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Nutritional and functional characterisation of alternative feed and food ingredients: emphasis on hemp-based products, co-products, microalgae and cultured meat

PhD candidate: Dr. Davide Lanzoni

Supervisor: Prof. Carlotta Giromini

Co-Supervisor: Prof. Eva Pěchoučková

Coordinator: Prof. Federica Cheli

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“La vita è una maratona,

e non si vince una maratona

senza mettersi i cerotti per le vesciche”

Preface

This PhD thesis represents experimental work carried out at the Department of Veterinary Medicine and Animal Sciences (DIVAS, University of Milan, Lodi, Italy), from January 2021 to December 2024 under the supervision of Professor Carlotta Giromini.

What you are about to read can be interpreted as a summary of these years, a journey that has explored various topics, which although so different, are united by a single common thread: ***the need to investigate new sustainable protein matrices for the feed and food sector.***

The success of the objective was possible thanks to valuable collaborations, in particular with the Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Science (Prague, Czech Republic), the Joint Research Centre (JRC) - European Commission (Ispra, Italy) and the University of Minho (Braga, Portugal).

Davide Lanzoni

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Abbreviations

AAs. Amino Acids

ABTS. 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid

AAE. Acid Ascorbic Equivalent

ACE. Angiotensin Converting Enzyme

ADF. Acid Detergent Fibre

ADL. Acid Detergent Lignin

AM. Additive Manufacturing

CAMC. Camelina Cake

CAP. Common Agriculture Policy payments

CBD. Cannabidiol

CBG. Cannabigerol

CC. Cardoon Cake

CK. Creatine Kinase

CM. Cardoon Meal

CP. Crude Protein

CS. Citrate Synthase

CV. *Chlorella vulgaris*

DLS. Dynamic Light Scattering

DM. Dry Matter

DMEM. Dulbecco's Modified Eagle's Medium

DPPH. 1,1-diphenyl-2-picryl-hydrazyl free radical

ECM. Extracellular Matrix

EE. Ethereal Extract

EFSA. European Food Safety Authority

EGF. Epidermal Growth Factor

EtOH. Ethanol

EU. European Union

FAMEs. Fatty Acid Methyl Esters

FAO. Food and Agriculture Organisation of the United Nations

FBS. Foetal Bovine Serum

FC. Folin-Ciocalteu

FCR. Feed Conversion Ratio

FDA. Food and Drug Administration

FGF. Fibroblast Growth Factor

FI. Feed Intake

FRAP. Ferric Reducing Antioxidant Power

FX. Flaxseed

F2F. Farm to Fork

GCCP. Good Cell Culture Practice

GelMA. Gelatin Methacroyl

GHGs. Greenhouse Gases

GM. Growth Medium

GMP. Good Manufacturing Practice

HLs. Hulls

HP. *Haematococcus pluvialis*

HPTLC. High-Performance Thin-Layer Chromatography

HS. Hempseed

HSC. Hempseed Cake

LDH. Lactate Dehydrogenase

MFL. Mix Flowers and Leaves

MPs. Microplastics

MPs. Microbial Proteins

MRF. Myogenic Regulatory Factors

MUFAs. Monounsaturated Fatty Acids

NASA. National Aeronautics and Space Administration

NDF. Neutral Detergent Fibre

NPs. Nanoplastics

ORAC. Oxygen Radical Absorbance Capacity

PdI. Polydispersity index

PE. Polyethylene

PS. Polystyrene

PUFAs. Polyunsaturated Fatty Acids

SCPL. Solvent Casting and Porogen Leaching

SDGs. Sustainable Development Goals

SDS. Sodium Dodecyl Sulphate

SFA. Singapore Food Agency

SFAs. Saturated Fatty Acids

SGF. Simulated Gastric Fluids

SIF. Simulated Intestinal Fluids

SLA. Stereolithography

SLS. Selective Laser Sintering

SOF. Simulated Oral Fluids

TAE. Tannic Acid Equivalent

TE. Trolox Equivalent

TF. Transcription Factor

THC. Δ9-tetrahydrocannabinol

TPC. Total Phenolic Content

TPTZ. 2, 4, 6-tripyridyl-s-triazine

UF. Undigested Fraction

USDA. US Department of Agriculture

WP. Whey Proteins

3DP. 3Dprinting

Abstract

The growth of the world population has led to an increase in demand for food, particularly meat and dairy products, further intensifying livestock production, resulting in an enhanced use of grain for animal feed and competition with crops for direct human consumption. In parallel, over time, the livestock sector has exerted considerable pressure on resources, with large areas of arable land dedicated to food cultures and grazing, as well as high water use. This highlights the urgent need for action with multidisciplinary approaches. For this reason, the aims of this thesis were to evaluate the nutritional and functional potential of alternative plant-based products, including hemp, cardoon, and camelina, by analysing their antioxidant properties, bioactive peptide profiles, and their impact on the quality of animal-derived products. It also aimed to optimize the culture medium of microalgae, in particular for *Euglena gracilis* to enhance its functional profile while investigating the risks of nanoplastics and microplastics contamination during cultivation under heterotrophic conditions. Additionally, the research sought to address key challenges in cultured meat production by developing alternatives to fetal bovine serum, scalable biofabrication methods, and the integration of waste-derived components to support cell proliferation and differentiation. These objectives collectively aimed to advance sustainable, ethical, and innovative practices in the agri-food sector.

Hemp based-products and their co-products have demonstrated high nutritional and functional profiles, as observed following *in vitro* digestion and solvent extraction methods. In support of this, an *in vivo* trial, conducted on laying hens, showed how the inclusion of a hemp co-product of up to 9% (leaves, non-standard hemp seeds, hulls and stems) improved performance while increasing the functional and nutritional profile of the eggs. However, it is important to emphasise that their applicability in the livestock sector requires a multidisciplinary approach, evaluating not only the positive effects on animal health but also taking into account limiting factors such as availability, high cost and variability.

Among the various possible alternatives, microbial proteins, in particular microalgae, are strong candidates. In particular, as demonstrated in this thesis, the modulation of the culture medium of *E. gracilis*, cultivated under heterotrophic conditions, led to improvements in the nutritional and functional profile. This result represents a milestone in the formulation of a

product that can be specifically designed for the needs of farm animals. However, the widespread use of plastic bioreactors for microalgae cultivation poses potential health challenges for the user. Indeed, plastics can release micro- and nanoplastics into the culture medium, which can accumulate in the biomass and enter the food chain, potentially impacting animal and human health.

Finally, a possible alternative of recent interest is the production of cellular foods. In this field, cultured meat is certainly the most discussed. Although scientific research has tried to find answers to the main questions concerning the production process and its sustainability, many open issues still remain. Among these, alternatives to foetal bovine serum that can guarantee the growth, proliferation and differentiation of muscle cells through an ethical and sustainable process deserve further and necessary investigation. As demonstrated in this thesis, possible alternatives involve co-products of food (e.g. whey proteins), which can support the viability, proliferation and differentiation of C2C12 murine muscle cells.

Although further studies are needed, all these alternatives represent fundamental steps towards the creation of resilient and environmentally friendly food systems. Despite promising results, further research is needed to standardise these approaches and address unresolved issues, ensuring their feasibility in the food, feed and livestock sectors. The exploration of these alternative proteins is in line with global efforts to create sustainable food systems and mitigate the environmental challenges posed by traditional agricultural practices.

Riassunto

La crescita della popolazione mondiale ha comportato un aumento della domanda di beni di prima necessità, in particolare di prodotti a base di carne e latticini, intensificando ulteriormente la produzione animale, con un conseguente maggiore utilizzo di cereali per l'alimentazione animale e una competizione diretta con le colture destinate al consumo umano. Parallelamente, nel tempo, il settore zootecnico ha esercitato una notevole pressione sulle risorse, con ampie aree di terra arabile dedicate alle colture alimentari e al pascolo, oltre a un elevato consumo di acqua. Ciò evidenzia l'urgenza di intervenire con approcci multidisciplinari. Per questo motivo, gli obiettivi di questa tesi sono stati valutare il potenziale nutrizionale e funzionale dei prodotti vegetali alternativi, tra cui canapa, cardo e camelina, analizzando le loro proprietà antiossidanti, i profili peptidomici bioattivi e il loro impatto sulla qualità dei prodotti di origine animale. Inoltre, si è puntato a ottimizzare il medium di coltura delle microalghe, in particolare per *Euglena gracilis*, al fine di migliorare il profilo funzionale, indagando nel contempo i rischi di contaminazione da nanoplastiche e microplastiche durante la coltivazione in condizioni eterotrofe. Inoltre, la ricerca ha affrontato le principali sfide nella produzione di carne coltivata, sviluppando alternative al siero fetale bovino, metodi di bio-fabbricazione scalabili e l'integrazione di componenti derivati da scarti per supportare la proliferazione e la differenziazione cellulare. Questi obiettivi hanno mirato collettivamente a promuovere pratiche sostenibili, etiche e innovative nel settore agro-alimentare.

I prodotti a base di canapa e i loro co-prodotti hanno dimostrato elevati profili nutrizionali e funzionali, come osservato a seguito della digestione *in vitro* e dei metodi di estrazione con solventi. A supporto di ciò, una prova *in vivo* condotta su galline ovaiole ha mostrato come l'inclusione di un co-prodotto della canapa fino al 9% (foglie, semi di canapa non standard, scafi e steli) abbia migliorato le performance aumentando il profilo funzionale e nutrizionale delle uova. Tuttavia, è fondamentale sottolineare che la loro applicabilità nel settore zootecnico richiede un approccio multidisciplinare, valutando non solo gli effetti positivi sulla salute animale, ma anche tenendo conto di fattori limitanti come la disponibilità, l'alto costo e la variabilità.

Tra le varie possibili alternative, le proteine microbiche, in particolare le microalghe, sono forti candidate. In particolare, come dimostrato in questa tesi, la modulazione del medium di coltura di *E. gracilis*, coltivata in condizioni eterotrofe, ha portato a miglioramenti nel profilo nutrizionale e funzionale. Questo risultato rappresenta una pietra miliare nella formulazione di un prodotto che può essere progettato specificamente per le necessità degli animali da allevamento. Tuttavia, l'uso diffuso di bioreattori in plastica per la coltivazione di microalghe presenta potenziali sfide per la salute dell'utente. Infatti, le plastiche possono rilasciare micro e nanoplastiche nel medium di coltura, che possono accumularsi nella biomassa e entrare nella catena alimentare, impattando potenzialmente sulla salute animale e umana.

Infine, una possibile alternativa di recente interesse è la produzione di prodotti cellulari. In questo campo, la carne coltivata è sicuramente la più discussa. Sebbene la ricerca scientifica abbia cercato di trovare risposte ai principali dubbi riguardanti il processo di produzione e la sua sostenibilità, molte problematiche rimangono ancora irrisolte. Tra queste, le alternative al siero fetale bovino in grado di garantire la crescita, la proliferazione e il differenziamento delle cellule muscolari attraverso un processo etico e sostenibile meritano ulteriori e necessari approfondimenti. Come dimostrato in questa tesi, possibili alternative riguardano i co-prodotti alimentari (ad esempio, le proteine del siero di latte), che possono supportare la vitalità, proliferazione e differenziamento delle cellule muscolari murine C2C12.

Sebbene siano necessari ulteriori studi, tutte queste alternative rappresentano passaggi fondamentali verso la creazione di sistemi alimentari resilienti e rispettosi dell'ambiente. Nonostante i risultati promettenti, è necessario proseguire con la ricerca per standardizzare questi approcci e affrontare le problematiche irrisolte, garantendo la loro fattibilità nei settori alimentare, mangimistico e zootecnico. L'esplorazione di queste proteine alternative è in linea con gli sforzi globali per creare sistemi alimentari sostenibili e mitigare le sfide ambientali poste dalle pratiche agricole tradizionali.

Introduction

1. World population growth: Consequences on the livestock and feed/food sector

The world population has undergone profound and radical changes over the last few centuries. As reported by Roser and Ritchie (2023), today's population is about 2.000 times higher than that recorded 12.000 years ago (approximately 4 million people). While the current global population figure is indeed alarming, what is even more concerning is the rapid pace of this growth. Historical data (Figure 1) illustrates this dramatic increase: in 1800, the world's population was approximately 1 billion; by 1950, it had risen to 2.5 billion; and as of 2023, it reached around 8 billion. Projections indicate that the population could soar to between 9-11 billion by 2050 (Röös et al., 2017; Roser and Ritchie, 2023).

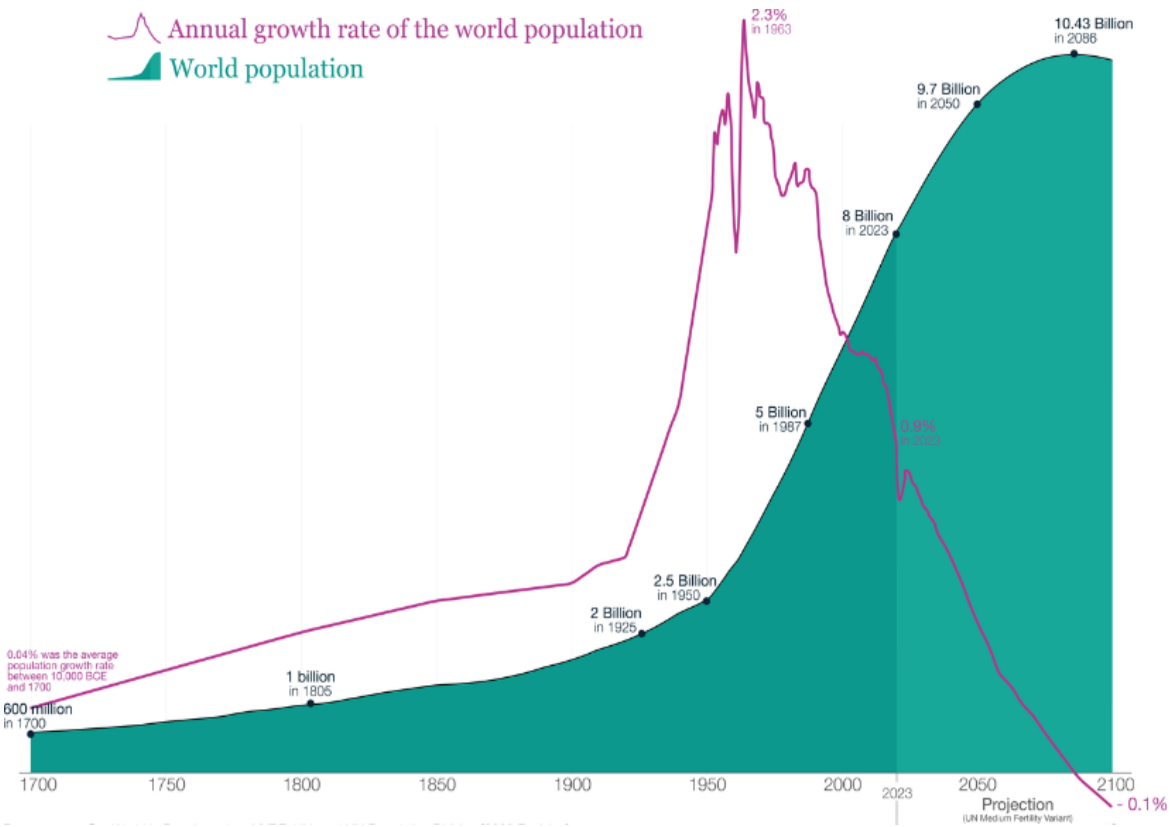

Figure 1. **World population growth from 1700 to 2100** (Roser and Ritchie, 2023).

These high rates brought about substantial changes in society, which had to adapt quickly to support these important transformations. Among these, the main one has certainly been faced by the food sector. As reported in the literature by Bellet and Rushton (2019), such a significant increase in the world population has caused in parallel an ever-increasing demand for food products, reaching 50% by 2030 and doubling by 2050, at which point it will be difficult to meet it without negatively impacting environmental health.

For sure, major products requested are those of animal origin, especially dairy products and meat. Only in 2012, the Food and Agriculture Organisation of the United Nations (FAO) estimated that world demand for meat will reach 455 million tonnes by 2050, an increase of 76% compared to 2005. This trend also applies to fish farming, where the amount of fish from aquaculture has increased from 4.7 to 66.6 million tonnes in just 32 years, and demand is expected to reach 140 million by 2054 (Bellet and Rushton, 2019; Rubio et al., 2020). For this reason, today's livestock system has faced profound changes by undergoing a process of intensification to satisfy an ever-increasing demand. In fact, as reported in Table 1, the livestock population (cattle, sheep, goats, pigs and poultry) is set to increase over the decades until 2050.

Table 1. **Projection of livestock population from 2020 up to 2050 in four decades**. Values are reported in billions (Yitbarek, 2019).

Livestock	2020	2030	2040	2050
Cattle	2.170	2.423	2.593	2.636
Sheep and Goat	2.359	2.566	2.677	2.939
Pigs	1.115	1.121	1.076	1.141
Poultry	24.760	28.819	32.423	37.030

It is easy to understand, how these growths and projections have caused and will continue to cause great pressure on the production of the feed sector, the main driver of livestock industry. Currently, as reported by Makkar (2018), about 800 million tonnes of cereals (about one third of total production) are destined for animal feed, reaching over 1.1 billion tonnes by 2050. Worrying data concern the monogastric sector, where in 2013 alone, it required about 155 million tonnes of feed protein with a projection of 52 million tonnes by 2030. The data just reported reveal an alarming problem: the feed/food competition. This entails significant trade-offs with the production of food for direct human consumption, as the supply of food of animal origin results in significant conversion losses (Makkar, 2018). However, alongside the problem of feed/food competition, the environmental impact of this

sector is causing concern in the scientific community. Globally, the production, processing and transport of feed for the livestock sector accounts for 45% of total greenhouse gas (GHG) production. At the same time, as reported by the same author, the area dedicated to feed cultivation accounts for 33% of the total arable land, while about 30% is occupied by grazing, with water consumption exceeding 90% (Makkar, 2018).

As previously reported, the food sector is directly affected by all these dynamics. More precisely, it is the main cause of resource depletion and negative ecological impact, being responsible not only for high land consumption, but also for the eutrophication of oceans and fresh waters (Vermeulen et al., 2012; Lindgren et al., 2018). At the same time, as reported by Benton et al. (2021) and Dalin and Rodriguez-Iturbe (2016), in the decade (2006-2017), GHGs produced by the food system accounted for 28.9% (20.4-37.3%) of the total global anthropogenic GHGs (52.0 ± 0.45%). More specifically, agriculture and land use accounted for about 4.9 ± 2.5% of all GHGs, ruminant and soil methane for about 4.0 ± 1.2%, fertilisers and manure for about 2.2 ± 0.7%, while transport, manufacturing and cooking accounted for 2.4 ± 4.8% (Benton et al., 2021).

2. European strategies for promoting a clean and circular economy

The above situation has prompted the European Union (EU) to work actively to ensure the long-term prosperity of a safe, green, clean and circular society. The achievement of this goal passes through the application of multiple strategies. Among these, Agenda 2030, One Health, Green Deal and Farm to Fork (F2F) play a major role.

Agenda 2030

The *2030 Agenda for Sustainable Development* is an action programme for people, planet and prosperity, signed in September 2015 by the governments of the 193 member countries of the United Nations. As shown in Figure 2, it consists of 17 Sustainable Development Goals (SDGs), in a broad action programme of 169 targets (United Nations, 2015).

Figure 2. **Sustainable Development Goal of Agenda 2030** (United Nations, 2015).

The official start of the SDGs took place in 2016, guiding the world on the road ahead over the next 15 years, with a target date of 2030 (United Nations, 2015). These goals follow up on the achievements of the Millennium Development Goals that preceded them. Within the food/feed sector, certainly the most prominent and important ones are Goals 2, 12, 13 and 15.

- Goal 2. End hunger, achieve food security, improve nutrition and promote sustainable agriculture

2.1) By 2030, end hunger and ensure that all people, especially the poor and most vulnerable, including infants, have secure access to sufficient nutritious food throughout the year (United Nations, 2015).

2.3) By 2030, double the agricultural productivity and incomes of small-scale food producers, particularly women, indigenous peoples, farm families, pastoralists and fishers, including through secure and equitable access to land, other productive resources and inputs, knowledge, financial services, markets and opportunities for value-added and non-agricultural employment (United Nations, 2015).

2.4) By 2030, ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, help protect ecosystems, enhance resilience to climate change, extreme weather, drought, floods and other disasters, and progressively improve soil quality (United Nations, 2015).

2.5) By 2020, maintain the genetic diversity of seeds, cultivated plants, farm and domestic animals and related wild species, including through diversified and appropriately managed national, regional and international seed and plant banks (United Nation, 2015).

- Goal 12. Ensuring sustainable patterns of production and consumption

12.2) By 2030, achieve sustainable management and efficient use of natural resources (United Nations, 2015).

12.3) By 2030, halve global per capita food waste at retail and consumer levels and reduce food losses during production and supply chains, including post-harvest losses (United Nations, 2015).

12.5) By 2030, substantially reduce waste generation through prevention, reduction, recycling and reuse (United Nation, 2015).

12.a) Support developing countries in strengthening their scientific and technological capacities to achieve more sustainable consumption and production patterns (United Nations, 2015).

❖ Goal 13. Promoting actions, at all levels, to combat climate change

13.1) Strengthen resilience and adaptation to climate-related risks and natural disasters in all countries (United Nations, 2015).

13.2) Integrate climate change measures into national policies, strategies and planning (United Nations, 2015).

One Health and Green Deal

The European One Health strategy represents an integrated and unifying approach designed to sustainably balance and optimise the health of people, animals and ecosystems. More specifically, it recognises that the health of humans, domestic and wild animals, plants and the environment (including ecosystems) is closely linked and interdependent (World Health Organisation, 2023). This approach includes different sectors, disciplines and communities at different levels of society to cooperate together with the aim of promoting well-being and addressing threats to health and ecosystems, while simultaneously responding to the collective need for clean water, energy and air, safe and nutritious food, action on climate change and contributing to sustainable development (World Health Organisation, 2023). Alongside this approach, on 24 June 2021, the European Commission presented the Green Deal, a roadmap designed to reduce GHG emissions by 55% by 2030 and achieve climate neutrality in Europe by 2050. This strategy will enable the EU to move closer to achieving its post-2050 negative emissions target (European Parliament, 2024).

Farm to Fork

Figure 3. Objectives of Farm to Fork strategy (European Commission, 2024).

The F2F strategy (Figure 3), an integral part of the European Green Deal, aims to make food systems fair, healthy and environmentally friendly. More precisely, as previously reported, today's food systems are responsible for almost one third of global GHGs emissions, high consumption of natural resources, causing in parallel loss of biodiversity and negative health impacts (due to both under- and over-nutrition). Furthermore, as described by the European Commission (2024), they do not allow for fair economic returns and livelihoods for all stakeholders, especially primary producers. In light of this, the F2F strategy aims to accelerate the transition to a sustainable food system through an integrated approach, as described below:

- Have a neutral or positive environmental impact;
- Help mitigate climate change by adapting to its impacts;
- Reverse biodiversity loss;
- Ensure food security, nutrition and public health by guaranteeing that everyone has access to sufficient, safe, nutritious and sustainable food;
- Preserving the affordability of food while generating fairer economic returns by promoting the competitiveness of the EU supply sector and fair trade (European Commission, 2024).

3. Alternative sustainable protein matrices for feed and food applications

In light of this, in line with European approaches, it is easy to understand why scientific research, supported by the livestock and feed/ food industry, is investigating new strategies to reduce the impact of the entire food chain. They include the use of plant protein matrices with a high nutritional and functional profile, which are simultaneously characterised by a low environmental impact; the valorisation of food scraps as co-products for livestock; the use of microbial proteins and the exploitation of new technologies for the production of cellular-foods products (Matassa et al., 2016; Chriki and Hocquette, 2020; Bailoni et al., 2021).

3.1 The evaluation of sustainable alternative protein matrices: the case of hemp

3.1.1 Hemp History

Hemp (*Cannabis sativa* L.) is an anemophilous herbaceous plant, the origin of which is still currently debated. As reported in the literature, the origin of hemp is attested in the territories of Asia without, however, identifying the exact region (Fike et al., 2019). The introduction of hemp into Europe, as shown in Figure 4, probably dates back to the second millennium B.C. (Fike et al., 2019). In Italy, hemp arrived in the 1st century B.C., and soon became 90.000 hectares cultivated in the 20th century (Sorrentino, 2021).

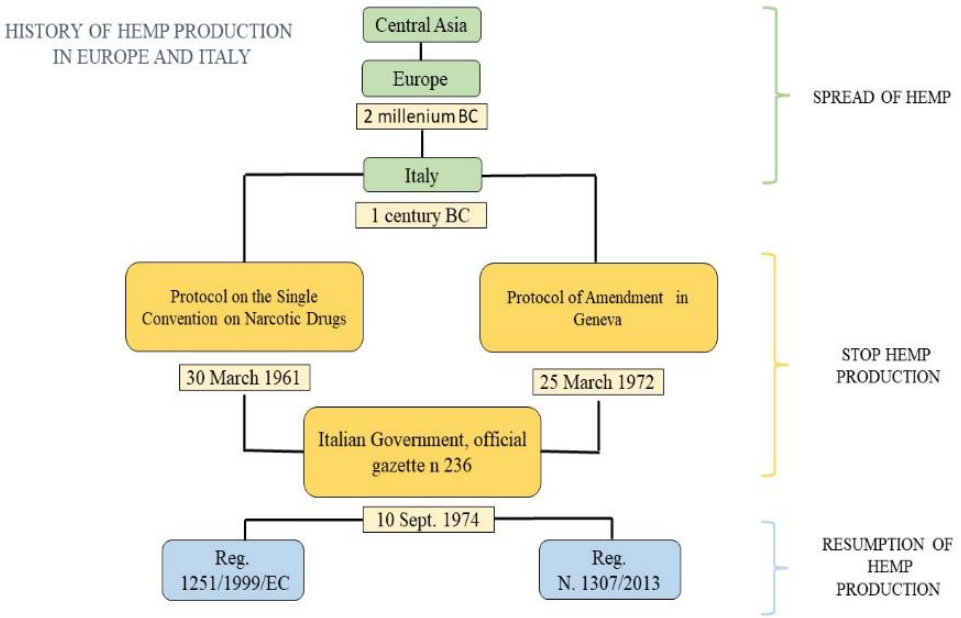

Figure 4. History of hemp production in Europe and Italy.

Despite this rapid expansion, between the 1940s and the end of the 1960s, the hemp trade suffered a sharp decline, disappearing from the market following the introduction of synthetic fibres, but above all due to Italy's adhesion to the Protocol on the Single Convention on Narcotic Drugs proposed by the United Nations, signed in New York on 30 March 1961, and subsequently to the Protocol of Amendment adopted in Geneva on 25 March 1972. The latter amendment was converted into law by the Italian Government with its publication in the Official Gazette n. 236 of 10 September 1974. This convention put an end to hemp cultivation in more than 183 countries (Sorrentino, 2021). However, as reported in Figure 4, at the beginning of the 21st century, at least in Europe, interest in hemp was revived with the introduction of two regulations: **(I)** EU Regulation 1251/1999/EC of 17/05/1999, which established a support system for all producers of particular types of seeds, including industrial hemp, and **(II)** Regulation (EU) 1370/2013, with which hemp cultivation was included among those eligible for Common Agriculture Policy payments (CAP) with the only condition that the seeds used for cultivation be of varieties registered in the European catalogue with a Δ9-tetrahydrocannabinol (THC) content of less than 0.2% (Sorrentino, 2021). Following the enactment of the two regulations, the cultivation of hemp began to spread in the main European countries, particularly France with approximately 18.000 hectares and Italy, the second largest producer, with 4.000 hectares (EIHA, 2018).

3.1.2 Hemp Classification

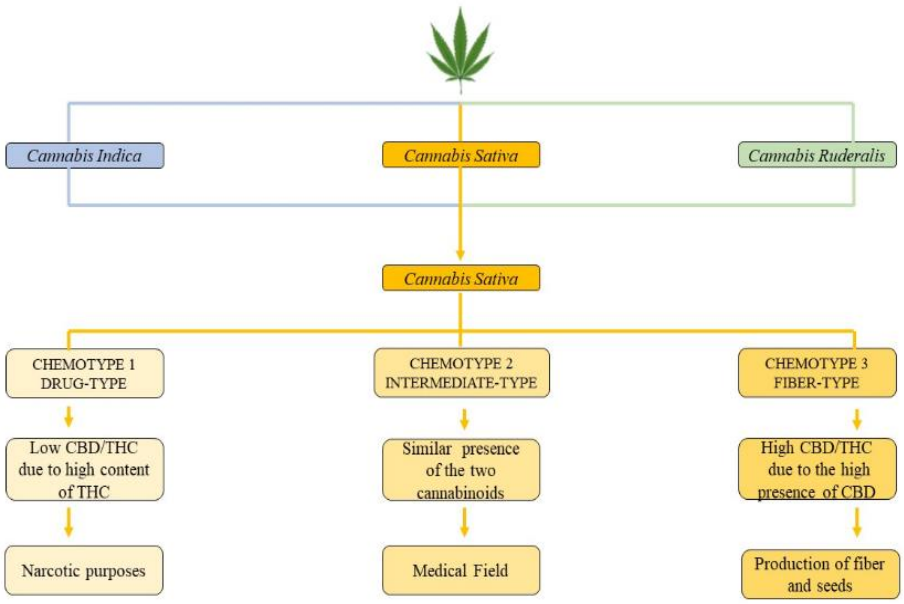

Figure 5. **Order, genus, species and chemotypes of *Cannabis sativa*.**

Hemp belongs to the order *Rosales*, family *Cannabaceae*, and genus *Cannabis*. As reported in Figure 5, this genus in turn comprises three species, *C. sativa*, *C. ruderalis* and *C. indica* (Farinon et al., 2020). To date, it is conventional to consider only one species (*C. sativa* L.), which comprises three subspecies: subsp. *sativa*, subsp. *indica* and, subsp. *ruderalis* (Farinon et al., 2020).

A common trait of all cannabis plants is the presence of secondary compounds, known as cannabinoids, or more precisely phytocannabinoids, produced by female plants at the level of trichomes (flowers). The profile and quantity of phytocannabinoids varies widely. To reflect this variability, it has been suggested that *Cannabis* varieties should be classified according to chemical phenotypes (chemotypes) with distinct cannabinoid profiles, specifically on the basis of the content of THC, known for its psychotropic effects, and cannabidiol (CBD), important for its antioxidant and anti-inflammatory activity (Farinon et al., 2020). More specifically, for the species *C. sativa*, the chemotypes outlined are the following: '*drug type*' (marijuana), '*fibre type*' (hemp) and '*intermediate type*' (medicinal use), according to the cannabinoid profile (Farinon et al., 2020), as shown in Figure 5. Many European countries, as anticipated, including Italy, have recognised and defined a legal and consequently commercial limit of 0.2% for THC. This value substantially defines the difference between hemp and marijuana (Petit et al., 2020).

3.1.3 Hemp Applications

The cultivation of fibre hemp, also known as industrial hemp, is gaining increasing interest. Today, the hemp market comprises more than 25.000 products (Rupasinghe et al., 2020). As reported in Figure 6, the sectors most involved are textiles, clothing, furniture, and energy, thanks to the ability to provide large quantities of cellulose-rich fibre (Rupasinghe et al., 2020; Rehman et al., 2021). In addition, hemp is used in the pharmaceutical and cosmetics industries due to the important medical value of CBD oil, extracted from flowers and characterised by an important functional profile (Farinon et al., 2020). In the last decade, hemp has been attracting a lot of attention in the food and feed industry, especially due to the high nutritional and functional properties of the seeds, previously considered a waste product of its processing (Rupasinghe et al., 2020). In addition to being involved in multiple sectors, hemp plays a key role in environmental protection and conservation. It is able to grow rapidly in various agro-ecological conditions (it can reach four metres in height), which

makes it an excellent candidate for carbon sequestration (nine-thirteen tonnes/ha). Its deep roots (approximately two metres long) prevent soil erosion and also withdraw nutrients and metals from the deeper layers of soil. In the first case, there is greater availability of nutrients; in the second case, hemp acts as a phytoremediator by storing metals in the soil such as nickel, cadmium, lead, copper, and selenium as well as molecules of a toxic-radioactive nature. Moreover, its cultivation does not require large amounts of water or herbicides, and it acts as an antagonist against weeds (Rehman et al., 2021; Rupasinghe et al., 2020).

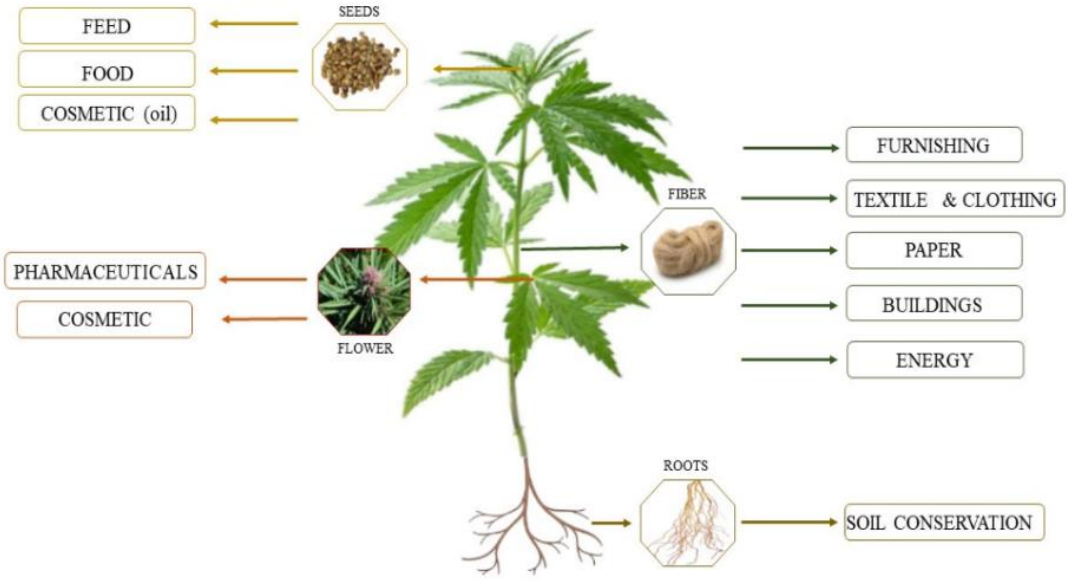

Figure 6. Hemp uses and applications. Figure adapted from Farinon et al. (2020).

