM) mode (Pérez-Palacios et al. 2015).

For the quantification, standard calibration curves of a mix of 22 amino acids were prepared in function of concentration level of each amino acid and the ratio of each amino acid peak area/norvaline (internal standard) peak area (Jiménez-Martín et al. 2012). Good correlation coefficients were obtained ($R^2$= 0.99-0.98). To this aim, each amino acid was dissolved in 0.1 M HCl (solution of 1000 µg/mL) and then mixed to obtain a solution of 200 µg/mL of each amino acid. From this solution seven dilutions were prepared ranging from 100 to 1 µg/mL. A standard stock solution (5 µg/mL) of norvaline internal standard was prepared in 0.1 M HCl. Then, 50 µL of each dilution was added of 50 µL of norvaline stock solution and dried under nitrogen flow, added of 50 µL dichloromethane and dried again. The derivatization was performed in the same condition of samples, injecting 1 µL in the GC-MS system. The limits of quantification and detection of the analytical method are reported elsewhere (Jiménez-Martín et al. 2012).

## Glucosinolates in beer

Glucosinolates were analysed in accordance with (Baenas et al. 2019; Abellán et al. 2021). The degassed beer was filtered on 0.45 µm Sartorius Regenerated Cellulose Membrane and injected (5 µL) in an Acquity Ultra Pressure Liquid Chromatographic H-class system (Waters Corporation, Milford, US), equipped with Photodiode Array Detector (PDA). The separation was carried out following Lin et al. (2014) on an analytical column UPLC CSH C18 (2.1 mm x 100 mm, 1.7 µm). The mobile phase was composed of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). The linear gradient was from 4 to 15% B (v/v) at 10 min, from 15 to 70% at 20 min, isocratic for 5 min, and decreased to 4% B (v/v) at 30 min. Flow rate was 0.3 mL/min, column oven was set at 35 °C and sample loading was carried out at 20 °C. PDA analysis was performed at 225 nm wavelength upon a spectrum scanning in the 210 - 500 nm range. Glucosinolates were identified by comparison of retention time and absorbance spectrum with pure standards (glucoraphanin, sinigrin and glucobrassicin). Their quantification was performed by external calibration. The calibration curves ranged from 1 to 100 µg/mL for glucobrassicin, 6-86 for sinigrin and 5-120 for glucoraphanin. Stock and working standards of glucoraphanin, glucobrassicin and sinigrin were prepared by dissolving the salts in deionised water. Good correlation coefficients ($R^2$) of 0.999 were obtained in all cases. The instrumental limit of detection (LOD) and quantification (LOQ), calculated at a signal-to-noise ratio of 3:1 and 10:1 respectively, were 23 and 75 (indole glucosinolates), 18 and 59 ng/mL (aliphatic glucosinolates).

## Lycopene in beer

Lycopene was extracted as reported by Chauveau-Duriot et al. (2010). Beer (350 µL) was deproteinized with the same volume of ethanol and carotenoids were extracted twice with *n*-hexane (800 µL). The mixture was vortexed (30 s) and centrifuged (1370 rpm, 10 min, 4°C). Organic phases were collected, evaporated under nitrogen and the residue was dissolved in acetone (100 µL) for injection (2 µL) injected in a Acquity Ultra Pressure Liquid Chromatographic H-class system (Waters Corporation, Milford, US), equipped with Photodiode Array Detector (PDA) and an Acquity column UPLC BEH C18 (2.1 mm x 100 mm, 1.7 µm) as reported by Nartea, Fanesi, et al. (2021a). The mobile phase was composed by phase A consisting of acetonitrile (75 %), dichloromethane (10 %) and methanol (15 %), and phase B consisting of acetate ammonium in water (0.05 M). Gradient started at 75:25 (A:B) till 10 min, 98:2 (A:B) from 10 to 11 min, 98:2 (A:B) till 20 min. Flow rate was 0.4 mL/min, column oven was set at 35 °C and sample loading was carried out at 20 °C. PDA analysis was performed at 450 nm wavelength upon a spectrum scanning in the 210 - 500 nm range. Lycopene was identified by comparison of retention time and absorbance spectrum with pure standards. The quantification was performed by external calibration. The calibration curve ranged from 0.05 to 100 µg/mL for lycopene. Good correlation coefficients ($R^2$) of 0.999 was obtained. The instrumental limit of detection (LOD) and quantification (LOQ), calculated at a signal-to-noise ratio of 3:1 and 10:1 was 5 and 18 ng/mL, respectively.

## Chlorogenic acid and caffeine in beer

QuEChERS-LC-DAD analysis was performed. For the extraction step, degassed beer (10 mL) was added of internal standard (100 µL syringic acid), acetic acid (50 µL), acetonitrile (2.5 mL), vigorously shaken (1 min), added again of $MgSO_4$ (3 g), NaCl (1.5 g), shaken (30 s) and centrifuged. For the clean-up an aliquot (1 mL) was transferred and added of $MgSO_4$ and d-SPE sorbent, shaken again (30 s) and centrifuged. An aliquot was injected (3 µL).

Chlorogenic acid and caffeine were analysed using an Agilent Poroshell 120 EC-C18 reversed phase column (2.7 µm particle size, 4.6 × 150 mm) on a Shimadzu Nexera UHPLC System (Shimadzu Nexera, Kioto, Japan) equipped with dual pump LC-30AD, on-line degasser DGU-20AS, column oven CTO-30A, auto sampler SIL-30AC, and diode array detector (SPD-M20A). Gradient separation was created from solvent A (2% acetic acid in water) and solvent B (acetonitrile) as follows: 0–0.1min, isocratic condition at 95% A; 0.1–15 min linear gradient from 5 to 15% B; 15–25 min, linear gradient from 15 to 50% B; 25–30 min linear gradient from 50 to 80% B; isocratic condition kept up to 35 min; 35 min back to initial condition at 95% A; isocratic step kept up to 40 min for column conditioning. The mobile phase flow rate was 450 µL/min. The column temperature was 30 °C. DAD analysis was performed at 330 and 280 nm wavelength upon a spectrum scanning in the 210 - 500 nm range for chlorogenic and caffeine, respectively. Compound were identified by comparison of retention time and absorbance spectrum with pure standards. Their quantification was performed by external calibration. Good correlation coefficients ($R^2$) of 0.999 were obtained. The calibration curves were y=6923x-8065 (chlorogenic acid) and y=5606x+75757 (caffeine).

## Volatile compounds by means of SPME-GC-MS

Degassed beer (8 mL) sample and 25 µL internal standard 4-methyl-2-pentanol (534 mg/L) were placed into a 20 mL glass vial for 5 min at 50°C. A HS-SPME fiber (100 µm, DVB/CAR/PDMS, 1 cm, Merck Life Science, Milan, Italy) was exposed for 40 min at 50°C. The fiber was desorbed of volatile compounds in the GC inlet at 250 ℃ for 5 min. Two columns were used (DBWAX, 60 m × 0.25 mm × 0.25 µm; DB-5MS 30m × 0.25 mm x 0.25 µm) for the untargeted volatile profile of beer for polar and non-polar compounds, respectively. An Agilent 7890 GC equipped with a 5977A MS (New York, USA), the carrier gas helium flowed at the rate of 1 mL/min. For the DBWAX untargeted volatile, the temperature program started at 40 °C, then increased to 120 °C (3 °C/min) and held for 5 min, and finally increased to 230 °C (5 °C/min) (Yu 2021). For the DB-5 untargeted volatile the initial temperature of the oven was 60°C for 1 min, increased to 120°C at 5°C/min rate, held for 3 min and raised to 200°C at a rate of 8°C/min. Finally, the temperature was increased to 250°C at a rate of 10°C/min and held for 1 min (Ji et al. 2021). The mass detector was set at 230 °C, and its electron ionization (EI) energy was 70 eV. The mass spectra were obtained in duplicate under full scan acquisition mode with a mass scan range of m/z 33-450 to achieve HS-SPME-GC-MS fingerprints to be used for PCA and PLS-DA elaboration.

## Statistical analysis

Data are reported as mean values ± standard deviation (SD) of three replicates. Data were analysed by one-way ANOVA and Tukey's mean comparison test at a significance level of $p < 0.05$ as well as principal component analysis (PCA) using R software version 3.5.0. and non-supervised partial least squares regression-discriminant analysis (PLS-DA) using latent variables (LVs) using Solo 8.6 chemometrics software from Eigenvector Research (Manson, WA, USA). The most appropriate number of LVs to build the PLS-DA model was established at the first significant minimum point of the Venetian blinds cross-validation (CV) error.

# Results and Discussion

## Physico-chemical parameters of beer

With respect to control beer, spent coffee ground and coffee powder gave the beer with higher pH value and % of acidity (Table 6). Special ingredients affected the colour with respect to ECB value of control beer and the highest value was recorded for coffee beer (51.1). Beers had IBUs range of 11.4-14.6 and an EBC range of 35.1-51.1. Alcohol and colour are within the range of the pale ale style while the bitterness is below the values of the style, but it can be adjusted with the hop quantities to fully enter the parameters beer style. In accordance, Abellán et al. (2021) found higher pH and acidity values in broccoli-supplemented beers.

*Table 6. Physico-chemical parameters of beer.*

| | pH | Acidity<br>% lactic acid | IBUs bitter<br>IBUs | Colour<br>EBC | Alcoholic grade<br>% | Total soluble solids<br>°Brix |
|---|---|---|---|---|---|---|
| Control beer | 4.20 ± 0.06 | 0.45 ± 0.04 | 13.5 | 35.1 | 6.1 | 5.69 |
| Tomatoe beer | 4.15 ± 0.03 | 0.46 ± 0.02 | 14.0 | 37.5 | 6.5 | 5.70 |
| Black cabbage beer | 4.21 ± 0.02 | 0.42 ± 0.03 | 14.5 | 42.3 | 6.6 | 5.80 |
| Coffee beer | 4.33 ± 0.03 | 0.55 ± 0.02 | 11.4 | 51.1 | 6.3 | 5.64 |
| Spent coffee beer | 4.29 ± 0.02 | 0.55 ± 0.02 | 14.6 | 41.3 | 6.1 | 5.65 |

## Bioactive compounds in beer

We explored different vegetable by-products (coffee, tomato, and brassica vegetables) rich in a wide spectrum of bioactive compounds. The plant material was directly added during the boiling step of wort at 10% level of inclusion for tomato and black cabbage, while coffee and spent ground coffee were added at the end of the boiling step as hot boiling extract to overcome problem of filtration because of fine powder of coffee (0.75 and 7.5 g/L, respectively). To explore the thermal treatment effect on bioactive compounds, the special ingredients were added during the boiling of wort as it is well known that heat can enhanced extractability of compounds from plat tissue and/or degrade molecules.

The levels of bioactive compounds after the experimental exploration found in beer are reported in Table 7. Tomato peels at 10% produced a slight increment of lycopene with a value of 5 mg/L ± 0.4. Coffee and spent ground coffee donated chlorogenic acids and caffeine to beer (Figure 13, Figure 14). Spent ground coffee is a

source of bioactive compounds as it produced a beer with less 23% of chlorogenic acids and half less of caffeine with respect to coffee beer. A glass of spent ground coffee beer (330 mL) brings 3.7 mg of caffeine, while a decaffeinate coffee cup brings 8 mg per coffee serving. While caffeine and chlorogenic acid contents of coffees ranged from 48–317 mg per serving and from 6–188 mg, respectively (Ludwig et al. 2014).