3.1.4 Nutritional and Functional profile of hempseeds

As previously reported, hempseed (HS) is the component of the plant mainly used in the food and feed industry due to its important nutritional and functional profile. It is an achene enveloped by a thin and hard pericarp characterised by a high amount of fibre (40-50% neutral detergent fibre (NDF), 30-35% acid detergent fibre (ADF) and 10-15% acid detergent lignin (ADL)); 23-30% proteins easy to digest and rich in essential amino acids (AAs), and 25-35% lipids with balanced fatty acids (Farinon et al., 2020; Fike et al., 2019). However, as reported in the literature, these concentrations depend on the genotype considered, the

environmental growth factors and, most importantly, the type of technological treatment the seed is subjected to (Fike et al., 2019; Leonard et al., 2020).

Fibre Content

The fibrous fraction of HSs accounts for almost the entire carbohydrate intake. More precisely, fibre content is characterised by a soluble:insoluble fibre ratio of 20:80, a similar value to that of other food/feed sources, such as flaxseeds (FXs), widely used in the livestock sector (Callaway, 2004; Farinon et al., 2020). However, due to treatments such as heat treatment and extrusion, it is possible to modify these values, making them more appreciable for food and feed applications (Amaducci et al., 2008).

Most of the phenolic compounds characteristic of HSs are located at the level of the fibrous fraction, particularly at the level of the hull. The main compounds identified within the seeds are lignans, phenols derived from shikimic acid biosynthetic pathways, also known as phenylpropionamides. These compounds belong to two main groups called phenolic amides and lignanamides (Farinon et al., 2020). Lignanamides are the main compounds found within HSs and include cannabisin B and N-trans-caffeoyltyramine, the main compounds in the hull fraction; catechin in the cotyledonary fraction; and cannabis A, F, I, Q, and grossamide (Farinon et al., 2020). However, fibre plays a direct role in the bioavailability of these phenolic compounds. Indeed, it can physically trap phenols and antioxidant compounds within a matrix by chemically interacting through hydrophobic interactions, hydrogen bonding (oxygen atoms from polysaccharide's glycosidic chains and hydroxyl groups from phenolic compounds), and covalent bonds. Thus, phenols reach the gut, particularly the colon where they can exert a beneficial effects (Leonard et al., 2020).

Protein Content

In HSs, the proteins are mostly located in the inner layer of the seed, with a low quantity in the hull. More precisely, 181 proteins have been identified and the most common are edestin, albumin, and β-conglycinin (Farinon et al., 2020). Edestin accounts for approximately 60-80% of the total protein content, albumin accounts for approximately 25%, and albumin accounts for approximately 5% (Farinon et al., 2020; Odani and Odani, 1998). Hempseeds provide a qualitatively and quantitatively high AA profile, enriched by the presence of essential AAs, resulting in a high biological value, although lysine, cysteine and tryptophan are limiting AAs, as reported in Table 2. Nevertheless, as described by Wang and Xiong

(2019) and Callaway (2004), the composition of AA is comparable to that of other high-quality proteins, such as soybean and rapeseed.

Table 2. **Amino acids composition of hempseed (25%), soybean (32%) and rapeseed (23%)**. Values are expressed in g/100. Table adapted by Callaway (2004).

Amino acids	Hempseed (25%)	Soybean (32%)	Rapeseed (23%)
Alanine	1.28	1.39	1.05
Arginine	3.10	2.14	1.49
Aspartic acid	2.78	3.62	1.82
Cystine	0.41	0.54	0.39
Glutamic acid	4.57	5.89	4.41
Glycine	1.14	1.29	1.28
Histidine	0.71	0.76	0.72
Isoleucine	0.98	1.62	1.00
Leucine	1.72	2.58	1.80
Lysine	1.03	1.73	1.49
Methionine	0.58	0.53	0.46
Phenylalanine	1.17	1.78	1.05
Proline	1.15	1.65	1.59
Serine	1.27	1.54	1.10
Threonine	0.88	1.35	1.13
Tryptophan	0.20	0.41	0.31
Tyrosine	0.86	1.14	0.69
Valine	1.28	1.60	1.26

In addition to AA composition, protein digestibility and bioavailability are also key parameters in determining the nutritional value of a protein. The high digestibility of HSs proteins was demonstrated by Mamone et al. (2019). The authors observed that, following *in vitro* digestion, only a few peptides resisted enzymatic digestion. Indeed, digestion of HSs proteins results in the release of peptides with a molecular weight below 10 kDa, which are thus able to escape enzymatic degradation within the gastrointestinal tract, being absorbed and introduced into the bloodstream (Farinon et al., 2020). In addition to being bioavailable, these peptides are characterised by high bioactivity, which includes antioxidant, antihypertensive, antiproliferative, hypocholestorolemic, anti-inflammatory and neuroprotective effects (Farinon et al., 2020). Another fundamental parameter to consider concerns allergenicity. Hempseeds are considered food products with a low allergen content. As reported by Decuyper et al. (2017), only five allergens have been identified in hemp plants. Only the RuBisCo protein in HSs. However, as reported by Mamone et al. (2019) this allergenic protein is completely degraded following the digestive process. In fact, as

observed by the authors, no protein fragments were present following gastrointestinal digestion.

Lipid Content

Table 3. **Fatty acid profile of hempseeds**. Values are expressed in % of oil. When several cultivars were analysed , the maximum and minimum values of the range of data obtained are indicated. When only one cultivar was analysed, the mean ± standard deviation is reported. SFAs: Saturated fatty acids; MUFAs: Monounsaturated fatty acids; PUFAs: Polyunsaturated fatty acids. Table adapted by Farinon et al. (2020).

SFAs	MUFAs	PUFAs	n-6/n-3	REF
n.a.	n.a.	84.0	2.5	Callaway (2004)
10.9±0.5	13.1±1.3	75.1±3.7	4	Siano et al. (2018)
10.9±0.7	17.5±6.3	72±4.3	3.2	Vecka et al. (2019)
9.6-10.3	n.a.	n.a.	n.a.	Vonapartis et al. (2015)
9.4-11.7	9.7-16.1	72.2-80.7	2.8-4.5	Galasso et al. (2015)
n.a.	n.a.	n.a.	3.9-5.5	Irakli et al. (2019)
n.a.	n.a.	72.0-78.6	3.2-5.0	Lan et al. (2019)

Over the past decade, the lipid content of HSs has attracted the attention of the feed and livestock industry due to its attractive nutritional profile, as shown in Table 3. More specifically, it is characterised by a low content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). Of these, the one most present is oleic acid (18:1, n-9), the amount of which (11.90 ± 0.35%) is highly comparable to that of other matrices used in the food and feed industry, such as FXs and chia seeds (Monserrat-de la Paz, 2014). At the same time, it has a high content of polyunsaturated fatty acids (PUFAs). Among the PUFAs, those most present are linoleic acid (18:2, n-6) (55.05 ± 0.47%) and α-linoleic acid (18:3, n-3) (16.70 ± 0.08%) (Farinon et al., 2020). In addition to being essential fatty acids, as they are introduced exclusively through the diet, they are precursors of long-chain PUFAs, including arachidonic acid (20:4, n-6), docosahexaenoic acid (22:6, n-3) and eicosapentaenoic acid (20: 5, n-3), which play key roles in brain development, cardiovascular health, obesity and the regulation of metabolic and inflammatory processes through the synthesis of prostaglandins and leukotrienes (Farinon et al., 2020). As shown in Table 3, interesting results are reported for n-6/n-3. Indeed, as described by EFSA (2009), values around 3.5, typical of the mediterranean diet, are responsible for maintaining health and physical well-being.

At the same time, HSs oil is also characterised by an interesting functional value, mainly due to its unsaponifiable matter. Although it accounts for less than 2% (1.8-1.9%) of the total oil

content, it is rich in antioxidant compounds, particularly tocopherols and sterols (Farinon et al., 2020; Leonard et al., 2020). Tocopherols are fat-soluble compounds characterised by marked antioxidant activity due to their ability to scavenge free radicals. They are divided into four classes, α-, β-, γ- and δ-tocopherol. In HSs, the predominant compound is γ-tocopherol (21.68 mg/100 g), followed by α- (1.82 mg/100 g), δ- and β-tocopherol (Skřivan et al., 2019). According to Skřivan et al. (2019), γ-tocopherol is easily absorbed and is able to accumulate within tissues where it plays a key role in inflammatory processes related to cyclooxygenase activity inhibition and its unsubstituted C-5 position appears to make it better able to capture lipophilic electrophiles, such as reactive nitrogen oxide species. γ-tocopherol is somewhat less potent in donating electrons than α-tocopherol. Therefore, the latter is generally considered to be more potent than γ-tocopherol as a chain-breaking antioxidant for inhibiting lipid peroxidation (Skřivan et al., 2019).

In addition to tocopherols, phytosterols are also part of the unsaponifiable matter. They are fat-soluble compounds that cannot be synthesised by humans or animals but only by plants. They have a structure similar to that of cholesterol. Due to this peculiarity, when ingested, phytosterols are able to reduce the solubility of cholesterol and compete with the absorption of free cholesterol, playing a key role in the prevention of diabetes and cardiovascular disease (Farinon et al., 2020; Leonard et al., 2020).

Hempseed oil is also rich in phenolic compounds, which contribute to the high oxidative stability. In particular, Smeriglio et al. (2016) reported that the oil contains a high content of polyphenols, particularly flavonoids such as flavones, flavonols, flavanols, and isoflavones, known for their potent antioxidant activity. More specifically naringenin, kaepferol-3-O-glucoside, epicatechin, and daidzein. Phenols also contribute and enhance this activity; these include dihydroxybenzoic acid, caffeoyl-tartaric acid isomers, p-cumaric acid, N-caffeoyltyramine, and N-feruoyltyramine (Smeriglio et al., 2016).

3.1.5 Limiting factors in the widespread use of hemp-based products in the food and feed sector

As described above, HSs are distinguished by a high nutritional and functional profile. Nevertheless, their characterisation, which is crucial for future applications in the feed and livestock sector, is still at an early stage, especially in Europe. This is mainly caused by stringent European regulations. More precisely, to date, the EU, through Regulation

2022/1104 of 1 July 2022, has only registered HSs, HS cake, HS oil, hemp flour and hemp fibre (both originating from stems) within the European catalogue of feed material (European Union, 2022), excluding, as will be described below, all other possible hemp co-products. This has caused a slow spread of hemp in Europe, compared to other countries. As argued by Muzyczek (2020), in fact, the main producers are China, South Korea, Russia, the United States and Canada. Only 25% of the global market is concentrated in Europe with France being the main producer, as shown in Figure 7.

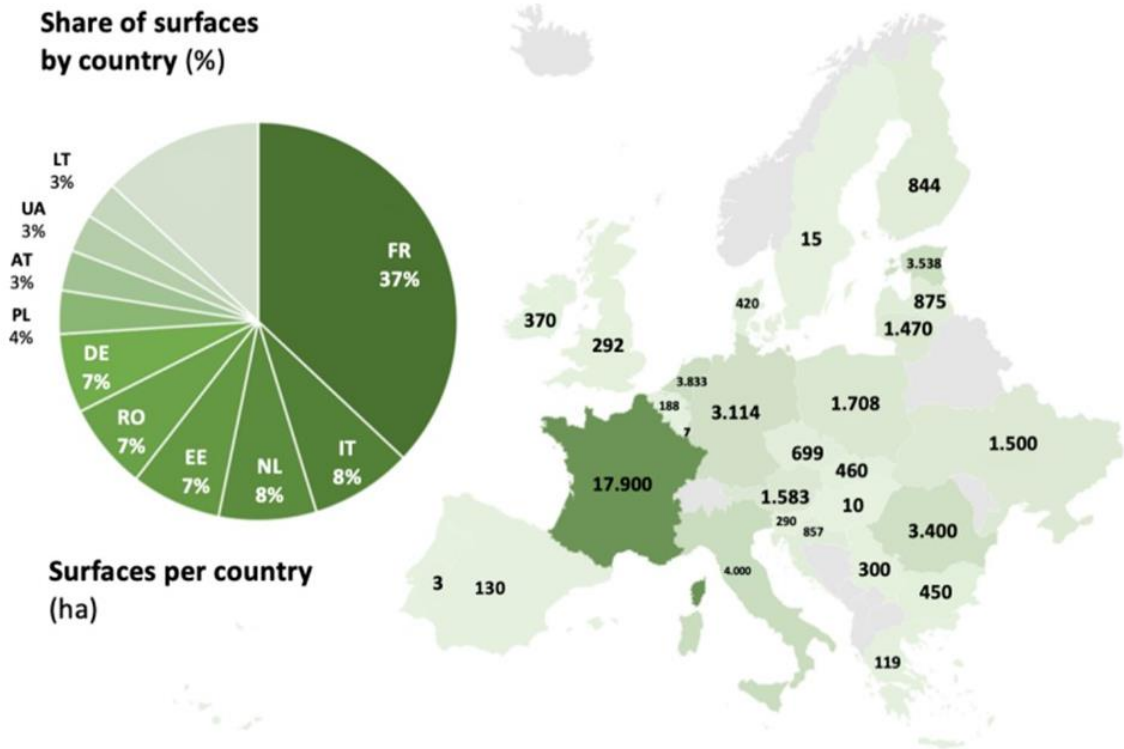

Figure 7. **European spread of hemp**. Results are reported in hectares (ha). Figure adapted from EIHA (2018).

This limited diffusion is the main cause of the elevated cost of HSs (2 euro/kg); much higher when compared to that of soybeans (0.5 euro/kg), the main protein source used for livestock sector, which does not allow, in conjunction with European regulations, an easy landing of hemp on the feed market. For this reason, it is necessary for scientific research to continue investigating this alternative matrix to ensure its possible application on a large scale.

3.2 The evaluation of sustainable alternative protein matrices: the case of co-products

The increase in food waste has been a major problem in recent decades. More precisely, as highlighted by FAO (2011), globally about 1.3 billion tonnes of food are wasted or lost every year. In this scenario, as previously reported, the European Commission initiated the F2F strategy with the ultimate goal of reducing losses along the food chain and ensuring the sustainability of food's production, processing and consumption (Vastolo et al., 2022). To achieve this, one possible approach could be the valorisation of food chain scraps as co-products of the livestock sector (Rakita et al., 2021). More precisely, as described by Pinotti et al. (2020), the term *co-product* refers to those products obtained through different agro-industrial processes, including the production of oil, protein extracts, sugar, fruit juices, and frozen vegetables. This strategy has multiple advantages. Primarily, it allows moving away from the linear economy and towards the circular one, while at the same time improving economic and environmental sustainability. This economy aims to develop a more efficient system that ensures a reduction in the use of natural resources and waste products (Toop et al., 2017). In addition, it guarantees a reduction in feed costs, an added value to animal products and an improvement in animal health (Correddu et al., 2020). To date, multiple co-products have been included in the animal diet, both fruit and vegetable scraps. Among the former, as shown in Table 4, those most investigated and with an interesting nutritional profile are co-products of apple, grape marcs and pomegranate (Vastolo et al., 2022).

Table 4. **Chemical composition of fruit co-products (% DM) (apple, grape and pomegranate)**. Data are reported as mean value. DM: dry matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; EE: ether extract; n.a.: not available. Table adapted from Vastolo et al. (2022).

Fruit	Co-product	CP	NDF	ADF	ADL	EE	Ash
Apple	Dried-pomace	5.10	67.2	46.0	15.0	6.00	n.a.
	Dried-pomace	6.74	44.2	35.4	13.4	3.71	n.a.
	Dried-pomace	2.65	34.0	23.8	n.a.	n.a.	1.75
	Fresh-pomace	5.60	45.3	38.0	n.a.	4.70	n.a.
	Fresh-pomace	7.20	43.3	32.3	n.a.	2.90	3.00
Grape	Fresh-residues	12.7	n.a.	n.a.	10.6	4.36	n.a.
	Dried-pomace	9.93	46.7	41.1	n.a.	5.71	6.69
	Dried-pomace	12.8	n.a.	50.2	n.a.	5.50	6.89
	Dried-pomace	12.4	38.8	37.5	n.a.	4.02	11.8
	Dried-pomace	9.40	28.4	25.0	n.a.	n.a.	n.a.
Pomegranate	Seed	15.4	68.0	49.0	n.a.	0.60	2.40
	Peel	3.6	28.0	15.1	n.a.	0.60	5.40
	Peel	8.4	n.a.	n.a.	n.a.	0.40	4.30
	Pomace	9.2	35.3	30.6	12.3	6.3	3.20
	Seed cake	14.9	71.6	50.5	10.9	0.95	4.10

The use of fruit co-products in the diet of livestock animals has to take the chemical composition into consideration. As reported by Mirzaei-aghsaghali et al. (2011), Chedea et al. (2017), Steyn et al. (2018), Atalay (2020) and Vastolo et al. (2022), grape, apple and pomegranate processing residues could be considered as structured carbohydrate sources with high amounts of neutral detergent fibres and low acid detergent lignin content. At the same time, as shown in Table 5, co-products of vegetable origin have been more widely used in the feed and livestock industry.

Table 5. Chemical composition of vegetables co-products (% DM) (tomato, pepper,, broccoli, cardoon, pumpkin, beet, canola). Data are reported as mean value. DM: dry matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; EE: ether extract; n.a.: not available. Table adapted from Vastolo et al. (2022) and Singh et al. (2023).

Vegetable	Co-product	CP	NDF	ADF	ADL	EE	Ash
Tomato	Dried-pulp	19.0	55.7	42.7	26.0	5.10	n.a.
	Dried-pomace	13.7	n.a.	n.a.	n.a.	0.90	11.1
	Dried-pomace	22.2	49.2	32.6	n.a.	15.0	n.a.
	Dried-pomace	18.9	45.2	13.4	5.30	4.14	7.56
	Fresh-residues	21.1	n.a.	n.a.	n.a.	13.2	3.38
Pepper	Core	19.2	31.1	22.2	5.50	6.70	n.a.
	Skin	9.90	75.3	64.1	38.3	3.30	5.10
	Fresh-residues	18.8	n.a.	n.a.	n.a.	8.18	n.a.
Broccoli	Fresh chaffed	27.20	24.3	21.9	3.30	5.12	5.78
Cardoon	Cardoon cake	21.1	46.8	36.0	6.43	7.72	5.57
Camelina	Camelina cake	35.2	38.5	20.3	n.a.	13.7	5.90
Pumpkin	Cake	19.9	41.1	32.6	10.7	14.0	6.57
Beet	Pulp	27.4	13.5	4.00	1.50	11.1	5.20
Canola	Meal	50.7	13.9	39.6	5.82	13.5	5.03
	Hulls	2.28	63.1	8.20	0.80	2.20	n.a.

However, co-products of new matrices such as hemp are emerging in recent years, due to their interesting nutritional and functional profile (Bailoni et al., 2021; Vastolo et al., 2022). As shown in Table 6, hemp co-products are characterised by a high level of fibre. As described by Kleinhenz et al. (2020), these matrices are poor sources of energy, suggesting a different use as a source of dietary fibre, especially in ruminants. In parallel, they have an interesting protein content, especially leaves, chaffs, HS cakes and hulls. However, European regulations (Regulation 2022/1104, European Union, 2022), as previously reported, limit their use in the feed and livestock sector, showing the need for further scientific research into their use in animal diets.

Table 6. Chemical composition of hemp co-products (Plant, leaves, stalks, chaffs, hempseeds cake, Extracted flowers, hulls). Values are expressed in % DM (Kleinhenz et al., 2020; Vastolo et al., 2022; Ely and Fike, 2022). CP: crude protein; ADF: acid detergent fibre; NDF: neutral detergent fibre; EX: extracted.

	Plant	Leaves	Stalks	Chaffs	HS cake	Ex. flowers	Hulls
CP	6.9	13.0	5.3	21.2	30.0-34.0	24.5	21.9
FAT	2.7	8.9	1.2	4.6	10.2-12.4	3.2	23.5
ADF	60.8	20.8	64.6	18.0	32.1-39.5	18.1	37.9
NDF	81.6	44.7	84.4	27.9	39.3-51.9	30.9	57.1

Although, co-products represent a valid strategy to pursue the achievement of a clean and circular economy, it must be emphasised that there are limitations in their application. Mainly, they present much variability in their composition, due to both the genotype considered, environmental factors, harvesting techniques and processing methods to which they are subjected (. At the same time, as reported by Salami et al. (2019) and Vastolo et al. (2022), they require preservation treatments that are essential for product stabilisation, particularly for co-products with high moisture and lipid values. All these constitute limiting factors for the large-scale application of co-products, prompting scientific research to further their characterisation to favour their use.

3.3 The evaluation of sustainable alternative protein matrices: the case of microalgae

Although the use of microorganisms for fermented foods dates back to early ancestors, the use of microorganisms as a direct food source has received attention recently. They, identified under the term *microbial proteins* (MPs), play a primary role in the transition to a more sustainable food production system, prompting scientific research to reconsider the current status of their use as an alternative to traditional and conventional food and feed sources (Ciani et al., 2021). Microbial proteins are characterised by a high protein content, the presence of all essential AAs, vitamins, minerals and other nutritionally essential substances. Within this group, as reported by Matassa et al. (2016), are microalgae, yeasts, bacteria and mycoproteins. This production system has multiple advantages over traditional open-field plant cultivation and today's farming system due to the efficient use of nutrients, stable growing conditions, low environmental impact, and the absence of pesticides and antibiotics (Ciani et al., 2021). As argued by Ciani et al. (2021), one of the main positive aspects associated with MPs is the efficient use of nitrogen and phosphorous, both of which are fully converted into consumable protein. This aspect differs from traditional agriculture, where the production of plant proteins only partially utilises nitrogen and phosphorous, dispersing them into the environment and consequently causing contamination of groundwater, eutrophication of surface waters, acidification of the oceans and GHGs emissions. At the same time, as previously mentioned, MPs are characterised by an interesting nutritional profile, otherwise comparable with that of soybean meal, one of the main matrices used in the feed industry (Table 7).

Table 7. **Average composition of protein obtained from different sources**. Table adapted from Alves et al. (2023); Banaszkiewicz, (2011). Range of values are due to the type of substrate, culture conditions and microorganism used. n.a.: not available. Values are expressed in %.

	Algae	Fungi/yeasts	Bacteria	Soybean meal
Protein	40-60	30-70	50-83	43.8-49.9
Total N	45-65	35-50	60-80	40-50
Lysine	4.6-7.0	6.7-7.8	4.3-5.8	2.99-3.22
Methionine	1.4-2.6	1.5-1.8	2.2-3.0	0.6-0.69
Fats/Lipids	5-10	5-13	8-10	0.55-3.0
Carbohydrate	9	n.a.	n.a.	33
Nucleic acids	4-6	9.70	15-16	n.a.
Mineral salts	7	6.6	8.6	n.a.
Amino acids	n.a.	54	65	21.9-24.9
Ash	3	n.a.	n.a.	5.6-7.2
Moisture	6.0	4.5-6.0	2.8	10.5-12.6
Fiber	3	n.a.	n.a.	4.3-7.2

For this reason, the food industry has increasingly investigated their application as an alternative source to traditional matrices, consequently expanding their market, as shown in Table 8.

Table 8. **Overview of production volumes and market size for different microbial proteins**. n.a. not available. Table adapted by Matassa et al. (2016).

Organism	Volume (ton DM/y)	Cost (eur/kg DM)	Global market (Billion Euro)	Yearly growth (% per year)
Yeast	3.000.000	n.a.	9.2	7.9
Microalgae	9.000	4-25	2.4	10
Mycoprotein	25.000	n.a.	0.214	20
Bacteria[1]	80.000	n.a.	n.a.	n.a.
Bacteria[2]	5000	n.a.	n.a.	n.a.

[1]Proflor ®; [2]Feedkin ®

Among the MPs, although microalgae are the most widely consumed resource, scientific research is increasingly investing in their use in the food/feed sector due to their important nutritional composition, as shown in Table 9.

Table 9. **Nutrient composition of different microalgae species** (Kusmayadi et al., 2021). Values are reported in %.

Microalgae	Composition (%)		
	Lipids	Protein	Carbohydrates
Botryococcus braunii	33	39.61	2.38
Chlorella vulgaris	14-22	51-58	12-17
Haematococcus pluvialis	15	48	27
Isochrysis galbana	12-14	50-56	10-17
Nannochloropsis sp.	22-31	33-44	8-14
Porphyridium cruentum	5.78-7.55	27.7-40.8	22.8-39.3
Scenedesmus quadricauda	1.9	47	21-52
Spirulina maxima	6-7	60-71	13-16
Synechococcus sp.	11	63	15
Tetraselmi smaculata	3	52	15

Lipid Content

The lipid content of the microalgae, as shown in Table 9, is highly variable depending on the strain considered. However, the lipid profile is enriched by the high presence of n-3 and n-6 PUFAs (Kusmayadi et al., 2021). In particular, as reported by Pulz and Gross (2004) and Choopani et al. (2016), they are characterised by the presence of α-linoleic acid (18:3, n-3), γ-linoleic acid (18:3, n-6), linoleic acid (18:2, n-6) and arachidonic acid, which are crucial in health defence mechanisms, including cellular and tissue healing and repair of wounds.

At the same time, they are characterised by the high presence of docosahexaenoic (22:6, n-3) and eicosapentaenoic acid (20:5, n-3), long-chain fatty acids, which are essential for the improvement of brain function, proper foetal development, and the prevention of inflammation, the management of cardiovascular diseases such as arrhythmia, hypertension and stroke, due to their ability to reduce oxidative stress and plasma triglycerides (Kusmayadi et al., 2021).

Protein Content

As previously described, since the late 1950s, microalgae have been consumed due to their high protein content. Proteins in microalgae are characterised by the presence of all essential AAs (leucine, isoleucine, methionine, lysine, threonine, valine and histidine), a profile that is comparable to that of other food sources of animal origin. At the same time, microalgae contain non-essential AAs (arginine, proline, glutamic acid, cysteine, serine, tyrosine and glutamic acid) that can provide important health benefits, including regulation of gene expression, cellular responses and antioxidant activity (Kusmayadi et al., 2021).

Carbohydrates Content

Microalgae are recognised to be a good source of carbohydrates, which are essential as structural components of cell walls and as an energy reserve (Markou et al., 2012). They are present in the cytosol and chloroplast of microalgae in the form of cellulose, sugars, starch and other polysaccharides (Kusmayadi et al., 2021). Certainly, among these, it is important to mention the paramylon. The latter is a high-molecular-weight polysaccharide (β-1,3-glucan), found exclusively in euglenoids and more specifically in *Euglena* species, whose role is to store energy and carbon (Gissibl et al., 2019). In particular, paramylon is characterised by immunomodulatory properties (Skov et al., 2012), finding its own market space in the nutraceutical sector, especially after Food and Drug Administration (FDA) released the certification as a food additive in 2017 (GRAS Notice No. 513, FDA, 2014). In general, the content of carbohydrates depends on the algal species considered, in particular on the photoconversion efficiency, reaching up to 50% of the total dry weight of carbohydrates, amount modulated by the availability of light, temperature, nutrients and saline concentration, during the cultivation period (Markou et al., 2012). In view of the above, it is important to choose algae species with high carbohydrate productivity and adequate sugar composition for the food and feed sector.

Vitamins and pigments

The nutritional profile of the microalgae is enriched by the presence of vitamins, the most important of which are vitamins A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid, biotin, pantothenic acid, thiamine, riboflavin, pyridoxine and cobalamin, which are essential for their positive effect on animal and human health (Villaruel-Lopez et al., 2017). At the same time, as reported by Villaruel-Lopez et al. (2017), microalgae are able to produce high amounts of pigments, classified as carotenoids (orange colouration), xanthophylls (yellowish colouration), phycobilin (red to blue colouration) and chlorophylls (green colouration). Carotenoids, the main pigments of microalgae, can be used as antioxidant molecules by acting as direct quenchers of reactive oxygen species, thereby protecting cells and tissues from oxidative damage (de Mello-Samapayo et al., 2013). Xanthophylls are the oxidation products of carotenes. Phycobilins (phycocyanin and phycoerythrin) are found in the stroma of the chloroplasts of Cyanobacteria, and are often used as food colourants (Koller et al., 2014). Finally, chlorophylls are divided into two isoforms (A and B). Of these, chlorophyll A is the main photochemical pigment with the important role of a light receiver, actively involved in photosynthesis (Villaruel-Lopez et al., 2017). As reported by the same authors, chlorophyll can be used also as pharmaceutical product due to its curative activity and its chemical structure, similar to that of haemoglobin, as well as its ability to stimulate tissue growth through the rapid exchange of carbon dioxide and oxygen.

Although the nutritional and functional profiles of microalgae are highly interesting, as described by Kusmayadi et al. (2021), they are easily modulated, mainly by culture conditions such as pH value, light intensity, nutrients supplied, temperature and CO_2 supply. For this reason, it is easy to understand why scientific research is investigating new strategies and approaches to modify growing media to provide the food and feed sector with more nutritious and healthy products.

3.4 The evaluation of sustainable alternative protein matrices: the case of cultured meat

Animal products (meat, milk, eggs and seafood) have been an integral part of people's diets and a strong cultural identity for millions of years (Wood et al., 2024). Although their high consumption has been linked to an increasing incidence of chronic diseases since the 1950s, global production in the last 50 years (1961-2020) has increased dramatically (Table 10).

Table 10. Consumption (kg/person/yr) of animal derived products for Word, Europe, China, North America and Africa in 1961 and 2020. Table adapted by FAO (2021).

	World		Europe		China		N. America		Africa	
	1961	**2020**	**1961**	**2020**	**1961**	**2020**	**1961**	**2020**	**1961**	**2020**
Meat	22.92	42.26	47.24	75.82	3.35	61.89	74.24	100.72	13.32	16.46
Milk	75.04	70.19	171.2	182.3	2.37	25.02	220.80	169.13	29.96	27.22
Fish	8.96	20.25	13.85	21.77	4.33	11.33	11.33	18.28	4.57	9.58
Eggs	4.52	10.33	8.96	13.90	2.06	14.44	14.44	15.78	1.24	2.14

In particular, meat and related products by 403% to 352 Mt, milk and dairy products by 167% to 918 Mt, fish and seafood by 355% to 176 Mt, and eggs by 513% to 92 Mt (FAO, 2021). As reported in Table 10, low- and middle-income countries, particularly in Asia, experienced the highest growth, driven by the increase in population and per capita consumption (Wood et al., 2024). As previously anticipated, the food chain (from farm to retail), is responsible for a high environmental impact, especially if GHGs emissions are taken into account, as shown in Table 11.

Table 11. **Estimates of greenhouse gas emission (Kg CO_2 equivalents) from production to retail of some agricultural products**. Table adapted by Wood et al. (2024).

Animals		**Plants**	
Per kg products			
Beef (beef herd)	99.5	Wheat	1.6
Beef (dairy herd)	33.3	Tomatoes	2.1
Pig meat	12.3	Peas	1.0
Poutry meat	9.9	Bananas	0.9
Milk	3.2	Other vegetables	0.5
Cheese	23.9	Potatoes	0.5
Farmed fish	13.6	Roat vegetables	0.4
Eggs	4.7	Nuts	0.4

Specifically, meat, dairy products, fish and eggs contribute about 56% of all emissions, with the farming phase dominating production, while subsequent activities contribute only a small part (Wood et al., 2024). In particular, Table 11, based on data collected from 38.700 farms in 119 different countries, shows that products of animal origin are associated with higher

emissions than those of plant source. Specifically, the highest emissions come from ruminants, where the main source is methane from enteric fermentation and manure processing (Wood et al., 2024). This represents a critical point, especially considering that the amount of methane in the atmosphere has increased over the last ten years and, although it has a shorter accumulation period than CO_2, it has an approximately 30-fold climate effect over a 100-year period (Wood et al., 2024). These data are also evident when considering the environmental impact (CO_2, N_2O and methane emissions) of different types of diets, as presented in Figure 8.

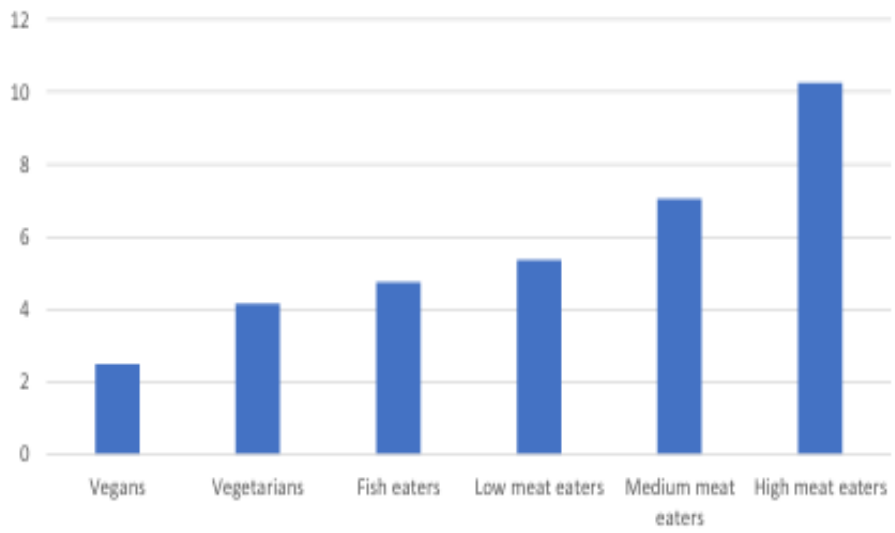

Figure 8. **GHGs (GWP $_{100}$ kg CO_2 equivalents/day) associated with different typologies of diets** (Wood et al., 2024).

In light of the above, the rationale behind the call for a reduction and subsequent consumption of animal products in high-income countries is evident. However, this clashes with the projections of low-to-middle income countries where consumption will increase in the coming decades (Wood et al., 2024). This rise is due both to an improvement in incomes, as the Western diet is a symbol of prosperity and economic growth, and also because animals are a key component of earnings in these realities and consequently of social inclusiveness (Wood et al., 2024; Parlasca and Qaim, 2022). Furthermore, as reported by Adesogan et al. (2020), it is important to emphasise that plant products in these regions are deficient in key body nutrients such as iron, zinc and vitamin B12, which is highly present in animal products. To date, as reported in this first part of the thesis, there are many solutions offered

by the scientific community to reduce the environmental impact of today's food industry. Among these, *cellular agriculture* would be an excellent candidate, however still subject to discussion and debate. Cellular agriculture involves the production of food products (meat, fish, dairy products) from individual cells/tissue samples rather than whole organisms, such as animals, with the ultimate goal of producing products that are molecularly and genetically similar to those obtained by traditional techniques (Eibl et al., 2021). However, as shown in Figure 9, the production processes are significantly different. This aspect is of particular importance when considering the production time: 18 months for traditional meat and around 3-4 weeks for cultured meat.

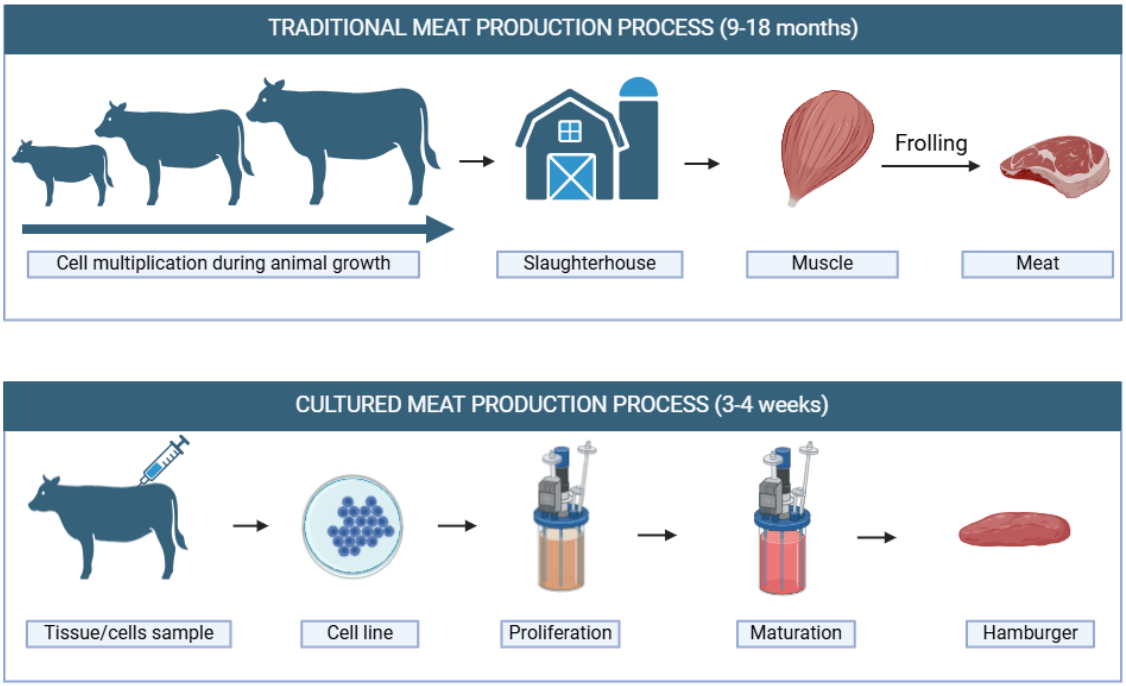

Figure 9. **Comparison between traditional meat production and cultured meat production**. Figure modified by Ding et al. (2021). Figure created with BioRender.com.

Of the major products, cultured meat is certainly the one that is best known and the source of debate today. Although it is of recent interest, the initial idea has ancient roots. More precisely, as early as 1931, Winston Churchill criticised farming systems by introducing an early form of the cultured meat concept, although still premature: *"We shall escape the absurdity of growing a whole chicken in order to eat its breast or wing, by growing these parts separately in suitable soil. In the future, of course, synthetic food will also be used"* (Churchill, 1032; Ford, 2011). However, the development of this new product did not garner

the interest of the scientific community until the late 20th century. Starting in these years, first with the first patent on the cultured meat process produced by Van Eelen et al. (1999), and later thanks to the National Aeronautics and Space Administration's (NASA) enterprise in culturing goldfish meat, cultured meat began to meet with great interest (Benjaminson et al., 2002).

The popularity of cultured meat, however, was only consolidated in 2013 with the live television presentation of the first synthetic hamburger by Dutch researcher Post (2014). From 2013 onwards, as reported in Figure 10, by Chriki at al. (2020), the number of scientific publications on cellular agriculture started to increase exponentially until the first cultured meat products were commercialised in December 2020 in Singapore (Treich, 2021).

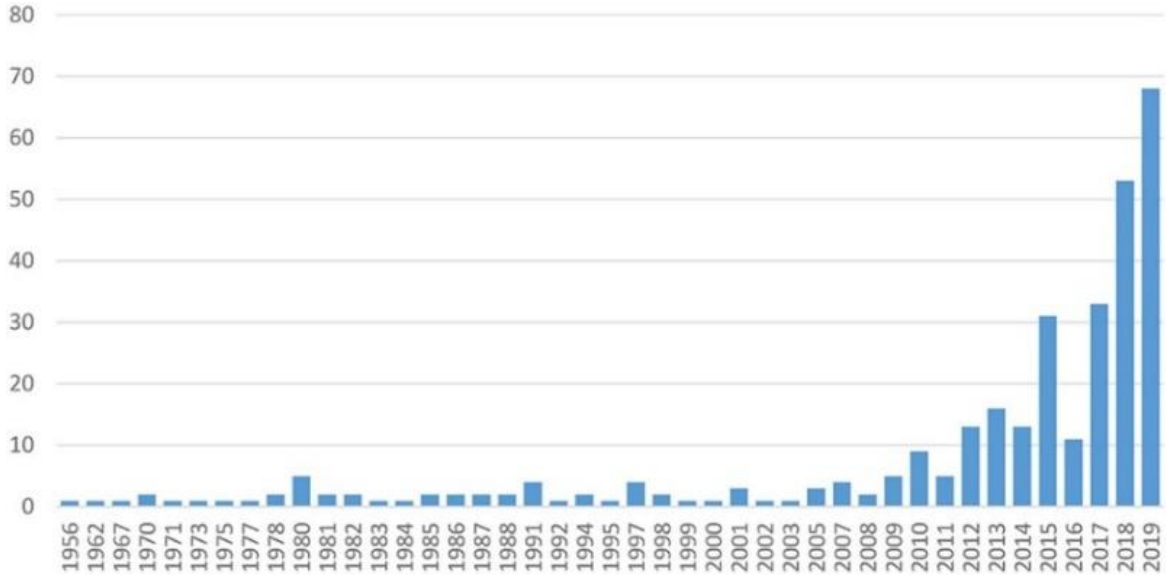

Figure 10. **Number of articles dealing with cultured meat registered per year (as of 31 December 2019) in the Web of Science bibliometric database**. (Chriki et al., 2020).

As described in Figure 10, the scientific community's interest in cultured meat has increased over the years, especially in the last decade. However, to date there are many doubts about this new production. The first certainly concerns the name. As reported in the literature, cultured meat is referred to using various terminologies, the most commonly used of these being: *in vitro* meat, clean meat, cultured meat, synthetic meat, fake meat, cell-based meat, artificial meat, lab-grown meat, animal-free meat, vegetarian meat, vegan meat, cell-based meat, slaughter-free meat and fake meat (Chriki et al., 2020). The name, which is not yet

universally defined, includes positive and negative connotations, depending on the interests of the stakeholders, as shown in Figure 11.

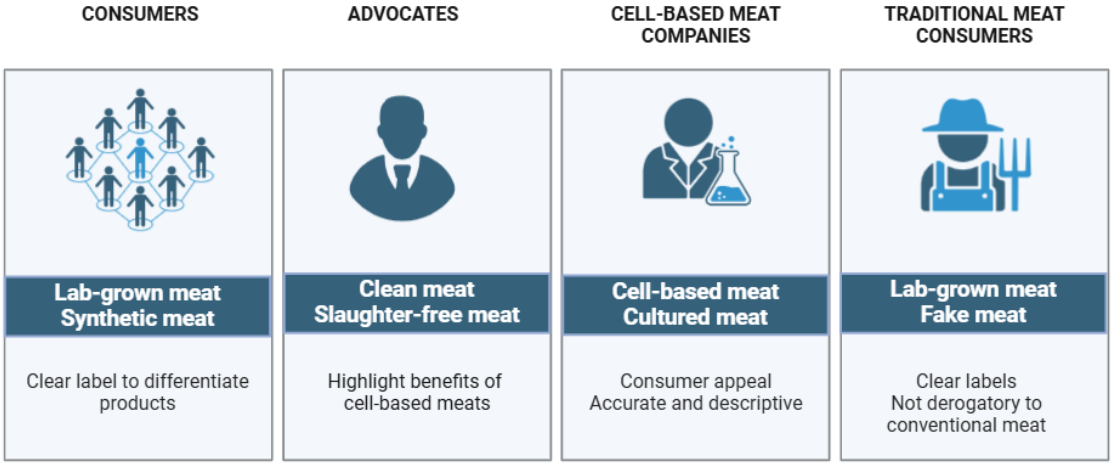

Figure 11. **Nomenclature of cellular meat: consumers, advocates, cellular meat companies and traditional meat producers**. Figure modified by Ong et al. (2021). Figure created with BioRender.com.

Other critical points as shown in Figure 12, concern the production process, environmental impact, sustainability, quality (nutritional and functional aspect), safety and the legislative aspect. All these points will be discussed in detail throughout this thesis.

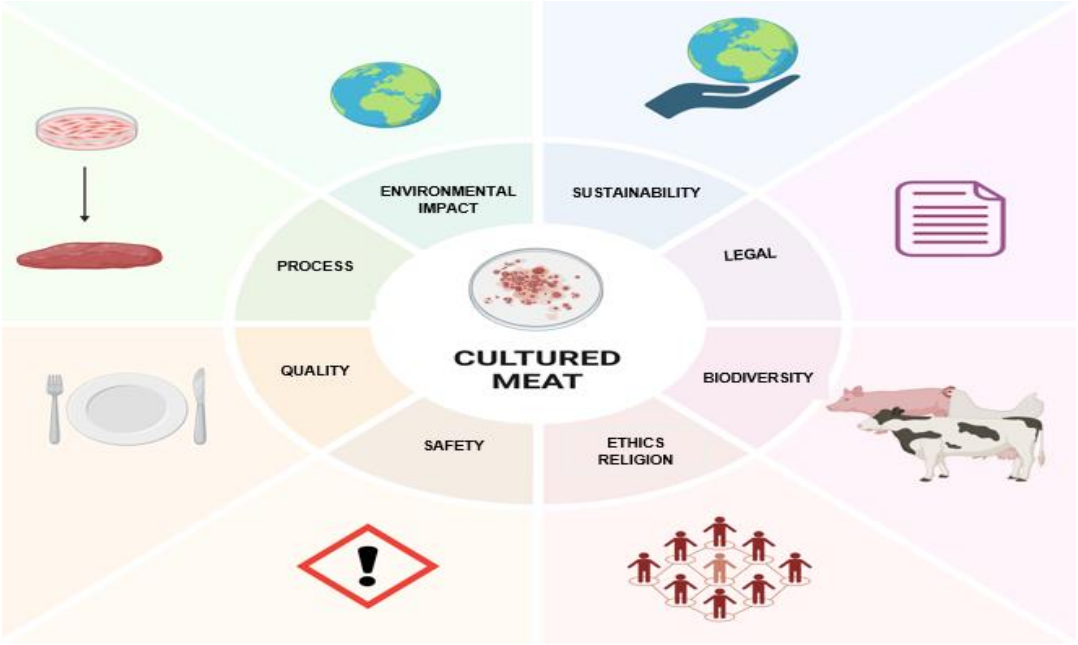

Figure 12. **Doubts and critical aspects of cultured meat**. Figure created with BioRender.com.

General aim

This doctoral thesis sought to address the urgent demand for sustainable food and feed systems in light of escalating global resource constraints and environmental challenges. The research focused on investigating different alternative protein sources, specifically hemp-based products, co-products, microalgae, and cultured meat, as innovative solutions to reduce the ecological footprint of food/feed production while ensuring nutritional adequacy. All these protein systems were examined, mainly by *in vitro* approach, for their potential to integrate seamlessly into existing agricultural and food production frameworks and align with European Union sustainability directives. A principal objective was to evaluate the capacity of these alternatives to enhance both animal productivity and human nutrition by analysing their nutritional profiles, functional properties, and potential to contribute to resilient and efficient food systems. The study also sought to identify and address key barriers to the scalability, economic viability, and regulatory approval of these innovative proteins, thereby facilitating their adoption on an industrial scale. This thesis adopted a multidisciplinary approach, leveraging advancements in biotechnology, agricultural sciences, and environmental policy to drive innovation. It emphasized fostering collaboration among researchers, policymakers, and industry stakeholders to ensure the translation of scientific knowledge into practical, impactful applications. Ultimately, the research aspired to contribute to the creation of sustainable, equitable, and robust food systems capable of meeting the complex demands of a rapidly growing global population.

Specific aims

The specific objectives are summarized below into three different chapters

Chapter I, entitled "*From characterisation to application: The role of hemp based-products and oilseed co-products in animal nutrition*" consists of:

- **Manuscript I**: *Nutritional aspects of hemp-based products and their effects on health and performance of monogastric animals*. To demonstrate the possible use of hemp-based products in the diet of monogastric animals (sows, piglets, broilers and laying hens) by a literature review (-2023).
- **Manuscript II**: *Total phenolic content and antioxidant activity of in vitro digested hemp-based products*. To characterise the nutritional and functional profile of hemp-based products, in particular the total phenolic content and antioxidant activity, following extraction with pure methanol and *in vitro* digestion, in order to study the behaviour of the molecules involved during the different stages of the digestive process, thus enabling new information on the functional aspect of these matrices to be acquired.
- **Manuscript III**: *Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and ex vivo digestion***.** To study the nutritional and functional profile of hemp co-products after solvent-extraction (food grade) and *ex vivo* digestion process for application in the feed industry.
- **Manuscript IV**: *Effects of dietary hemp co-products inclusion on laying hens performances and on egg nutritional and functional profile*. To evaluate how different inclusion levels (3%, 6%, and 9%) of a hemp co-product, resulting from the cleaning of whole hempseeds (leaves, non-standard hempseeds, hulls, and stems), in the diet of laying hens, are able to modulate production performance and the nutritional/functional profile of egg yolks.
- **Manuscript V**: *Antioxidant capacity and peptidomic analysis of in vitro digested Camelina sativa L. Crantz and Cynara cardunculus co-products*. To investigate the functional characteristics, in particular the antioxidant profile of cardoon and camelina co-products during the *in vitro* digestion process and to deepen the correlation with the main bioactive peptides resulting from the digestive process.

Chapter II, entitled "*Enhancing and challenging microalgal profiles: studies on nutrients and nanoplastic contaminants*", includes:

- **Manuscript VI**: *Functional characterisation of Euglena gracilis following growth medium enrichment*. To evaluate how the supplementation of microelements and AAs in the culture medium of *E. gracilis* was able to modulate its nutritional and functional profile. The evaluation was carried out following both chemical extraction (food grade) and an *ex vivo* digestion process.
- **Manuscript VII**: *Impact of Nanoplastics on the Functional Profile of Microalgae Species Used as Food Supplements: Insights from Comparative In Vitro and Ex Vivo Digestion Studies*. To assess the impact of single polystyrene (PS) and polyethylene (PE) and mixed nanoplastics (NPs) (PS+PE) exposure on the functional profile of two microalgae species *C. vulgaris* and *H. pluvialis* used as food supplements, before and after *in vitro* and *ex vivo* digestion. In parallel, the behaviour of the NPs was also assessed by determining the particle size (hydrodynamic diameter), polydispersity index (PdI) and surface charge (ζ), using Dynamic Light Scattering (DLS).

Chapter III, entitled "*Cultured meat development: exploring biological advances, regulatory hurdles, and alternative growth media*" comprises:

- **Manuscript VIII**: *Biotechnological and technical challenges related to cultured meat production*. To outline and discuss the critical steps and challenges involved in cultured meat production, focusing on biological and technical strategies, such as the selection of animal species, animal-free alternatives to FBS, cell-biomaterial interactions, and scalable, sustainable biofabrication methods. The objective is to emphasize that while cultured meat holds significant promise, many of these challenges are still at a preliminary stage, necessitating further research to enable efficient large-scale production.
- **Manuscript IX**: *Cultured meat in the European Union: Legislative context and food safety issues*. To provide an overview of the current legislative, food safety, technical but also economic challenges of cultured meat. In particular, the paper intends to examine first of all the legislative regulations governing the marketing of this product, with a focus on the EU context. In parallel, the document promotes an in-depth examination of food safety issues and the need to provide a comprehensive and careful analysis on this

point. At the same time, the main critical issues in the modulation of organoleptic and nutritional properties that can guarantee a product similar to the conventional one are highlighted. Finally, the paper aims to illustrate possible future markets for cultured meat, with a focus on consumer acceptance.

- **Manuscript X**: *Milk whey as a sustainable alternative growth supplement to fetal bovine serum in muscle cell culture.* To evaluate the potential of whey proteins (WP) in supporting myoblast proliferation in the absence of foetal bovine serum (FBS) and its ability to facilitate myotube formation in the C2C12 myoblast model. To this end, the effect of WP on myoblast proliferation in the absence of FBS was initially assessed, followed by the induction of myotube formation using a standard low-mitogen differentiation medium. The objective is to determine whether WP can be an effective alternative to FBS in promoting myoblast growth and differentiation.

Chapter I

From characterisation to application: The role of hemp based-products and oilseed co-products in animal nutrition.

The research presented in this chapter explores the potential of innovative plant-based and plant co-products, particularly from hemp, for their application in animal nutrition and function feed development. In parallel, the research also focused on cardoon and camelina co-products. The overarching aim is to address contemporary challenges in sustainable agriculture and animal husbandry by investigating the nutritional and functional properties of these matrices. By examining their total phenolic content, antioxidant activity, and bioactive peptide profiles during different stages of digestion, this research sheds light on their behaviour and functional potential within the digestive process. Additionally, the studies evaluate the practical implications of incorporating hemp co-products into animal diets, focusing on their effects on production performance and the nutritional and functional qualities of animal-derived products, such as egg yolks. This first chapter of the thesis provides critical insights into the versatility and applicability of plant-derived products, emphasizing their potential to enhance sustainability, animal health, and product quality in the livestock industry.

Manuscript I

Review: Nutritional aspects of hemp-based products and their effects on health and performance of monogastric animals

Davide Lanzoni, Eva Skrivanova, Luciano Pinotti, Raffaella Rebucci, Antonella Baldi and Carlotta Giromini

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Review: Nutritional aspects of hemp-based products and their effects on health and performance of monogastric animals

D. Lanzoni[a], E. Skrivanova[b,c], L. Pinotti[a], R. Rebucci[a], A. Baldi[a] and C. Giromini[a,d]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamycka 129, 165 00 Prague, Czech Republic; [c] Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Sciences, Pratelstvi 815, 104 00 Prague, Czech Republic; [d]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy.

ABSTRACT: Sustainable agriculture aims to produce food and feed that ensure food security and play a key role in environmental protection. For this, producers, supported by scientific research, are investigating new protein alternatives for animals that guarantee high performance and preserve their health. Among these, hemp (*Cannabis sativa* L.) is gaining great success, both for its active role in environmental conservation and for the high nutritional profile of the seeds (20–30% carbohydrates, 25–30% proteins easy to digest and rich in essential amino acids, and 25–35% lipids with a balanced fatty acid composition), also ensured by the co-products, particularly seed cakes (30–34% proteins and 10–12% lipids). However, the last scientific report by the European Food Safety Authority for the use of hemp-based products in the feed sector now dates back to 2011. For this reason, the objective of this review, in addition to outlining the nutritional profile of hempseeds (HSs) and co-products, aims to investigate their use in the monogastric sector, particularly in the diets of pigs, broilers, and laying hens, by summarising the main works in the literature up to 2023, investigating the effects on animal health and performances. The reported results showed that the addition of 50 g/kg of HSs and HS oil improved the nutritional profile of milk and colostrum in lactating sows, particularly the lipid profile, positively affecting the health of piglets. For broilers, the inclusion of HSs (20 g/kg) resulted in better values on growth performance. This was not matched by the addition of HS oil (up to 60 g/kg). In particular, although a better polyunsaturated fatty acid profile was observed, the results on growth performance were contradictory. The same trend was observed for HSs cakes with 50, 150, and 200 g/kg inclusion. For laying hens, the inclusion of HSs (up to 250 g/ kg), HS oil (up to 300 g/kg), and HSs cake (up to 150 g/kg) increased the nutritional and functional profile of the eggs, safeguarding performance and animal welfare. However, despite the promising results, the function of hemp-based products in the diet of monogastric animals needs to be further investigated to identify the optimal level of inclusion and timing of administration, necessary to ensure high performance and health of the animals.

Keywords: Co-products; Hemp; Laying Hens; Pig; Poultry

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IMPLICATIONS

Feed from sustainable agriculture is a key issue in the livestock sector. Producers, supported by research, are investigating new alternatives that are able to satisfy their principles. Hemp (*Cannabis sativa* L.) represents a viable alternative, both for its high nutritional profile and its low environmental impact. For this reason, the aim of the following review was to investigate the use of hemp-based products in addition to the diet of monogastric animals, demonstrating the potential of these products on the health and performance of swine, broilers and laying hens.

INTRODUCTION

Sustainable agriculture is the production of agricultural products at an environmental cost that does not jeopardise food/feed security and animal health. For this reason, scientific research is investigating new alternatives to classical protein sources to ensure these principles. This objective is strongly pursued by the European Union through the One Health approach, and subsequently with the Green Deal, dated 1 December 2019, which recognise that human, animal, and environmental health are inter connected, promoting efficient use of resources through a circular and clean economy (Wolf et al., 2021). Among the possible alternatives, *Cannabis sativa* L., commonly known as hemp, is gaining increasing curiosity in the food/feed sector, not only due to the high nutritional and functional properties of hempseeds (HSs) but also for the role it plays in environmental protection. As well as being able to grow rapidly in different agroecological conditions (it can reach four metres in height) while requiring limited amounts of water and herbicides, it is an excellent candidate for carbon sequestration (nine to thirteen tonnes/ha). In addition, its deep roots (about two metres long) prevent soil erosion and also take up nutrients and metals from the deeper soil layers (Rupasinghe et al., 2020; Rehman et al., 2021). In particular, interest in hemp has grown following the European Regulation 1251/1999/EC, which established a support system for all producers of particular types of seeds, including industrial hemp, and European Regulation 1370/2013, with which hemp cultivation was included among those eligible for Common Agriculture Policy payments with the only condition that the seeds used for cultivation be of varieties registered in the European catalogue with a Δ9-tetrahydrocannabinol content of less than 0.2-0.3% (Sorrentino, 2021).

To ensure sustainable agriculture, one possible approach involves recycling nutrients from residues and unusable crop co-products and adding them to the diet of livestock animals, among these, hemp co-products are attracting great interest, in particular the whole plant, leaves and flowers (obtained before/after the cannabinoid extraction process), stems, stalks, whole seed heads, and seed hulls, as reported by Kleinhenz et al. (2020), Vastolo et al. (2022), and Ely and Fike (2022).

However, the last report by the European Food Safety Authority (EFSA) on the use of hemp-based products in feed sector, entitled Scientific Opinion on the safety on hemp (*Cannabis* genus) for use as animal feed, dates back to 2011 (EFSA, 2011). More precisely, in this

report, EFSA suggests inclusion levels based on the work published until then. As might be expected, the number of scientific publications investigating the use of hemp-based products in the diets of monogastric animals has increased since 2011. For this reason, the objective of the following review, in addition to outlining the nutritional profile of HSs and co-products, aims to investigate their use in the monogastric sector (pigs, broilers, and laying hens) by summarising the main works in the literature up to 2023, investigating the effects on animal health and performances.

Nutritional properties of hempseeds

Hempseed is the main component of the plant used in the food and feed sector due to its very promising nutritional and functional properties. It is an achene, enveloped by a thin and hard pericarp characterised by a high amount of fibre, in particular 40–50% NDF, 30–35% ADF, and 10–15% ADL, 23–30% proteins easy to digest and rich in essential amino acids (AAs), 25–35% lipids with balanced fatty acids and 5% of ashes (Farinon et al., 2020; Fike, 2019; Lanzoni et al., 2023). These values, as shown in Table 1, are similar to those of flaxseeds, an important matrix in the food/ feed industry.

Table 1. **Nutritional profile of hempseeds (HSs) compared to flaxseed** (% w/w on DM basis). Data are presented as mean ± SEM. EE = ether extract. Table adapted by Lanzoni et al. (2023).

	DM	CP	EE	NDF	ADF	ADL	ASHES
HSs	94.6±0.12	23.1±0.57	27.9±0.75	44.6±0.21	33.2±0.31	14.4±0.32	5.8±0.10
FXs	91.6±0.14	23.0±0.45	36.0±0.07	41.5±1.27	19.5±0.35	9.2±0.41	2.7±0.20

However, as reported in the literature, these percentages depend on the plant genotype, environmental growth factors and, above all, the type of technological treatment the seed is subjected (Fike, 2019; Leonard et al., 2020; Lanzoni et al., 2023).

The fibrous fraction of HSs is characterised by a soluble:insoluble fibre ratio of 20:80, a similar value to that of other food/feed sources, such as flaxseeds (Callaway, 2004; Farinon et al., 2020). However, due to treatments such as heating and extrusion, it is possible to concentrate fibre fraction, making them more appreciable for food and feed applications (Amaducci et al., 2008).

In HSs, the proteins are mostly located in the inner layer of the seed, with a low fraction in the hull. To date 181 proteins have been identified, the most common of which are edestin, albumin, and b-conglycinin (Farinon et al., 2020). Edestin accounts for approximately 60–80% of the total protein content, albumin accounts for approximately 25%, and b-conglycinin accounts for approximately 5% (Farinon et al., 2020; Odani and Odani, 1998). Edestin, whose molecular weight is 300 kDa, is made up of six identical subunits, each of which reorganises itself into an acidic and a basic subunit, held together by sulphur bonds, which make it less flexible than albumin. However, the main difference between these two proteins is their AA composition. Edestin is characterised by a greater number of aromatic, branched-chain, and above all, sulphur-rich AAs such as cysteine and methionine, which

provide greater nutritional value than albumin (Wang and Xiong, 2019). In general, HSs provide a qualitatively and quantitatively high AA profile, enriched by the presence of essential AAs, resulting in a high biological value, although lysine, cysteine and tryptophan are limiting AAs. However, as demonstrated by Callaway (2004), the AA composition is comparable to that of other high-quality proteins, such as soybeans and rapeseeds.

The lipid content of HSs, called also oil, is characterised by a valuable nutritional profile, as reported in Table 2.

Table 2. **Fatty acid profile of hempseeds**. Values are expressed in % of oil. When several cultivars were analysed, the maximum and minimum values of the range of data obtained are indicated. When only one cultivar was analysed, the mean ± SD is reported. PA: Palmitic acid; SA: Stearic acid; OA: Oleic acid; LA: Linoleic acid; GLA: γ-Linoleic acid; ALA: α-Linolenic acid; SDA: Stearidonic acid; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; n.a. not available. Table reported by Farinon et al. (2020).

	Callaway (2004)	Siano et al. (2018)	Vecka et al. (2019)	Vonapartis et al. (2015)	Galasso et al. (2016)	Irakli et al. (2019)	Lan et al. (2019)
PA	5.0	7.0±0.3	5.6±0.5	6.7-7.0	6.7-7.3	7.1-9.1	6.1-7.8
SA	2.0	2.8±0.4	3.9±0.4	2.1-2.8	2.3-3.5	2.1-2.8	2.3-4.0
OA	9.0	12.7±1.3	16.2±6.2	9.4-13.0	9.2-15.7	10.3-17.9	12.2-18.8
LA	56.0	56.2±3.5	54.7±4.1	55.6-56.6	55.0-58.2	51.6-54.2	53.9-59.0
GLA	4.0	2.9±0.4	n.a.	2.6-4.5	0.6-4.5	1.9-5.0	3.5-6.2
ALA	22.0	15.0±1.1	16.2±4.0	14.7-17.3	12.6-19.6	10.5-15.3	12.3-18.9
SDA	2	n.a.	0.5±0.01	n.a.	0.2-1.5	n.a.	n.a.
SFAs	n.a.	10.9±0.7	10.9±0.7	9.6-10.3	9.4-11.7	n.a.	n.a.
MUFAs	n.a.	13.1±1.3	17.5±6.3	n.a.	9.7-16.1	n.a.	n.a.
PUFAs	84.0	75.1±3.7	72.0±4.3	n.a.	72.2-80.7	n.a.	72.0-78.6
n-6/n-3	2.5	4	3.2	n.a.	2.8-4.5	3.9-5.5	3.2-5.0

It is distinguished by a low content of saturated fatty acids (SFAs) (10.9 ± 0.7%) and a high content of unsaturated fatty acids, in particular polyunsaturated fatty acids (PUFAs). The latter, as shown in Table 2, accounts for about 75.1 ± 3.7% of the entire oil fraction, reaching up to 84% (Callaway, 2004; Siano et al., 2018). Of the PUFAs present, the most prevalent are linoleic acid (18:2, n-6) and a-linoleic acid (18:3, n-3, ALA), defined as essential fatty acids. They are the precursors of long-chain PUFAs, arachidonic acid (20:4, n-6), docosahexaenoic acid (22:6, n-3), and eicosapentaenoic acid (20:5, n-3), which play key roles in brain development, cardio vascular health, obesity, and in the regulation of metabolic and inflammatory processes, via the synthesis of prostaglandins and leukotrienes (Farinon et al., 2020). High levels of linoleic acid and ALA present within the lipid component of HSs mean that the n-6/n-3 ratio, responsible for physical well-being, is low, approximately 3.5, defined by the EFSA as the ideal intake (Leonard et al., 2020; EFSA, 2009). In addition to linoleic acid and ALA, HS oil also contains their respective biological metabolites c-linolenic Acid (18:3, n-6) and stearidonic acid (18:4, n-3), which allow the first critical enzymatic step of d-6-desaturasetobebypassed,facilitating conversion into the biologically active form of long-chain PUFAs (Farinon et al., 2020). Among the monounsaturated fatty

acids (MUFAs),the most prevalent is oleic acid (18:1,n-9) which is present in quantities comparable to flaxseeds and greater than chia seeds (Montserrat-de la Paz et al., 2014).

Although, as previously reported, HSs are characterised by a high nutritional profile, it is important to underline the presence of anti-nutritional factors as well. The main anti-nutritional factors in HSs are phytic acid, trypsin inhibitors, condensed tannins, saponins, and non-starch polysaccharides. Although phytic acid plays an important role in preventing lipid peroxidation, it can alter the activity of digestive enzymes, impairing the bioavailability of minerals and proteins(Russo and Reggiani, 2015). The phytic acid content in HSs is found to be around 4–8% of the dry weight of defatted matter, a higher concentration than that found in soy beans(2%) (Russo, 2013; Russo and Reggiani, 2015). Condensed tannins are phenolic compounds and therefore have a high antioxidant, anti-inflammatory, and anti-microbial activity. However, they are considered to be anti-nutritional factors as they form insoluble complexes with minerals and proteins, thus affecting their absorption by the body (Mattila et al., 2018). Although, within HSs, trypsin inhibitors are present in a lower concentration than in soybeans, they play a negative role during the digestive process. In particular, as reported by Farinon et al. (2020), these anti nutritional factors inhibit the action of proteinases in the stomach and trypsin and chymotrypsin in the intestine, not allowing the degradation of protein chains, thus reducing AA bioavailability. Saponins are able to cause gastritis and haemolysis of red blood cells, only when ingested in high quantities. However, the concentration of these anti-nutritional factors is lower than in common matrices such as flaxseeds, quinoa seeds and soybeans (Russo and Reggiani,2015). Finally, HSs are characterised by the presence of non-starch polysaccharides, albeit little explored in the literature. As reported by Hetland et al. (2004), non-starch polysaccharides are compounds that can resist the animal's digestive enzymes, creating a highly viscous intestinal environment, consequently affecting the digestion and absorption of nutrients. As reported by House et al. (2010), the main non-starch polysaccharides in HSs are Rhamnose (1.14±0.17mg/g), Arabinose (2.99±0.32mg/g), Xylose (74.74±15.74mg/g), Mannose (1.23±0.21mg/g), Galactose (2.61±0.28mg/g) and Glucose (76.52±19.84mg/g). Although these values are elevated, processes involving HSs such as dehulling can drastically reduce these values, emphasising that non-starch polysaccharides are mainly present in the seed hull (Houseetal.,2010). In light of the above, although HSs are characterised by the presence of anti-nutritional factors, their content is not only highly comparable to that of other matrices used in the feed industry but can also be modulated with treatments as observed for non-starch polysaccharides, consequently increasing the nutritional profile.