*Table 7. Level of target bioactive compounds determined in beer samples. Data are reported as mean value of triplicates ± standard deviation. LOD (limit of detection). *Sinigrin, glucobrassicin and glucoraphanin.*

| | Lycopene | Glucosinolates* | Chlorogenic acids | Caffeine |
|---|---|---|---|---|
| Control beer | <LOD | <LOD | <LOD | <LOD |
| Tomato beer | 5.0 mg/L ± 0.4 | <LOD | <LOD | <LOD |
| Black cabbage beer | <LOD | <LOD | <LOD | <LOD |
| Coffee beer | <LOD | <LOD | 3.0 ± 0.3 µg/mL | 24.1 ± 1.9 µg/mL |
| Spent coffee beer | <LOD | <LOD | 2.3 ± 0.2 µg/mL | 11.4 ± 0.4 µg/mL |

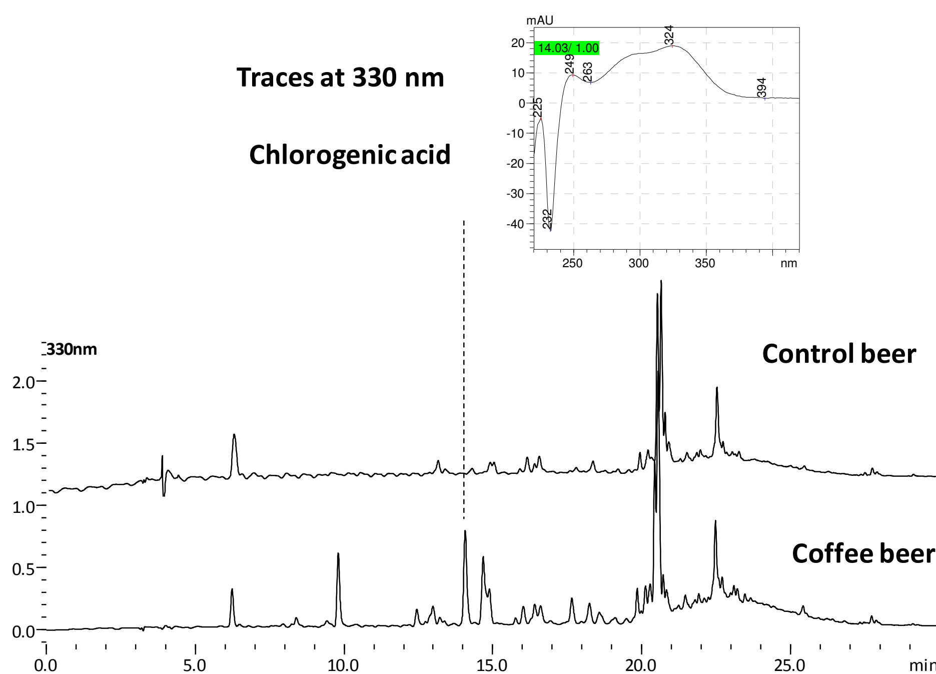



*Figure 13. Trace LC-PDA of chlorogenic acids of control beer and coffee beer.*

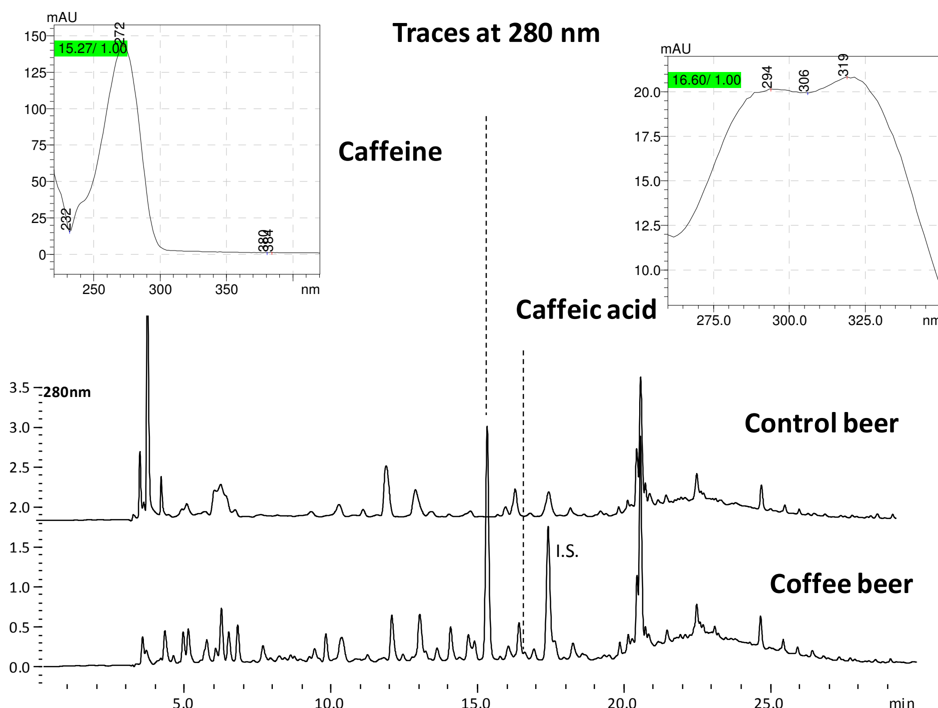



*Figure 14. Trace LC-PDA of caffeine of control beer and coffee beer.*

Considering the glucosinolates, beer made with black cabbage stalks did not contain detectable levels of total glucosinolates (sinigrin, glucobrassicin and glucoraphanin). As well Abellán et al. (2021) developed a broccoli-supplemented beer with high concentrations of the bioactive isothiocyanates as sulforaphane (18.85 μmol/L), while the inactive glucosinolate precursor (glucoraphanin) was not detected. The limit of our study is that we did not quantify the isothiocyanates, but it will be performed after the indications of this prior exploration. Boiling of black cabbage could have provoked the loss of glucosinolates after leaching into surrounding water, enzyme breakdown as myrosinase converting glucosinolates in isothiocyanates, or thermal breakdown. Wu et al. (2021) found a decrement of 32.36% for total glucosinolates after boiling in red cabbage and the authors observed that sinigrin were more thermal sensitive than other glucosinolates. In boiling, the potential major cause for losing glucosinolates is the heat deactivating the myrosinase and degrading thermally the compounds leading to the formation of volatile compounds such as nitriles and other compounds (Hanschen et al. 2012; Wu et al. 2021).

## Free amino acids profile

The concentration of free amino acids in beers depends on various factors; amino acids are the building blocks of proteins in the raw materials used for beer production, they influence the activity of cereal proteolytic enzymes in the course of various mashing procedures, as well as the microorganisms involved in the fermentation process and they contribute to beers final taste properties (Kabelová et al. 2008).

The content of total free amino acid in beer samples ranged from 370 to 680 mg/L (Table 8). The major amino acids in beer samples were proline, arginine, phenylalanine, valine, and alanine. Black cabbage and tomato beer displayed the lowest value of total essential amino acids and the highest values of total non-

essential amino acids with respect to the other beer samples. The PCA, Principal Component Analysis (Figure 15), accounted for 63% (PCA 1) and for 22% (PCA 2). The vegetable by-products affected the amino acid profile of beer, especially black cabbage and tomato with similar effect.

The amino acid content of a beer affects its aroma and flavour, and it is related to proteolysis of raw material during brewing such as barley, hops and other cereals during brewing. The results were in agreement with previous studies. The content of free amino acids ranged from 49−3,902 mg/L and proline, asparagine, arginine and aspartic acid were the most abundant amino acids in twenty-seven beer samples (Baigts-Allende et al. 2021). As well Redruello et al. (2017) found proline as the most abundant amino acid with concentrations ranging from 238 to 688 mg/L (mean 421.33 mg/L).

The assimilation during fermentation step of the released amino acids by microorganisms influenced their profile in beer. Isoleucine and leucine promote bitter flavours, while proline and valine the bittersweet one. The most important substrates for beer flavour in terms of higher alcohols are leucine (yielding isoamyl alcohol, 3-methylbutanol), isoleucine (yielding active amyl alcohol, 2-methylbutanol), and valine (yielding isobutanol, 2-methylpropanol) (Baigts-Allende et al. 2021; Redruello et al. 2017; Kabelová et al. 2008).

*Table 8. Free amino acid in beer samples. Data are reported as mean of triplicates ± standard deviation. *Essential amino acids. LOD, limit of detection.*

| mg/L | Control beer | Black cabbage beer | Tomatoe beer | Coffee beer | Spent coffee beer |
|---|---|---|---|---|---|
| Alanine | 14.1 ± 1.6 | 3.7 ± 0.7 | 2.6 ± 0.4 | 13.7 ± 3.4 | 13.9 ± 6.1 |
| Glycine | 10.3 ± 0.7 | 3.3 ± 0.1 | 4.2 ± 1.7 | 37.0 ± 8.6 | 45.4 ± 0.1 |
| Valine* | 36.7 ± 8.5 | 6.6 ± 0.2 | 7.1 ± 0.5 | 24.1 ± 3.7 | 25.4 ± 3.7 |
| Leucine* | 6.7 ± 2.0 | 3.0 ± 0.1 | 3.6 ± 0.0 | 8.5 ± 0.7 | 8.3 ± 0.8 |
| Isoleucine* | 11.2 ± 2.8 | 2.1 ± 0.0 | 2.9 ± 0.3 | 9.7 ± 1.1 | 10.4 ± 2.0 |
| Proline | 374.8 ± 48.2 | 331.1 ± 20.7 | 367.0 ± 20.9 | 336.9 ± 104.9 | 381.0 ± 10.4 |
| Methionine* | 1.7 ± 0.6 | 1.1 ± 0.0 | 1.3 ± 0.3 | 3.6 ± 1.5 | 5.2 ± 0.1 |
| Serine | 1.4 ± 0.5 | 1.3 ± 0.0 | 1.1 ± 0.1 | 1.8 ± 0.0 | 1.8 ± 0.4 |
| Threonine* | 4.1 ± 1.1 | 2.6 ± 0.1 | 3.1 ± 0.8 | 5.8 ± 0.3 | 5.7 ± 0.9 |
| Phenylalanine* | 20.6 ± 4.2 | 4.2 ± 0.1 | 4.8 ± 1.0 | 13.6 ± 2.8 | 13.3 ± 0.1 |
| Aspartic acid | 2.5 ± 0.5 | 1.3 ± 0.0 | 1.8 ± 0.2 | 3.1 ± 0.2 | 3.1 ± 0.4 |
| Hydroxyproline | <LOD | <LOD | <LOD | <LOD | <LOD |
| Cysteine | <LOD | <LOD | <LOD | <LOD | <LOD |
| Glutamic acid | 2.1 ± 0.4 | 1.2 ± 0.1 | 1.2 ± 0.5 | 2.2 ± 1.5 | 3.3 ± 0.6 |
| Asparagine | 2.0 ± 0.6 | 2.2 ± 0.1 | 1.8 ± 0.4 | 0.7 ± 1.0 | 0.6 ± 0.9 |
| Lysine* | <LOD | <LOD | <LOD | <LOD | <LOD |
| Glutamine | <LOD | <LOD | <LOD | <LOD | <LOD |
| Arginine | 18.1 ± 1.3 | 19.9 ± 2.9 | 58.6 ± 2.9 | 65.4 ± 57.8 | 107.9 ± 17.5 |
| Histidine* | <LOD | <LOD | <LOD | <LOD | <LOD |
| Tyrosine* | 15.9 ± 0.5 | 2.4 ± 0.1 | 2.1 ± 0.1 | 8.1 ± 0.6 | 8.0 ± 1.0 |
| Tryptophan | 1.7 ± 1.3 | <LOD | 3.7 ± 3.0 | 5.9 ± 8.3 | 10.2 ± 4.4 |
| Cystine | <LOD | <LOD | <LOD | <LOD | <LOD |
| Tot essential | 96.9 ± 14.0 | 21.8 ± 0.2 | 25.0 ± 1.7 | 73.4 ± 7.7 | 76.2 ± 8.3 |
| Tot non-essential | 426.9 ± 53.1 | 363.8 ± 23.1 | 442.0 ± 24.0 | 466.7 ± 183.2 | 567.2 ± 38.8 |
| TOT | 523.8 ± 39.1 | 385.7 ± 23.3 | 467.0 ± 22.3 | 540.1 ± 175.5 | 643.4 ± 47.1 |

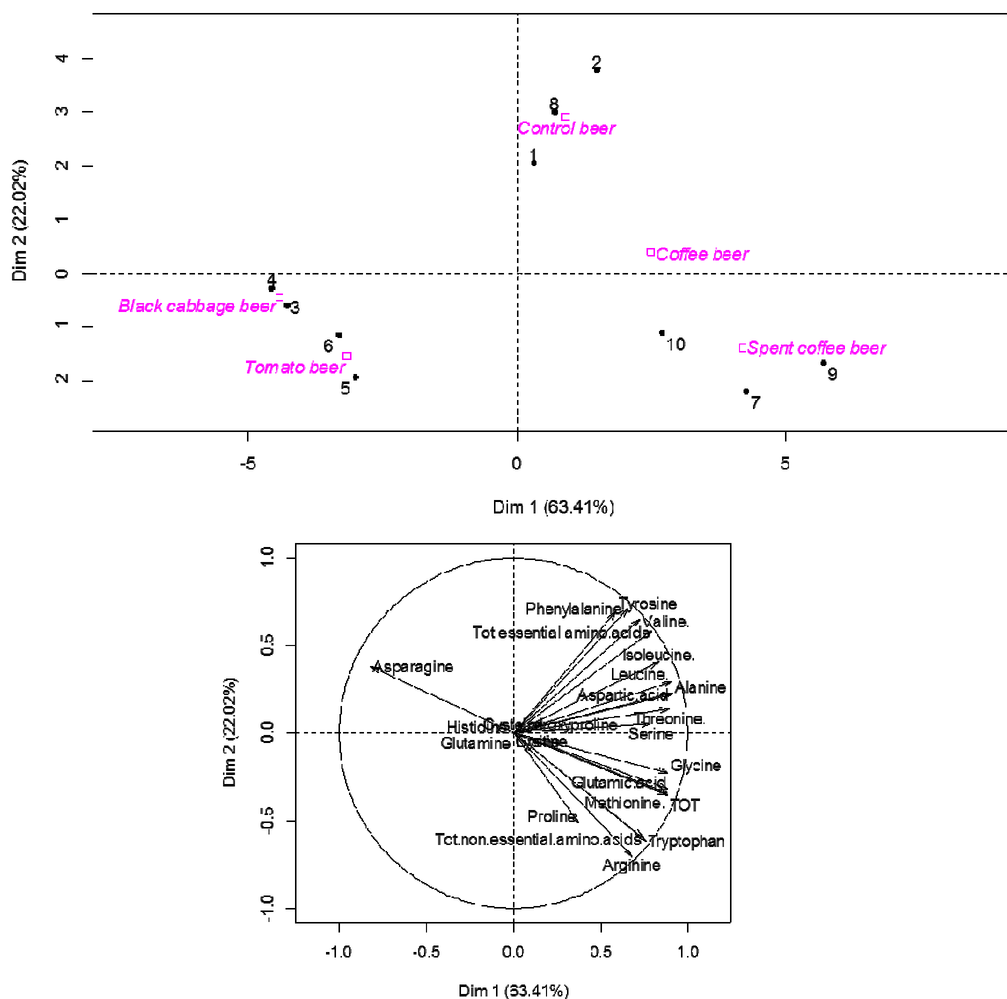



*Figure 15. PCA, Principal Component Analysis of free amino acids in beer sample. Score plot (up) and loading plot (down) are reported.*

## Volatile compounds by means of untargeted SPME-GC-MS

For the volatile profile of beer, PLS processing was performed using all signals *vs* time processing as a non-targeted approach. The elaboration was conducted by processing the signals *vs* time requiring two chromatographic columns having different polarity, Wax column and DB-5 column. Scatter plots of scores from principal components (PCs), in PCA, and latent variables (LVs), in PLS-DA, were used to study the distribution of samples, to reveal similarities and differences in the volatile profile.

In Figure 16, the PLS-DA for the untargeted volatile compounds on a wax column pointed out that the inclusion of vegetable by-products produces a variation in beer volatile profile, producing differences among samples. The greatest impact was recorded for the black cabbage beer and spent ground coffee both for the untargeted volatiles organic compounds analysed on Wax or DB-5 column (Figure 17). Coffee beer was not such differentiated from control beer as well as tomato beer. Considering that the type and content of the volatile components in beer are strongly influenced by raw materials, brewing process and storage parameters, it is easy to understand that vegetable by-products can have an important impact on the volatile and beer flavour. Among all samples, vegetables discards from back cabbage had the great impact on the

differentiation of untargeted volatiles compounds. In order to identify which variables (chemical descriptors) were responsible for the observed discrimination, compounds identification need to be implemented after this exploration.

Alcohols, aldehydes, isothiocyanates, nitriles, sulphides and other volatiles were found in brassica vegetables (Wieczorek and Jelen 2019). The boiling of black cabbage by-products for one hour during brewing process may alter glucosinolates recovery in beer as well as volatile profile also as result of glucosinolate degradation in nitriles (Hanschen et al. 2012; Wu et al. 2021). In brassica vegetables, Wieczorek and Jelen (2019) found that cooking caused a decrement in the contents of aldehydes and alcohols.

A preliminary sensorial evaluation of a non-expert panel group was performed to testify beer acceptance, but further investigation is required by an expert panel group to confirm volatile compounds by GC-MS.

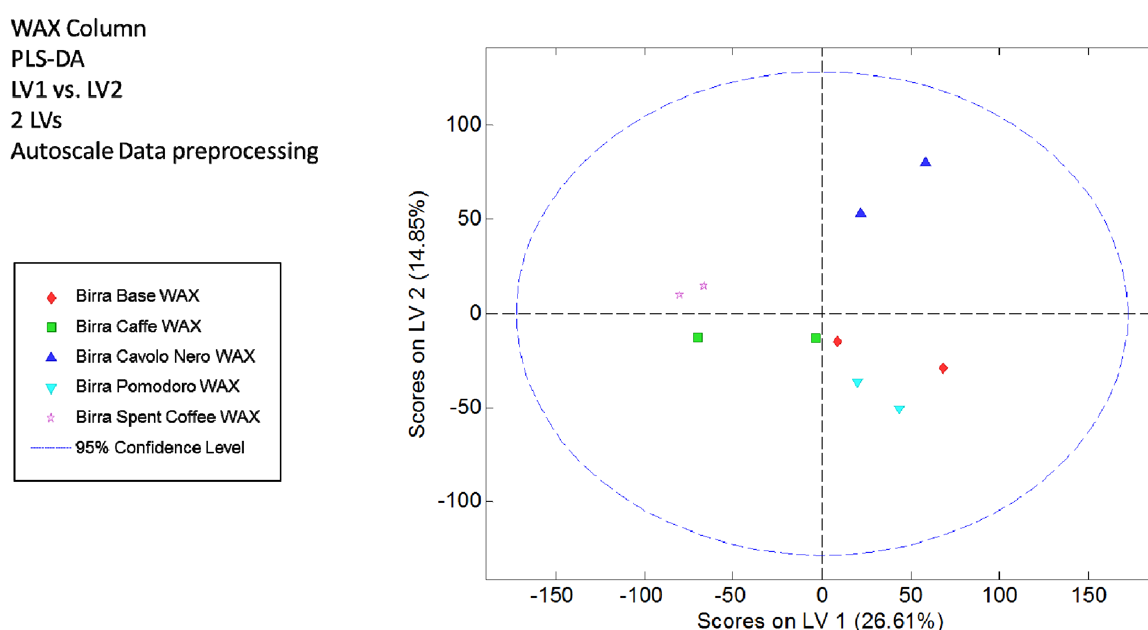
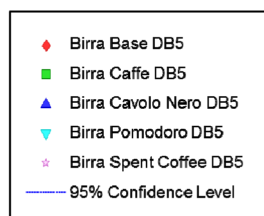



*Figure 16. PLS-DA Wax column untargeted compounds of beer samples. Red = control beer, green = coffee beer, blue = black cabbage beer, light blue = tomato beer, pink = spent ground coffee beer.*



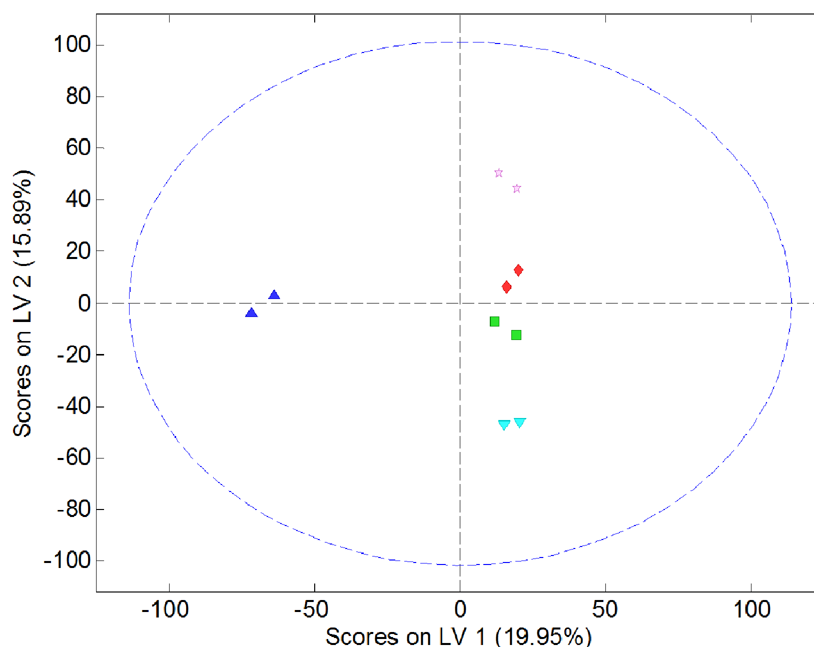




*Figure 17. PLS-DA DB-5 column untargeted compounds of beer samples. Red = control beer, green = coffee beer, blue = black cabbage beer, light blue = tomato beer, pink = spent ground coffee beer.*

## Conclusions

For the first time a preliminary exploration on brewing beer with vegetable by-products as drivers of bioactive compounds was carried out. The results can help research and food industries in designing and developing food application such as beer enriched with functional ingredients to meet market and sustainability needs. Vegetable by-products affected physicochemical parameters of beer, as well as free amino acid profile and volatile profile. Tomato peels produced a slight increment of lycopene in beer. Spent ground coffee is valuable source for chlorogenic acid enriched beer. A glass of spent ground coffee beer (330 mL) contains an equivalent of caffeine to half decaffeinate coffee cup. Black cabbage discards need to be optimized to in order to recover glucosinolates or the active metabolites (isothiocyanates) in beer. Along the untargeted volatile profile, satisfactory differentiation by PLS-DA score plots was obtained.

# Chapter 3

# Modulation of nutritional value of *Hermetia illucens* larvae by feeding microalgae-enriched coffee silverskin: amino acids, fatty acids, and carotenoids


## Abstract

Black Soldier Fly (BSF) larvae, *Hermetia illucens* (Diptera, Stratiomydae), represent a sustainable alternative to convert large quantities of coffee silverskin (Cs), an industrial waste, into valuable ingredients for aquafeed. BSF larvae composition shows high lipid and protein content, but its fatty acid profile is characterized by high amounts of saturated fatty acids (SFA) and low amounts of unsaturated ones (PUFA). Thus, the aim of this work was to modulate the nutritional composition of BSF larvae by enriching the control diet (Cs) with different percentage of microalgae (5, 10, 15, 20% of *Arthrospira platensis*) which is rich in proteins, carotenoids, linoleic and γ-linolenic acids. Larval growth and developmental time (from six-days old larvae to prepupae) were monitored. The effects of dietary composition on BSF larvae crude protein, amino acids, fatty acid profile and carotenoids were also assessed. Diet affected BSF larval development, and the higher dietary inclusion level of microalgae supported a larval development time (21 days) shortening and increasing weight gain (100.02 ± 7.70 mg). The dietary levels of microalgae did not affect the larval crude protein content and the amino acid profile, except for threonine, lysine, histidine, and tyrosine. The larval lipid content raised by increasing dietary microalgae levels. Feeding with 15% of microalgae led to larvae with the highest PUFA content (1600.7 ± 62.7 mg/100g dry weight). A significant increase of carotenoids (zeaxanthin and β-carotene) was observed in larvae fed on Cs enriched with microalgae, reaching the maximum bioconversion on diet containing 20% of microalgae up to 74.2 ± 0.1 and 274.9 ± 1.6 mg/kg dry weight, respectively. In conclusion, the BSF larvae used in this study can be successfully grown on diets composed of coffee by-products enriched with microalgae.