Nutritional properties of hemp co-products

The use of co-products is a valid strategy to ensure the sustainability of the feed sector. As reported above and shown in Table 3, the main hemp co-products investigated in the scientific literature are: whole plant, leaves, flowers, stems, stalks, seeds cake, chaffs, seed heads and hulls.

Table 3. Chemical Composition of hemp co-products. (whole plant, leaves, seed cake, seed heads, chaff, extracted flower, whole plant SHB, stem and leaves SHB, hemp hulls). Values are expressed in % DM. Table adapted by Kleinhenz et al. (2020); Vastolo et al. (2022) and Ely and Fike (2022). SHB: Spent hemp biomass.

	CP	FAT	ADF	NDF
Whole Plant	6.9	2.7	60.8	81.6
Leaves	13.0	8.9	20.8	44.7
Stalks	5.3	1.2	64.6	84.4
Seeds Cake	30.0-34.4	10.2-12.4	32.1-39.5	39.3-51.9
Seed heads	23.0	13.2	29.6	53.2
Chaffs	21.2	4.6	18.0	27.9
Extracted flower	24.5	3.2	18.1	30.9
Whole plant SHB	22.4	4.3	32.3	40.1
Stems & Leaves SHB	19.2	7.5	17.6	23.4
Hemp hulls	21.9	23.5	37.9	57.1

The percentages of these nutrients vary depending on the cultivation techniques, environmental conditions, and genotype analysed.

In particular, the CP content varies from 5.4% to 34.4%, with higher values reported for HSs cake (30-34.4%), due to the high level of the protein content following the oil extraction process. At the same time, as reported by Vastolo et al. (2022), this product has a high protein digestibility, tested following the *in vitro* digestion process, showing values highly comparable to tobacco co-products. Interesting values include seed heads (23%), chaffs (21.2%), hulls (21.9%), and exhausted biomasses (Kleinhenz et al., 2020; Vastolo et al., 2022 and Ely and Fike, 2022). As reported by Ates (2021), the total protein content of co-products, obtained as a result of the cannabinoid extraction process, is similar to alfalfa, with higher values for the lipid content.

As shown in Table 3, hemp co-products are characterized also by high level of fibre. The NDF ranges from 23.4% to 84.4%, which is lower for co-products rich in leaf and flower components. The latter have higher digestibility than corn stalks, oat or barley straw. However, as reported by Kleinhenz et al. (2020), such co-products are poor sources of energy within livestock diets, suggesting a different use as a source of dietary fibre, especially in ruminants.

Furthermore, as reported by Ely and Fike (2022), being an annual crop that grows when most cool-season fodder slows down, hemp could have potential as an alternative summer fodder or used in emergencies, although to date there are few studies available that evaluate its use as fodder. However, at the same time, hemp co-products have some limitations. These include the high variability of nutrients due to different processing methods and the need for essential preservation treatments to stabilise the product, especially those with high moisture and lipid values (Ely and Fike, 2022).

Use of hemp-based products in monogastric animals

Swine

The use of HSs and hemp-based products in the swine sector is still at an early stage. As shown in Table 4, the main studies concern the use of these products in diets for lactating sows and piglets, which are key steps for the success of the entire production cycle.

Table 4. Effects of the inclusion of hemp-based products (HSs, HSs oil, HSs meal) in the swine (sows and piglets) diets. HSs: Hempseeds: FA: Fatty acids

Animals	Last	Treatments	Effects	Ref
10 sows and 96 suckling piglets	21 days	Control group; Sows (50 g/kg of HSs); Piglets (15 g/kg of HSs).	Increased n-3 FAs profile; Decreased n-6/n-3 in colostrum and milk; Increased Milk Yield; Increased Average Daily Gain during first week.	(Habeanu et al., 2018)
24 sows	35 days	Control group; 50 g/kg HSs oil; 25 g/kg HSs oil + 25 g/kg soy beans oil).	Increased n-3 FAs profile; Decreased n-6/n-3 in colostrum and milk; Decreased n-6/n-3 in plasma of sows and piglets; Decreased sows final BW; Increased of total and live born piglets; Increased weight gain of piglets during first week.	(Vodolazska and Lauridsen, 2020)
7 sows	28 days	Control group; 16 g/kg of HSs oil.	No differences in live born piglets; Increased final BW of piglets; Increased Average Daily Gain; Increased sows final BW.	(Idriceanu et al., 2021)
10 sows and 16 suckling piglets	31 days	Control group; Sows (20 g/kg of HSs meal before farrowing; 50 g/kg of HSs throughout the lactation period). Piglets (15 g/kg of HSs meal).	Increased anti-oxidative status of sows; Increased anti-oxidative status of piglets.	(Palade et al., 2019)

Weaning piglets represents the most delicate and crucial time for the health of these animals. During this phase, the animals face environmental (separation from the mother), social (union of different broods), and nutritional (transition from mother's milk to solid feed) changes. All these factors act as stressors, simultaneously causing both a decrease in feed

intake (FI) and the development of gastric and intestinal problems, resulting in severe physiological and immunological disorders (Palade et al., 2019; Vodolazska and Lauridsen, 2020). For this reason, it is necessary that the energy and nutritional intake provided through breast milk is high, to overcome this critical phase. Although piglets are born with an energy deficit and low fat reserves, the absorption of lipids and fatty acids from colostrum and milk is efficient (Palade et al., 2019; Vodolazska and Lauridsen, 2020). For this, animal producers supported by research are trying to intervene through diet to safeguard their health. One of the possible alternatives, still little explored in the literature, concerns the use of hemp-based products in sows rations to study how piglets performance varies.

As shown in Table 4, the addition of HSs (up to 50 g/kg) in the diets of lactating sows resulted in positive results on the growth performance of piglets. This result, as reported by Habeanu et al. (2018), is related both to the direct consequence of lipid supplementation in the diet, which allows for an increase in milk fat and thus a higher energy intake, and to the high nutritional profile, especially the lipid content of HSs. Indeed, Habeanu et al. (2018) observed that the addition of HSs increased the n-3 PUFAs content in the milk of treated sows (6.66% of total content) compared to the control group (soybean meal) (4.35%), which resulted in a lower n-6/n-3 ratio (6.91 vs 10.52, respectively). Similarly positive results were obtained with the inclusion of 50 g/kg HSs oil in the diet (Vodolazska and Lauridsen., 2020). Vodolazska and Lauridsen (2020) found high concentrations of ALA (C18:3 n-3), arachidonic acid (C20:4 n-6), and stearidonic acid (C18:4 n-3), resulting in an even lower n-6/n-3 ratio (4.30) in the milk of treated sows. Positive growth performance was also observed with a lower (16 g/kg) inclusion level of HSs oil (Idricenau et al., 2021).

As previously reported, in the pig sector, particular attention is focused on the weaning period. However, at this stage, according to the literature, sows can lose an average of 15 kg to 40 kg during the entire lactation period (Hansen, 2012). For this reason, it is necessary to select a diet that guarantees both the health of the piglets and that of the sows.

Although, as previously shown, the addition of two different concentrations of HSs oil resulted in the same positive effects on piglet performance, the inclusion of 50 g/kg HSs oil affected the total weight of the lactating sows (320 ± 0.18 kg), compared to the control group (346±11.4 kg) (Vodolazska and Lauridsen, 2020). This trend was not observed with the inclusion of 16 g/kg HSs oil (Idricenau et al., 2021). Most probably, while a high lipid content in the diet of lactating sows allows an improvement in the growth performance of piglets, high milk production with a high lipid content affects the performance of sows, resulting in weight loss (Hansen, 2012).

Another determining factor in weight loss is the oxidative stress faced by sows mainly related to the process of lipid peroxidation, which alters the antioxidant status of the animal. In particular, oxidative stress affects the ability of sows to produce milk, which, as previously reported, negatively limits the growth and performance of piglets. Persistent stress conditions lead to the formation of a large amount of free radicals and reactive oxygen species, which can affect the endogenous antioxidant system. In these situations, poor

nutrient intake may be one of the main causes of systemic oxidative stress in sows (Palade et al., 2019).

In the study conducted by Palade et al. (2019), however, the inclusion of a dietary treatment with HSs (20 g/kg HSs meal before farrowing; 50 g/kg during lactation) led to an improved antioxidant profile in the plasma of sows and piglets. According to the same authors, the positive correlation between HSs and antioxidant activity is due to the high content of PUFAs, which are known not only for their high nutritional profile, but also for their ability to increase gene expression and the activity of several antioxidant/detoxifying enzymes (Palade et al., 2019). This hypothesis was also confirmed in the work of Vodolazska and Lauridsen (2020), in which the addition of HSs oil (50 g/kg) resulted in elevated levels of eicosapentaenoic acid (C20:5 n-3) and docosapentaenoic acid (C22:5 n-3), in the plasma of sows and piglets, as a result of the conversion of ALA and stearidonic acid to longer chain n-3 PUFAs. In fact, as reported by Farinon et al. (2020), these fatty acids are characterised not only by high inflammatory activity, but also by an important antioxidant activity.

As reported earlier, there are few studies in the literature focusing on the use of hemp-based products in the pig sector, mainly considering the weaning period. As shown, inclusion levels of up to 50 g/kg of HSs in pregnant sows resulted in improved growth performance of treated piglets due to a better nutritional profile of the milk, richer in n-6 and n-3 PUFAs. Similar results were obtained with 50 g/kg of HSs oil, although this inclusion led to a reduction in sow weight during pregnancy, a phenomenon not observed with the inclusion of 16 g/kg, suggesting that lower levels may benefit both lactating sows and piglet growth performance.

However, although all these results suggest that hemp-based products could be included in pig diets, further studies are needed to identify the optimal level of inclusion for each product to ensure the best animal health and performance.

Broilers

Interesting results were reported for broilers.

Table 5. Effects of the inclusion of hemp-based products (HSs, HSs oil, HSs cake, HSs expellers, hemp plant top) in the broiler diet. HSs: Hempseeds; FCR: Feed Conversion Ratio; FI: Feed Intake; PUFAs: polyunsaturated fatty acids.

Animals	Last	Treatments	Effects	Ref
160 broiler chicks	42 days	Control group; 5, 10, 20 g/kg of HSs.	Increased final BW gain (20 g/kg HSs); Increased FCR (20 g/kg HSs); Decreased FI (20 g/kg HSs); No differences in mortality; Increased profit per chick..	(Khan et al., 2010)
50 broiler chicks	49 days	Control group; 10, 20, 30, 40 g/kg of HSs hearts.	Increased final BW gain (20 g/kg HSs); Increased FCR (20 g/kg HSs).	(Parr et al., 2020)
192 broiler chicks	42 days	Control group; 2 and 3 g/kg of HSs.	Decreased BW gain and FI in the first 3 weeks; Decreased total cholesterol and triglyceride in serum.	(Vispute et al., 2019)
540 cockerels	35 days	Control group; 40 g/Kg HSs.	No differences in BW; No differences in FI; No differences in FCR; No differences in PUFAs; Increased bone health.	(Skřivan et al., 2020)
150 broiler chicks	21 days	Control group; 30 and 60 g/kg of HSs oil).	No differences in BW; No differences in FI; No differences in FCR; Increased n-3 PUFAs in thigh and breast meat.	(Jing et al., 2017)
200 broiler chicks	21 days	Control group; 61 g/kg HSs oil.	Decreased BW gain; Decreased FCR; Decreased FI; Increased level of PUFAs; No differences in cholesterol and triglycerides.	(Kanbur, 2022)
1200 chicks	70 days	Control group; 100 g/Kg (starter), 200 g/kg (grower) of HSs cake.	No differences in FCR; No differences in FI; No differences in live BW; No differences in mortality rate.	(Eriksson and Wall; 2012)
75 cockerels	35 days	Control group; 150 and 50 g/kg of HSs cake.	Decreased final BW; Decreased in FCR; No differences in % of breast and leg muscle.	(Stastnik et al., 2015)
60 cockerels	27 days	Control group; 25 g/kg of HSs expellers; 10 g/kg of hemp plant tops.	No differences in final BW; No differences in carcass yield; No differences in microbial colonization.	(Stastnik et al., 2016)

More precisely, as observed in the study by Khan et al. (2010), the inclusion of 20 g/kg of HSs resulted in a better feed conversion ratio (FCR) with a higher slaughter weight compared to the control group (2087.2±10.25 g/kg and 1861.4±32.2 g/kg). As explained by the authors, this trend is due to the high lipid and AA profile of the HSs, confirming the results reported by Parr et al. (2020), where the inclusion of 20 g/kg proved to be better than 10 g/kg, 30 g/kg, and 40 g/kg to modulate the growth performance of broilers.

However, the inclusion of 40 g/kg, as reported by Skrivan et al. (2020), resulted in comparable results in terms of performance and product quality to flaxseeds, but added at 60 g/kg, confirming the high nutritional profile of HSs. This trend was also observed for bone health, a key parameter for animal welfare, especially under intensive livestock conditions. Most probably, as reported by Skrivan et al. (2020), this result is positively correlated with the presence of α-tocopherol, which is higher in both the diet and the meat of animals that received the HSs diet. Indeed, α-tocopherol supplementation tends to induce an increase in osteogenic bone mass in the vertebral secondary cancellous bone, where active bone remodelling occurs (Skrivan et al., 2020). However, although these results showed that an inclusion of up to 40 g/kg of HSs in broiler diets leads to positive results, it is necessary to consider how in the first weeks of the production cycle, the high fibre and mineral content could affect the health of the animals. Indeed, as reported by Vispute et al. (2019), at this stage, chickens have an underdeveloped mucosa, unable to produce sufficient enzymes capable of digesting high cellulose content.

The use of HSs oil led to similar results with different concentrations. In particular, Jing et al. (2017) showed that the inclusion of 30 g/kg and 60 g/kg of HSs oil in a control diet (based on corn oil) did not change the performance (BW, FI and FCR) of the treated animals, but increased the n-3 PUFAs content in the meat (71.4±14.8 g/100 g and 148.9±14.8 g/100 g, respectively). Result partially confirmed by Kanbur (2022). However, the author strongly advises against the use of HSs oil, especially in the first three weeks of rearing, having recorded negative values for growth performance. In contrast to the reports of Vispute et al. (2019), where it was the high fibre content that affected growth performance, in this case, dealing with a lipid matrix is difficult to speculate on the causes. However, as reported by Fouad and El-Senousey (2014), a high inclusion of PUFAs in the diet of broilers can lead to fatty acid β-oxidation by reducing fat deposition.

The use of hemp co-products in the diets of monogastric animals is attracting increasing interest, however, to date there is limited work in the literature. Stastnik et al. (2016) tested the use of 25 g/kg of HSs expellers and 10 g/kg hemp plant tops (flowers, HSs and a bit of shives) in the diet of broilers, observing no difference on carcass yield at slaughter, compared to the control diet (maize diet). However, the use of these hemp-based products has not yet been authorised by the European Union (EU, 2017), suggesting the need for further studies on the valorisation of these co-products. In parallel, the European Union approved the use of HSs cake, although conflicting results were observed for broilers. More specifically, Eriksson and Wall (2012) demonstrated how the inclusion of 200 g/kg of HSs cakes

registered highly comparable values in growth performance parameters (FI, FCR, final weight) and mortality to diets based on soy bean cakes, rapeseed cakes and peas. Although these results suggest the use of HSs cakes as an alternative to soy bean cakes, Stastnik et al. (2015) reported opposite results with inclusions of 50 g/kg and 150 g/kg, causing a worsening in final weight compared to the control diet (soy bean cake). Most probably, these effects can be attributed to the presence of anti-nutritional factors, especially non-starch polysaccharides, whose content at the fibrous level increases with the removal of the lipid fraction.

As shown in Table 5, the use of hemp-based products led to contrasting results. The inclusion of HSs recorded better values on growth performance (final BW) with an inclusion of 20 g/kg. Studies conducted on the inclusion of oil (up to 60 g/kg) in the diet of broilers revealed a highly improved PUFAs profile in meat, although growth performance are often unaffected or depleted. The same applies to hemp co-products. Seed cake showed discordant results with the inclusion of 50 g/kg, 150 g/kg, and 200 g/kg on FCR and final BW, suggesting further investigation of these matrices to identify the best inclusion levels.

Laying Hens

As shown in Table 6, the use of hemp-based products has been widely explored in the laying hens sector.

Table 6. Effects of the inclusion of hemp-based products (HSs, HSs oil, HSs cake) in the laying hens diet. HSs: Hempseeds; SFAs: saturated fatty acids; PUFAs: polyunsaturated fatty acids; MUFAs: monounsaturated fatty acids.

Animals	Last	Treatments	Effects	Ref
60 hens	21 days	Control group; 150 g/kg, 200 g/kg, 250 g/kg of HSs.	Decreased cholesterol level in egg yolk; Decreased SFAs in egg yolk; Increased PUFAs in egg yolk; Increased n-3 in egg yolk.	(Shahid et al., 2015)
240 hens	84 days	Control group; 30 g/kg, 60 g/kg, 90 g/Kg of HSs.	Decreased cholesterol level in yolk egg; Increased tocopherol level in egg yolk; Increased yellowness with an inclusion of 30 g/kg and 60 g/kg of HSs respectively; Increased laying rate for 30 g/Kg HSs; Decreased shell thickness; Increased tibia strength.	(Skřivan et al., 2019)
108 hens	84 days	Control group; 150 g/kg of raw HSs; 150 g/kg heat-treated HSs.	Increased PUFAs in egg yolk; Decreased SFAs (only for heat-treated HSs) in egg yolk; Decreased MUFAs in egg yolk; Increased egg yolk colour in raw HSs; No differences in egg weight; No differences in laying rate.	(konka et al., 2019)

40 hens	42 days	Control group; 40 g/kg, 80 g/kg of HSs oil.	No differences in MUFAs in egg yolk; No differences in SFAS in egg yolk; Increased levels on n-3 in egg yolk; No differences in laying rate.	(Jing et al., 2017)
48 hens	84 days	Control group; 100 g/kg, 200 g/kg, 300 g/kg of HSs; 45 g/kg, 90 g/kg of HSs oil.	No differences in SFAs in egg yolk; Decreased MUFAs in egg yolk; Increased n-3 PUFAs in egg yolk.	(Neijat et al., 2016)
48 hens	84 days	Control group; 100 g/kg, 200 g/kg, 300 g/kg of HSs; 45 g/kg, 90 g/kg of HSs oil.	No differences in egg weight; No differences in eggshell thickness; No differences in laying rate.	(Neijat et al., 2014)
48 hens	84 days	Control group; 40 g/kg, 80 g/kg, 120 g/kg HSs oil; 100 g/kg, 200 g/kg HSs).	Increased n-3 PUFAs in egg yolk; Increased egg weight only for 200 g/kg of HSs; No differences in egg mass production; No differences laying rate.	(Gakhar et al., 2012)
48 hens	84 days	Control group; 40 g/kg, 80 g/kg, 120 g/kg of HSs oil; 100 g/kg, 200 g/kg of HSs.	Increased n-3 PUFAs; Increased n-6 PUFAs; Increase egg yolk redness and yellowness.	(Goldberg et al., 2102)
30 hens	35 days	Control group; 16.8 g/kg of HSs oil.	Decreased total cholesterol level and triglycerides in serum; Increased egg yolk colour; Increased laying rate.	(Park et al., 2014)
102 hens	28 days	Control group; 50 g/kg, 100 g/kg, 200 g/kg of HSs cake.	No differences in egg weight; No differences in laying rate.	(Silversides and Lefrançois, 2005)
216 hens	168 days	Control group; 50 g/kg, 100 g/kg, 150 g/kg of HSs cake.	No differences in egg weight; Increased egg mass production.	(Halle and Schone, 2013)
800 hens	112 days	Control group; 100 g/kg, 200 g/kg, 300 g/kg of HSs cake.	No differences in laying rate.	(Rajasekhar et al., 2021a)
800 hens	112 days	Control group; 100 g/kg, 200 g/kg, 300 g/kg of HSs cake.	Decreased MUFAs in egg yolk; Increased PUFAs; No detection of cannabinoids.	(Rajasekhar et al., 2021b)
120 hens	70 days	Control group; 80 g/kg of HSs; 200 g/kg of HSs cake.	No differences in cholesterol level in egg yolk; Increased tocopherol lever in egg yolk for HSs; Increased egg weight with inclusion of HSs; No differences in laying rate.	(Mierliță, 2019)

The major studies, in fact, focus on the functional and nutritional characterisation of eggs as well as the evaluation of qualitative and quantitative parameters. In parallel, aspects such as production performance and animal welfare have been investigated.

Regarding the functional aspect of eggs, although Mierliță (2019) observed no differences in the total cholesterol content of egg yolk from hens treated with HSs (80 g/kg and 200 g/kg), studies by Shahid et al. (2015) and Skřivan et al. (2019) reported different trends. More specifically, Shahid et al. (2015) reported that increasing the inclusion of HSs (150 g/kg, 200 g/kg, and 250 g/kg) reduced the cholesterol content with significant differences (16.91±0.01 mg/g; 14.29±0.01 mg/g; 11.65±0.01 mg/g) compared to the control (corn and soy bean meal) (19.27±0.01 mg/g), respectively. Identical trends were observed by Skřivan et al. (2019), even with lower inclusions of HSs (30 g/kg, 60 g/kg, and 90 g/kg). This is in agreement with the work of Mahmoudi et al. (2015) and Vispute et al. (2019). Most probably, this positive effect is related to the presence of phytosterols, particularly β-sitosterol, which is able to reduce hypercholesterolaemia by blocking the absorption of cholesterol through coprecipitation and crystallisation. Furthermore, phytosterols with low water solubility compared to cholesterol can misplace cholesterol from intestinal micelles (Shahid et al., 2015).

Functionality also depends on the presence of antioxidant compounds. In this regard, it is important to point out that Skřivan et al. (2019) demonstrated that the inclusion of HSs resulted in an increase in tocopherol, an important antioxidant involved in the prevention of lipid peroxidation during the storage period. This result was also confirmed by Mierliță (2019). Indeed, as reported by the same author, eggs obtained from HSs-treated animals not only showed higher levels of α-tocopherol, but also a lower level of malondialdehyde (a marker of lipid peroxidation) than the control group.

Another aspect to consider is the nutritional one. In general, eggs from animals treated with hemp-based products showed a better lipid profile. More precisely, hemp-based products tend to decrease the level of SFAs and MUFAs within the yolk in favour of PUFAs, as reported by Shahid et al. (2015). These results are mainly due to the high level of n-3 PUFAs following the treatments with 150 g/kg (7.79±0.13 mg/g), 200 g/kg (10.29±0.09 mg/g), and 250 g/kg (15.11±0.81 mg/g) of HSs compared to the control (2.66±0.17 mg/g), resulting in an improved n-6/n-3 ratio (shahid et al., 2015). Similar results were observed both by Jing et al. (2017) with the inclusion of HSs in hemp diets of 40 g/kg and 80 g/kg and by Neijat et al. (2016). The latter observed that the inclusion of up to 300 g/kg of HSs and up to 90 g/kg of HSs oil, although not yielding statistically significant differences in total SFAs content, did decrease MUFAs content by increasing PUFAs content, particularly n-3 PUFAs, improving the n-6/n-3 ratio. Specifically, as reported by Gakhar et al. (2012), the main n-3 fatty acid subject to an increase is ALA. The authors showed how treatments with 100 g/kg and 200 g/kg HSs resulted in higher levels of this fatty acid of 51.7±3.23 mg/g and 91.3±3.23 mg/g, respectively, compared to the control 15.8±3.23 mg/g. Results also confirmed in diets with inclusion of 40 g/kg, 80 g/kg and 120 g/kg HSs oil. The high ALA content plays a key

role, as ALA can be converted to docosahexaenoic acid by the metabolism of chickens, the positive role of which has been explained above. Specifically, docosahexaenoic acid is derived from ALA, following the sequential reaction of the enzymes desaturase and elongase and β-oxidation, with eicosapentaenoic acid and Docosapentaenoic acid as intermediates. These reactions explain why high levels of docosapentaenoic acid, eicosapentaenoic acid and docosahexaenoic acid are found in the eggs of these animals, despite the fact that HSs lack them. In fact, as shown by Gakhar et al. (2012), treatments with HSs (100 g/kg and 200 g/kg) resulted in an increase in eicosapentaenoic acid (0.9±0.08 mg/g, 1.2±0.08 mg/g, respectively) compared to control (diet based on soy bean meal and corn oil) (0.2±0.08 mg/g) and docosahexaenoic acid (39.2±1.64 mg/g, 47.4±1.64 mg/g) compared to control (17.1±1.64 mg/g). These results are also confirmed in studies conducted by Goldberg et al. (2012) and Rajasekhar et al. (2021b). Interesting observations were made by Konca et al. (2019).

Other aspects to consider are the physical parameters of eggs, such as colour, weight, shell thickness and breakage. Although colour is only a minor aspect, it is of fundamental importance in the consumer's perception. However, it is complicated to identify colour as a quality parameter, as it depends on individual preferences (Konca et al., 2019). As shown in Table 6, the addition of hemp-based products resulted in changes in egg yolk colour (Skřivan et al., 2019; Konca et al., 2019; Goldberg et al., 2012; Park et al., 2014). As reported by Goldberg et al. (2012), the intensity of yolk colour is mainly due to the presence of yellow and red oxycarotenoids, or xanthophyllic pigments, in the diet of hens. Depending on the level of these compounds in the diet, the colour of the yolk can vary from almost absent to an intense orange. However, it is important to emphasise that where changes in yolk colour are statistically significant, the perception to the human eye may be relatively insignificant and these results cannot infer consumer preferences.

Egg weight, on the other hand, represents a quality parameter. Most likely, the increase in egg weight is directly related to the increase in yolk weight, at the expense of other egg components. As shown in Table 6, the addition of hemp-based products not only resulted in comparable results to the control eggs (Halle and Shone, 2013; Silversides and Lefrançois, 2005; Neijat et al., 2014), but also in better results. More specifically, in the study conducted by Mierliță (2019), the supplementation of 80 g/kg HSs resulted in higher weights (61.7±0.937 g) than eggs from animals treated with 200 g/kg HSs cake (58.3±0.937 g) and control animals. Similar results were also observed by Gakhar et al. (2012). Indeed, animals treated with an inclusion of up to 200 g/kg HSs produced eggs characterised by a weight of 60.57±1.14 g, higher than those treated control (56.2±1.14 g).

In parallel, eggshell thickness is another key parameter. Plasma Ca and P in laying hens are interrelated. The Ca and P content in the diet has an effect on the body's effort to regulate blood pH with a consequence on the acid-base balance (Keshavarz, 1994). In laying hens, maintaining an optimal acid-base balance is necessary for bicarbonate homeostasis and eggshell formation. Differences in eggshell quality have been attributed to changes in the

mineral profile of the hens' blood plasma (Keshavarz, 1994). However, as reported by Neijat et al. (2014), it is difficult to find a direct link between dietary treatment and the content of these minerals in the blood. Most likely, as the authors argue, their levels reflect changes in demand with age. For example, Ca requirements are highest during peak egg production and may be reflected by serum/plasma calcium levels. The increase over time, irrespective of diet, may reflect a relative excess of Ca and P intake over metabolic requirements as the hens exceed peak production. However, as shown in Table 6, treatment with HSs had discordant results. While Park et al. (2014), Neijat et al. (2014) observed no difference compared to the control groups, Skrivan et al. (2019) showed a negative correlation as HSs inclusion increased. In particular, the control group showed an eggshell thickness of 363±1.5 μm, which was comparable to the diets containing 30 g/kg (357±1.5 μm) and 60 g/kg (357±1.5 μm) of HSs, but higher than the diet with 90 g/kg of HSs (350±1.5 μm). These minerals also play a key role in bone health. As observed by Skřivan et al. (2019), the higher tibial strength is in agreement with the higher Ca level in groups fed HSs-enriched diets, up to 90 g/kg. At the same time, Cannabidiol and α-tocopherol also produce positive results. The former improves fracture healing by acting on collagen cross-linking, while the latter, as previously reported, tends to induce an increase in osteogenic bone mass in vertebral secondary cancellous bone, where active bone remodelling takes place (Skřivan et al., 2019).

As far as the production parameters of laying hens are concerned, the laying rate is certainly the most important. As shown in Table 6, the results obtained are very interesting, but require further investigation. In general, the inclusion of hemp-based products has enabled production rates to be matched or improved, with no negative trends. If in the studies conducted by Jing et al. (2017), Neijat et al. (2014), Gakhar et al. (2012), Mierliță (2019), Konca et al. (2019), Rajasekhar et al. (2021a), no differences were observed compared to the control groups, interesting results were recorded by Skřivan et al. (2019); Park et al. (2014) and Halle and Shone (2013). More precisely as reported by Skřivan et al. (2019), the laying rate was improved following the 30 g/kg HSs treatment (93.6±0.59%) compared to the control group and the 60 g/kg and 90 g/kg treatments (88.7±0.59%, 86.4±0.59%, 89.49±0.59%, respectively). These results were also confirmed by Halle and Shone (2013) with a supplementation of 100 g/kg and 150 g/kg HSs cake compared to flaxseeds cake. Park et al. (2014) showed that 16.8 g/kg HSs oil increased the deposition rate (97.86±0.250%) compared to the control group (96.07±0.250%). Although it is difficult to hypothesise the explanation for this effect, it is very likely that hemp-based products have nutrigenomic value by modulating the genes responsible for oviposition rates.

As just reported, the use of hemp-based products has resulted in largely positive results in laying hens, both in the functional and nutritional aspect of eggs, animal health and growth performance. These positive effects were recorded with different levels of HSs inclusion (up to 250 g/kg). In parallel, the use of HSs oil increased the nutritional profile of eggs by raising the PUFAs present, with an inclusion of up to 300 g/kg. Interesting results were also reported for hemp co-products, in particular HSs cake (up to 150 g/kg) improving laying hens production. These findings add to the knowledge reported by EFSA (2011), in which the use

of HSs and HSs cake were confirmed up to 20% and up to 12% for HSs oil, without altering the performance of the animals and the sensory characteristics of the eggs.

CONCLUSION

The inclusion of hemp-based products in the diets of monogastric animals has led to different results depending on the species considered. As reported, the use of these products need further investigation, especially in pigs and broilers, due to limited studies. However, for these species, this review increased the data shown in the last EFSA report by reporting for the first time considerations for the use of new matrices such as HSs oil. For laying hens, on the other hand, the large number of scientific studies has made it possible to identify and confirm hemp-based products as effective and safe matrices, able of positively modulating animal health and performance, while simultaneously enhancing the nutritional and functional profile of eggs. However, even in this case, the choice of the optimal inclusion of hemp-based products requires further evaluation.

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Manuscript II

Total Phenolic Content and Antioxidant Activity of *In Vitro* Digested Hemp-Based Products

Davide Lanzoni, Eva Skrivanova, Raffaella Rebucci, Antonio Crotti, Antonella Baldi, Luca Marchetti and Carlotta Giromini

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Total Phenolic Content and Antioxidant Activity of *In Vitro* Digested Hemp-Based Products

D. Lanzoni[a], E. Skrivanova[b,c], R. Rebucci[a], A. Crotti[a], A. Baldi[a], L. Marchetti[a] and C. Giromini[a,d]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamycka 129, 165 00 Prague, Czech Republic; [c] Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Sciences, Pratelstvi 815, 104 00 Prague, Czech Republic; [d]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy.

ABSTRACT: The growth of the world population has prompted research to investigate new food/feed alternatives. Hemp-based products can be considered excellent candidates. Hemp (*Cannabis sativa* L.) is an environmentally sustainable plant widespread worldwide. Following the reintroduction of its cultivation, hemp is attracting interest, especially in the food/feed industry. To date, scientific research has mainly focused on its nutritional aspect. Therefore, the aim of the work was also to investigate the functional profile (total phenolic content (TPC) and antioxidant activity (Ferric- reducing antioxidant power (FRAP) and 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) of hemp-based products (hempseeds (HSs), flowers, and HS protein extract), following methanol extraction and *in vitro* digestion, to study the behaviour of the molecules involved. The results show an interesting nutritional value, even when compared to matrices used in the food/feed industry, such as soy and flaxseeds. The functional profile revealed a very interesting TPC following methanol extraction for HSs, flowers, and HS protein extract, respectively, (550.3 ± 28.27; 2982.8 ± 167.78; and 568.9 ± 34.18 mg Tannic Acid Equivalent (TAE)/100 g). This trend was also confirmed for FRAP (50.9 ± 4.30; 123.6 ± 8.08; and 29.73 ± 1.32 mg Ascorbic Acid Equivalent (AAE)/100 g), recording values similar/higher than soy protein extract and flaxseeds (17.4 ± 1.55; and 10.4 ± 0.44 mg AAE/100 g). The results were also maintained following physiological digestion. These results, although promising, need further investigation, confirming what has been observed with different antioxidant activity assays and identifying individual molecules involved in functional pathways. This information will be necessary to gain a better understanding of the functional characteristics of these matrices for use in food/feed formulations.

Keywords: Antioxidant; Folin-Ciocalteu; Functional Profile; Hemp; *In vitro* digestion; Phenolic compounds.