## Introduction

The European Commission (Regulation 2017/893/EC) allows and regulates the use of a list of insect species in the composition of feed. Among the various orders of insects, Diptera have the best properties for use in aquaculture feed production (Barroso et al. 2014), and one of the species of greatest interest is *Hermetia illucens*, commonly named black soldier fly (BSF). The effects of BSF meal have been assessed in sea and freshwater fish (Osimani et al. 2021; Zarantoniello et al. 2021) in replacement of fish meal and fish oil, the main components of aquafeed. Other studies exploited the use of BSF meal as the only ingredient in fish diet (Vargas et al. 2018). Larvae or prepupae of BSF are likely to grow on agro-industrial waste at fast development rates providing a sustainable intake of proteins (30-60% of dry matter) in aquafeed. BSF larvae show an essential amino acid profile similar to fish meal (Müller, Wolf, and Gutzeit 2017; Belghit et al. 2019) and adequate protein content (32%) (Nogales-Mérida et al. 2019; Caligiani et al. 2018). BSF is also rich in lipids (7-38% dry matter) (Barragan-Fonseca, Dicke, and van Loon 2017) but the higher amount of saturated fatty acids (SFA) at the expense of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) could comprise the fish health. Lauric (38.43% of total fatty acids), palmitic (15.71%) and myristic (12.33%) are the most abundant fatty acids (Ushakova et al. 2016). However, the fatty acid composition of BSF can be modulated through the rearing substrate to reduce SFA content (Barragan-Fonseca, Dicke, and van Loon 2017). For this purpose, a newest possibility consists in the use of coffee silverskin (Cs) as rearing substrate for insects (Truzzi et al. 2020; Osimani et al. 2021), which in a circular economy point of view, allows a bioconversion of undervalued organic wastes (Meneguz et al. 2018), into insect biomass, valuable for feed formulation. In fact, CS is the most abundant solid by-product generated during roasting of coffee processing. It is considered an excessive waste as the total production of coffee worldwide went up to 168,68 million of bags (60 Kg each) in 2019/20 (ICO 2020). Cs is a thin tegument, enveloping the coffee bean, constituted of dietary fiber (~60%), proteins (~20%), minerals (~8%), lipid (lower than 3%), antioxidants compounds (chlorogenic acids and melanoidins), vitamins, and caffeine (Borrelli et al. 2004; Santos et al. 2021). Due to the high organic content and the presence of compounds such as caffeine, tannins, and polyphenols, Cs has a remarkable and negative environmental impact (Toschi et al. 2014). Solutions, including the application of Cs as rearing substrate for insects, are being proposed to valorise Cs as source of bioactive compounds (i.e., phenolic compounds, tocopherols and melanoidins) instead of landfill disposal and fuel (Santos et al. 2021).

Despite the high potential of Cs as rearing substrate for insects, it is considered incomplete substrate since PUFA and MUFA are present in very low amounts. Moreover, larvae require high fatty diets to complete the development (Tomberlin, Sheppard, and Joyce 2002) but Cs contains only around 3% of lipids. Thus, some authors had proposed to enrich Cs with different levels of microalgae (*Schizochytrium sp* or *Isochrysis sp*), know to provide high contents of n-3 and n-6 PUFA and of essential amino acids (Zarantoniello et al. 2020; Osimani et al. 2021; Truzzi et al. 2020). Microalgae are good source of proteins, lipids, and vitamins. For reference, spirulina contains all essential amino acids particularly leucine, isoleucine, and valine with proteins amount of 50-70% (dry weight), γ-linolenic acid and carotenoids such as zeaxanthin and β-carotene

(provitamin A activity) (Vaz et al. 2016). Spirulina, till date, to the best of our knowledge, has not been used to enrich *Hermetia illucens* Cs-based diet.

In this scenario, the aim of the present study was to modulate the nutritional composition of BSF larvae in order to use the larvae as high value ingredient in aquafeed. Cs was used as basal rearing substrate for BSF larvae and it was enriched with increasing level (5, 10, 15, 20%) of marine microalga (spirulina, *Arthrospira platensis*) as source of essential amino acids, PUFA and carotenoids with provitamin activity A (Andrade 2018; Vaz et al. 2016). Vitamin A is an essential dietary requirement for fish physiological development (Hernandez and Hardy 2020). The larval growth performance, total biomass, mature larvae (prepupae) nutrient composition, and bioconversion efficiency of BSF were assessed.

## Materials and methods

### Diet preparation

The main component of the insect feeding substrate consisted of coffee silverskin (Cs) provided by Saccaria Caffe' S.R.L. (Marina di Montemarciano, Ancona, Italy). Cs (moisture 55%) was grinded (0.4 ± 2 mm particle size), divided in sealing bags (30 x 40 cm) of 500 g each, vacuum-packed and stored at -20°C. Diets were formulated including four different concentrations of *Arthrospira platensis* (S) (5, 10, 15 and 20%) to Cs (Table 9). The freeze-dried Spirulina sp. was provided by Spimpro S.P.A. (Fossombrone, PU, Italy) and stored at 4°C until use. Insect feeding substrate was added with distilled water to reach a final moisture of ~70% (Makkar et al. 2014). Feed mixtures of Cs:S were used as experimental diets (n=4) and Cs was used as control (Table 9). Samples of Cs and S were stored at -20 °C for further analysis.

*Table 9. Insect diet formulations based on coffee by-products (Cs) and different level of A. platensis (S).*

| Diets | Cs (%) | S (%) |
|---|---|---|
| A | 95 | 5 |
| B | 90 | 10 |
| C | 85 | 15 |
| D | 80 | 20 |
| E | 100 | 0 |

### Experimental design

Insects were reared in a climatic chamber at 27 ± 1 °C, relative humidity of 65 ± 5% (Spranghers et al. 2017), in continuous darkness. Six days old larvae (purchased from Smart Bugs s.s. Ponzano Veneto (TV), Italy) were sieved with a mesh diameter of 1.18 mm, to ensure a uniform size and mass of the young larvae, and only those that managed to pass through the sieve by themselves were used (Tschirner and Simon 2015). Twelve replicates of 100 larvae each (Meneguz et al. 2018) were carried out for each diet (n=5), with a total of 6000 specimens. Larvae were placed in a plastic box (19 × 19 cm), screened with fine mesh cotton gauze

(30 x 30 cm) (Sideris and Tsagkarakis 2017) and covered with a lid provided with a single ventilation hole (4.5 cm Ø) (Spranghers et al. 2017). Larvae were reared at a density of 0.3/$cm^2$ (Barragan-Fonseca, Dicke, and van Loon 2017). Each larva was provided with a feeding rate of 100 mg/day. Prepupae were identified by the change in tegument colour from white to black. The larvae were divided in two different groups (six replicates for each diet for each experiment) to perform different experiments

Experiment A

Larvae were used to conduct analysis on larval growth performances (larval survival rate, larval development time and prepupae weights). A group of 100 newly emerged larvae were placed directly on the rearing substrate and 20 larvae for each replicate were randomly selected to be weighted at the beginning of the experiment. The diets were replaced once a week (larvae were counted and transferred by using fine forceps into another box containing the new week feeding ratio) until 40% of the larvae reached the prepupal stage. Every week 20 random larvae, from each replicate, were individually weighted and replaced in the box. At the end of the experiment, the prepupae were collected, washed, counted, dried, weighted one by one and stored at −20 °C.

Experiment B

Larvae were used to determine the Growth Rate (GR), Waste Reduction Index (WRI), Efficiency of Conversion of Digested food (ECD) (Meneguz et al. 2018) and Food Conversion Ratio (FCR) (Oonincx et al. 2015). Each experimental diet was placed in one specific plastic container and a group of 100 larvae was weighted and placed directly on the rearing substrate. The diets were added once a week (70 g for each box). Once in each replicate the 40% of the larvae reached the prepupal stage, insects were isolated from the diet, cleaned from substrate residues, washed in water, dried, and counted. The total insect final biomass and the litters (excrements from larvae mixed with substrate residues) were weighted. Diets, litters and prepupae samples were stored at -20°C for further analysis.

## Growth performance and conversion efficiency of the BSF larvae

The larval development time was calculated as the number of days elapsed between the start of the experiment and the day when the 40% of larvae reached the prepupa stage. Larval survival rate was calculated as: final number of insect (prepupae + larvae) / initial number of larvae * 100. Larval growth was measured by biomass gain and expressed in Growth rate (GR) and indicate daily weight gain in mg of each individual larva. GR was calculated as: larvae average final body weight (mg) – larva initial body weight (mg) / days of trial (d). Higher GR indicates a faster growth of larvae. Feed conversion efficiency can be expressed in different ways. The most common measure in insect rearing is the Feed Conversion Ratio (FCR), which is the amount of feed needed (in g) to obtain one g of weight increase of larvae. FCR was calculated as: weight of ingested food / weight gained and obtained on a fresh matter basis (Oonincx et al. 2015). Larvae ability to reduce substrate was calculated by Waste reduction index (WRI) with the equation $WRI = [(W – R) / W] / \text{days of trial (d)} * 100$, where W was the total amount of rearing substrate distributed

during the experiment (g) and R was the residue substrate (g). WRI was the percentage of substrate consumed by the larvae daily. High value of WRI indicate a better larvae ability of reducing substrate.

### Insect diets and larva chemical composition analysis

The dry-microalgae biomass, the Cs substrate, the different experimental diets and the corresponding insect larvae were analysed. Except for microalgae biomass, all samples were freeze-dried before being analysed for proximate composition. The proximate analyses consisted of analytical determinations of water (moisture), dry matter, crude protein, total lipid, and ash. Moisture content was determined by difference after freeze-drying all the samples and dry matter was defined in oven at 105°C for 24 hours. Crude protein content (N x 6.25; CP) was measured according to the Kjeldahl method (proc. 2001.11; AOAC 2005) after acid digestion and total lipid content according to Blight and Dyer method as modified by Burja et al. (2007). Finally, ash content was determined in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany) by combustion at 550 °C for 4 h (proc. 942.05; AOAC 2005). All these analyses were done in duplicate.

### Chemicals and reagents

Fatty acids standards (>98 %; tridecanoic and nonadecanoic acid methyl esters), carotenoids standards (>95 % purity; zeaxanthin, lutein, α-carotene, β-carotene), L-amino acids analytical standards (alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, hydroxyproline, glutamic acid, arginine, asparagine, lysine, glutamine, histidine, tyrosine and DL-norvaline as internal standard, 98.5 % purity), derivatization agent (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide, MTBSTFA, >99 % purity), solvents HPLC grade, hydrochloric acid (HCl), 37 %, boron trifluoride-methanol solution ($BF_3$-MeOH, 14 % in methanol) were purchased by Merck (Darmstadt, Germany). Anhydrous sodium sulphate was purchased by ITW Company (Darmstadt, Germany). MilliQ water was purified with Millipure System (Millford, SC, USA).

### Extraction of total lipids and fatty acid profile

Total lipids were isolated as described by Folch, Lees, and Sloane Stanley (1957). Minced freeze-dried insects and diets (2 g) dissolved in chloroform: methanol (2:1, v/v, 40 mL) were added of tridecanoic and nonadecanoic acid methyl esters as internal standards (500 µL of a 10 mg/mL solution in *n*-hexane), agitated (5 min) and centrifuged (3000 rpm, 10 min, 4 °C). The organic phase was washed with distilled water (5 mL), filtered through Whatman filter paper (Grade 4, 90 mm, Merck KGaA, Darmstadt, Germany) over anhydrous sodium sulphate (3 g) and evaporated with rotary evaporator (30 °C), and the fat yield was calculated.

Fatty acid methyl esters (FAME) were obtained from total lipids through transmethylation by $BF_3$-MeOH reagent (Medina et al. 1992). Briefly, 20 mg of fat were added of *n*-hexane (0.5 mL), $BF_3$-MeOH solution (0.5 mL) and vortexed. After 15 min at 100 °C, the reaction was stopped with distilled water (0.5 mL) and

the mixture was centrifuged (4500 rpm, 3 min). The organic phase was analysed by capillary gas chromatography as reported by (Balzano et al. 2017).

## Carotenoids determination

Carotenoids were extracted from freeze-dried samples and analysed by liquid chromatography as reported by Nartea et al. (2021). The quantification was performed by external calibration. Good correlation coefficients ($R^2$) of 0.999 were obtained in all cases. The instrumental limit of detection (LOD) and quantification (LOQ) were as follows: lutein and zeaxanthin, 5 and 16, β-carotene, 5 and 18 ng/mL.

## Amino acids determination

An aliquot (20 mg) of freeze-dried sample was hydrolysed with 6 M HCl (500 µL) at 110 °C for 24 h under vacuum. The hydrolysate was taken to dryness under nitrogen flow, reconstituted with 0.1 M HCl (5 mL) and centrifuged (4500 rpm, 3 min, 4 °C). Then, 50 µL of the extract was added of DL-norvaline as internal standard (50 µL of a 50 mg/mL solution in 0.1 M HCl), dried, added of dichloromethane (50 µL), dried again and derivatized as reported by Jiménez-Martín et al. (2012).