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INTRODUCTION

The steady growth of the world population will reach 9–11 billion by 2050. At the same time, there will be a dramatic growth in demand for food, reaching 50% by 2030 and doubling by 2050 at which point it will be difficult to supply the demand without negatively impacting

the health of the environment (Roos et al., 2017; Bellet and Rushton, 2019; Rischer et al., 2020). The main products required will be those of animal origin, in particular meat and dairy products. According to statistics from the Food and Agriculture Organization of the United Nations (FAO), the world demand for meat will reach 455 million tonnes by 2050, an increase of 76% compared to 2005 (Bellet and Rushton, 2019). As a result, there has been a substantial increase in the need for feed for the livestock sector, opening the feed/food competition debate. Today, around 800 million tonnes of cereal are used within the feed sector, a value that will exceed 1.1 billion tonnes by 2050. Expansion of the monogastric sector will lead to an increase in demand for maize and coarse grains, accounting for almost half of the cereals produced in 2050. In 2000 alone, pigs and poultry consumed about 78% of feed grains. More precisely, in 2013, the monogastric sector consumed 155 million tonnes of proteins, and by 2030, an additional 52 million will be needed to satisfy the sector's demand. Most of these are in direct competition with human nutrition (Makkar, 2018). The necessity has therefore arisen to satisfy the nutritional requirements of a rapidly increasing population through sustainable and environmentally friendly processes. This goal is strongly pursued by the European Union (EU), which, first with the One Health approach and later with the Green Deal (1 December 2019), aims to recognize that human, animal, and environmental health are interconnected, promoting efficient use of resources towards a clean and circular economy (Wolf et al., 2021; Sinclair, 2019). For this reason, to ensure food safety and environmental protection, research in recent years has been largely focused on finding new food/feed alternatives that meet the principles proposed by the EU.

Among the possible alternatives, hemp (*Cannabis sativa* L.) is attracting great interest. Hemp, originated in Central Asia and considered to be one of the oldest crops, is a dicotyledonous annual, herbaceous, and angiosperm plant widespread throughout the world due to its easy adaptability (Rehman et al., 2021; Petit et al., 2020). It is a low environmental impact crop, traditionally cultivated for fibre production. Despite its rapid expansion, the hemp trade declined sharply between the 1940s and the late 1960s, disappearing from the market, as a result of both the introduction of synthetic fibres and, above all, the entry into force of the Protocol on the Single Convention on Narcotic Drugs proposed by the United Nations, signed in New York on 30 March 1961 and subsequently the Protocol of Amendment adopted in Geneva on 25 March 1972. This convention put an end to the cultivation of hemp in more 183 countries (Sorrentino, 2021). However, at the start of the 21st century, interest in hemp was relaunched thanks to the introduction of EU Regulation 1251/1999/EC of 17/05/1999, which instituted a support system for all producers of particular types of arable crops, among them industrial hemp. However, it was only with Regulation (EU) No 1307/2013 that hemp cultivation was also included among those qualifying for Common Agricultural Policy (CAP) payments, with the only condition being that the seeds used for cultivation be of varieties registered in the European catalogue with a Δ9-tetrahydrocannabinol (THC) content (i.e., the plant's recognised psychoactive compound) of less than 0.2% (Sorrentino, 2021; EFSA, 2011). The introduction of these laws allowed hemp to reach the market, finding use in a wide range of sectors, from

construction and energy to cosmetics and pharmaceuticals. More precisely, to date, the hemp market comprises more than 25,000 products (Rupasinghe et al., 2020). In recent decades, interest in hemp and hemp-based products has increased, also involving the food and feed sector, as reported by EFSA (2011). In particular, the food industry has investigated the use of hempseeds (HSs) to obtain oil, protein, and dietary fibre, facilitating their incorporation into food products, such as yoghurt and baked goods (Rupasinghe et al., 2020). In addition, Guang and Wenwei (2010) used HS flours to produce functional food that aid disease prevention by increasing high-density lipoprotein levels and stabilising the level of other triglycerides and lipoproteins. As shown, the interest is in the seeds of this plant, previously considered as a waste product in fibre production, but characterised by high nutritional and functional profiles (Farinon et al., 2020), prompting researchers to deepen their potential beneficial effects in human and animal diets. To date, scientific research has focused more on the nutritional characterisation. The functional aspect, however, is still little explored. For this reason, the aim of the study was to characterise not only the nutritional, but above all, the functional profile of hemp-based products, in particular total phenolic content (TPC) and antioxidant activity, following extraction with pure methanol and *in vitro* digestion, to study the behaviour of the molecules involved during different phases of the digestive process, thus enabling new information on the functional aspect of these matrices to be acquired.

More specifically, in our study, the characterisation of the nutritional profile involved the analysis of the protein, lipid, fibre, and ash contents, as well as the determination of the digestibility level. The functional profile characterisation, instead, entailed quantification of the TPC and antioxidant capacity.

MATERIALS and METHODS

Materials

Hempseeds (*C. sativa* L, variety Futura) were purchased from a Czech company (Chrastice, Czech Republic). Specifically, the hemp sowing took place in April/May 2021, and the harvest was carried out at 70% maturity. After collection, the seeds were dried at 40°C to a final moisture content of 7% and stored at 10–12°C in the dark. Cannabinoid analysis was performed at the Institute of Animal Science (Czech Republic) showing THC and cannabidiol (CBD) contents of 0 µg/g and 30 µg/g, respectively. HS samples, before each analysis, were stored at 4°C in the dark.

The hemp flowers (*C. sativa* L, variety Carmagnola) were provided by a local company (CN, Italy). Specifically, they were collected from plants sown from May to October 2021 and harvested in October 2021. Manual harvesting with the removal of the cut plants was followed by slow drying in a closed, ventilated environment, without direct light, to a humidity between 12 and 14°C. Subsequently, the flowers were analysed for THC (<0.2%) and CBD (7.24%) contents. The samples, before each analysis, were stored at room temperature (RT) in the dark.

HS protein extract, soy protein extract, and flaxseeds (*Linum usitatissimum* L.) were provided by a commercial supplier.

Chemical Analysis

The chemical analysis of the samples (HSs, flowers, HS protein extract, soy protein extract, and flaxseeds) was performed according to official methods (AOAC, 2005; European Commission, 2009), and the fibrous fraction was analytically measured as reported by Van Soest et al. (1991).

Methanol Extraction

For methanol extraction, each sample (HSs, flowers, HS protein extract, soy protein extract, and flaxseeds) was ground and weighed (5 ± 0.5 g) and mixed with 30 mL methanol (100%) for 48 h at RT in the dark. Subsequently, each sample was filtered with filter paper (Whatman 54, Florham Park, NJ) in accordance with Castrica et al. (2018) and Attard (2013).

Total Phenolic Content and Antioxidant Activity

Subsequently, the chemical extracts were analysed for TPC and antioxidant activities (ABTS and FRAP).

Total Phenolic Content

For the total quantification of phenols, the protocol of Attard (2013) was followed. Tannic acid, methanol, Folin–Ciocalteu (FC) reagent, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid was prepared in seven 1:2 dilutions, from 960 µg/mL down to 0 µg/mL. The FC reagent was diluted 1:10 with distilled water, while sodium carbonate was prepared as a 1 M solution. Then, 100 µL of each sample was added to 500 µL of FC and 400 µL of sodium carbonate and incubated in the dark at RT for 20 min. At the end of incubation period, samples were read at 630 nm. Appropriate solvent blanks were run in each assay. TPC was expressed in terms of tannic acid equivalent (mg TAE/100 g).

ABTS Assay

ABTS 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay was performed following the protocol of Re et al. (1999). In particular, 2.5 mM Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Sigma Chemical Co.) (St. Louis, MO, USA) was used as antioxidant standard. Fresh working standards were prepared daily on dilution with ethanol. ABTS, provided by Sigma Chemical Co. (St. Louis, MO, USA), was dissolved in distilled water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study of antioxidant activity, the $ABTS^{\bullet+}$ solution was diluted with ethanol to reach an absorbance value of 0.70 (±0.02) at 734 nm. Then, 20 µL of sample was added to 2.0 mL of diluted $ABTS^{\bullet+}$ solution (A_{734nm} = 0.700 ± 0.020), incubated for 6 min at RT in the dark, and

read to 734 nm. Appropriate solvent blanks were run in each assay (Regmi et al., 2009). Values were expressed in terms of Trolox equivalent (mg TE/100 g).

Ferric-Reducing Antioxidant Power (FRAP) Assay

FRAP assay was performed following the protocol of Abdelaleem and Elbassiony (2021) with minor adaptations. In particular, ascorbic acid was used as antioxidant standard and prepared in seven dilutions from 1000 µL to 0 µL. a) Acetate buffer (300 mM; pH 3.6) was prepared using 2.69 g sodium acetate trihydrate (Sigma Chemical Co. (St. Louis, MO, USA)) in 16 mL of glacial acetic acid and made the volume to 1.0 L with distilled water. b) TPTZ (2, 4, 6-tripyridyl-s- triazine), provided by Sigma Chemical Co. (St. Louis, MO, USA), was obtained dissolving 31.2 mg in 10 mL of 40 mM of HCl. c) $FeCl_3$ (Sigma Chemical Co. (St. Louis, MO, USA)) was obtained dissolving 0.054 g in 10 mL of distilled water. Then, 10 µL of each sample was added to 300 µL of FRAP reagent and incubated at RT for 10 min in the dark and read at 595 nm. Appropriate solvent blanks were run in each assay. Values were expressed in terms of acid ascorbic equivalent (mg AAE/100 g).

In Vitro Digestion and Digestibility

In vitro digestion was performed according to Regmi et al. (2009), with minor adaptations reported by Castrica et al. (2019). The protocol aims to simulate the three phases of digestion. At the end of each digestive phase (oral, gastric, ½ intestinal, and intestinal), aliquots (1 mL) corresponding to the soluble fraction were taken and frozen at −80 °C and used to monitor the TPC and antioxidant activities during *in vitro* digestion. In addition, at the end of digestion, an undigested fraction (UF) was also obtained. The UF was subsequently harvested in a filtration unit using a porcelain filtration funnel covered with pre-weighed filter paper (Whatman 54 Florham Park, NJ). The UF, together with the filter paper, was dried overnight at 65 °C. The UF was used to determine the *in vitro* digestibility Equation:

$$\text{Digestibility (\% dry matter; DM)} = (\text{sample DM} - \text{UF DM})/\text{sample DM} \times 100$$

The described procedure was performed in triplicate (n =3). Following *in vitro* digestion, the samples were analysed for total phenolic content and antioxidant activity (ABTS and FRAP), as described above.

Statistical Analysis

All data were analysed by one-way Anova followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are expressed as means ± standard error of the mean (SEM) of at least three independent experiments. Values are considered statistically significant for a 95% confidence interval (p-value = 0.05).

RESULTS and DISCUSSION

Chemical Analysis and In Vitro Digestibility

The chemical composition and digestibility of the matrices under study are given in Table 1.

Table 1. Chemical composition and digestibility of study matrices (*% w/w* on DM basis). Data are presented as mean ± standard error of mean (SEM). (*n = 3*). DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; HS = hempseeds; PE = protein extract; DIG = digestibility. Different superscript letters in columns indicate statistically significant differences (*p<0.05*).

	DM	CP	EE	NDF	ADF	ADL	ASHES	DIG.
HS	94.6± 0.12^{a}	23.1± 0.57^{a}	27.9± 0.75^{a}	44.6± 0.21^{a}	33.2± 0.31^{a}	14.4± 0.32^{a}	5.8± 0.10^{a}	53.4± 0.78^{a}
Flowers	98.4± 0.03^{b}	18.9± 0.29^{b}	12.9± 0.25^{b}	37.0± 0.67^{b}	23.7± 0.35^{b}	11.7± 0.79^{b}	16.3± 0.08^{b}	37.4± 1.87^{b}
HS PE	95.7± 0.02^{c}	45.1± 0.84^{c}	9.2± 0.09^{c}	40.4± 1.80^{a,b}	20.2± 1.02^{c}	9.5± 0.62^{b,d}	8.3± 0.08^{c}	65.5± 0.67^{c}
Soy PE	91.1± 0.22^{d}	51.2± 0.57^{d}	1.2± 0.06^{d}	48.2± 0.97^{a,c}	8.8± 0.14^{d}	2.2± 0.10^{c}	6.6± 0.20^{d}	74.1± 3.59^{c}
Flaxseeds	91.6± 0.14^{d}	23.0± 0.45^{a}	36.0± 0.07^{e}	41.5± 1.27^{a,b}	19.5± 0.35^{c}	9.2± 0.41^{d}	2.7± 0.20^{e}	24.2± 2.08^{d}

As previously reported, HSs can be considered as one of the most complete sources from a nutritional perspective due to their high nutritional characteristics (Farinon et al., 2020), as shown in Table 1. In the literature, many authors show high variability in HS composition due to genotype and environmental growth factors (Lan et al., 2019; Mattila et al., 2018; House et al., 2010). However, as reported by Leonard et al. (2020); Farinon et al. (2020); and Callaway (2004), they typically contain 94% dry matter, 20–25% protein, 25–35% lipids, 20–30% carbohydrates (mostly dietary fibre), and 5–6% ash. These values, as show in Table 1, are comparable to those obtained for flaxseeds, one of the most important oilseed crops for industrial as well as food, feed, and fibre purposes (Zajac et al., 2021; Singh et al., 2011). HSs show a good level of digestibility, higher than that of flaxseeds, showing statistically significant differences ($p < 0.05$). Zhou et al. (2020) demonstrated that *in vitro* digestion of flaxseed polysaccharides did not lead to their degradation (no change in molecular weight), suggesting that ingested polysaccharides would reach the large intestine intact. Similar results were reported by Marambe et al. (2012). The authors observed that following the *in vitro* digestion process, the protein digestibility of flaxseeds was only 12.61%, values that tended to increase following the removal of the fibrous and lipid contents. In the light of the results obtained, and as reported in the literature, to improve the digestibility of these two matrices, it is preferable not to use the whole seed, but only after treatment (heat, extrusion, and dehulling) to reduce the fibre level (Leonard et al., 2020) or to prefer the meal to reduce the fat content, thus making the seeds more suitable for food and feed applications.

Hemp flowers are an important component of the plant. They are currently used for ornamental purposes as well as in cosmetics and pharmaceutical. The essential oil, rich in functional properties (antioxidant and anti-inflammatory) (Farinon et al., 2020) can be obtained from their processing. Although the use of flowers within the food/feed sector is not thoroughly documented in the literature, they have an interesting nutritional profile, especially in terms of protein content, suggesting their possible use within the feed sector, as confirmed by Kleinhenz et al. (2020). However, the high fibre and mineral contents adversely affect final digestibility. Furthermore, the presence of phytocannabinoids makes it necessary to study their effects on animal and/or human health. For this reason, further investigations are needed to assess their possible inclusion within the food and feed sector.

In our experiment data, the digestibility of HS protein extract showed no statistically significant differences with soy protein extract. Mamone and colleagues (2019) studied how HS protein is characterised by high digestibility. The authors demonstrated how, following *in vitro* digestion, only a few peptides withstood the digestive process. This finding is also confirmed by Wang et al. (2008). They not only observed that HS protein was highly digestible, but that the level of digestibility was also higher than that of soy. Protein digestibility is a crucial parameter, as together with amino acid composition and bioavailability, it determines the nutritional value of a protein source (Farinon et al., 2020). HS protein is characterised by a good amino acid content, greater than or similar to that of soy, with the exception of aspartic acid, glutamic acid, and lysine. In addition, it contains all essential amino acids (Farinon et al., 2020). This suggests that HS protein can be used as a viable substitute to soy protein, the main protein source used in the feed industry due to its important nutritional value (Tang et al., 2009).

Total Phenolic Content and Antioxidant Activity of Methanol Extracts

In Table 2, the values for TPC and antioxidant activities (ABTS and FRAP) of methanol extracts are reported. In our study, chemical extraction was performed in methanol to ensure the best possible yield, as demonstrated by Kalinowska et al. (2022).

Table 2. Total phenolic content (TPC) and antioxidant activity (ABTS and FRAP) of methanol extracts. TAE = tannic acid equivalent; TE = Trolox equivalent; AAE = ascorbic acid equivalent; HS = hempseeds; PE = protein extract. Data are presented as mean ± standard error of mean (SEM). ($n = 3$). Different superscript letters in column indicate statistically significant differences ($p < 0.05$).

Sample	TPC (mg TAE/100 g)	ABTS (mg TE/100 g)	FRAP (mg AAE/100g
HS	550.3±28.27[a]	205.14±3.37[a]	50.9±4.30[a]
Flowers	2982.8±167.78[b]	6122.1±249.52[b]	123.6±8.08[b]
HS PE	568.9±34.18[a]	174.5.±4.30[a]	29.73±1.32[c]
Soy PE	792.3±0.28[a]	209.6±10.73[1]	17.4±1.55[c,d]
Flaxseeds	634.0±18.95[a]	73.2±3.32[a]	10.4±0.44[d]

Total Phenolic Content of Methanol Extracts

As shown in Table 2, the TPC of HS is comparable to that of flaxseed (550.3 ± 28.27 vs. 634.0 ± 18.95), known for their high phenol content, mainly lignans, flavonols, flavanones, flavones, and phenolic amides (Alasalvar et al., 2021). This result is partially confirmed by Galasso et al. (2016). The authors investigated the TPC of HS and flaxseed and reported that the levels were slightly higher in the former. This difference can be explained by the influence of several biotic and abiotic factors involved in the biosynthetic pathway of phenols, among which, genotype, year of cultivation, and the interaction between these factors (Irakli et al., 2019). More precisely, HSs used in this study are characterised by both a different year of cultivation, altitude and, above all, harvesting at a different stage of maturity, most likely factors responsible for this difference. Phenolic compounds are secondary metabolites, which plants produce as a defensive weapon against biotic and abiotic stresses, among them predator attack and UV radiation. They are known for their intrinsic antioxidant effect, which means they can protect cells from oxidative damage, thus limiting degenerative diseases associated with oxidative stress (Sorrentino, 2021). HSs as reported in the literature are characterised by a high phenol content, localised more in the hull than in the kernel (Mattila et al., 2018; Pojic et al., 2014), as well as in oil (flavanones, flavonols, flavanols, and isoflavones) (Smeriglio et al., 2016). In particular, the main phenolic compounds identified in HSs are the lignans, phenols derived from the shikimic acid biosynthetic pathway, also called phenylpropionamides thanks to their particular chemical structure. They are part of two major groups, known as phenolic amides and lignanamides (Irakli et al., 2019). Lignanamides are those most commonly found within HS, including cannabisin B and N-trans-caffeoyltyramine, which are the main phenolic compounds in the hull fraction; catechin, in the cotyledonary fraction (Irakli et al., 2019), as well as cannabisin A, F, I, and Q and grossamide (Pojic et al., 2014; Smeriglio et al., 2016; Bourjot et al., 2017).

In the literature, the characterisation of TPC related to hemp flowers is little studied. However, as shown in Table 2, they have a higher TPC than the other analysed matrices, showing statistically significant differences. As reported by Izzo et al. (2020), hemp flowers can be regarded as a promising new source of phenols for nutraceutical formulations. The authors demonstrated through their characterisation that this part of the plant is distinguished by a high content of lignanamides, specifically cannabisin A, B, and C. Additional phenolic compounds include hydrozycinnamic acids (caffeic acids, chlorogenic acid, *p*-coumaric acid, and ferulic acid) and flavonoids. Of the latter, those most commonly present are flavones, in particular, cannflavin A and B, which are characterised by important functional properties and are 10–100 times more abundant than in other parts of the plant, most likely explaining the reason for such a high TPC (Izzo et al., 2020).

As shown in Table 2, the TPC of the HS protein extract is highly comparable to that of the whole seed (550.3 ± 28.27 vs. 568.9 ± 34.18). This result can be attributed to the fact that the protein extract tested is not pure but has similar fibre concentrations as the whole seed,

a component where most of the phenolic compounds might be located. At the same time, the protein extract of HS is comparable to that of soy. The values show no statistically significant differences, although that of soy is higher. As reported by Chatterrjee et al. (2018), isoflavones, one of the main bioactive compounds in soy, are bound to the protein component, resulting in a greater value. However, it must be considered that non-phenolic compounds, such as some vitamins and their derivatives (L-ascorbic acid, folic acid, folinic acid, retinoic acid, and thiamine), nucleotide bases (e.g., guanine), amino acids (tyrosine, tryptophan, and cysteine), and simple inorganic ions (Fe^{+2}, Mn^{+2}, I^{-}, and SO_3^{-2}), react as well as with the Folin–Ciocalteu reagent. Nevertheless, the assay is commonly applied for the study of the TPC in food products (Kalinowska et al., 2022).

Antioxidant Activity of Methanol Extracts

The antioxidant activity of phenolic acids is related to the quantity, number, and position of hydroxyl groups in the molecule. Phenolic compound can act in many ways: (I) chelating metals, such as iron and copper; (II) breaking the chain of reactions triggered by free radical; and (III) slowing down or accelerating enzyme activity (Biskup et al., 2013). Given the different modes of action of phenolic compounds, the antioxidant activity was assayed by two different methods: (I) ABTS, allows the quantification of free radical scavenging capacity and (II) FRAP, allows the quantification of compounds capable of reducing the complex of ferric ions (Fe^{+3}) ligand to ferrous complex (Fe^{+2}).

As shown in Table 2, HSs are characterised by good antioxidant activity, highly comparable to that of flaxseeds. No statistically significant difference was observed with the ABTS method, unlike that reported for FRAP. However, as can be seen, HSs show greater scavenging activity than metal chelation. HSs, in fact, are characterised by the presence of cannabisin B, N-trans-caffeoyltyramine, and high concentrations of tocopherol, compounds capable of disrupting free radical chain reactions by capturing them (Chen et al., 2012; Engin, 2009). However, as suggested by Chen et al. (2012), the antioxidant activity is not so much related to the type of molecule present, but to the concentrations of the individual compounds within the matrix analysed. As reported in Table 2, hemp flowers show a greater antioxidant activity than all other matrices studied, showing statistically significant differences. Again, the scavenging activity of the antioxidant molecules is higher. This result is not only due to the presence of phenolic compounds, but also to CBD within the flowers, the only component of the plant to have it. CBD, in fact, is characterised by multiple properties, including anti-inflammatory and antioxidant properties. In particular, it is able to inhibit oxidant molecules by interrupting the chain reactions triggered by free radicals, capturing them or transforming them into less active forms (Atalay et al., 2019). It can also promote a direct reduction in oxidant levels as well as modify the redox balance by changing the level and activity of antioxidant molecules (Peres et al., 2018; Costa et al., 2007). The antioxidant activity of HS protein extract appears to be comparable to that of soy. In the literature, the functional activity of these protein matrices is not very extensive. However, as shown by Farinon et al. (2020) and Chatterrjee et al. (2018), they acquire high antioxidant

value only, and following the process of hydrolysis by digestive enzymes are capable of fragmenting the protein structure by releasing bioactive peptides.

Total Phenolic Content and Antioxidant Activity of In Vitro Digested Samples

A further step in the study was the evaluation of TPC trends and antioxidant activity during the different phases of *in vitro* digestion (end of oral phase, end of gastric phase, ½ intestinal phase, and end of intestinal phase) to assess the bioaccessibility of these compounds.

Total Phenolic Content of In Vitro Digested Samples

Figure 1 shows the TPC trend of all analysed matrices.

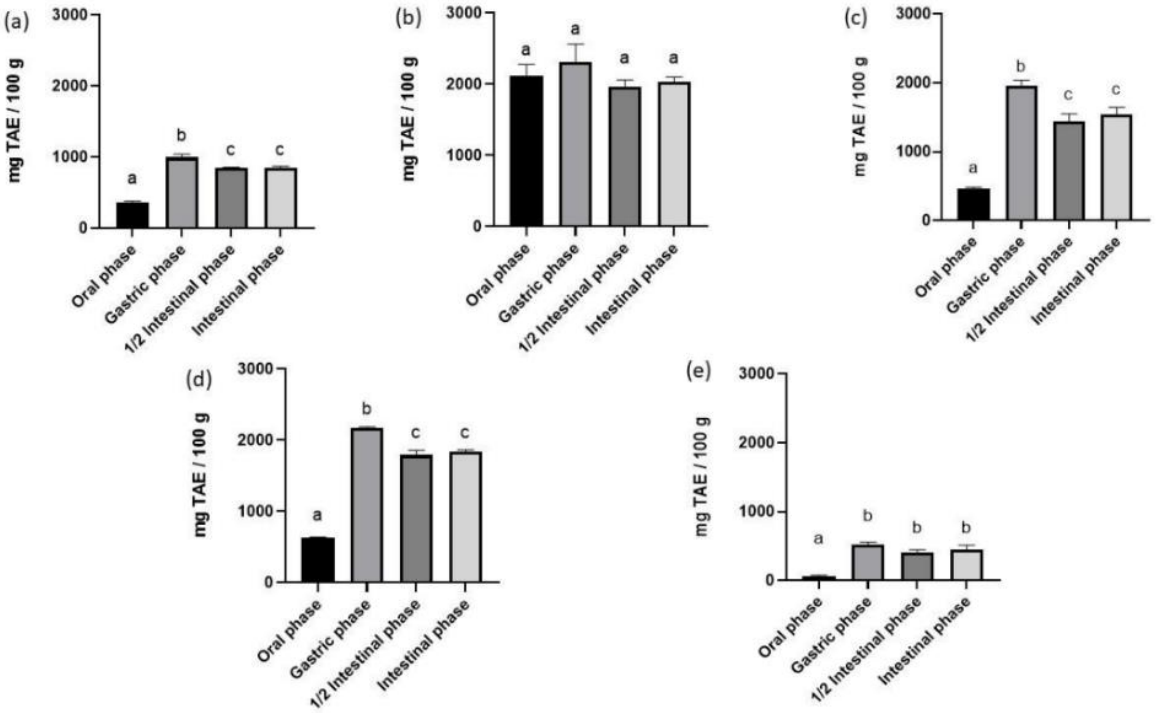

Figure 1. Total phenolic content (TPC) of *in vitro* digestion. TAE = Tannic acid equivalent. Data are presented as mean ± standard error of mean (SEM). (n = 3). Different superscript letters in columns indicate significant different data ($p < 0.05$). (**a**) hempseeds; (**b**) flowers; (**c**) hempseed protein extract; (**d**) soy protein extract; and (**e**) flaxseeds. End of oral phase, end of gastric phase, ½ intestinal phase, and end of intestinal phase.

The nature of phenolic components is important in assessing the antioxidant activity of a sample (Giromini et al., 2020). As shown in Figure 1, the TPC within the analysed matrices shows the same trend, with higher values for hemp flowers, most probably due to the ready bioavailability of phenolic compounds. HS shows a higher content than flaxseed, while the two protein matrices are highly comparable. These results are confirmed in other studies in the literature conducted on plant matrices (bamboo leaves, Butia, and Carob fruits). At the end of the oral phase, there is an initial and partial degradation of the phenolic compounds (Ma et al., 2020; Goulas and Hadjisolomou, 2019; Vinholes et al., 2018). Furthermore, as

reported by Leonard et al. (2020), there is a strong interaction between fibre and phenols. In fact, in addition to being able to trap them, it reacts chemically through hydrophobic interactions, hydrogen bonding (oxygen atoms from polysaccharide's glycosidic chains and hydroxyl groups from phenolic compounds), and covalent bonds, thus reducing the bioavailability of phenolic compounds within the analysed matrices. However, as suggested by Ginsburg et al. (2012), saliva could play a key role in the solubilisation of phenolic compounds by substantially increasing their availability (Ginsburg et al., 2012).

At the end of the gastric phase, an increase in TPC can be observed in all analysed matrices. This increase is the result of a low pH value (2 ± 0.05) that can promote the release of phenols following the breaking of bonds within the matrix including polysaccharides and proteins (Goulas and Hadjisolomou, 2019).

Subsequently, in the middle of the intestinal phase, the TPC trend decreases. This result, also confirmed by Friedman and Jurgens (2000), is a direct consequence of the instability of phenols at high pH values (6.8 ± 0.05). At the end of the intestinal phase, there is a slight increase in TPC, as the phenolic compounds are, most likely, transformed into other structural forms, formed as a result of the cleavage of specific bonds in their structure (Pinacho et al., 2015). These molecules, detected by the assay, thus result in an enhanced concentration of TPC.

In conclusion, TPC during the *in vitro* digestion process is thus influenced by the nutritional components, especially fibre, but above all by the physiological conditions of the digestion process. Concerning the individual matrices, it can be observed that hemp flowers also in this case showed the highest TPC. HS showed a slightly higher TPC than flaxseed, as did the HS protein extract compared to soy.

Antioxidant Activity of In Vitro Digested Samples

For the evaluation of the effect of *in vitro* digestion on the antioxidant capacity of hemp-based products, the ABTS and FRAP assays of digested samples were determined and are shown in Figure 2.

As reported in Figure 2, HS shows higher (ABTS) and highly comparable (FRAP) values than flaxseed, further confirming the important functional aspect of this matrix. The same applies to HS protein extract when compared to soy protein. As previously reported, HS protein is not characterised by high bioactivity. However, the hydrolysis process in the protein structure leads to the formation and release of bioactive peptides. Bioactive peptides can be defined as isolated small fragments of protein, which provide some physiological health benefits (Giromini and Cavalleri, 2022). In particular, HS peptides are characterised by high functional properties, including antioxidant, antihypertensive, antiproliferative, cholesterol-lowering, anti-inflammatory, and neuroprotective properties (Teh et al., 2016; Zanoni et al., 2017; Malomo and Aluko, 2016). This shows that the peptides are encapsulated within the native protein structure and are only released during the hydrolysis process. In particular, Tang and coworkers (2006) and Wang and colleagues (2009) demonstrated that

HS hydrolysates with a greater degree of hydrolysis possessed greater antioxidant activity *in vitro*, particularly with regard to radical scavenging and iron chelation capacity (Tang et al., 2006; Wang et al., 2009). Hemp flowers, although they once again confirm their great antioxidant power, show a different trend from those we studied and from those reported in the literature. For this reason, it is necessary to investigate this aspect further for their possible use within the food and feed sector.

The antioxidant activity of the samples under study showed different trends for the two methods used (ABTS and FRAP). The values obtained for FRAP, the method used in other plant matrices, as reported by Koocheki et al. (2022) are in line with those observed for TPC. As can be seen in Figure 2, there is a peak in antioxidant activity following the gastric phase. As explained above, this may lead to the release of phenolic compounds during the digestive process. These results are also confirmed in studies by Gonzalez et al. (2016) and Ma et al. (2020), where different matrices, such as Maqui berry and bamboo leaves, showed the same trend. In the middle of the intestinal phase there is a decrease in antioxidant activity, because as reported by Ma and colleagues, a large part of the phenolic compounds is degraded at this stage of the digestive process (Ma et al., 2020). At the end of the digestive process, a slight increase in antioxidant activity can be observed, due to the release of additional compounds with high antioxidant activity, such as flavonoids, tocopherols, carotenoids, pigments, and ascorbate (Pinacho et al., 2015).

As far as the antioxidant activity recorded by the ABTS method is concerned, the graphs in Figure 2 all show the same trend, with the exception of the hemp flowers, which record a decrease in antioxidant activity at the end of the gastric phase.

For all the other matrices, a peak is observed at the end of the intestinal phase, confirming what was previously reported. Based on the evidence of Pinacho et al. (2015), phenolic compounds are sensitive to the alkaline pH of the intestinal phase and for this reason may be converted into other structural forms, either unknown or difficult to detect, that exert their antioxidant activity at the end of digestion.

These values, coupled with the functional profile, emphasise how hemp-based products can be considered as a viable alternative to flaxseeds and soy, the main crops used in the food and feed industry. In agreement with the WWF, each kilogram of chicken, pork, eggs, and meat contains 575, 263, 307, and 173 g of soy, respectively (WWF, 2017). As reported in the literature, soy has a high environmental impact (Erikson et al., 2018). Hemp, which is characterised by an important role in environmental health, could mitigate this problem.

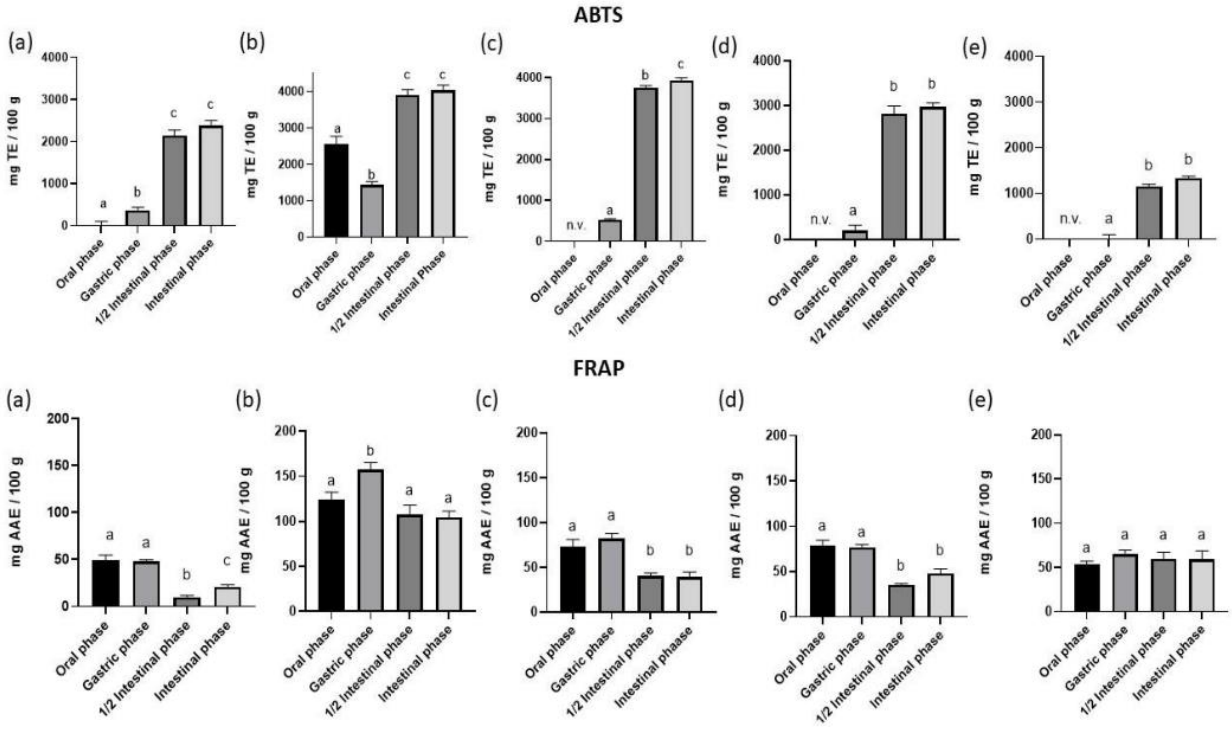

Figure 2. **Antioxidant activity (ABTS and FRAP) of *in vitro* digestion**- TE = trolox equivalent; AAE = ascorbic acid equivalent. Data are presented as mean ± standard error of mean (SEM). (n = 3). Different superscript letters in columns indicate significant different data ($p < 0.05$). (**a**) hempseeds; (**b**) flowers; (**c**) hempseed protein extract; (**d**) soy protein extract; and (**e**) flaxseeds. End of oral phase, end of gastric phase, ½ intestinal phase, and end of intestinal phase. n.v.: not valuable.