All samples were injected (1 µL) in a GC/EI-MS (Thermo Scientific, USA) system, equipped with a split/splitless injector, single quadrupole, and a fused silica capillary column MDN-5 (30 m, 0.25 mm, 0.25 µm, Supelco, Bellefonte, PA). The chromatographic conditions were in accordance with Fico et al. (2018). Identification and quantification were carried as reported by (Pérez-Palacios et al. 2015).

For the quantification, standard calibration curves of a mix amino acids (from 80 to 0.1 µg/mL) in 0.1M HCl were prepared in function of concentration level of each amino acid and the ratio of each amino acid peak area/norvaline (internal standard) peak area (Jiménez-Martín et al. 2012). Good correlation coefficients were obtained ($R^2$= 0.99-0.98). A standard stock solution (5 µg/mL) of norvaline internal standard was prepared in 0.1 M HCl. Then, 50 µL of each dilution was added of 50 µL of norvaline stock solution and dried under nitrogen flow, added of 50 µL dichloromethane, dried and derivatized (Jiménez-Martín et al. 2012).

## Statistical analysis

Statistical analysis was performed using the R statistical programming. Data were compared by means of Kruskal-Wallis test or by one-way analysis of variance (ANOVA), according to their distribution. The pairwise Wilcoxon post-hoc test or the Tukey-Kramer's Honestly Significant Difference (HSD) multiple comparison post-hoc test, respectively, were applied for the mean separation ($p < 0.05$) between the tested diets. Data normality was assessed by the Shapiro-Wilk test and multiple testing correction was performed using the Benjamini-Hochberg (BH) method.

# Results and Discussion

The effect of utilizing a coffee by-product enriched with the four levels of microalga inclusion on BSF growth performance and quality attributes is reported in Table 10 and Table 11.

## Experiment A

*Table 10. Growth performance and survival rate of BSF larvae reared on different feeding substrates. Data are reported as mean ± SD (n=6). Different letters indicate significant differences (p< 0.005).*

| Diets | Development time (days) | Prepupal weight (mg) | Survival rate (%) |
|---|---|---|---|
| A | 32.0 ± 3.1 a | 78.54 ±7.39 a | 80.83 ± 10.91a |
| B | 29 ± 1.1 ab | 83.29 ± 7.53 ab | 88.00 ± 8.37 b |
| C | 27.8 ± 0.4 b | 88.10 ± 9.71 b | 83.00 ± 8.79 a |
| D | 21.0 ± 0.0 c | 100.02 ± 7.70 c | 74.17 ± 7.09 c |
| E | 35.7 ± 1.0 d | 44.31 ± 3.02 d | 71.67 ± 6.44 c |

The development time was significantly lower ($p<0.005$) for all diets respect control. In diet D we had the fastest development ($p<0.005$) while the slowest was found in diet A and diet B. About prepupal weight, diet D induced the greatest increase ($p<0.005$). There were no differences ($p>0.005$) in weight gain between diet A and diet B and between diet B and diet C. All diets induced significant weight gain ($p<0.005$) compared to control. Considering survival rate, a significantly higher survival ($p<0.005$) was observed in diet B than in all other diets. A significantly lower value ($p<0.005$) was observed in diet D and diet E while diet A and diet C presented no differences ($p>0.005$). Diet D allowed the larvae to pass to the prepupal stage in shorter times but induced a high mortality. Among all diets, the highest mortality was observed in the first week of the experiment. Diet B was the one with the highest survival rate. There were no differences between diet A and diet C except that in diet C, where the larvae reached the pupal stage few days earlier. Diet D was the fastest to reach the prepupal stage and increased weight earlier, at the expense of a high mortality rate. Diet B was the one that induced the greatest weight gain. Diet C induced weight gain similar to diet A but reached pupal stage about a week before diet B and A.

## Experiment B

*Table 11. Conversion efficiency and survival rate of BSF larvae reared on different feeding substrates. Data are reported as mean ± SD (n=6). Different letters in the same column indicate significant differences (p < 0.005).*

| Diet | GR (mg/day) | Prepupal weight (mg) | WRI | FCR | Survival % |
|---|---|---|---|---|---|
| A | 3.2 ± 0.1 a | 92.10 ± 3.89 a | 1.350 ± 0.033 a | 12.65 ± 0.98 a | 93.17 ± 4.96 a |
| B | 4.0 ± 0.4 b | 114.32 ± 11.84 b | 1.412 ± 0.066 a | 10.55 ± 1.02 b | 94.00 ± 3.35 abc |
| C | 4.3 ± 0.1 b | 109.45 ± 3.72 b | 1.537 ± 0.050 b | 10.27 ± 0.57 b | 98.33 ± 1.97 b |
| D | 5.0 ± 0.1 c | 106.65 ± 1.88 b | 1.868 ± 0.049 c | 8.20 ± 0.43 c | 96.33 ± 2.94 ab |
| E | 2.6 ± 0.5 d | 93.92 ± 15.49 a | 1.070 ± 0.037 d | 16.96 ± 1.87 d | 86.33 ± 5.68 c |

The results exhibited that GR (growth rate) was significantly higher ($p<0.005$) for all diets respect control. In diet D we had a greater increase ($p<0.005$) than in all other diets. Diet A was the one that induced the least

increase ($p<0.005$) while there were no differences between diet B and C ($p>0.005$). Considering WRI (waste reduction index), diet E showed a significantly lower value ($p<0.005$) respect all other diets. The higher value was observed in diet D and there were no significant differences ($p>0.005$) in diets A and B that induced the least increase between diets with microalgae inclusion. Prepupal weight was significantly higher ($p<0.005$) in all diets respect diet E except for diet A. No differences were observed in diet B, diet C and diet D ($p>0.005$). Regarding FCR (Feed Conversion Ratio), there was significantly lower ($p<0.005$) in all diets respect control. Diet D exhibited the lower value, diet A the higher ($p<0.005$). There were no differences between diet B and diet C ($p>0.005$). Survival rate was significantly lower ($p<0.005$) in the diet E than in all other diets except for diet B. The highest survival ($p<0.005$) was observed in diet C and diet D.

Our study investigated, through two different experiments, the effects of different rearing substrates on larval growth performance, waste reduction efficiency and nutritional composition of BSF larvae.

The composition of rearing substrate is very important for BSF larvae because, during the adult stage, BSF do not eat (Sheppard et al. 1994) and therefore, accumulate nutrients only during larval stage. A lack of nutrient during this stage (in particular fat), increases development times to reach prepupa stage (Nguyen, Tomberlin, and Vanlaerhoven 2015). Furthermore, larval development and weight gain positively correlates with the protein content of the diet (Oonincx et al. 2015).

Regarding prepupal weight, all experimental diets with inclusion of *A. platensis* showed a higher value if compared to the control diet. Unfortunately, our results in experiment A were in disaccording to other studies presents in literature (Tomberlin, Sheppard, and Joyce 2002; Nguyen, Tomberlin, and Vanlaerhoven 2015) and only diet with higher concentration of microalgae (diet D) reported similar values, to other authors (Meneguz et al. 2018). In experiment B, we founded the same discordant results but for every diet was reported an improvement in terms of final weight. These results could be explained by the different technique using during the trials: the removal of larvae which took place weekly to change the diet (handled) negatively affect the weight gain of larvae (Nguyen, Tomberlin, and Vanlaerhoven 2015).

The results of survival rate were very different in the two experiments. In experiment B we reach the higher value in all diets when compared with the same diet in experiment A. The results of microalgae enriched substrate in experiment B were in according to values reported by authors in literature (Bava et al. 2019; Raimondi et al. 2020). This difference could be explained by the same reason that a decrease in weight was found. Handling larvae could increase mortality and, consequently, decrease survival rate.

Considering development time, our results showed a positive correlation between concentration of microalgae and time to reach the prepupal stage. There was a positive trend through the diets, and we reached the shorter larval development time in BSF larvae reared on diet D. The results of diet D (21 days) were similar to that reported by (Barragan-Fonseca, Dicke, and van Loon 2017), and other diets showed a shorter development time (28-32 days) if compared to several authors presents in literature (Manurung et al. 2016; Oonincx et al. 2015). Our results confirmed that a higher lipid and protein content of the diet is correlated to a faster development time (Nguyen, Tomberlin, and Vanlaerhoven 2015; Oonincx et al. 2015). This information could better explain the improvements obtained in the larval growth performance in diets

that contain *Arthrospira platensis*, one of the richest microalgae in terms of essential fatty acid and amino acid content (Zhang et al. 2020).

In both experiments, the diet E (without microalgae) showed the worst results in terms of prepupa final weight, development time and survival rate. These results could derive from the presence of anti-nutritional factors in the Cs that inhibit the intake of proteins in the digestive system of insects, such as trypsin inhibitors. In addition, even the high concentration of phenols contained in the coffee silverskin (Borrelli et al. 2004) could negatively affect the growth performance of the larvae, raising mortality levels.

In experiment B we analysed the growth rate and the waste reduction efficiency of the larvae, and in all parameters, we recognized an improvement in the values as the concentration of *A. platensis* increases. To have an optimal waste reduction efficiency (high value of WRI and low value of FCR) is necessary that the larvae can effectively use the rearing substrate.

The Waste Reduction Index (WRI) reported in this study was resulted much lower than WRI values reported by other authors in literature (Meneguz et al. 2018; Bava et al. 2019) but higher respect to others (Permana and Ramadhani Eka Putra 2018; Manurung et al. 2016). Only Parra Paz et al. (2015) showed a WRI values similar to ours.

Regarding Feed Conversion Ratio (FCR), the values reported in this work are similar to Giannetto et al. (2020); but much higher if compared with several authors (Rehman et al. 2017; Oonincx et al. 2015). Diet E showed the higher value, and this could be explained by a lack of nutrients in the rearing substrate (protein and lipid), which leads to an increase in FCR values (Oonincx et al. 2015)

The Growth Rate (GR) resulted much higher in diet D and lower in diet E, showing an improvement trend in relation to microalgae concentration. the values in this study remain much lower if compared with others work in literature (Meneguz et al. 2018) but better respect to Permana et al. (2018) and Giannetto et al. (2020).

## Composition of diets

To validate the enrichment of coffee silverskin with different percentages of microalgae the gross and chemical composition was determined for diets (A, B, C, D, E) and *A. platensis*, as reported in Table 12 and Table 13, respectively.

The *A. platensis* dry-microalgae biomass alone showed a higher crude protein (CP) and lipid content than that observed in the coffee silverskin alone (corresponding to Diet E). The nutrient composition of the diets used for insect rearing was affected by the increasing *A. platensis* microalgae biomass inclusion. Both CP and lipid content were found highest in the D diet were 20% of *A. platensis* was included to the coffee substrate.

In detail, the rearing coffee silverskin substrate showed an amino acid profile different to *A. platensis* microalgae biomass. The ranking based on % profile was aspartic acid > leucine > glutamic acid > valine > lysine for *A. platensis* and hydroxyproline > arginine > proline > valine > leucine for diet E. The inclusion of microalgae biomass to coffee sikverskin substrate generated diets with higher amounts of amino acids with

respect to diet E, not in all cases statistically significant, but with a linear increasing trend. Σ essential amino acids for fish feeding went up from 53.7±1.2 (diet E) to 177.9±10.6 g/kg dried weight (diet D with 20% of microalgae addition). To notice the percentages of amino acids did not vary among the different diets. Diet D showed the highest contents of essential amino acids such as leucine, valine, phenylalanine, threonine, lysine, histidine, tyrosine, and isoleucine, while diet C was the richest in arginine and methionine.

As concern the lipid fraction, Diet E mainly presents saturated fatty acids (SFA, 53.1%, 168.3 mg/100g dry weight), followed by polyunsaturated fatty acids (PUFA, 30.8%, 97.5 mg/100g dry weight and monounsaturated fatty acids (MUFA, 16.1%, 51.2 mg/100g dry weight). *A. platensis* was rich in palmitic (42.2%), linoleic (22.1%) and γ-linolenic acids (13.7%). Accordingly, with previous studies (Osimani et al. 2021; Truzzi et al. 2020), the inclusion of *A. platensis* in different percentage (5, 10, 15 and 20%) to coffee wasted silverskin caused a significant increase of fatty acids. In specific, positive linear correlations were found for PUFA ($R^2$ = 0.989, y = 61.37x + 24.78), SFA ($R^2$ = 0.926, y = 99.28x + 33.66), linoleic ($R^2$ = 0.983, y = 39.85x + 50.18) and γ-linolenic acids ($R^2$ = 0.919, y = 21.52x + 25.40).