CONCLUSION

In the light of these reports, HSs and HS protein extract proved to be products with a high nutritional profile. Interesting results were also reported for flowers, where the high protein content suggests their possible application in the food and feed sector. However, while the nutritional aspect of hemp-based products has already been adequately described in the literature, the functional one is still little investigated. For this reason, the aim of this work was to further explore the functional characterisation of hemp-based products following extraction with pure methanol and the *in vitro* digestion process, obtaining new information on TPC and antioxidant activities. The reported results show that the flowers are characterised by a high functional profile and that the HSs and HS protein extract have similar or higher values than those reported for other food/feed matrices, such as soy and flaxseeds. The high nutritional aspect combined with the functional one suggests that hemp-based products can be considered as viable alternatives for the food and feed industry. However, these results, although promising, need further investigation, confirming what has been observed with different assays of antioxidant activity and identifying the individual molecules involved in the functional pathway. This information will be necessary to gain a better understanding of the functional characteristics of these matrices for use in food/feed formulations.

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Manuscript III

Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and *ex vivo* digestion

Davide Lanzoni, Francesca Mercogliano, Raffaella Rebucci, Corinne Bani, Luciano Pinotti, Chiara Di Lorenzo, Giovanni Savoini, Antonella Baldi and Carlotta Giromini.

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Phenolic profile and antioxidant activity of hemp co-products following green chemical extraction and *ex vivo* digestion

D. Lanzoni[a], F. Mercogliano[b], R. Rebucci[a], C. Bani[b], L. Pinotti[a,c], C. Di Lorenzo[b,c], G. Savoini[a], A. Baldi[a] and C. Giromini[a,c]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milano, Italia; [c]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy.

ABSTRACT: The valorisation of biomass generated along the agri-food chain into co-products represents a valid approach to produce alternative and sustainable feed ingredients. Although to date, some co-products have already been included in the diets of livestock animals, the characterisation of hemp co-products (Cannabis sativa L.) is still at an early stage. For this, the aim of this this work was to investigate the nutritional and functional profile [total phenolic content (TPC) and antioxidant activity (ABTS, FRAP)] of hemp co-products [mix flowers and leaves (MFL), hulls (HLs) and hempseeds cake], after green chemical extraction (0% EtOH; 50% EtOH; 100% EtOH) and *ex vivo* digestion process. High-Performance Thin Layer Chromatography was performed on 50% EtOH chemical extracts to identify the main phenolic compounds. The results reported an interesting nutritional profile, although dependent on the processing to obtain the co-product. The functional aspect of the chemical extracts showed a higher TPC for MFL, especially after 50% EtOH extraction (3551.12 ± 98.54 mg TAE/100 g) identifying Quercetin, Kaempferol, Rutin, Chlorogenic acid, Ferulic acid, Cannabigerol and α-tocopherol as the main compounds present, a profile similar to that of HLs. This was also observed for ABTS (6950.10 ± 546.82 mg TE/100 g) and FRAP (130.05 ± 4.67 mg FeSO4/100 g). *Ex vivo* digestion confirmed the high functional profile of the two matrices. Despite this, it is important to emphasise that processing co-products are characterised by a high variability that may lead to different results and effects on the health and performance of monogastric animals.

Keywords: Antioxidant; Extraction; Digestion; Hemp; Phenols

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HIGHLIGHTS

- The high environmental impact of the food/feed sector has prompted scientific research to valorise the co-products of the food chain.
- Hemp co-products are characterised by an interesting nutritional profile, particularly in terms of protein and fat content.
- Hemp co-products, particularly hulls and mix flowers/leaves are characterised by high functional activity.

INTRODUCTION

The high environmental impact of the food and especially feed sector is a highly debated topic in recent years, which is attracting increasing interest from the scientific community, particularly from the perspective of food-feed competition. Globally, the production, processing, and transport of feed for the livestock sector accounts for 45% of the total production of greenhouse gases (Makkar, 2018). Simultaneously, as reported by the same author, the area dedicated to feed-crop cultivation accounts for 33% of the total arable land, while about 30% is occupied by grazing, with more than 90% water consumption (Makkar, 2018). This scenario is bound to worsen if considering that the world's current population will exceed 9 billion people in the next 30 years, leading to an ever-increasing need for food and food production, which could have a further impact on climate change, as shown by projections for 2050 by the EAT-Lance commission (Willet et al., 2019).

For this reason, the European Union (EU) is working to ensure a sustainable future through the implementation of strategies such as the Green Deal and One Health, the aim of which is the transformation of the EU into a prosperous society with a competitive, clean, and circular economy that recognises the close link between human, animal, and environmental health (Fetting, 2020; Sinclair, 2019; Pinotti et al., 2023). In this context, the Farm to Fork (F2F) strategy, the heart of the European Green Deal, plays a major role. F2F is a 10-year plan devised by the European Commission to guide the agricultural transition towards more equitable, healthy, and environmentally friendly food systems, making them more sustainable than they are today (European Union, 2020). To achieve these goals, one possible approach is to utilise the huge amount of non-edible biomass produced along the entire food chain (about 1.3 billion tonnes) as co-products for livestock (FAO, 2011; Rakita et al., 2021). As reported by Pinotti et al. (2020), the term co-product refers to any product obtained from different agro-industrial processes.

To date, several co-products such as beet pulp, soybean meal, soybean molasses, sunflower meal, and grape marcs (GMs) have already been included in animal diets due to their interesting nutritional profile, but many others could be used (Vastolo et al., 2022; Mirzaei-Aghsaghali and Maheri-Sis, 2008). Among these, the co-products of industrial hemp (*Cannabis sativa* L.) are attracting increasing interest. Hemp is a dicotyledonous, annual, herbaceous, angiosperm plant that is widespread worldwide due to its easy adaptability (Lanzoni et al., 2023; Lanzoni et al., 2023a). It is considered a low environmental impact plant as it does not require large amounts of water, use of pesticides and acts as an antagonist to weeds (Rupasinghe et al., 2020). At the same time, as well as being able to grow rapidly in different agro-ecological conditions, it is an excellent candidate for carbon sequestration due to its height (it can reach up to four metres) (Farinon et al., 2020; Rehman et al., 2021; Rupasinghe et al., 2020). Following EU Regulation No. 1307/2013, which allows the cultivation of hempseeds (HSs), on condition that they are registered in the European catalogue and have a Δ9-tetrahydrocannabinol (THC) content of less than 0.2%, this plant has been attracting the interest of several industries (EFSA, 2011; Lanzoni et al., 2023). In particular, the food and feed industry has studied the properties of HS, previously considered

a waste product from the processing of this plant, recognising it as a feed material with important nutritional and functional properties (Lanzoni et al., 2023a; Farinon et al., 2020). In the F2F contest, hemp shows as a valuable opportunity to valorise the co-products of its processing. As reported by Ely and Fike (2022), Vastolo et al. (2021), and Lanzoni et al. (2023a), the hemp co-products can be summarised as follows: whole plant, stems, chaffs, stalks, hulls (HLs), HS cake (HSC), leaves (before and after cannabinoid extraction), and flowers (before and after cannabinoid extraction). As reported by the same authors, these co-products are characterised by an interesting nutritional profile, particularly regarding to protein content, which starts at 5% for stalks and reaches values of more than 20% for HSC, chaffs, flowers, and HLs. Interesting results are reported also for the lipid content (Ely and Fike, 2022; Vastolo et al., 2021).

However, their nutritional and especially functional profile is still poorly characterised. This is probably due to the fact that hemp cultivation is still small-scale, even though Europe, as reported by Horne (2020), is one of the main countries in terms of hemp production area. Furthermore, as reported by Ely and Fike (2022), this is because the various hemp industries are in their early stages, without significant markets capable of absorbing large quantities of hemp scraps and consequently generating large volumes of co-products. To date, the EU, through Regulation 2022/1104 of 1 July 2022, has registered only HS, HSC, HS oil, hemp flour and hemp fibre (both originating from stems) in the European Catalogue of feed materials (EU, 2022).

On this basis, the aim of this work was to investigate the nutritional and functional profile of hemp co-products (mix flowers/leaves (MFL), HLs and HSC), also in comparison to GMs, matrices highly described in the literature, to assess their potential for livestock feed. In particular, total phenolic content (TPC) and antioxidant activity [2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (*ABTS); Ferric Reducing Antioxidant Power (FRAP);* 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH)] were monitored following a green chemical extraction, also identifying the main phenolic compounds by High-Performance Thin-Layer Chromatography (HPTLC). In parallel, TPC, ABTS, and FRAP were also evaluated following an *ex vivo* digestion protocol.

MATERIALS and METHODS

Materials

- Hulls: HLs are derived from the dehulling process of HSs using sieves and airflow (*C. sativa* L, variety Bialobrzeskie). The HSs were supplied by a Czech company (Chrastice, Czech Republic). Specifically, the HSs were sown in May 2022 and harvested in early October 2022.

- Mix flowers/leaves: The MFL (*C. sativa* L, variety Carmagnola) was provided by a local company (CN, Italy). After hand-harvesting, the sample was dried slowly in the dark in a closed and ventilated environment in order to safeguard the functional compounds.

Subsequently, the cannabidiol (CBD) content was recorded and resulted in a concentration of between 2-3%.

- HSs cake: The HSC (*C. sativa* L, variety Carmagnola) was supplied by a local company (CN, Italy). Specifically, the seeds were sown in May 2022 and harvested in October 2022 by threshing. Following the harvesting process, the seeds were dried to stabilise the moisture content. Finally, they were cold-pressed for the production of HSs oil. The remaining fraction obtained (HSC) was harvested and stored in a dry and ventilated environment.

- Grape marcs: The GMs (from red wine industry, also containing seeds) were provided by a local producer (PD, Italy).

Methods

Chemical analysis

Chemical analysis was performed following the Official Methods of Analysis (AOAC, 2005). In particular, dry matter (DM) and ashes were determined according to AOAC method 942.05. Protein content and ether extract were assessed with the AOAC method 2001.11 and DM 21/12/1998, respectively. Finally, the fibrous fractions were identified according to Van Soest et al. (1991).

Green Chemical extractions

Green chemical extractions were performed using three different ratios of water:ethanol (H_2O:EtOH) (0% EtOH; 50% EtOH; 100% EtOH). More precisely, the protocol developed by Brighenti et al. (2017) was performed with minor modifications (Lanzoni et al., 2024). Following grinding (diameter of 1 mm, rotor mill Retsch Mod. zm 200, Hann, Germany) of the samples (0.150±0.05 g), 5 mL of solvent (H_2O:EtOH) were added and incubated for 1 h at room temperature (RT), under shaking conditions in the dark. At the end of the incubation, the samples were centrifuged at 4000 rpm for 5 min obtaining two fractions: (I) supernatant was recovered and stored at 4°C, (II) precipitate, corresponding to the residual fraction, was extracted twice, obtaining a final volume of extract equal to 15 mL per sample. The final extract obtained was stored at -20°C until further analysis (TPC and antioxidant activity). Each extraction was performed in triplicate (n=3). For each biological replicate, the technical duplicate was considered.

Total phenolic content and Antioxidant Activity (ABTS and FRAP assays)

The protocol of Attard (2013) with minor modifications (Lanzoni et al., 2023) was used to test TPC. Specifically, 200 µL of each sample were incubated with 1.0 mL of Folin-Ciocalteu reagent and 800 µL of sodium carbonate in the dark at RT for 20 min. At the end of the incubation, the samples were read with a spectrophotometer at a wavelength of 630 nm. Values were expressed in terms of Tannic acid equivalent (mg TAE/100 g of dried material).

At the same time, antioxidant activity was assessed using the ABTS (Re et al., 1999) and FRAP (Abdelaleem and Elbassiony, 2021) methods. For ABTS, 10 µL of sample were added to 1.0 mL of diluted $ABTS^{\circ+}$ solution (A_{734nm} = 0.700±0.020), incubated for 6 min at RT in the dark, and read at 734 nm. Data were expressed as Trolox equivalent (mg TE/100 g of dried material). For FRAP, the working solution (FRAP reagent) was prepared as follows: a) 25 mL of acetate buffer (300 mM; pH 3.6); b) 2.5 mL of 2,4,6-tripyridyl-s- triazine (10 mM); c) 2.5 mL of $FeCl_3$ (20 mM). Next, 10 µL of each sample were added to 300 µL of FRAP reagent and incubated at RT for 10 min in the dark and read at 595 nm. Results were expressed as mg $FeSO_4$/100 g of dried material.

High-Performance Thin Layer Chromatography and DPPH of green chemical extractions

In this study, the HPTLC technique was used to analyse flavonoids, phenolic acids, Cannabigerol (CBG) and α-tocopherol after chemical extraction with 50% EtOH, as a high TPC was obtained with this ratio. Gallic acid, Chlorogenic acid, Caffeic acid, Ferulic acid, Ruthin, Epicatechin, and α-tocopherol standards were purchased from Sigma Aldrich (Steinheim, Germany), while Quercetin 3-O-glucoside, Kaempferol, and Catechin were purchased from Extrasynthese (Genay, France). Cannabigerol by Linnea Natural Pharma Solutions (Riazzino, Switzerland). In particular, following the extraction process, 5 mL of each sample were centrifuged at 3000 rcf at 4°C for 10 min (5810 R, Eppendorf, Hamburg, Germany). After centrifugation, the samples were filtered using 0.45 µm PTFE filters (VWE) and dried with nitrogen flow to concentrate the compounds and solubilise them in 200 µL of methanol (VWR International, Fontenay-sous-Bois, France), resulting in a final concentration of 250 mg/mL. Subsequently, 10 µL of each sample were loaded onto an HPTLC silica-gel plate 60 F254 (10×20 cm, Merck, Darmstadt, Germany) with 5 µL of Quercetin 3-O-glucoside, Rutin, Catechin, Epicatechin, Gallic acid, Caffeic acid, Chlorogenic acid, Ferulic acid, CBG and α-tocopherol standard solution, by a semi-automatic sample applicator (Linomat 4, CAMAG, Muttenz, Switzerland). For all standards used, the concentration was 200 µg/mL, with the exception of CBG (1 mg/mL). After the chromatographic run, where the mobile phase consisted of 10 mL of acetone:toluene:formic acid (4.5:4.5:1 v/v/v) (VWR International, Fontenay-sous-Bois, France), the plate was exposed to UV light at 254 and 366 nm. Subsequently, the plate was derivatized with a 0.05% DPPH methanolic solution (Sigma Aldrich Steinheim, Germany), kept in the dark for 30 min, and then examined under visible light using VisionCats software (CAMAG, Muttenz, Switzerland).

Ex vivo digestion process

The digestion process was adapted from Devle et al. (2014) and performed as reported by Lanzoni et al. (2024). More precisely, gastric and intestinal fluids were collected from pigs (n=20) at slaughter between 50 and 110 days of age. Then, in order to remove the undigested fraction, the fluids were centrifuged for 10 min at 4000 rcf and used to pool gastric and intestinal fluids to reduce variability. The resulting fluids were frozen at -20°C for up to 48h. Before digestion, pH and enzyme activity were checked and adjusted if necessary to match

gastric and intestinal conditions. As reported in Figure 1, at the beginning of the gastric phase (0h), at the end of the gastric phase (2h), and at the end of the intestinal phase (4h), aliquots (1 mL) were taken to measure TPC and antioxidant activity (ABTS and FRAP) during *ex vivo* digestion. The digestion process was replicated three times (n=3) taking two technical replicates for each digestion step.

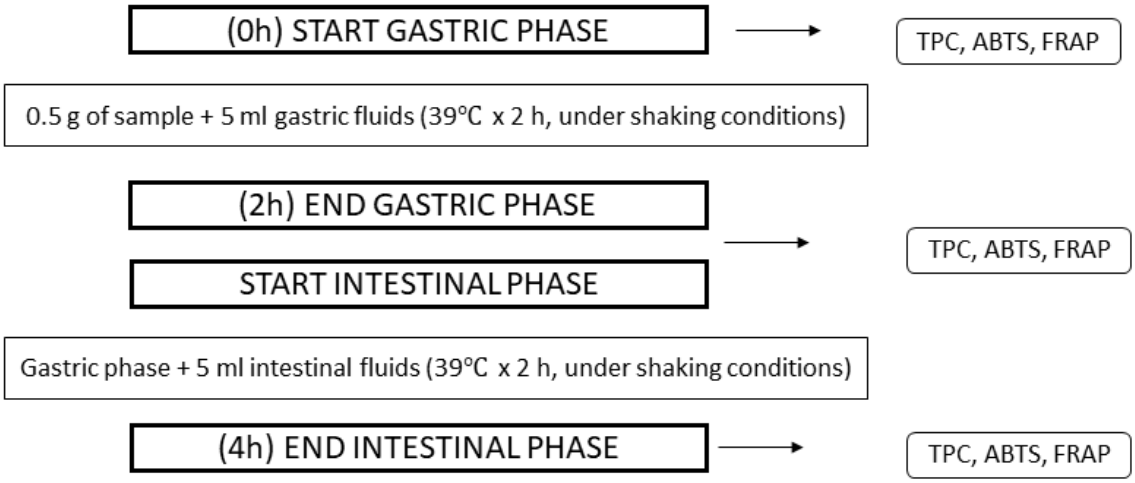

Figure 1. Workflow of *ex vivo* digestion protocol (Devle et al., 2014; Lanzoni et al., 2024). TPC: Total Phenolic Content.

Statistical Analysis

Total phenolic content and antioxidant activities of the green chemical extracts and *ex vivo* digestion were analysed by two-way Anova (*EtOH concentration x Co-products*; *Time x Co-products*, respectively) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are reported as means ± standard error of the mean (SEM) of at least three independent experiments. Values are considered statistically significant for a 95% confidence interval (*p-value* = 0.05).

RESULTS and DISCUSSION

Chemical analysis

The nutritional profile of HLs, MFL, HSC, and GMs is shown in Table 1.

Table 1. Chemical composition of MFLs (mix flowers/leaves), HLs (hulls), HSC (HS cake) and GM (grape marcs). Data are presents as mean ± standard error of mean (SEM). (n = 3). DM = dry matter; CP = crude protein; EE = ether extract; NDF=neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin. Ashes, CP, EE, NDF, ADF and ADL are expressed in % w/w on DM basis.

Sample	DM	ASHES	CP	EE	NDF	ADF	ADL
MFL	94.58±0.43	17.40±0.19	15.79±0.02	12.02±0.37	42.06±0.31	18.05±0.72	5.34±0.15
HLs	91.28±2.11	12.03±0.31	14.49±0.75	9.85±0.04	44.70±1.47	29.30±0.50	10.67±0.11
HSC	94.46±0.05	8.27±0.85	32.54±0.71	7.69±0.05	52.59±1.84	29.00±1.59	12.14±0.95
GMs	94.02±0.02	7.57±0.14	10.69±0.27	8.33±0.62	55.49±0.66	47.54±0.12	35.47±0.47

As reported in Table 1, hemp co-products showed an interesting nutritional profile, even when compared to GMs, a co-product of the wine industry, which is highly used in the feed sector. In general, the results obtained in this study are partially confirmed in the literature (Ely and Fike, 2022; Vastolo et al; 2022; Kleinhenz et al; 2020). As reported by Vastolo et al. (2022), these differences are probably due, not only to the genotype of the plant, but also to harvesting, agrological, and environmental conditions.

In more detail, MFL showed a high ash content. Kleinhenz et al. (2020) demonstrated that hemp leaves are characterised by an ash content of 21.2% while flowers at 14.1%, suggesting that a combination of them results in a reduction of the final percentage (intermediate in our case). The high ashes values are due to the presence of minerals such as calcium, phosphorus, magnesium, potassium, and sulphur, which are known to be high in hemp leaves and flowers (Kleinhenz et al., 2020) and which, in turn, may influence the digestibility of hemp, as shown by Lanzoni et al. (2023). As reported in Table 1, HSC showed a high protein content (32.54±0.71%). This feature is related to the treatment adopted for obtaining the specific co-product. In this case, the removal of oil (one of the main components of whole HSs) led to an increase in the total protein content (from 30% to 50%), as demonstrated by House et al. (2010) and Ely and Fike (2022). In parallel, HLs and MFL were characterised by a highly comparable protein content (14.49±0.75%; 15.79±0.02%, respectively), however lower than reported by Ely and Fike (2022). This difference, as previously reported, is due to the variability factors listed by Vastolo et al. (2022). It is important to emphasise that, although HLs are a matrix obtained following the removal of the fibrous outer layer of HSs (dehulling process), they showed a high protein content. As reported by House (2021), this could be due to the fact that, during the dehulling process, part of the HSs endosperm (rich in protein and lipids) is also inevitably removed, thus increasing the final protein content.

This also explains the high lipid content observed in HLs (9.85±0.04%), highly comparable with GMs (8.33±0.62%), confirming the values reported by House et al. (2010) and Baumgärtel et al. (2007). Among the matrices studied, the MFL showed the highest lipid content (12.02±0.37%), confirming the values observed by Ely and Fike (2022). This result could be justified by the presence of essential oil in the hemp flowers, which is highly used in the cosmetic and pharmaceutical sectors for its important functional properties (Farinon et al., 2020). All analysed matrices presented high fibre content, expressed as Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL). Highest values for all analysed fractions were obtained for HSC. In particular, NDF measured in HSC was close to GMs, confirming, at least partially, what was observed by Halle and Shone (2013). More precisely, the authors reported a percentage of NDF, ADF, and ADL of 45%, 30% and 12% (expressed on DM basis) respectively, values comparable with those reported in Table 1. In parallel, among the tested samples, MFL showed the lowest values for NDF (42.06±0.31%), ADF (18.05±0.72%), and ADL (5.34±0.15%). Although there is no data in the literature on the nutritional composition of the MFL, it is evident that the values observed in the present study are a combination of the results obtained from the analysis of the matrices tested individually, as reported by Kleinhenz et al. (2020) and Ely and Fike (2022). Finally, the HLs showed a lower content of NDF, ADF, ADL than observed by House et al. (2010), Kim and Nyachoti (2017), and Kim et al. (2018). More precisely, the authors showed that NDF in these matrices can reach 50-65% and 38-50% of ADF (on DM basis).

Again, the observed differences can be attributed to genotype, collection, agrological and environmental differences. Although high levels of fibre may affect the total digestibility, it is important to emphasise that its adequate consumption may have a functional, especially probiotic, role, as reported by Farinon et al. (2020).

Total phenolic content and Antioxidant activity (ABTS and FRAP) of green chemical extracts

Scientific research is increasingly investigating the properties of phenolic compounds due to their associated benefits, which can be exploited in the formulation of functional foods and nutraceuticals (Abdalla et al., 2007).

The properties of these compounds can be influenced by the type of extraction. To date, solvent-based extraction is one of the most widely used on a laboratory scale due to its speed and cost-effectiveness. The efficacy of this type of extraction depends mainly on the solvent used, identified by Rotta et al. (2017) as one of the main critical parameters due to the different chemical characteristics and polarity of the phenolic compounds.

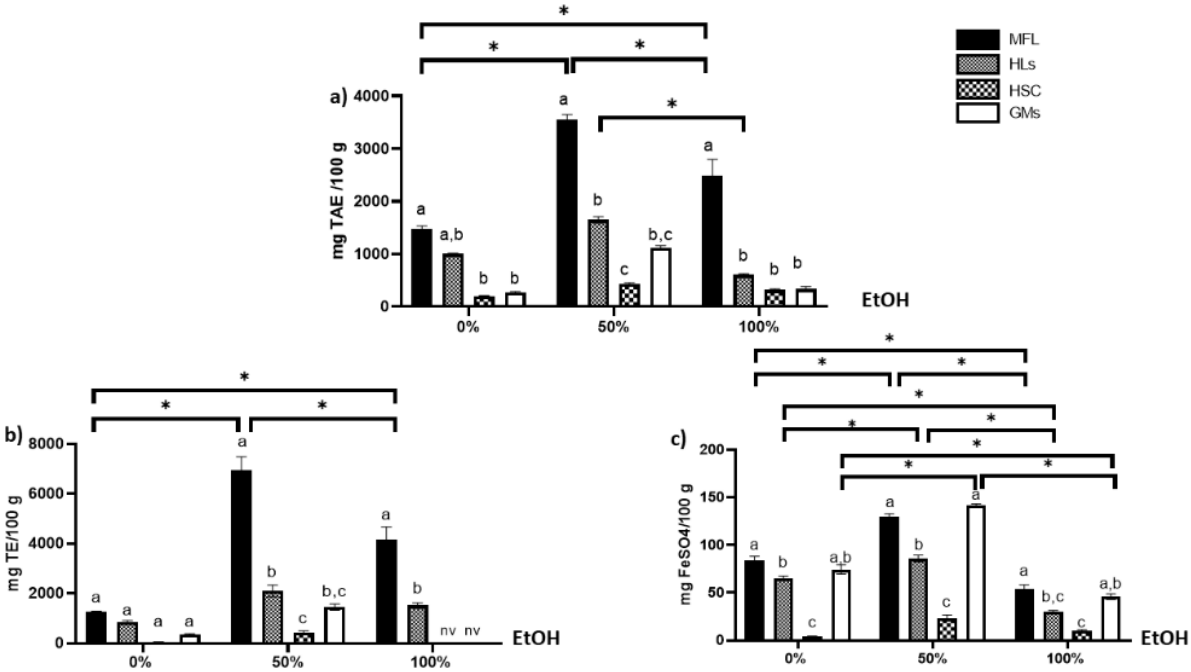

Figure 2. Total phenolic content and Antioxidant activity (ABTS and FRAP) of green chemical extracts. TAE: Tannic Acid Equivalent, TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant differences ($p<0.05$) between the samples within each extraction. Bars indicate significant differences ($p<0.05$) between the same samples in different extractions. (**a**) Total Phenolic content; (**b**) ABTS assay; (**c**) FRAP assay.

For this reason, as shown in Figure 2a, we decided to study the TPC of hemp co-products, using three different ratios of H_2O:EtOH, as previously reported.

As shown, 50% EtOH extraction (3551.12±98.54 mg TAE/100g) resulted in significantly higher TPC (*p<0.05*) for MFL. This was also observed for HLs (1647.11±60.53 mg TAE/100g) compared to 100% EtOH (600.53±23.55 mg TAE/100g). This trend, although not statistically significant compared to other ratios, was also reported for HSCs and GMs, partially confirming what has been reported in the literature (Prasad et al., 2009; Ozbek et al., 2020). More specifically, Prasad and colleagues (2009) showed that the extraction rate of longan fruit pericarp increased with increasing EtOH concentration from 25% to 50% and decreased for concentrations higher than 75%. A similar result was obtained by Ozbek et al. (2020) on pistachio HLs. Most probably, this trend can be explained by the "*like dissolves like principle*", where H_2O allows polar compounds to dissolve, while EtOH, an organic solvent, dissolves less polar ones, resulting in a higher extraction rate (Lim et al. 2019). Although Ozbek et al. (2020) reported a lower extraction rate with 100% EtOH than with 100% H_2O, it is evident in Figure 2a, how this depends on the matrix analysed. Indeed, as reported by Robbins (2003), in addition to the solvent used, sample-solvent ratio, pH, temperature, and extraction time, the nature of the compounds are important factors to consider, as they can influence the extraction rate. Indeed, considering the individual matrices (Figure 2a), MFL showed significantly higher values (*p<0.05*) in each concentration tested, except in comparison with HLs for 0% EtOH extraction. Although, the

phenolic profile of the leaves is little explored in the literature, that of the flowers has been characterised by Izzo et al. (2020). The authors reported how hemp flowers can be used as new resources for nutraceutical formulations, thanks to the high presence of phenols, in particular hydroxycinnamic acids (chlorogenic, p-coumaric, ferulic acids) and especially flavonoids. More precisely, Izzo and colleagues (2020) demonstrated how these compounds are 10 to 100 times more present in flowers than in any other part of hemp, thus explaining the high TPC levels, expressed as mg TAE/100 g, shown in Figure 2a. At the same time, the results obtained for HLs in each extraction (999.65±13.58 mg TAE/100g; 1647.1±50.53 mg TAE/100g; 600.53±24.55 mg TAE/100g, for 0%, 50% and 100% EtOH, respectively), highly comparable with those of GMs, showed higher values than those obtained in a previous our publication for HSs (550.3±28.27 mg TAE/100g) extracted in pure methanol, a stronger solvent than EtOH (Lanzoni et al., 2023). This result is most probably due to the fact that phenolic compounds are more concentrated in the hull than in the kernel fraction of HSs, and the dehulling process leads to a higher concentration of phenols (Chen et al., 2012). Finally, as shown in Figure 2a, HSC showed no significant difference in each ratio tested. These results partially confirmed what was observed by Teh et al. (2014). More specifically, the authors reported a TPC for HSC, extracted with 100% EtOH, of 351.33±2.08 mg GAE/100 g, highly comparable to that observed in this work (315.67±22.42 mg TAE/100 g). With regard to GMs, on the other hand, binary extraction was not significantly different between 0% EtOH and 100% EtOH, although an upward trend was observed, partially confirming what was reported by Spigno et al. (2007).

Rotta et al. (2017) reported the importance of measuring the antioxidant property using at least two methods based on different reaction mechanisms. Therefore, as shown in Figures 2b and 2c, the antioxidant capacity was evaluated using the ABTS and FRAP assays. More precisely, Figures 2b and 2c show that the antioxidant activity presents the same trend as the TPCs, confirming the active role of phenolic compounds in antioxidant properties, as reported by Ozbek et al. (2020) on pistachio HLs. At the same time, the authors demonstrated how concentrations between 70% and 100% EtOH reduced antioxidant capacity. In our study, this effect only occurred for MFLs in the ABTS assay, whereas for FRAP in all matrices except HSC. This distinction indicates that the type of bioactive compounds and the relative amounts recovered are influenced by the ratios of H_2O and EtOH. Indeed, with the polarity of the solvent varying from very polar (100% H_2O) to less polar (0% H_2O), the ability of the solvent to dissolve selected groups of antioxidants also varies and this has an implication on the subsequent antioxidant activity (Lim et al., 2019).

Thus, as reported, solvent extraction is the most common means of recovering phenolic compounds from plant-derived materials due to the simplicity of the technique, its efficiency, and the wide range of possible applications. Although, as reported in the literature, there are several solvents that can be used (methanol, hexane, acetone, ethyl acetate) (Sun et al., 2015), the possibility of combining EtOH with H_2O in any ratio, the different polarity of both solvents, and their acceptability for human consumption, make their mixtures the most

suitable solvents to obtain phenolic extracts from plants, without leaving toxic residues (Conde-Hernandez and Guerrero-Beltran, 2014).

High-Performance Thin Layer Chromatography and DPPH of green chemical extracts

Because of the interesting results obtained with the 50% EtOH extraction, the next step was to characterise the phenolic composition by HPTLC analysis. In this study, HPTLC (Figure 3) was used to semi-quantitatively characterize flavonoids, phenolic acids, CBG, and α-tocopherol content in the samples. The HPTLC analysis was also useful for assessing the contribution of various types of phenolic compounds (such as flavonols, flavan-3-ols and phenolic acids) to the overall antioxidant activity, which can be directly related to specific molecules in the samples.

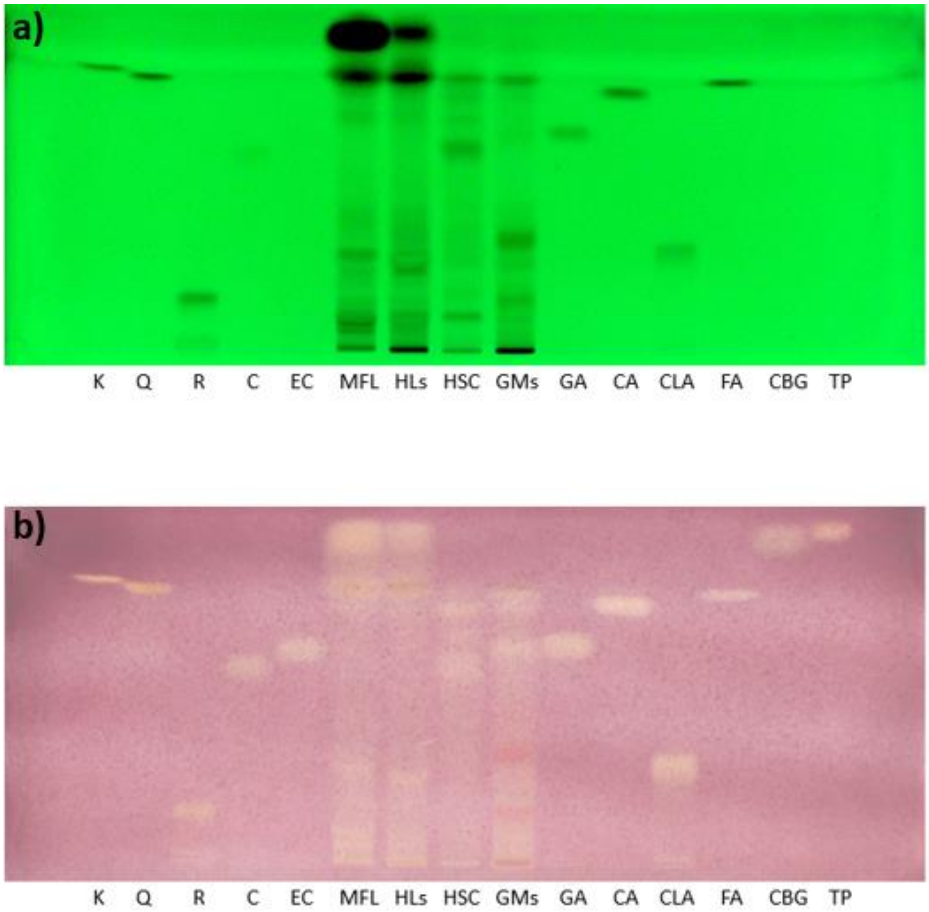

Figure 3. HTPLC plate of standards and samples detected 254 nm (a) and after derivatization with DPPH detected at visible light (vis) (b). K: Kaempferol, Q: Quercetin 3-O-glucoside, R: Ruthin; C: Catechin; EC: Epicatechin; GA: Gallic acid; CA: Caffeic acid; CLA: Chlorogenic acid; FA: Ferulic acid; CBG: Cannabigerol; TP: α-tocopherol; MFL: Mix Flowers/leaves; HLs: Hulls; HSC: Hemp Seed Cake; GMs: Grape Marcs.