*Table 12. Proximate composition (n=2), fatty acids (n=3), amino acids (n=2) and carotenoids (n=3) of the freeze-dried diets: E= 100% coffee; A= 95% coffee + 5% A. platensis; B= 90% coffee + 10% A. platensis; C= 85% coffee + 15% A. platensis; D= 80% coffee + 20% A. platensis.*

| | **Diet E** | | | | **Diet A** | | | | **Diet B** | | | | **Diet C** | | | | **Diet D** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **% DM** | | | | | | | | | | | | | | | | | | | |
| **Moisture** | 5.3 | | | | 6.5 | | | | 6.5 | | | | 4.5 | | | | 6.0 | | | |
| **Crude protein** | 19 | | | | 21 | | | | 23 | | | | 24.0 | | | | 26 | | | |
| **Lipid** | 6.7 | | | | 6.9 | | | | 7.3 | | | | 7.6 | | | | 8.3 | | | |
| **Ash** | 7.7 | | | | 7.8 | | | | 7.9 | | | | 7.1 | | | | 6.9 | | | |
| **Amino acids** | g/kg DM | | | | | | | | | | | | | | | | | | | |
| Alanine | 2.6 | ± | 0.5 | a | 4.2 | ± | 0.4 | ab | 7.6 | ± | 0.5 | bc | 7.2 | ± | 0.5 | bc | 10.7 | ± | 1.7 | c |
| Glycine | 5.2 | ± | 1.4 | a | 7.9 | ± | 0.7 | ab | 13.8 | ± | 1.0 | ac | 15.6 | ± | 5.2 | a | 9.4 | ± | 6.1 | a |
| Valine* | 8.4 | ± | 0.0 | a | 10.7 | ± | 1.6 | ab | 17.7 | ± | 0.3 | bc | 19.7 | ± | 4.2 | c | 23.0 | ± | 0.2 | c |
| Leucine* | 8.0 | ± | 0.5 | a | 12.6 | ± | 0.1 | ab | 19.5 | ± | 1.0 | bc | 15.6 | ± | 2.0 | c | 41.2 | ± | 2.8 | d |
| Isoleucine* | 5.8 | ± | 0.1 | a | 7.5 | ± | 1.0 | ab | 11.8 | ± | 0.1 | ac | 12.7 | ± | 3.3 | bc | 17.2 | ± | 0.1 | c |
| Proline | 9.7 | ± | 0.1 | a | 14.9 | ± | 0.4 | ab | 22.7 | ± | 5.6 | ac | 23.3 | ± | 7.1 | a | 30.5 | ± | 14.5 | a |
| Methionine* | 0.5 | ± | 0.3 | a | 0.1 | ± | 0.1 | a | 1.9 | ± | 0.4 | a | 3.5 | ± | 3.6 | a | 2.6 | ± | 1.9 | a |
| Serine | 2.3 | ± | 0.6 | a | 2.7 | ± | 0.0 | a | 4.2 | ± | 0.4 | a | 2.6 | ± | 2.9 | a | 6.5 | ± | 2.6 | a |
| Threonine* | 4.9 | ± | 0.7 | a | 3.4 | ± | 0.5 | a | 4.7 | ± | 0.5 | a | 7.1 | ± | 3.0 | a | 8.4 | ± | 2.0 | a |
| Phenylalanine* | 6.5 | ± | 1.2 | a | 8.7 | ± | 0.7 | ab | 10.0 | ± | 0.3 | ac | 14.8 | ± | 1.0 | bc | 16.7 | ± | 4.2 | c |
| Aspartic acid | 6.1 | ± | 2.1 | a | 9.8 | ± | 0.7 | ab | 13.6 | ± | 1.4 | b | 13.9 | ± | 0.4 | b | 24.9 | ± | 1.7 | c |
| Hydroxyproline | 12.8 | ± | 0.2 | a | 7.7 | ± | 1.6 | a | 10.7 | ± | 0.9 | a | 12.8 | ± | 2.7 | a | 20.5 | ± | 8.8 | a |
| Glutamic acid | 3.8 | ± | 0.9 | a | 6.9 | ± | 0.8 | ab | 10.2 | ± | 1.0 | bc | 11.7 | ± | 1.0 | c | 18.4 | ± | 1.0 | d |
| Asparagine | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | |
| Lysine* | 4.4 | ± | 0.2 | a | 6.7 | ± | 1.1 | ab | 10.3 | ± | 1.5 | ac | 13.2 | ± | 2.7 | bc | 15.1 | ± | 2.9 | c |
| Glutamine | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | |
| Arginine* | 10.9 | ± | 1.5 | a | 29.7 | ± | 6.6 | a | 35.2 | ± | 9.9 | a | 50.8 | ± | 15.2 | a | 40.3 | ± | 20.8 | a |
| Histidine* | 1.4 | ± | 0.1 | a | 1.7 | ± | 0.3 | a | 2.8 | ± | 0.8 | ab | 3.4 | ± | 0.2 | bc | 4.7 | ± | 0.2 | c |
| Tyrosine* | 2.9 | ± | 0.6 | a | 4.3 | ± | 0.4 | ab | 4.7 | ± | 0.3 | ac | 7.9 | ± | 0.7 | bc | 8.7 | ± | 2.1 | c |

| | | | | | |
|---|---|---|---|---|---|
| **Σ essential** | **53.7 ± 1.2 a** | **85.4 ± 11.0 ab** | **118.7 ± 10.7 bc** | **148.5 ± 21.3 cd** | **177.9 ± 10.6 d** |
| **Σ non-essential** | **42.7 ± 4.1 a** | **54.1 ± 1.4 a** | **82.7 ± 4.9 ab** | **87.0 ± 10.9 b** | **120.9 ± 24.1 a** |
| **Total** | **96.4 ± 7.6 a** | **139.5 ± 12.3 b** | **201.4 5.8 c** | **237.7 7.3 c** | **298.9 ± 13.5 d** |
| **Fatty acids** | mg/100 g DM | | | | |
| C8:0 | 0.0 ± 0.0 a | 14.9 ± 0.0 ab | 13.7 ± 1.0 ab | 43.4 ± 9.2 c | 29.5 ± 0.2 bc |
| C14:0 | 10.7 ± 2.2 b | 8.6 ± 0.2 ab | 5.6 ± 0.5 a | 8.7 ± 0.2 ab | 29.4 ± 0.5 c |
| C15:0 | 8.1 ± 0.2 a | 9.9 ± 0.6 a | 13.7 ± 0.5 b | 16.5 ± 1.2 b | 20.0 ± 0.7 c |
| C16:0 | 102.1 ± 1.2 a | 149.1 ± 2.9 ab | 193.3 ± 4.9 b | 293.9 ± 15.5 c | 411.5 ± 21.5 d |
| C18:0 | 28.0 ± 4.8 a | 29.3 ± 0.5 a | 18.8 ± 0.4 a | 24.5 ± 5.6 a | 57.3 ± 5.8 b |
| C20:0 | 19.3 ± 2.3 a | 22.3 ± 0.7 ab | 22.2 ± 2.5 ab | 25.1 ± 0.7 ab | 28.0 ± 1.9 b |
| ∑ SFA | 168.3 ± 1.8 a | 234.2 ± 4.9 ab | 267.3 ± 3.1 b | 412.1 ± 32.4 c | 575.8 ± 25.8 d |
| C16:1Δ9c | 0.0 ± 0.0 a | 7.0 ± 0.5 b | 12.2 ± 0.4 c | 16.6 ± 2.0 d | 16.6 ± 0.7 d |
| C18:1Δ9c | 38.9 ± 2.5 a | 45.7 ± 0.4 ab | 36.4 ± 2.7 a | 66.3 ± 12.6 b | 46.1 ± 0.1 ab |
| C20:1Δ9c | 12.3 ± 5.2 a | 19.2 ± 0.3 ab | 19.1 ± 1.4 ab | 22.8 ± 0.8 b | 26.6 ± 1.0 b |
| **∑ MUFA** | **51.2 ± 7.7 a** | **72.0 ± 0.2 ab** | **67.7 ± 1.6 ab** | **105.7 ± 13.8 c** | **89.3 ± 1.6 bc** |
| C18:2Δ9c,12c ω6 | 97.5 ± 1.2 a | 127.6 ± 0.6 ab | 161.5 ± 10.6 bc | 202.9 ± 8.6 c | 259.1 ± 19.8 d |
| C18:3Δ6c,9c,12c ω6 | 0.0 ± 0.0 a | 6.2 ± 0.0 a | 41.9 ± 1.4 b | 74.0 ± 0.9 c | 73.7 ± 8.6 c |
| **∑ PUFA** | **97.5 ± 1.2 a** | **133.8 ± 0.6 b** | **203.4 ± 9.2 c** | **277.0 ± 9.5 d** | **332.8 ± 11.2 e** |
| **∑ PUFAω6** | **97.5 ± 1.2 a** | **133.8 ± 0.6 b** | **203.4 ± 9.2 c** | **277.0 ± 9.5 d** | **332.8 ± 11.2 e** |
| **Carotenoids** | mg/100 g DM | | | | |
| zeaxanthin | <LOD | 37.63 ± 0.17 a | 69.43 ± 0.52 b | 115.15 ± 1.82 c | 178.32 ± 0.74 d |
| lutein | 9.21 ± 0.0 c | 8.94 ± 0.02 b | 9.11 ± 0.07 c | 8.13 ± 0.03 a | 11.47 ± 0.14 d |
| β-carotene | <LOD | 126.76 ± 2.06 a | 228.51 ± 1.57 b | 373.69 ± 3.82 c | 597.60 ± 4.89 d |

Results represents means value ± standard deviation. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds. Different letters in the same row means statistical difference (ANOVA, $p<0.05$). LOD, limit of detection. DM, dry matter. *Essential amino acids for fish.

*Table 13. Proximate composition (n=2), carotenoids (n=3), fatty acids (n=3) and amino acids (n=2) of freeze-dried A. platensis microalgae biomass.*

| **Proximate composition** | % |
|---|---|
| Moisture | 6.7 |
| Crude | 68 |
| Lipid | 9.4 |
| **Carotenoids** | mg/kg DM |
| Zeaxanthin | 147.20 ± 0.08 |
| Lutein | <LOD |
| β-carotene | 523.33 ± 8.54 |

| **Fatty acid** | mg/100g DM | % |
|---|---|---|
| C8:0 | 417.5 ± 16.1 | 12.1 ± 0.1 |
| C10:0 | 21.7 ± 2.4 | 0.6 ± 0.1 |
| C12:0 | <LOD | <LOD |
| C14:0 | <LOD | <LOD |
| C15:0 | <LOD | <LOD |
| C16:0 | 1461.2 ± 112.9 | 42.2 ± 2.1 |
| C17:0 | <LOD | <LOD |
| C18:0 | 36.3 ± 3.6 | 1.1 ± 0.1 |
| C20:0 | <LOD | <LOD |
| ∑ **SFA** | 1936.8 ± 123.0 | 56.0 ± 2.0 |
| C16:1Δ9c | 148.7 ± 7.2 | 4.3 ± 0.1 |
| C18:1Δ9c | 136.6 ± 9.0 | 3.9 ± 0.1 |
| C20:1Δ9c | <LOD | <LOD |
| ∑ **MUFA** | 285.3 ± 16.3 | 8.2 ± 0.2 |
| C18:2Δ9c,12c ω6 | 763.4 ± 36.4 | 22.1 ± 1.7 |
| C18:3Δ6c,9c,12c ω6 | 473.8 ± 4.5 | 13.7 ± 0.5 |
| C18:3Δ9c,12c,15c ω3 | <LOD | <LOD |
| C20:5Δ5c,8c,11c,14c,17c ω3 (EPA) | <LOD | <LOD |
| ∑ **PUFA** | 1237.2 ± 40.9 | 35.8 ± 2.2 |
| ∑ **PUFAω6** | 1237.2 ± 40.9 | 35.8 ± 2.2 |

| **Amino acids** | g/kg DM | % |
|---|---|---|
| Alanine | 82.6 ± 10.2 | 5.1 ± 0.1 |
| Glycine | 63.5 ± 4.9 | 4.3 ± 0.5 |
| Valine* | 172.8 ± 15.2 | 10.4 ± 0.7 |
| Leucine* | 189.9 ± 20.1 | 11.4 ± 0.8 |
| Isoleucine* | 107.0 ± 2.3 | 7.2 ± 0.6 |
| Proline | 88.0 ± 10.3 | 5.6 ± 0.1 |
| Methionine* | 14.3 ± 0.7 | 2.2 ± 1.8 |
| Serine | 31.7 ± 1.6 | 1.6 ± 0.5 |
| Threonine* | 68.7 ± 4.3 | 5.3 ± 1.4 |
| Phenylalanine* | 51.8 ± 5.3 | 3.8 ± 0.8 |
| Aspartic acid | 254.1 ± 22.0 | 15.3 ± 1.0 |
| Hydroxyproline | 1.2 ± 0.1 | 0.2 ± 0.2 |
| Glutamic acid | 178.8 ± 31.2 | 10.4 ± 1.2 |
| Asparagine | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Lysine* | 105.2 ± 6.5 | 7.1 ± 0.7 |
| Glutamine | 0.2 ± 0.0 | 0.0 ± 0.0 |
| Arginine* | 115.9 ± 9.3 | 6.5 ± 1.1 |
| Histidine* | 17.4 ± 0.7 | 1.1 ± 0.0 |
| Tyrosine* | 45.5 ± 1.3 | 2.4 ± 0.7 |
| **Σ essential amino acids** | **892.9 ± 51.8** | **57.3 ± 2.0** |
| **Σ non-essential amino acids** | **663.7 ± 16.9** | **42.7 ± 2.0** |
| **Total** | **1589.6 ± 81.6** | |

Results represents means value ± standard deviation. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds. LOD, limit of detection. *Essential amino acids for fish.