As shown in Figure 3a, hemp co-products showed a complex and rich profile. More specifically, the band patterns in the chromatographic run of the MFL and HLs samples showed a greater degree of similarity than those observed in the HSC and GMs samples. In

particular, as shown in Figure 3b, MFL and HLs showed similar bands with same Ratio frontis (*Rf*) to those used for the standards, namely Quercetin (*Rf*=0.79), Kaempferol (*Rf*=0.83), Rutin (*Rf*=0.16), Chlorogenic acid (*Rf*=0.29), and Ferulic acid (*Rf*=0.78) (*Rf* are shown in Table 2). These phenolic compounds are characterised by high antioxidant activity, as shown in Figure 3b, confirming the results reported by Izzo et al. (2020a), Nagy et al. (2019), and Aloo et al. (2023). At the same time, the MFL revealed matches for the α-tocopherol band, an isoform of vitamin E (*Rf*=0.96), supporting the results shown by Beleggia et al. (2023). In particular, the authors, characterising the phytochemical profile of hemp flowers, detected the high presence of tocopherol. Of this, the main isoform present was α- (86.6-89.4 %), followed by β+γ- (8.1-11.2 %) and δ-tocopherol (1.5-2.3 %) (Beleggia et al., 2023). The presence of α-tocopherol was also found in HLs. As reported by Izzo et al. (2020b) and Engin (2009), this molecule is highly present in HSs oil where it covers a key role in preserving the oxidative stability of oils, acting as a chain-breaker, and consequently slowing down the lipoperoxidation process, confirming the results reported in Figure 3b. What has just been observed plays an important role in that, even following the dehulling process, the lipid profile in the endosperm of the HSs is not altered, thus improving the nutritional and functional profile of the HLs. In parallel, MFL showed a match for CBG, an important phytocannabinoid, confirming the reports of Farinon et al. (2020) and Nagy et al. (2019). Phytocannabinoids are produced, collected, and stored at the level of pedunculated glandular trichomes, small specialised secretory epidermal glands present and abundant on flowers, in fewer numbers on leaves and stems while they are absent at the level of seeds, so the latter organs should not contain cannabinoids (Farinon et al., 2020). However, HLs also showed bands for CBG. Most probably, this is caused by contamination with other parts of the plant during harvesting. In fact, as reported by Farinon et al. (2020), the presence of cannabinoids in HSs represents an accidental contamination due to both the considered cultivar and the seed cleaning process. Most probably, the confirmed presence of CBG, a highly antioxidant molecule as shown in Figure 3b, influenced the results previously reported with the ABTS and FRAP assays following chemical extraction for HLs, resulting in an increased antioxidant profile. For HSC, bands were observed for Quercetin, Ferulic acid, Caffeic acid *(Rf*=0.75), and Catechin (*Rf*=0.57), partially confirming the results reported by Teh et al. (2014). More specifically, the authors reported the presence of Caffeic acid and Quercetin in HSC, but the absence of Ferulic acid and Catechin, although the latter, as shown in Figure 3b, is more evident following derivatisation with DPPH. As reported by Ingallina et al. (2019), the presence/absence and relative concentrations of phenolic compounds depend on environmental conditions and seasonality, resulting in different profiles even when considering the same matrix. These compounds are characterised by important antioxidant activity, as shown in Figure 3b, particularly Caffeic acid and Catechin, confirming the results reported by Gülçin (2006) and Grzesik et al. (2018).

Table 2. HTPLC standards with their correspondent Ration frontis (Rf) and γ (nm) values. Vis: visible light (380-700 nm).

Standard	Abbreviations	Ratio frontis (Rf)	γ (nm)
Kaempferol	K	0.83	254, vis
Quercetin 3-O-glucoside	Q	0.79	254, vis
Rutin	R	0.16	254, vis
Catechin	C	0.57	vis
Epicatechin	EC	0.62	vis
Gallic acid	GA	0.63	254, vis
Caffeic acid	CA	0.75	254, vis
Chlorogenic acid	CLA	0.29	254, vis
Ferulic acid	FA	0.78	254, vis
Cannabigerol	CBG	0.94	vis
α-tocopherol	TP	0.96	vis

Total phenolic content and antioxidant activity of hemp co-products ex vivo digested

Figure 4 shows graphs of the TPC, ABTS, and FRAP of hemp co-products following *ex vivo* digestion.

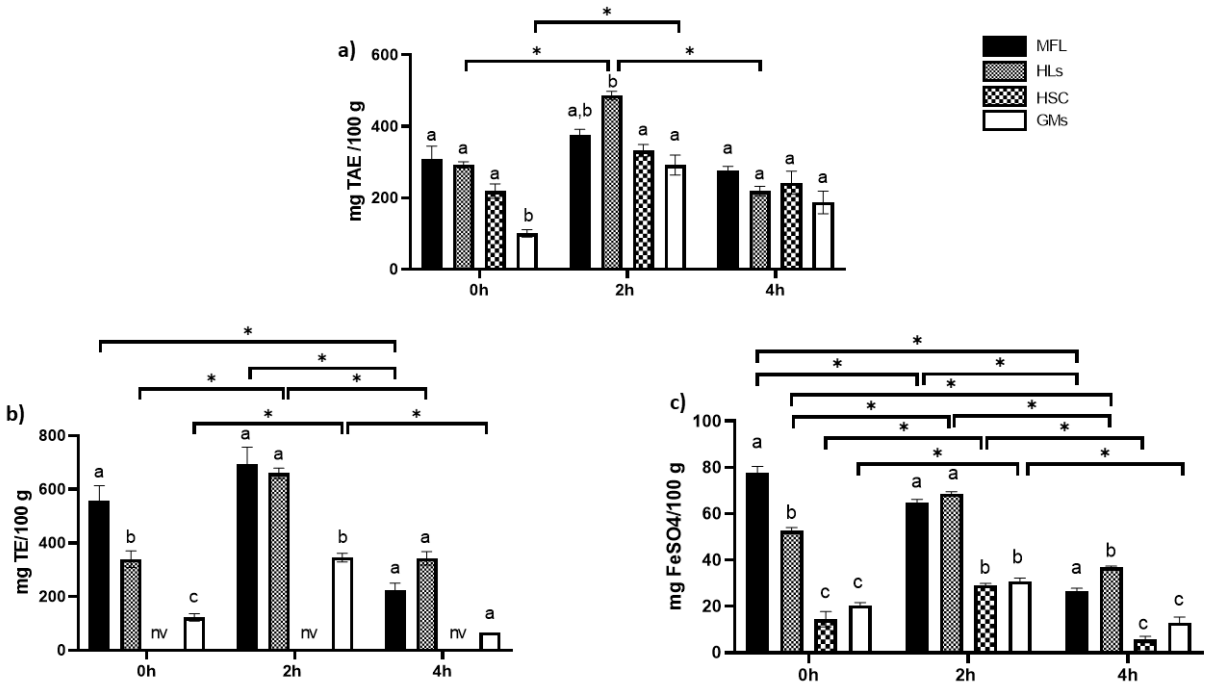

Figure 4. Total phenolic Content and Antioxidant activity (ABTS and FRAP) of *ex vivo* digestion of hemp co-products. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), (n=3). Different superscript letters in columns indicate significant differences ($p<0.05$) between the samples within each digestion phase. Bars indicate significant differences ($p<0.05$) between the same samples in different digestion phase. (a) Total Phenolic content: (b) ABTS assay; (c) FRAP assay. 0h: start of gastric phase; 2h: end of gastric phase; 4h: end of intestinal phase.

As reported in Figure 4a, all the matrices analysed showed the same trend, characterised by an increase in TPC after the end of the gastric phase (2h), although it was only statistically significant ($p<0.05$) for HLs and GMs. This trend is confirmed on matrices digested with *in vitro* digestion protocols (Lanzoni et al., 2023; Ma et al., 2020; Goulas and Hadjisolomou, 2019). In general, as reported in the literature, the behaviour of phenols during the digestive process is highly influenced by both the food matrix and the digestion conditions (Olivas-Aguirre et al., 2017). More specifically, in the stomach, the acidic pH (2.0-3.0) allows the release of phenolic compounds from the food matrix following the breaking of protein and polysaccharide (fibre) bonds (Goulaas and Hadjisolomou, 2019), increasing their availability, thus confirming the results obtained in this study. In parallel, as shown by Pineda-Vadillo et al. (2016), the acid pH protects phenols during gastric digestion, not altering their quantification. As shown in Figure 4a, at the end of the intestinal phase (4h), there was a decrease in TPC compared to the gastric phase, although statistically significant ($p<0.05$) only for HLs. This trend is caused by the instability of phenolic compounds at the alkaline pH (>7.0), typical of the intestinal environment, especially during pancreatic action, which leads to the transformation of phenols into unknown structural forms with different chemical properties, different bioaccessibility, bioavailability, and biological activity (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, interesting results are reported for HLs (486.57±11.56 mg TAE/100 g) at the end of the gastric phase, with statistically ($p<0.05$) higher differences compared to HSC (332.28±17.10 mg TE/100 g) and GMs (291.68±27.86). Although, dietary fibres are the main carriers of phenolic compounds, thus influencing their bioaccessibility, as reported by Wojtunik-Kulesza et al. (2020), it is also true that in HSs, as previously observed, the phenols are mainly localised in the HL, and following the gastric phase, the disintegration of the food matrix results in a high release, leading to a greater concentration than in other samples (House, 2021).

In parallel, as shown in Figures 4b and 4c, the ABTS and FRAP (with the exception of MFL) assays showed the same trend as the TPC, confirming that phenolic compounds are actively involved in antioxidant activity, as reported above. As phenolic compounds, the antioxidant capacity is also influenced by digestion conditions. Wojtunik-Kulesza et al. (2020) reported that the low pH, typical of the gastric phase, increases the antioxidant power of the compounds due to an increase in their ability to donate electrons. In parallel, the decrease observed for each sample at the end of the intestinal phase could be attributed either to a structural reorganisation of some phenolic compounds due to their sensitivity to alkaline pH or to the fact that, during digestion, some of them are able to bind other constituents of the food matrix, forming complexes that inhibit their antioxidant power (Wojtunik-Kulesza et al., 2020). Considering the individual matrices, MFL and HLs showed statistically significant differences from HSC and GMS, with the exception of the ABTS assay at the end of the intestinal phase, confirming what was observed following the green chemical extraction. Although these results can be attributed to the high concentration of phenols, compounds such as α-tocopherol and CBG play a major role in functional activity, as shown before. At the same time, multiple biological functions are correlated with CBG, including

anti-inflammatory, antibacterial, antifungal, redox balance regulation, and as a neuromodulator (Jastrzab et al., 2022). However, the presence of phytocannabinoids, particularly THC, despite their positive effects, raises concerns about possible bioaccumulation in animal products with a resulting health risk for the consumer. For this reason, it should be emphasised that the supplementation of hemp-based products in the diet of broilers did not result in traces of THC in the breast, liver, lungs, and thighs, confirming the safety of these compounds (Jing et al., 2017). As shown in Figure 4c, MFL showed a different trend at 0h, suggesting a ready availability of compounds with antioxidant capacity to chelate metals, the principle of action of the FRAP assay.

In Figure 4b, the values for HSC were not shown, as the ABTS assay obtained non-detectable values at each stage of the digestive process. This result could most likely be related to the behaviour of the phenols during digestion. As reported above, HSC is mainly characterised by the presence of caffeic acid, ferulic acid, quercetin and catechin. However, as reported by Wojtunik-Kulesza et al. (2020), not all phenols are able to perform the same antioxidant activity. More precisely, as shown by Khokhar and Apenten (2003), some conditions such as alkaline pH or the presence of oxygen can inhibit the antioxidant action and induce a pro-oxidant behaviour of some phenols, especially to the damages of small compounds, among them caffeic acid. In parallel, ferulic acid appears to be one of the main phenolic compounds linked to lignin and non-starch polysaccharides in HSs; factors that could inhibit its antioxidant action (Wojtunik-Kulesza et al., 2020). However, results obtained by FRAP assay showed highly comparable values with GMs at every stage of the digestive process. This result could be related to the fact that Ferulic acid, Caffeic acid, Quercetin, and Catechin are phenolic compounds also characterised by metal chelating antioxidant activity (Zdunska et al., 2018; Gülçin, 2006; Leopoldini et al., 2006; Kashima, 1999).

Hemp co-products were characterised by an adequate nutritional profile and a high functional potential. However, to date, the use of hemp-based products in the feed sector is limited, due both to the current regulatory status, the different processes on matrices, the cost and limited volumes produced (Salami et al., 2019; Vastolo et al., 2022). More precisely, as shown by Moscariello et al. (2021), the price of HSs, the main product of hemp for food and feed purposes, is around 1.33/1.99 €/kg with a production of 0.6-0.9 ton/ha per year, a price significantly higher than that of soybeans (0.42/0.50 €/kg) (Granaria Commodity Trading Association Milano, 2024), the main protein source used in the diet of livestock animals. However, although cost is a critical point, the interesting nutritional but above all functional profile of hemp-based products compared to soy products, as also demonstrated in our previous work (Lanzoni et al., 2023), suggest their use in the diets of monogastric animals. For these reasons, to stimulate the use of hemp-based products and their co-products, it is necessary to increase cultivation and production by creating new markets for hemp, thus ensuring the core principles of the Green Deal, One Health and F2F.

CONCLUSIONS

Hemp co-products showed an interesting nutritional profile, especially in terms of protein and lipids, comparable or superior to that of GMs, a matrix widely used in the feed industry. For HLs and MFL, valuable results were obtained in terms of TPC and antioxidant activity (ABTS, FRAP and DPPH), both following green chemical extraction and *ex vivo* digestion. Extraction with 50% EtOH showed to be the most effective for extracting phenolic compounds and antioxidants from plant-derived matrices. This work represents a preliminary report on the functional profile of hemp co-products for their future application in the feed industry. *In vivo* trial will be necessary to evaluate their efficacy on the health and growth performance of farm animals, taking into account the high variability of these matrices. The presented data support the potential future growth of hemp processing industries, thus creating markets capable of absorbing large quantities of scraps and consequently valorising it into co-products for the feed industry.

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Manuscript IV

Effects of dietary hemp co-product inclusion on laying hens performances and on egg nutritional and functional profile

Davide Lanzoni, Milos Skřivan, Michaela Englmaierova, Elena Petrosillo, Luca Marchetti, Vera Skřivanová, Valentino Bontempo, Raffaella Rebucci, Antonella Baldi and Carlotta Giromini.

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Effects of dietary hemp co-product inclusion on laying hens performances and on egg nutritional and functional profile

D. Lanzoni[a], M. Skrivan[b], M. Englmaierová[b], E. Petrosillo[a], L. Marchetti[a], V. Skřivanová[b], V. Bontempo[a], R. Rebucci[a], A. Baldi[a] and C. Giromini[a,c]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Department of Nutritional Physiology and Animal Product Quality, Institute of Animal Science, Pratelstvi 815, 104 00 Prague, Czech Republic; [c]Institute for Food Nutrition and Health, University of Reading, Reading, RG6 5EU, UK.

ABSTRACT: The European Union promotes a circular economy by valorising food industry scraps as co-products for animal feed. Hemp (*Cannabis sativa* L.) co-products are promising for their nutritional and functional profile, but their use in animal nutrition remains limited. This study evaluated the effect of increasing levels (0%, 3%, 6%, 9%) of a hemp co-product (variety Futura) from hempseed cleaning, included in laying hens' diets (control, T_3, T_6, T_9), on performance and the nutritional/functional quality of egg yolks, focusing on total phenolic content (TPC) and antioxidant activity (FRAP and ABTS assays), also assessed after green chemical extraction and *in vitro* digestion. Including up to 9% hemp co-product in diets did not affect dry matter and protein digestibility but improved the functional profile. A 9% inclusion significantly increased egg mass production ($p<0.01$) and reduced saturated fatty acid content (T_9: 7672 mg/100 g; T_6: 8534 mg/100 g; T_3: 8837 mg/100 g; control: 8742 mg/100 g), alongside an improved n-6/n-3 fatty acid ratio. Cholesterol levels were significantly lower than the control in all concentrations tested, with better results for T9 ($p<0.01$). Antioxidant activity, measured via ABTS after *in vitro* digestion, increased significantly (control: 601.12±42.40 mg TE/100 g; T_3: 773.17±43.77 mg TE/100 g; T_6: 765.64±38.71 mg TE/100 g; T_9: 843.48±38.71 mg TE/100 g; $p<0.05$), despite no differences in TPC. In conclusion, the inclusion of 9% hemp co-product in laying hens' diets improved egg production and yolk quality, highlighting its potential for animal feed. However, the composition variability requires further evaluation to ensure future large-scale application.

Keywords: Antioxidant, Digestion, Hemp, Poultry, Yolks.

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HIGHLIGHTS

- Farm to Fork strategy aims to guide the agri-sector to sustainable food system.
- Hemp co-products are featured by an interesting nutritional and functional profile.
- The 9% inclusion of the hemp co-product improved the performance of laying hens and the nutritional and functional profile of eggs.

INTRODUCTION

In recent decades, the European Union has been actively working to ensure a clean and circular economy (Garcia et al., 2020; Beltran et al., 2022). In this panorama, the Farm to Fork strategy plays an important role as it aims to guide the agricultural sector towards healthier and more environmentally friendly food systems, making them more sustainable than the current ones (European Union, 2020). To achieve this, one possible strategy is to use the large amount of non-edible biomass produced along the food chain and to valorise it as co-product for the livestock sector (FAO, 2011; Rakita et al., 2021).

To date, scientific research is deepening the use of co-products derived from hemp (*Cannabis sativa L.*) (Bailoni et al., 2021; Vastolo et al., 2021; Lanzoni et al., 2023a). Hemp is well-known in the food/feed sector for the important nutritional (25-35% lipid content, 20-25% protein rich in essential amino acids and 20-30% carbohydrates, a major part of which is dietary fibre) and functional profile (presence of phenols and antioxidant molecules) of its seeds (Lanzoni et al., 2023b; Farinon et al., 2020). Despite this, the use of hemp co-products is still in an early stage. As demonstrated in literature, hemp co-products can be summarised as: whole plant, stems, chaff, stalks, hulls, HSs cakes, leaves and flower (before and after cannabinoid extraction) (Vastolo et al., 2021; Ely and Fike, 2022). As shown by Ely and Fike (2022) and Vastolo et al. (2021), hemp co-products are characterised by an interesting nutritional and functional profile, which unfortunately varies widely due to many factors such as environmental, agro-ecological conditions, genotype, harvesting techniques and the transformation processes. To date, through Regulation 2022/1104 of 1 July 2022, the European Union only authorised HSs, HSs oil, HSs cake, hemp flour and hemp fibre (both originated by stems) for animal feeding, as reported in the European Catalogue of Feed Materials (European Union, 2022). For this reason, it is important to investigate the use of new hemp-based matrices, which are able to guarantee high animal performances and improve the nutritional aspect of animal products. The starting point for future research is related to the fact that important results have been obtained with the incorporation of HSs in the livestock industry, particularly in the laying hen sector. Indeed, HSs are able not only to increase the performance of the treated animal, but at the same time to improve the nutritional and functional profile of the eggs (Goldberg et al., 2012; Gakhar et al., 2012; Neijat et al., 2014; Shahid et al., 2015; Neijat et al., 2016; Skrivan et al., 2019; Konca et al., 2019; Mierlita, 2019). In the light of the above, it is possible to assume that such important results can also be achieved through the enhancement of hemp co-products.

For this reason, the aim of this work was to evaluate how different levels of inclusion (3%, 6% and 9%) of a hemp co-product, deriving from the cleaning of the whole HSs (leaves, non-standard HS, hulls and stems), in the diet of laying hens are able to modulate the production performance and nutritional/functional profile of egg yolks. Specifically, in addition to assessing the fatty acid profile and cholesterol content, the presence of bioactive compounds such as tocopherol, retinol, β-carotene, lutein and zeaxanthin were analysed. Finally, the total phenolic content (TPC) and anti-oxidant profile of egg yolks were evaluated following green chemical extraction and *in vitro* digestion.

MATERIAL and METHODS

Material

The hemp co-product (*C. sativa* L, variety Futura) (BIOHEMP, Prague, Czech Republic), harvested at the end of September 2022, was generated during the cleaning of whole HSs using sieves and airflow. Hemp co-product was characterised by the presence of leaves, non-standard HSs, HSs hulls and stems. The resulting sample was stored in the dark at room temperature.

Experimental design

240 hens (Bovans Brown) aged 21 weeks took part in the experiment. The trial lasted nine weeks (two weeks of adaptation and seven of experiment), which were comprehensive of the peak production phase, as from week 30, production gradually decreases (Jin et al., 2011; Gonzalez-Uarguin et al., 2021). The hens were divided into four dietary treatments [control, T_3, T_6 and T_9) (selected on the basis of our previous work; Skrivan et al., 2019) with increasing inclusion of the hemp co-product in the diet (0%, 3%, 6% and 9%, respectively), as shown in Table 1.

Table 1. Composition of the dietary treatments fed to laying hens (Control, T3, T6 and T9). Values are reported in %.

	Control	T_3	T_6	T_9
Ingredient (%)	0%	3%	6%	9%
Wheat (11% CP)	39.10	37.70	36.20	34.10
Corn	19.60	19.60	19.60	19.60
Soybean meal (44%)	24.40	24.10	23.70	23.70
Wheat bran	4.10	2.70	1.30	0.00
Hemp co-product	0.00	3.00	6.00	9.00
Rapeseed oil	1.97	2.34	2.79	3.48
CaH_2PO_4	0.78	0.75	0.72	0.69
Sodium bicarbonate	0.30	0.30	0.30	0.30
Sodium chloride	0.20	0.20	0.20	0.20
Limestone-grit (1-2 mm)	8.90	8.60	8.40	8.10
L-Lysine hydrochloride	0.00	0.02	0.06	0.07
DL-Methionine	0.14	0.16	0.18	0.20
L-Threonine	0.01	0.03	0.05	0.06
AMINOVITAN	0.50	0.50	0.50	0.50

All diets, as reported in Table 2, were formulated to contain similar levels of metabolisable energy and protein content, therefore, following increasing concentrations of the hemp co-product featured by contained energy levels, vegetal oil (rapeseed oil) was gradually increased to maintain isoenergetic diets among groups.

Table 2. Chemical composition of dietary treatments fed to laying hens (Control, T3, T6, T9). HCP: Hemp co-product; MEN: Metabolisable energy.

	Control	T_3	T_6	T_9	
Nutrient content	0%	3%	6%	9%	HCP
Dry matter (g/kg)	899	901	902	902	889
Ashes (g/kg)	124	142.8	128.9	132.7	148.3
Crude protein (g/kg)	173	174	170	175	156
Ether extract (g/kg)	36.9	37.7	39.1	40.7	64.8
NDF (g/kg)	190	207	208	193	423
ADF (g/kg)	33.7	39.1	46.7	53.7	281
ADL (g/kg)	7.2	8.1	11.9	13.4	107.1
ME_N (MJ/kg)	10.9	11.0	10.9	10.9	5.2
Calcium (g/kg)	36.2	36.0	36.3	36.2	32.2
Phosphorus (g/kg)	5.69	5.73	5.65	5.74	5.25
α-Tocopherol (mg/kg)	40.1	42.0	44.0	47.3	19.2
γ-Tocopherol (mg/kg)	14.1	16.9	17.4	18.5	12.0
Retinol (mg/kg)	1.89	2.10	2.40	3.08	-
Lutein (mg/kg)	2.46	3.36	4.32	5.54	26.4
Zeaxanthin (mg/kg)	2.21	1.92	1.62	1.57	1.02
β-Carotene (mg/kg)	0.267	0.689	1.173	1.071	4.90

More precisely, the hens were housed in three-floor enriched cages and divided into ten hens/cages for a total of six cages per group ensuring optimal microclimatic and technological conditions for the considered genotype, with a daily light cycle length of 16 h (02.30-18.30). The cages had a surface area of 7.560 cm^2. A nest box, feeder (12 cm per hen) and three nipple water dispensers were included in each cage. Additionally, the cages were equipped with a perch (15 cm per hen), a dust bath and equipment for claw abrasion. Room temperature was maintained at 20 – 22°C, and the light intensity was approximately 10 lx in the central storey.

At the beginning and end of the experiment all animals were weighed. The health status and mortality rate were assessed daily, while the feed intake per cage and group was recorded weekly. The number of eggs laid was registered daily.

Methods

Chemical analysis of co-product and diets

The chemical analysis of the hemp co-product and diets was carried out following the official methods reported by AOAC (2005). In particular, DM was obtained by drying the sample in a forced-air oven at 65°C for 24 h (AOAC method 942.05), while ashes by incinerating the samples in a muffle at 550°C for 3 h (AOAC method 942.05). In parallel, CP content was measured by the Kjeldahl method (AOAC method 2001.11) and the ether extract by extraction in petroleum ether, using a Tecator 1045 Soxtec Extraction Unit (DM 21/12/1998). Finally, the fibrous fractions were detected using the protocol reported by Van Soest et al. (1991). Calcium content was measured in hydrochloric acid by atomic absorption spectrometry using the ContrAA 700 F instrument (Analytik Jena AG, Jena, Germany), as described by Skrivan et al. (2019), while phosphorus content was determined using the vanadate-molybdate reagent, as reported by AOAC (2005; method 956.17). The fatty acid profile [saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs)] were classified following chloroform and methanol extraction of the total lipid fraction as shown by Folch et al. (1957). Subsequently, alkaline trans-methylation of fatty acids was performed as reported by Raes et al. (2003). More specifically, the fatty acid methyl esters (FAMEs) were determined by gas chromatography (HP chromatograph 6890, Agilent Technologies, Inc.) with a 60 m DB-23 capillary column (150-230°C) and a flame ionisation detector, using specific temperature programmes, as featured in the Supplementary Materials. Finally, the fatty acids were identified by their retention times compared with standards (PUFA 1, PUFA 2, PUFA 3 and a 37-component FAME mixes (Supelco, Bellefonte, PA, USA)).

The concentration of α-tocopherol, γ-tocopherol, β-carotene and retinol was identified following saponification and extraction in diethyl ether in accordance with European standards (European Committee for Standardisation, 2000). Lutein and zeaxanthin contents were calculated using HPLC equipped with a diode-array detector (VP series; Shimadzu, Kyoto, Japan) as reported by Skrivan et al. (2019) and Froescheis et al. (2000). A wavelength of 450 nm was applied for the detection. More specifically, a Kinetex C18 column (100 x 4.6 mm; 2.6 um (Phenomenex, Torrance, USA) was used. After preparing the eluents [(A): acetonitrile:water:ethyl acetate (88:10:2) and (B): acetonitrile:water:ethyl acetate (88:0:15)], the samples were analysed by comparing with appropriate calibration standards (Sigma-Aldrich, Prague, Czech Republic). The analysis for the identification of lutein and zeaxanthin was of 18 min.

In vitro digestion and digestibility of hemp co-product and diets

The hemp co-product and diets were digested following Regmi et al. (2009) with minor modifications (Lanzoni et al., 2023b), as reported in Supplementary Materials.

At the end of digestion, the samples were vacuum filtered through paper filters (Whatman 54 Florham Park, NJ), collecting the undigested fraction (UF). UF was then incubated overnight at 50°C and used to determine the DM digestibility.

$$\text{Digestibility (\% DM)} = (\text{sample DM - UF DM})/\ \text{sample DM} \times 100)$$

For diets only, filters were used to assess protein digestibility, using the Kjeldahl method (AOAC, 2005).

In parallel, aliquots (around 2 mL) were taken every hour of digestion for the hemp co-product, while for the diets at the end of digestive process, to monitor TPC and antioxidant activity, as described below.

Determination of physical parameters of eggs

The physical parameters of the eggs were determined at the end of the trial. A whole day of egg production was analysed and the values were averaged per cage (n=6). More precisely, following the measurement of the total weight and surface, the eggs were broken and the individual fractions were isolated. For each component, the weight was determined and based on this, the percentages of white, yolk and shell were subsequently identified. The shell's breaking strength was determined in the vertical axis using an Instron 3360 apparatus (Instron, Norwood, MA, USA). After removal of the shell membranes, the thickness was measured using a micrometer. Finally, the yolk colour was analysed using DSM yolk colour fan (DSM Nutritional Products, Basel, Switzerland) and Minolta CR-300 colorimeter (Konica Minolta, Osaka, Japan) (Skrivan et al., 2019).

Nutritional and functional profile of egg yolks

Eggs for determination of fatty acid, cholesterol, vitamin and carotenoid content in the yolks were collected at the end of the trial (3 yolks (= 1 sample) from each cage, n=6). The content and lipid profile were determined as previously reported (Folch et al., 1957; Raes et al., 2003) by gas chromatography (HP chromatograph 6890, Agilent Technologies, Inc). The fatty acids were identified by their retention times compared with standards. PUFA 1, PUFA 2, PUFA 3 and 37-component FAME mixes (Supelco, Bellefonte, PA, USA) were used as standards.

In parallel, the atherogenic and thrombogenic indices were quantified as described by Ulbricht and Southgate (1991) and the peroxidation index in accordance with Cortinas et al. (2003). The α-tocopherol, γ-tocopherol, β-carotene, retinol, lutein and zeaxanthin present in egg yolks were analysed as indicated above (European Committee for Standardisation, 2000; Froescheis et al., 2000; Skrivan et al., 2019), using HPLC (VP series; Shimadzu, Kyoto, Japan) equipped with a diode array detector. The yolks for vitamin determination were

subjected to alkaline saponification with 60% potassium hydroxide followed by the appropriate extraction with diethyl ether.

Finally, the cholesterol content was measured as reported by Skrivan et al. (2019). Briefly, following saponification of the lipid component, the non-saponified fraction was subjected to extraction in ethyl ether. The derivatives thus obtained were quantified using a gas chromatograph equipped with a SAC-5 capillary column (Supelco, Bellefonte, USA) in isothermal conditions with a temperature of 285°C.

Green chemical extraction and in vitro digestion of egg yolks

Before the extraction and digestion process, the yolks of each individual egg were subjected to the freeze-drying process (3 yolks (=1 sample) from each cage, n=6). After this, pools were created for each dietary treatment (control, T_3, T_6, T_9). More precisely, the biphasic green chemical extraction (50:50; H_2O:EtOH) was performed as described in supplementary materials, following the protocol of Brighenti et al. (2017) with minor modifications as reported by Lanzoni et al. (2024).

In vitro digestion was performed following the Infogest protocol (Minekus et al., 2014; Brodkorb et al., 2019) (Supplementary materials). At the end of digestion, the samples were used for the evaluation of TPC and antioxidant activity with the Ferric Reducing Antioxidant Power (FRAP) and 2,2'-azinobis-(3-ehtylbenzothiazoline-6-sulfonic acid (ABTS) assays.

Total phenolic content and antioxidant activity of hemp co-product, diets and eggs

Total phenolic content was assessed with the Folin-Ciocalteu assay, as reported by Attard (2013). In parallel, antioxidant activity was evaluated by FRAP and ABTS method, according to the protocols of Abdelaleem and Elbassiony (2021) and Re et al. (1999), respectively, with minor modifications as reported by Lanzoni et al. (2024). The results were expressed as mg tannic acid equivalent (TAE)/100 g, mg $FeSO_4$/100 g and mg Trolox equivalent (TE)/100 g, for TPC, FRAP and ABTS respectively. The methodology is detailed in the Supplementary materials.

Statistical Analysis

Data on animal performances, physical characteristics of eggs, egg yolks lipid profile, cholesterol content and antioxidant molecules were analysed using one-way Anova with GLM procedure in SAS software (Version 9.3; 2003). Differences between the groups were tested by the Duncan test. The main effect was the dose of hemp co-product. The mathematical statistical model was as follows:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

Where Y_{ij} was the value of trait; μ was the overall mean; α_i was the effect of hemp co-product inclusion (i = 0%, 3%, 6%, 9%) and eij was the random residual error. The cage was the experimental unit (n=6). At the same time, DM/protein digestibility, TPC, ABTS and FRAP data of hemp co-product, diets and egg yolks were analysed by one-way Anova followed by

Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are reported as mean ± SEM. Values are considered statistically significant for a 95% confidence interval (p-value = 0.05).