## Proximate composition of insects

The insect nutrient composition on the crude protein content was unaffected by the dietary levels of *A. platensis* while the lipid content raised in parallel to the microalgae biomass proportion present in the diet Table 14.

*Table 14. Proximate composition (% of dry matter, DM) of the freeze-dried insect larvae reared on different diets. Data of n=2 samples.*

| | Moisture % | Crude protein | Lipid | Ash |
|---|---|---|---|---|
| **Insects** | | | | |
| E | 11.0 | 53 | 8.7 | 20.5 |
| A | 17.1 | 52 | 11.8 | 21.0 |
| B | 14.4 | 53 | 13.1 | 18.8 |
| C | 9.2 | 51 | 13.4 | 16.4 |
| D | 11.3 | 51 | 14.7 | 15.4 |

Experimental diets and insects reared larvae abbreviations: E= 100% coffee; A= 95% coffee + 5% *A. platensis*; B= 90% coffee + 10% *A. platensis*; C= 85% coffee + 15% *A. platensis*; D= 80% coffee + 20% *A. platensis*

## Fatty acid profile of insects

The fatty acid profile of HI larvae reared on Cs substrate without *A. platensis* (diet E) was characterized by the dominance of SFA (C12:0 > C16:0 > C18:0 > C14:0), reflecting the profile of coffee silverskin diet (Table 15). Although the γ-linolenic (C18:3 Δ6c,9c,12c ω6), α-linolenic (C18:3 Δ9c,12c,15c ω3) and the eicosapentaenoic (C20:5Δ5c,8c,11c,14c,17c ω3, EPA) acids were not detected in the diets, they were found HI. Thus, fatty acid composition of HI E reflects only in part the composition of the correspondent rearing substrate. 15% of microalgae addition to coffee silverskin gave the best results by generating HI with the highest contents of MUFA and PUFA, 1366 and 1600 mg/100g dry weight insect biomass, respectively, and lowest percentage in SFA (31.0%). To notice that the percentage of PUFA compounds increased linearly ($R^2 = 0.918$, $y = 6.22x + 13.74$) in function of % of microalgae till 15%, while at 20% of spirulina the bioconversion was less efficient even than the diet at 10% of microalgae. EPA was highly present in the nutritionally poorest substrate (diet E). Higher lauric acid content (C12:0) is considered a positive indicator for the goodness of the growing substrate, in terms of carbohydrates (Spranghers et al. 2017). Increasing levels of spirulina in HI feed resulted in a significant (except sample A) positive correlation ($y= 34.077x + 276.95$, $R^2= 0.9537$), with the maximum level of 986.1±55.0 mg/100g dry weight (HI D), 2.8-fold more than sample HI E (351.2 mg/100 g dry weight, 17.7% total fatty acids).

Our results confirmed that the inclusion of microalgae in a pour substrate of lipids and proteins such as Cs (Costa et al. 2018; Vargas et al. 2018) enhanced the lipid accumulation in the insect body. In particular, lauric acid increments were in compliance with Truzzi et al. (2020), obtained for HI grown on Cs enriched with *Schizochytrium sp* or *Isochrysis sp* microalgae (5-25%), selected for their long chain fatty acids profile.

In their experiment C12:0 increased till 2.3-fold more with respect to 100% Cs substrate (14.1 g/100g fatty acids. However, to consider spirulina a good enrichment substrate, apart from C12:0, lipid and unsaturated fatty acids and protein content need to be increased in HI. Thus, the best results in our experiment were recorded at 15% level of spirulina inclusion to Cs. Truzzi et al. (2020) optimal results were found at 10% of *Schizochytrium sp.* Several studies have demonstrated the great influence of lipid composition of the substrate on the lipid profile and content of insects (Barroso et al. 2014; Osimani et al. 2021; Spranghers et al. 2017; Truzzi et al. 2020). Data regarding lipid influence of spirulina diet on HI are missing. We demonstrated that HI is good bioconvertor of essential γ-linolenic acid from microalgae and organic waste (Cs). Spirulina is of interest in the aquafeed industry as many studies used it as dietary supplement for fishes and crustaceans, even if the production cost limits its use in commercial applications (Ragaza et al. 2020). For reference, *Schizochytrium sp,* added to Cs to reduce the environmental impact of aquafeed production, is more convenient (Truzzi et al. 2020), but spirulina nutritional value could be a valuable solution once its cost production will be lowered. Ragaza et al. (2020) concluded that a small inclusion levels (1–10%) of spirulina increased the nutritional value of aquaculture diets (not insect-based) improved growth and health performance of fish.

*Table 15. Fatty acid compositions of different insects (A, B, C, D, E) expressed as mg fatty acid/100g dry weight and weight % of total fatty acid.*

| | E | A | B | C | D | E | A | B | C | D |
|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | mg/100g dry weight | | | | | % | | | | |
| C10:0 | 10.0 ± 0.4 a | 12.0 ± 0.6 a | 24.1 ± 2.7 b | 40.4 ± 2.2 c | 35.3 ± 0.7 c | 0.5 ± 0.0 | 0.7 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.0 | 0.8 ± 0.0 |
| C12:0 | 351.2 ± 8.0 a | 366.2 ± 19.0 a | 584.9 ± 24.5 b | 800.2 ± 1.5 c | 986.1 ± 55.0 d | 17.7 ± 0.6 | 20.1 ± 0.4 | 21.4 ± 1.0 | 18.6 ± 0.3 | 22.4 ± 0.5 |
| C14:0 | 74.9 ± 1.6 ab | 66.8 ± 6.5 ab | 61.2 ± 17.0 a | 100.8 ± 10.6 bc | 135.5 ± 2.8 c | 3.8 ± 0.0 | 3.7 ± 0.2 | 2.2 ± 0.6 | 2.3 ± 0.3 | 3.1 ± 0.0 |
| C15:0 | 33.5 ± 0.9 b | 25.3 ± 2.0 ab | 19.5 ± 1.5 a | 37.5 ± 6.3 b | 30.7 ± 0.7 ab | 1.7 ± 0.0 | 1.4 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.0 |
| C16:0 | 250.4 ± 10.7 bc | 228.1 ± 3.7 b | 172.8 ± 8.7 a | 270.3 ± 6.3 c | 447.2 ± 9.7 d | 12.6 ± 0.4 | 12.5 ± 0.2 | 6.3 ± 0.3 | 6.3 ± 0.0 | 10.2 ± 0.1 |
| C17:0 | 18.7 ± 2.5 a | 27.4 ± 0.7 b | 26.5 ± 2.1 b | 28.2 ± 0.2 b | 31.1 ± 1.0 b | 0.9 ± 0.1 | 1.5 ± 0.1 | 1.0 ± 0.1 | 0.7 ± 0.0 | 0.7 ± 0.0 |
| C18:0 | 82.5 ± 5.5 c | 54.1 ± 0.6 b | 33.7 ± 1.0 a | 45.2 ± 4.0 ab | 71.2 ± 1.6 c | 4.2 ± 0.2 | 3.0 ± 0.1 | 1.2 ± 0.0 | 1.1 ± 0.1 | 1.6 ± 0.0 |
| C20:0 | 25.2 ± 0.0 c | 11.3 ± 0.2 a | 11.4 ± 0.4 a | 11.5 ± 0.5 a | 15.0 ± 0.7 b | 1.3 ± 0.0 | 0.6 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| **∑ SFA** | **846.5 ± 12.8 ab** | **791.3 ± 29.0 a** | **934.0 ± 10.6 b** | **1334.1 ± 9.0 c** | **1752.1 ± 69.4 d** | **42.7 ± 0.2** | **43.4 ± 0.2** | **34.1 ± 0.5** | **31.0 ± 0.4** | **39.8 ± 0.2** |
| C16:1Δ9c | 253.6 ± 6.2 a | 293.1 ± 8.6 a | 386.8 ± 25.0 b | 654.8 ± 14.0 c | 575.3 ± 36.1 c | 12.8 ± 0.5 | 16.1 ± 1.0 | 14.1 ± 0.9 | 15.2 ± 0.0 | 13.1 ± 0.4 |
| C18:1Δ9c | 533.4 ± 4.4 b | 205.5 ± 17.9 a | 513.8 ± 19.2 b | 711.2 ± 3.4 c | 684.9 ± 7.0 c | 26.9 ± 0.1 | 11.3 ± 0.6 | 18.8 ± 0.6 | 16.5 ± 0.4 | 15.6 ± 0.4 |
| **∑ MUFA** | **787.0 ± 1.8 b** | **498.6 ± 9.3 a** | **900.7 ± 44.2 b** | **1366.0 ± 10.6 c** | **1260.2 ± 43.2 c** | **39.7 ± 0.5** | **27.3 ± 0.3** | **32.9 ± 1.5** | **31.8 ± 0.4** | **28.7 ± 0.0** |
| C18:2Δ9c,12c ω6 | 289.2 ± 8.3 a | 421.8 ± 13.7 a | 668.2 ± 5.7 b | 1180.4 ± 65.7 c | 1045.0 ± 38.4 c | 14.6 ± 0.3 | 23.1 ± 0.0 | 24.4 ± 0.3 | 27.4 ± 1.0 | 23.8 ± 0.0 |
| C18:3Δ6c,9c,12c ω6 | 5.8 ± 0.3 a | 83.3 ± 8.9 b | 208.0 ± 17.1 c | 391.5 ± 5.9 e | 312.6 ± 2.1 d | 0.3 ± 0.0 | 4.6 ± 0.3 | 7.6 ± 0.7 | 9.1 ± 0.3 | 7.1 ± 0.2 |
| C18:3Δ9c,12c,15c ω3 | 29.5 ± 0.7 b | 17.2 ± 1.4 a | 20.2 ± 1.7 a | 27.4 ± 0.9 b | 21.1 ± 1.1 a | 1.5 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.0 | 0.5 ± 0.0 |
| C20:5Δ5c,8c,11c,14c,17c ω3 (EPA) | 26.6 ± 3.2 c | 12.7 ± 2.4 b | 6.2 ± 1.3 ab | 1.4 ± 2.0 a | 5.7 ± 0.8 ab | 1.3 ± 0.1 | 0.7 ± 0.2 | 0.2 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.0 |
| **∑ PUFA** | **351.0 ± 10.5 a** | **535.1 ± 18.9 b** | **902.6 ± 22.4 c** | **1600.7 ± 62.7 e** | **1384.4 ± 40.7 d** | **17.7 ± 0.3** | **29.3 ± 0.1** | **33.0 ± 1.0** | **37.2 ± 0.7** | **31.5 ± 0.2** |
| **∑ PUFAω3** | **56.1 ± 2.5 b** | **29.9 ± 3.7 a** | **26.4 ± 0.4 a** | **28.8 ± 2.8 a** | **26.8 ± 0.2 a** | **2.8 ± 0.1** | **1.6 ± 0.3** | **1.0 ± 0.0** | **0.7 ± 0.1** | **0.6 ± 0.0** |
| **∑ PUFAω6** | **294.9 ± 8.0 a** | **505.1 ± 22.6 b** | **876.2 ± 22.8 c** | **1571.9 ± 59.8 e** | **1357.6 ± 40.5 d** | **14.9 ± 0.2** | **27.7 ± 0.4** | **32.0 ± 1.0** | **36.5 ± 0.7** | **30.9 ± 0.2** |

Results represents means value ± standard deviation (n = 2). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds. Different letters in the same row means statistical difference (ANOVA, $p<0.05$).

## Carotenoids in insects

To design a balanced aquafeed, not only protein and lipid fraction should be considered, but also vitamins such as vitamin A. Many studies have focused their attention on the modulation of black soldier fly in terms of lipids and proteins, to be optimal for food and feed, but, as good bioconvertors, those insects could provide from targeted diets also carotenoids such as β-carotene, emblematic precursor of vitamin A. Vitamin A is an essential dietary nutrient for fish involved in physiological functions such as vision, reproduction, embryogenesis, growth and differentiation and maintenance of epithelial cells (Hernandez and Hardy 2020).

The enrichment of HI substrate with microalgae biomass produced in all samples a significant increase of zeaxanthin and β-carotene, reaching the highest (74.2 and 274.9 mg/kg dried weight, respectively) bioconversion in sample HI D reared on 20 % of *A. platensis* + 80% coffee silverskin (Table 16). Zeaxanthin showed an exponential trend ($R^2 = 0.966$, $y = 0.6155e^{0.9697x}$) while β-carotene displayed a polynomial function of second degree (y = 23.662x2 - 79.582x + 65.622, $R^2 = 0.954$).

Our results demonstrated that HI are good bioconvertors of carotenoids with provitamin A activity (β-carotene) and non (zeaxanthin). Spirulina carotenoids profile was found in agreement with literature, as β-carotene and zeaxanthin were found the major carotenoids (Park et al. 2018). The bioaccumulation reached a maximum of 44% of β-carotene and of 40% of zeaxanthin, when rearing HI on the diet composed by 20% of spirulina. Borel et al. (2021) found that black soldier fly larvae can bioaccumulate significant amounts of provitamin A, recycling them from fruits and vegetable waste. Vitamin A requirements for fish species (juvenile stages principally) of commercial aquaculture importance ranges 1,000 to 20,000 international unit (IU)/kg body weight day (Hernandez and Hardy 2020), corresponding for reference to 0.6-12 β-carotene µg/kg body weight day. BSF values ranged from around 15 (diet A, 5% spirulina) to 270 (diet C, 20%) β-carotene mg/kg dry insect, providing hypothetically good levels of vitamin A. Moreover, in juvenile rainbow trout, β-carotene supplementation improved growth performance and skin carotene level (Kelestemur and Coban 2016) (30-70 mg/kg).

*Table 16. Carotenoids (mg/kg dried weight) of freeze-dried insects reared on the corresponding diets. E=100% coffee; A= 95% coffee + 5% A. platensis; B= 90% coffee + 10% A. platensis; C= 85% coffee + 15% A. platensis; D= 80% coffee + 20% A. platensis.*

| mg/kg DM | Zeaxanthin | | | Lutein | | | β-carotene | | |
|---|---|---|---|---|---|---|---|---|---|
| **Insects** | | | | | | | | | |
| E | 1.2 | ± 0.0 | a | 2.1 | ± 0.0 | a | <LOD | | |
| A | 5.5 | ± 0.0 | b | 2.1 | ± 0.0 | a | 14.5 | ± 0.7 | a |
| B | 16.0 | ± 0.0 | c | 3.0 | ± 0.0 | b | 57.8 | ± 0.4 | b |
| C | 23.4 | ± 0.2 | d | 2.8 | ± 0.3 | b | 88.6 | ± 0.5 | c |
| D | 74.2 | ± 0.1 | e | 5.9 | ± 0.1 | c | 274.9 | ± 1.6 | d |

Data are expressed as mean of three replicates ± standard deviation (SD). LOD, limit of detection. Different superscript letters (a, b, c, d, e) in the same column and matrix mean a significant statistical difference analysed by a one-way ANOVA (p< 0.05).

## Amino acids in insects

Along fish dietary requirements, the essential amino acids are as follows: threonine, valine, leucine, isoleucine, methionine, tryptophan, lysine, histidine, arginine, phenylalanine. The non-essential amino acids cystine and tyrosine can be synthetized from methionine and phenylalanine, respectively, thus the dietary requirement for those non-essential amino acids is dependent on the levels of the respective non-essential amino acids within the diet (FAO 1987).

Table 17 reports the amino acid composition of HI larvae, which in general did not vary among insects reared on substrates with increasing levels of crude proteins. The inclusion of microalgae biomass to substrate of coffee silverskin generated increasing but not significant levels of Σ essential amino acids and total amino acid content. Significant variations were recorded for some essential amino acids such as threonine, lysine, histidine, and tyrosine, as HI D reared on diet D reported higher values than HI E, corresponding to diet E without *A. platensis*. Σ non-essential amino acid value showed a significant difference between HI D and HI E (80.3±8.9 vs 129.9±19.1 g/kg dried weight), reflecting the difference in terms of alanine, proline, serine, aspartic and glutamic acid. The essential amino acids, histidine and tyrosine accounted for 14.2±3.3 and 17.9±4.9 g/kg dried weight, respectively, as the highest values in HI D.

In agreement with our results, Müller, Wolf, and Gutzeit (2017) have found no evidence that the amino acid composition can be altered significantly by different feeding regimes. Our outcomes showed that the dominant amino acid in HI E was proline (32,5±7,7 g/kg dried weight). Differently, Liland et al. (2017) demonstrated that the most abundant amino acids in larvae reared on seaweed were aspartic acid (non-essential) and glutamic acid (essential amino acids). The predominance of glutamic acid was also found by Müller, Wolf, and Gutzeit (2017) and by Giannetto et al. (2020). The choice of substrate (organic waste) significantly affected the concentration of limiting and non-limiting amino acids in HI, but in general and for the most limiting amino acids like lysine, methionine, isoleucine and tyrosine no significant substrate effect was found (Shumo et al. 2019).

## Conclusions

The BSF larvae can be grown coffee by-product diets and the nutritional value of feed made of edible insects can be modulated to recover coffee silverskin. All diets had improved the growth performances of larvae. The enrichment with spirulina of coffee by-product diet had positive effect on the modulation of fatty acids (γ-linolenic acid), zeaxanthin and β-carotene. The dietary levels of microalgae did not affect the larval crude protein content. Diet D with 20% of spirulina had the higher impact on some essential amino acid. The application is designed for aquafeeds.

*Table 17. Amino acids profile of insects reared on different diets (E, A, B, C, D). Data are reported as mean of two replicates ± standard deviation (SD), expressed as g/kg dried weight and. LOD, limit of detection.*

| | **E** | | | | **A** | | | | **B** | | | | **C** | | | | **D** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | g/kg DM | | | | | | | | | | | | | | | | | | | |
| Alanine | 8.8 | ± | 1.1 | a | 13.1 | ± | 1.4 | ab | 13.2 | ± | 1.4 | ab | 9.8 | ± | 1.2 | ab | 15.4 | ± | 3.1 | b |
| Glycine | 15.3 | ± | 0.6 | | 19.4 | ± | 0.7 | | 18.1 | ± | 1.3 | | 17.1 | ± | 0.6 | | 20.9 | ± | 4.8 | |
| Valine* | 21.6 | ± | 4.8 | | 32.1 | ± | 3.8 | | 35.0 | ± | 3.5 | | 25.7 | ± | 3.8 | | 33.7 | ± | 6.2 | |
| Leucine* | 17.0 | ± | 3.6 | | 31.1 | ± | 10.5 | | 30.5 | ± | 2.9 | | 27.5 | ± | 5.5 | | 26.4 | ± | 4.4 | |
| Isoleucine* | 11.1 | ± | 3.5 | | 18.4 | ± | 2.5 | | 18.3 | ± | 1.5 | | 14.4 | ± | 1.8 | | 16.0 | ± | 3.4 | |
| Proline | 32.5 | ± | 7.7 | ab | 30.0 | ± | 1.5 | a | 28.9 | ± | 1.9 | ab | 29.0 | ± | 0.6 | ab | 44.7 | ± | 3.2 | b |
| Methionine* | 6.2 | ± | 1.5 | b | 4.5 | ± | 0.9 | ab | 1.8 | ± | 0.3 | a | 3.6 | ± | 0.9 | ab | 4.5 | ± | 0.7 | ab |
| Serine | 4.9 | ± | 0.0 | b | 5.4 | ± | 0.4 | b | 4.6 | ± | 0.2 | b | 2.2 | ± | 0.7 | a | 7.4 | ± | 0.8 | c |
| Threonine* | 12.5 | ± | 3.9 | a | 10.6 | ± | 0.2 | a | 10.5 | ± | 0.2 | a | 8.8 | ± | 0.0 | a | 19.3 | ± | 0.4 | b |
| Phenylalanine* | 17.4 | ± | 1.5 | | 21.7 | ± | 0.8 | | 17.7 | ± | 0.9 | | 15.3 | ± | 0.6 | | 17.5 | ± | 5.0 | |
| Aspartic acid | 9.9 | ± | 0.5 | a | 16.2 | ± | 4.8 | ab | 23.0 | ± | 2.3 | b | 23.7 | ± | 4.0 | b | 21.9 | ± | 3.2 | b |
| Hydroxyproline | 3.0 | ± | 1.2 | | 2.1 | ± | 0.3 | | 1.4 | ± | 0.0 | | 2.7 | ± | 0.5 | | 2.7 | ± | 0.2 | |
| Glutamic acid | 8.8 | ± | 0.1 | a | 14.6 | ± | 3.2 | ab | 16.3 | ± | 1.7 | ab | 17.4 | ± | 2.7 | b | 19.5 | ± | 4.0 | b |
| Asparagine | 0.7 | ± | 0.7 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | |
| Lysine* | 15.9 | ± | 3.0 | a | 27.4 | ± | 1.1 | b | 31.1 | ± | 2.7 | b | 23.3 | ± | 5.0 | ab | 29.2 | ± | 7.1 | b |
| Glutamine | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | | 0.0 | ± | 0.0 | |
| Arginine | 30.5 | ± | 7.4 | | 15.2 | ± | 5.9 | | 26.9 | ± | 2.9 | | 19.1 | ± | 0.6 | | 15.3 | ± | 21.6 | |
| Histidine* | 0.8 | ± | 0.8 | a | 4.3 | ± | 3.7 | ab | 13.8 | ± | 1.4 | c | 10.7 | ± | 3.1 | bc | 14.2 | ± | 3.3 | c |
| Tyrosine* | 12.2 | ± | 1.4 | a | 9.9 | ± | 0.1 | a | 11.6 | ± | 0.6 | ab | 10.9 | ± | 1.1 | ab | 17.9 | ± | 4.9 | b |
| Σ essential amino acids | 133.0 | ± | 15.2 | | 165.3 | ± | 25.9 | | 185.5 | ± | 15.7 | | 148.5 | ± | 19.5 | | 176.0 | ± | 8.1 | |
| Σ non-essential amino acids | 80.3 | ± | 8.9 | a | 98.8 | ± | 9.7 | ab | 104.1 | ± | 8.8 | ab | 99.1 | ± | 8.6 | ab | 129.9 | ± | 19.1 | b |
| Total | 229.2 | ± | 27.4 | | 276.1 | ± | 35.5 | | 302.7 | ± | 25.2 | | 261.2 | ± | 28.7 | | 326.5 | ± | 32.2 | |

*Essential amino acids for fish. Different letters in the same row mean statistical difference (one-way ANOVA, p>0.05).

# Final remarks of the thesis

This research provides indication for developing functional bakery products (glucosinolates, vitamin A and phytosterols) for food industries from cauliflower by-products. To meet market and sustainable needs towards healthier food products, brewing beer with vegetable by-products can be a novel strategy to drive health effects to the consumers (i.e., chlorogenic acids). Moreover, vegetable waste (coffee silverskin) could be a sustainable solution to the production of valuable fish species through the bioconversion of edible insects. The potentialities, within an explorative and multidisciplinary approach, of brassica and coffee waste streams were confirmed in bakery products, in beverages and feeds.

These multi-opportunities can be exploited by food industries by applying the findings of this thesis for *ad-hoc* solutions that could meet the market trends towards functional and sustainable foods.

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