RESULTS

Nutritional and functional profile of in vitro digested hemp co-product and diets

As shown in Table 2, the hemp co-product had an interesting nutritional profile, mainly due to its high protein content (156 g/kg). At the same time, it presented a discrete lipid level (64.8 g/kg), enriched by the high presence of PUFAs (1225.61 mg/kg) compared to MUFAs (263.47 mg/kg) and SFAs (430.61 mg/kg), which consequently improved the lipid profile of the diets, as reported in Table 3. In parallel, it showed a high content of ashes (148.3 g/kg) and fibre (423 g/kg, 281 g/kg and 107.1 g/kg, respectively for NDF, ADF and ADL), causing a reduced DM digestibility (36.09±1.29%). Despite this, increasing inclusions of hemp co-product in the diet (control, 3%, 6% and 9%) did not result in statistically significant differences in DM (64.21±0.97%; 64.06±1.39%; 63.85±1.16%; 62.14±1.40%) and protein (81.13±6.62%; 79.10±2.60%; 77.71±7.06%; 78.82±3.43%) digestibility, respectively, for control, T_3 (3%), T_6 (6%) and T_9 (9%).

Table 3. Fatty acid profile of hemp co-product and dietary treatments. Values are expressed in mg/100 g. HCP: Hemp co-product; CLA: Conjugated Linoleic acid.

	Control	T_3	T_6	T_9	
	0%	3%	6%	9%	HCP
Caproic acid (C6:0)	0.056	0.07	0.10	0.11	
Caprylic acid (C8:0)	0.21	0.21	0.23	0.24	
Capric acid (C10:0)	0.42	0.43	0.45	0.50	
Lauric acid (C12:0)	1.87	2.76	3.18	3.41	1.55
Trydecylic acid (C13:0)	0.08	0.12	0.13	0.15	
Myristic acid (C14:0)	3.35	7.14	9.10	10.29	42.69
Myristoleic acid (C14:1-n5)	0.22	0.44	0.56	0.80	4.18
Pentadecylic acid (C15:0)	1.82	1.82	2.18	2.20	1.76
Palmitic acid (C16:0)	388.02	381.15	450.82	449.34	254.65
Palmitoleic acid (C16:1, n-7)	7.28	7.99	9.92	11.88	10.32
Margaric acid (C17:0)	3.12	3.20	3.97	4.07	3.20
Stearic acid (C18:0)	84.56	89.45	119.03	123.52	94.57
Olenic acid (C18:1, n-9)	1318.03	1409.32	1853.58	2126.27	214.59
Vaccenic acid (C18:1, n-7)	66.07	71.56	92.42	112.32	26.02

Linoleic acid (C18:2, n-6)	1479.78	1490.07	1687.67	1729.22	856.32
CLA (C18:2 (9,11))	0.61	0.64	0.73	0.83	15.46
CLA ((C18:2 (10,12))	0.21	0.23	0.34	0.41	0.22
γ-linoleic acid (C18:3, n-6)	3.11	3.40	7.96	9.25	43.02
α-linolenic acid (C18:3, n-3)	241.35	256.47	334.91	381.73	290.06
Arachidic acid (C20:0)	14.89	19.05	24.86	29.76	31.40
Gondoic acid (C 20:1, n-9)	27.62	29.55	39.34	46.13	8.36
Eicosadienoic acid (C20:2, n-6)	2.45	2.45	2.78	3.28	3.23
Heneicosylic acid (C21:0)	0.97	0.93	1.01	0.92	
Dihomo-γ-linoleic acid (C20:3, n-6)	0.28	0.28	0.57	0.78	
Arachidonic acid (C20:4, n-6)	0.49	0.67	0.90	0.82	0.83
Dihomo-γ-linolenic acid (C20:3, n-3)	0.16	0.44	0.69	0.85	
Behenic acid (C22:0)	0.30	0.38	0.39	0.46	0.79
Eicosapentaenoic acid (C20:5, n-3)	4.07	5.33	8.41	10.19	11.03
Erucic acid (C22:1, n-9)	0.77	0.84	0.94	1.34	
Tricosylic acid (C23:0)	0.27	0.31	0.36	0.44	
Adrenic acid (C22:4, n-6)	0.38	0.85	1.57	1.87	
Lignoceric acid (C24:0)	0.20	0.25	0.47	0.49	
Clupanodic acid (C22:5, n-3)	5.23	6.33	9.79	11.80	
Nervonic acid (C24:1, n-9)	0.55	0.59	0.73	0.80	
Docosahexaenoic acid (C22:6, n-3)	4.27	4.82	7.65	9.64	5.44
Total	3663.07	3799.54	4677.74	5086.11	1919.70
Saturated fatty acids	500.52	508.12	617.85	627.77	430.61
Monounsaturated fatty acids	1420.54	1520.29	1997.49	2299.54	263.47
Polyunsaturated fatty acids	1742.01	1771.13	2062.40	2158.80	1225.61
Polyunsaturated fatty acids (n-3)	255.08	273.39	361.45	414.21	306.53
Polyunsaturated fatty acids (n-6)	1486.93	1497.74	1700.95	1744.59	903.40
n-6/n-3	5.83	5.48	4.71	4.21	2.95

As previously reported, aliquots of the hemp co-product were collected every hour during the digestion process to investigate the behaviour of phenolic (Figure 1a) and antioxidant compounds (Figure 1b, 1c). The hemp co-product presented an increasing trend in TPC (Figure 1a) from the beginning of the digestive process (1072.66±115.82 mg TAE/100 g), peaking at the end of the gastric phase (1752.16±66.73 mg TAE/100 g) ($p<0.05$). At the start of the intestinal incubation (1127.37±40.17 mg TAE/100 g), a statistically significant

($p<0.05$) momentary decrease was observed, although it declined at the end of the digestive process, resulting in a value highly comparable to that previously registered at the end of the gastric phase (1507.50±9.72 mg TAE/100 g).

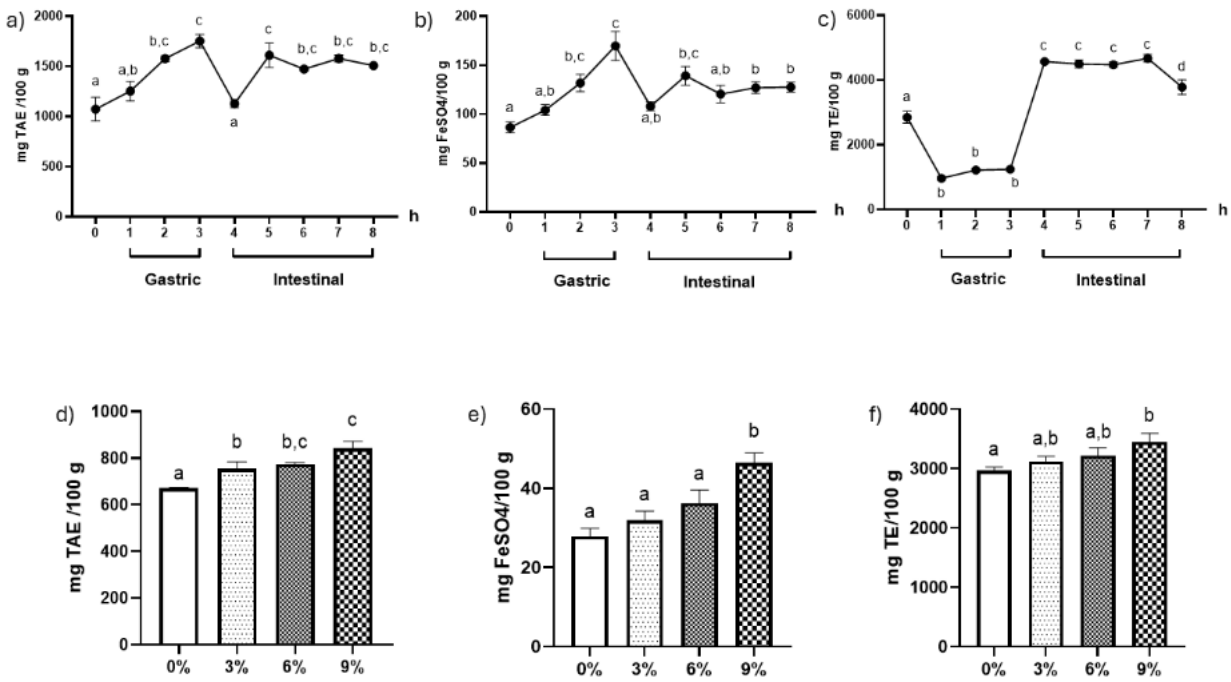

Figure 1. **Total phenolic content and antioxidant activity (FRAP and ABTS) of hemp co-product and dietary treatments**. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicates. Different superscript letters indicate significant different data ($p<0.05$). a) Total phenolic content of hemp-co product monitored each hour; b) FRAP assay of hemp-co product monitored each hour; c) ABTS assay of hemp-co product monitored each hour, d) Total phenolic content of dietary treatments; e) FRAP assay of dietary treatments; f) ABTS assay of dietary treatments.

The same trend was also obtained for the FRAP assay (Figure 1b), confirming the peak at the end of gastric digestion (169.80±14.84 mg $FeSO_4$/100 g), significantly higher ($p<0.05$) than at any other timepoint. In contrast, the ABTS method (Figure 1c) reported an opposite course. More specifically, the beginning of the gastric phase (969.07±56.51 mg TE/100 g) showed a significant ($p<0.05$) decrease in antioxidant activity compared to the beginning of digestion (2852.03±183.27 mg TE/100 g). Although no appreciable differences were observed during the gastric incubation, the start of the intestinal phase showed a marked ($p<0.05$) increase (4573.77±39.38 mg TE/100 g), which remained constant over the next three hours, before declining in the last hour of incubation (3782.09±223.58 mg TE/100 g) ($p<0.05$). As presented in Figure 1d, increasing inclusions of the hemp co-product improved the TPC of T_3 (3%), T_6 (6%) and T_9 (9%) compared to the control (0%). More precisely, T_3 showed a significantly ($p<0.05$) higher phenolic content (755.53±29.07 mg TAE/100 g) than the control (670.42±3.88 mg TAE/100 g) and highly similar to T_6 (773.68±7.37 mg TAE/100 g). Better results were obtained for T_9 (841.89±29.96 mg TAE/100 g) although comparable

with T_6. Similar trends were observed for the FRAP assay (Figure 1e), with statistically higher values ($p<0.05$) for T_9 (46.48±2.48 mg $FeSO_4$/100 g) compared to the control (27.89±2.01 mg $FeSO_4$/100 g), T_3 (31.99±2.29 mg $FeSO_4$/100 g) and T_6 (36.23±3.42 mg $FeSO_4$/100 g).

In parallel, the antioxidant activity assayed by the ABTS method (Figure 1f) confirmed better results for the 9% (3451.51±143.44 mg TE/100 g) inclusion of the co-product in the diet, significantly higher ($p<0.05$) than the control (2963.76±62.04 mg TE/100 g) and comparable with the inclusion of 3% and 6% (3125.50±78.73 mg TE/100 g; 3221.29±129.15 mg TE/100 g, respectively). However, although an increasing trend was observed from control to T_6, no statistically significant difference was observed between any of the diets considered.

Laying hens performances

Table 4. Laying hen performances. Different superscript letters indicate significant different data ($p<0.05$)

	Control	T_3	T_6	T_9		
	0%	3%	6%	9%	SEM	P-value
Body weight 25th week (g)[1]	1839	1773	1800	1797	13.6	NS
Body weight 32nd week (g)[2]	1868	1802	1831	1840	15.6	NS
Feed consumption (g/hen/day)	122.3	118.7	118.3	120.7	0.73	NS
Mortality (%)	0	0	0	0	-	-
Hen-day egg production (%)	92.7^a	89.1^b	89.0^b	94.6^a	0.54	<0.001
Feed consumption (g/egg)	132.2	134.6	133.6	128.2	1.12	NS
Egg mass production (g/hen/day)	56.2^b	53.7^c	54.2^c	59.0^a	0.37	<0.001

As reported, the increasing inclusion of the hemp co-product in the diet of the laying hens did not result in changes in their final weight both following the adaptation period and at the end of the experimental trial. The same results were also observed for feed consumption (g/hen/day). In parallel, the T_9 group (94.6%) reported a hen-day egg production comparable to that of the control (92.7%) and statistically superior ($p<0.01$) to the T_3 (89.1%) and T_6 (89.0%), although feed consumption (g/egg) was similar between all dietary treatments. In addition, the 9% inclusion resulted in statistically ($p<0.01$) higher egg mass production (59.0 g/hen/day) then 0%, 3% and 6% inclusion (56.2 g/hen/day, 53.7 g/hen/day and 54.2 g/hen/day, respectively).

Egg physical parameters

Table 5. Physical characteristic of eggs. Different superscript letters indicate significant different data (*p<0.05*)

	Control	T_3	T_6	T_9		
	0%	3%	6%	9%	SEM	P-value
Egg surface (cm^2)	73.0[b]	72.6[b]	72.8[b]	74.3[a]	0.368	<0.001
Egg weight (g)	60.6[b]	60.2[b]	60.9[b]	62.4[a]	0.37	<0.001
Albumen weight (g)	40.6[b]	40.2[b]	40.6[b]	42.2[a]	0.17	<0.001
Yolk weight (g)	14.9	14.9	14.8	15.1	0.06	NS
Shell weight (g)	6.34	6.27	6.26	6.34	0.024	NS
Albumen percentage (%)	65.7[b]	65.4[b]	65.7[ab]	66.3[a]	0.10	0.015
Yolk percentage (%)	24.1	24.4	24.1	23.7	0.09	NS
Shell percentage (%)	10.3[a]	10.2[a]	10.2[a]	10.0[b]	0.03	0.006
Egg yolk/egg albumen (%)	36.8	37.4	36.8	35.9	0.20	NS
Shell thickness (µm)	375[a]	370[ab]	368[b]	368[a]	1.0	0.047
Shell deformity (mm)	0.29	0.29	0.28	0.29	0.0023	NS
Shell strength	4754	4769	4667	4744	32.2	NS
Shell strength (N)	48.5	48.6	47.6	48.4	0.33	NS
DSM yolk colour fan	4.48[d]	5.14[c]	5.72[b]	6.35[a]	0.045	<0.001
Redness (a*)[1]	1.63[d]	2.74[c]	3.83[b]	4.32[a]	0.062	<0.001
Yellowness (b*)[2]	36.9[d]	42.5[c]	46.7[b]	48.7[a]	0.27	<0.001

[1]redness (-100 green; 100=red). [2]yellowness (-100=blue; 100=yellow)

As shown in Table 5, the 9% inclusion of the hemp co-product showed a significantly (*p<0.01*) greater egg surface (74.3 cm^2) and egg weight (62.4 g) than the control (73.0 cm^2; 60.6 g), 3% (72.6 cm^2; 60.2 g) and 6% inclusion (72.8 cm^2; 60.9 g), respectively. No differences were observed in the weight of each egg component, with the exception of albumen, with significantly (*p<0.01*) greater values for T_9 (42.2 g) than control (40.6 g), T_3 (40.2 g) and T_6 (40.6 g). These values resulted in a significantly (*p<0.05*) higher albumen percentage for T_9 (66.3%) than for control (65.7%) and T_3 (65.4%), but comparable to T_6 (65.7%). Although no difference was observed for percentage of yolk, the percentage of control shell (10.3%) was similar to T_3 (10.2%) and T_6 (10.2%); all significantly (*p<0.05*) superior to T_9 (10.0%). Despite this, the shell thickness, deformity and strength showed no appreciable difference between the groups. Finally, as reported in Table 5, interesting results were obtained for egg yolks colour. More specifically, increasing levels of inclusion of hemp co-product resulted in significant (*p<0.01*) rises between each group in the redness (1.63;

2.74; 3.83; 4.32) and yellowness (36.9; 42.5; 46.7; 48.7) of the yolks, for control, inclusion at 3%, 6% and 9%, respectively.

Nutritional and Functional profile of egg yolks

The nutritional profile of egg yolks is shown in Table 6. The 9% inclusion (7672 mg/100 g) of the hemp co-product resulted in a significant reduction (*p<0.01*) in the SFAs content compared to the control (8742 mg/100 g), 3% (8837 mg/100 g) and 6% inclusion (8524 mg/100 g). The same trend (*p<0.05*) was recorded for MUFAs (12849 mg/100 g; 13331 mg/100 g; 12966 mg/100 g; 12152 mg/100 g, for control, T_3, T_6 and T_9, respectively). Total PUFAs values were significantly (*p<0.05*) higher for T_3 (6145 mg/100 g), compared to control (5431 mg/100 g) and T_6 (5620 mg/100 g), but highly comparable with T_9 (5897 mg/100 g). No difference was observed between control and inclusion at 3% and 9%.

Table 6. Fatty acid profile (mg/100 g) and cholesterol content (g/kg) of egg yolks. Different superscript letters indicate significant different data (p<0.05). CLA: Conjugated Linoleic acid; HH: Hypocholesterolemic/hypercholesterolemic fatty acid ratio.

	Control	T_3	T_6	T_9		
	0%	3%	6%	9%	SEM	P-value
Caproic acid (C6:0)	1.07^{c}	1.29^{b}	1.62^{a}	1.70^{a}	0.052	<0.001
Caprylic acid (C8:0)	2.48^{b}	2.62^{b}	2.95^{a}	2.70^{ab}	0.052	0.009
Capric acid (C10:0)	1.70^{b}	2.39^{a}	1.91^{b}	1.83^{b}	0.068	<0.001
Lauric acid (C12:0)	5.13^{b}	5.95^{a}	4.84^{c}	4.57^{d}	0.102	<0.001
Trydecylic acid (C13:0)	0.84^{d}	1.08^{c}	1.33^{b}	1.57^{a}	0.055	<0.001
Myristic acid (C14:0)	96.2^{a}	95.4^{a}	77.5^{b}	72.9^{b}	2.07	<0.001
Myristoleic acid (C14:1, n-5)	18.0^{a}	18.8^{a}	10.6^{b}	10.3^{b}	0.77	<0.001
Pentadecylic acid (C15:0)	18.5^{c}	20.9^{b}	26.5^{a}	25.1^{a}	0.63	<0.001
Cis-10-pentadecycli acid (C15:1, n-5)	12.3	13.4	12.7	13.2	0.23	NS
Palmitic acid (C16:0)	6311^{ab}	6379^{a}	6062^{b}	5511^{c}	74.8	<0.001
Palmitoleic acid (C16:1, n-7)	714^{a}	673^{b}	554^{c}	514^{d}	15.9	<0.001
Margaric acid (C17:0)	49.1^{b}	58.6^{a}	52.7^{b}	44.0^{c}	1.18	<0.001
Stearic acid (C18:0)	2233^{a}	2243^{a}	2262^{a}	1980^{b}	30.3	<0.001
Olenic acid (C18:1, n-9)	11453^{ab}	11988^{a}	11774^{a}	11036^{b}	118.0	0.018
Vaccenic acid (C18:1, n-7)	568^{a}	562^{a}	531^{b}	505^{b}	6.7	0.001
Linoleic acid (C18:2, n-6)	4078	4082	4374	4187	47.9	NS
CLA (C18:2 (9,11))	5.02^{b}	5.86^{a}	6.29^{a}	5.04^{b}	0.122	<0.001
CLA ((C18:2 (10,12))	1.63^{c}	1.87^{b}	2.27^{a}	2.10^{a}	0.054	<0.001

γ-linoleic acid (C18:3, n-6)	32.0[b]	30.6[b]	41.9[a]	41.6[a]	1.07	<0.001
α-linolenic acid (C18:3, n-3)	319[c]	315[c]	367[b]	418[a]	8.4	<0.001
Arachidic acid (C20:0)	6.22[b]	6.11[bc]	8.26[a]	5.60[c]	0.204	<0.001
Gondoic acid (C 20:1, n-9)	73.1[a]	65.8[b]	72.2[a]	63.5[b]	1.06	<0.001
Eicosadienoic acid (C20:2, n-6)	44.2[b]	50.8[a]	50.0[a]	42.7[b]	0.86	<0.001
Heneicosylic acid (C21:0)	2.63[c]	3.95[b]	4.41[a]	4.49[a]	0.151	<0.001
Dihomo-γ-linoleic acid (C20:3, n-6)	25.8[b]	27.1[b]	33.4[a]	33.1[a]	0.70	<0.001
Arachidonic acid (C20:4, n-6)	505[b]	559[a]	581[a]	515[b]	8.5	0.001
Dihomo-γ-linolenic acid (C20:3, n-3)	4.97[c]	5.72[b]	5.63[b]	7.26[a]	0.168	<0.001
Juniperonic acid (C20:4, n-3)	4.51[a]	3.85[b]	3.62[b]	4.18[ab]	0.114	0.023
Behenic acid (C22:0)	3.86[c]	4.40[b]	5.04[a]	4.64[b]	0.095	<0.001
Eicosapentaenoic acid (C20:5, n-3)	5.05[c]	6.43[b]	7.08[a]	6.09[b]	0.153	<0.001
Erucic acid (C22:1, n-9)	3.31[a]	3.56[a]	3.74[a]	2.39[b]	0.128	<0.001
Docosadienoic acid (C22:2, n-6)	10.89[a]	6.90[d]	8.22[b]	7.47[c]	0.289	<0.001
Tricosylic acid (C23:0)	4.26[b]	3.89[b]	4.73[a]	4.33[ab]	0.089	0.005
Adrenic acid (C22:4, n-6)	8.1[b]	11.6[b]	33.6[a]	33.0[a]	2.25	<0.001
Lignoceric acid (C24:0)	4.03[c]	5.51[b]	6.16[a]	5.65[b]	0.157	<0.001
Clupanodic acid (C22:5, n-3)	51.1[c]	54.4[c]	89.6[a]	74.1[b]	2.93	<0.001
Nervonic acid (C24:1, n-9)	7.92[a]	6.77[b]	7.94[a]	7.56[a]	0.121	<0.001
Docosahexaenoic acid (C22:6, n-3)	335[c]	459[b]	542[a]	521[a]	15.7	<0.001
Total	27023[ab]	27788[a]	27636[a]	25721[b]	269.4	0.019
Saturated fatty acids	8742[a]	8837[a]	8524[a]	7672[b]	104.4	<0.001
Monounsaturated fatty acids	12849[a]	13331[a]	12966[a]	12152[b]	132.7	0.009
Polyunsaturated fatty acids	5431[b]	5620[b]	6145[a]	5897[ab]	76.6	0.002
Polyunsaturated fatty acids (n-3)	720[c]	844[b]	1015[a]	1031[a]	25.0	<0.001
Polyunsaturated fatty acids (n-6)	4705[b]	4768[b]	5122[a]	4859[ab]	57.4	0.044
n-6/n-3	6.54[a]	5.65[b]	5.05[c]	4.72[d]	0.127	<0.001
HH	2.61[d]	2.70[c]	2.89[b]	3.00[a]	0.029	<0.001
Atherogenic index	0.367[a]	0.358[b]	0.334[c]	0.322[d]	0.0034	<0.001
Thrombogenic index	0.790[a]	0.753[b]	0.696[c]	0.653[d]	0.0098	<0.001
Peroxidation index	38.2[d]	41.6[c]	47.2[b]	48.2[a]	0.76	<0.001
Cholesterol	11.4[a]	10.7[b]	10.4[bc]	10.0[c]	0.13	<0.001

The same tendency was observed for n-6 PUFAs. In contrast, increasing inclusions of the hemp co-product increased the amount of n-3 PUFAs. More specifically, T_3 (844 mg/100 g) showed significantly (*p<0.01*) higher values than the control (720 mg/100 g) and lower content than T_6 (1015 mg/100 g) and T_9 (1031 mg/100 g). No difference was recorded between T_6 and T_9. These results yielded an n-6/n-3 dependent on inclusion levels, with statistically significant differences (*p<0.01*) between each group (6.54; 5.65; 5.05; 4.72, respectively for control, T_3, T_6 and T_9). The same tendency was observed for the hypocholesterolemic/hypercholesterolemic fatty acid ratio, atherogenic, thrombogenic and peroxidability indices.

Finally, interesting results were obtained for cholesterol content. More specifically, the egg yolks of hens fed with 9% hemp co-product (10.0 g/kg) showed a similar level to the T_6 (10.4 g/kg) group, but significantly lower than T_3 (10.7 g/kg) and control (11.4 g/kg). At the same time, T_3 and T_6 were significantly higher than the control. As reported in Table 7, although no difference was reported for α-tocopherol, the inclusion of the hemp co-product at 6% (42.1 mg/kg) and 9% (45.1 mg/kg) significantly increased (*p<0.01*) the γ-tocopherol content compared to the control (28.7 mg/kg) and inclusion at 3% (33.6 mg/kg). Interesting results were reported for β-carotene and lutein values. In the first case, higher inclusions of hemp co-product corresponded to lower β-carotene values in egg yolks with higher values for the control (0.0608 mg/kg), although comparable to T_3 (0.0605 mg/kg), T_6 (0.0578 mg/kg), but significantly higher (*p<0.05*) than T_9 (0.0531 mg/kg). In contrast, lutein content was dependent on hemp inclusion percentages, with significant (*p<0.01*) differences between each group (5.8 mg/kg, 10.6 mg/kg, 16.0 mg/kg and 21.5 mg/kg, respectively for control, inclusion at 3%, 6% and 9%).

Table 7. Content of α-tocopherol, γ-tocopherol, retinol, β-carotene, lutein and zeaxanthin in egg yolks. Values are expressed in mg/kg DM. Different superscript letters indicate significant different data (*p<0.05*).

	Control	T_3	T_6	T_9		
	0%	3%	6%	9%	SEM	P-value
α-tocopherol	231	256	261	252	4.4	NS
γ-tocopherol	28.7^{c}	33.6^{b}	42.1^{a}	45.1^{a}	1.28	<0.001
Retinol	9.8	10.3	10.0	10.1	0.12	NS
Lutein	5.8^{d}	10.6^{c}	16.0^{b}	21.5^{a}	1.09	<0.001
Zeaxanthin	5.15	5.07	5.16	5.95	0.163	NS
β-carotene	0.0608^{a}	0.0605^{a}	0.0578^{ab}	0.0531^{b}	0.001	0.029

Following the green chemical extraction process, egg yolks from hens fed the hemp co-product had a significantly (*p<0.05*) higher TPC (Figure 2a) than those receiving the basal diet (295.09±22.18 mg TAE/100g; 458.25±30.93 mg TAE/100g; 489.45±38.10 mg TAE/100 g; 414.36±24.53 mg TAE/100 g, respectively for control, T_3, T_6 and T_9). This trend was also confirmed for the antioxidant activity measured by the ABTS method (Figure 2c). The FRAP assay (Figure 2b) showed that, although the control (38.65±2.87 mg FeSO4/100 g) was comparable to the inclusion at 6% (49.01±1.26 mg FeSO4/100 g) and 9% (48.74±2.97 mg

FeSO4/100 g), it was significantly (*p<0.05*) lower than 3% (52.53±3.07 mg FeSO4/100 g), while no difference was noted between T_3, T_6 and T_9.

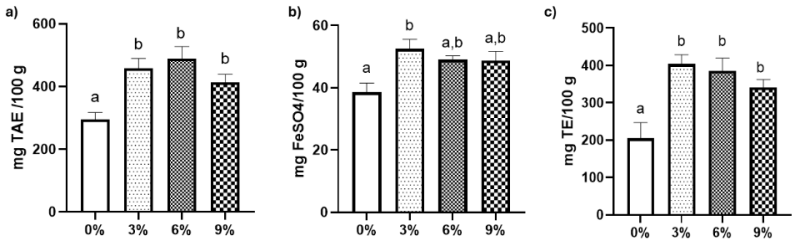

Figure 2. Total phenolic content and antioxidant activity (FRAP and ABTS) of egg yolks after green chemical extraction. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicated. Different superscript letters indicate significant different data (*p<0.05*). a) Total phenolic content; b) FRAP assay; c) ABTS assay.

As shown in Figure 3, following the digestive process, although no difference was observed in the TPC (Figure 3a), the ABTS data confirmed what was previously reported (Figure 3c). More specifically, each inclusion of the hemp co-product recorded significantly (*p<0.05*) higher values than the control (601.12±42.40 mg TE/100 g; 773.17±43.77 mg TE/100 g; 765.64±38.71 mg TE/100 g; 843.48±38.71 mg TE/100 g, for 0%, 3%, 6% and 9%, respectively). For FRAP (Figure 3b), higher results were obtained for T_6 (83.70±4.92 mg FeSO4/100 g), comparable with T_3 (70.36±3.06 mg FeSO4/100g) and T_9 (74.62±4.65 mg FeSO4/100g), but significantly higher than the control (60.37±5.85 mg FeSO4/100 g). No difference was observed between control, T_3 and T_9.

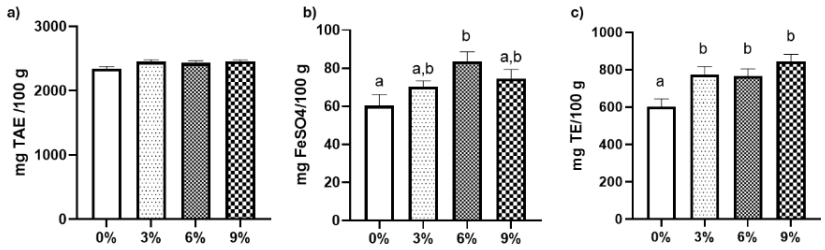

Figure 3. Total phenolic content and antioxidant activity (FRAP and ABTS) of egg yolks after *in vitro* digestion. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± SEM of at least three replicates. Different superscript letters indicate significant different data (p<0.05). a) Total phenolic content; b) FRAP assay; c) ABTS assay.

DISCUSSION

Although it is difficult to compare the nutritional profile of the hemp co-product used in this trial with values found in the literature, mainly due to the harvesting method and processing, it is evident that it presents a high nutritional profile. More precisely, it can be assumed that the interesting protein content derives from the contribution of HSs, hulls, stems and leaves, as reported in the literature (Kleinhenz et al., 2020; Ely and Fike, 2022, Vastolo et al. 2022; Lanzoni et al., 2023b). At the same time, it is distinguished by a lipid profile typical of hemp-based matrices, characterised by a higher content of PUFAs, compared to MUFAs and SFAs (Callaway, 2004; Vonapartis et al., 2015). More specifically, n-6 PUFAs present a higher percentage than n-3, resulting in an n-6/n-3 PUFAs (2.95) similar to that reported by Vesak et al. (2019) and Galasso et al. (2016) (3.2 and 2.8-4.5, respectively). However, as previously noted, the high ashes and especially the fibre content affected its digestibility. In spite of this, increasing inclusions of up to 9%, did not alter the digestibility of DM and protein in the diets, demonstrating its potential use.

At the same time, the hemp co-product showed an interesting functional profile. In particular, the behaviour of phenolic and antioxidant compounds was studied every hour to understand at what stage of digestion they are released and can benefit the animal health. Precisely, as previously described, from the beginning of the oral phase, the co-product showed an increasing TPC reaching a peak at the end of the gastric phase (3 h). This trend is confirmed in our previous study, in which hemp-based products (HSs, HSs protein, flowers) were digested with the same *in vitro* protocol (Lanzoni et al., 2023b). These results could be explained by the action of the acidic pH of the stomach, which causes the release of phenolic compounds as a result of the breaking of protein and polysaccharide bonds, particularly of fibre, the component with the highest phenol content (Goulas and Hadjisolomou, 2019). Furthermore, as reported by Pineda-Vadillo et al. (2016), the stomach's pH is able to protect the structure of the phenols, enabling their exact quantification. Later, following the addition of pancreatin (4 h), a steep decrease was recorded. As explained by Wojtunik-Kulesza et al. (2020), this is a consequence of the instability of phenols at alkaline pH, particularly during the pancreatic phase. More precisely, this condition causes a structural reorganisation of phenols compounds that modulates their function and bioaccessibility. However, as previously described, the final TPC showed values comparable to those at the end of the gastric phase. As demonstrated by Tarko et al. (2013), only phenols that are released from the food matrix during the digestive process and reach the gut can be absorbed (Tarko et al., 2013). Although 10% of these compounds remain bound to the food matrix, approximately 48% are digested in the small and 42% in the large intestine (Tarko et al., 2013). This is of paramount importance, as their absorption can exert multiple positive functions in the body, such as antioxidant, anti-inflammatory and improving intestinal health (Mahfuz et al., 2021). As just reported, phenols are characterised by antioxidant activity. In support of this, the FRAP assay showed the same trend as the TPC. However, the ABTS method reported a completely opposite course. As described by Zhou et al. (2016), such differences between the antioxidant assays can be observed as the sample contains a high antioxidant profile with

different molecules with diverse mechanisms of action. As shown, higher values were obtained in the ABTS assay.

This is in line with the content of α,γ-tocopherol, lutein, β-carotene, and zeaxanthin observed in the hemp co-product. More precisely, as reported by Farinon et al. (2020), tocopherols are fat-soluble compounds with a high antioxidant capacity that can protect the lipid fraction from oxidation due to their high ability to scavenge free radicals, the mechanism of action of ABTS. Of these, as described by Porto et al. (2015), γ-tocopherol has been indicated as the most active antioxidant in lipids and consequently is the one that most contributes, together with other antioxidant compounds (e.g. phenols), to providing high oxidative stability in hemp-based products. At the same time, the hemp co-product showed good carotenoid values, with higher concentrations for lutein, β-carotene and zeaxanthin respectively, confirming the observations of Farinon et al. (2020). Again, these compounds are characterised by a greater free radical scavenging mode of action than metal chelation, the mechanism of action of FRAP assay (Sindhu et al., 2010).

As shown so far, the high functional properties of the hemp co-product have improved that of diets, especially T_9, demonstrating that the increased inclusion not only preserves digestibility parameters, but also increases the functional value.

These results can be correlated with the performance of the treated animals. As previously described, the body weight of the treated animals showed no difference during both the adaptation period and the entire trial. This finding confirms both the value of the diets digestibility and the possibility of including the co-product up to 9% without any negative aspects for the growth performances of the animals, despite higher values for ashes and fibre content than the control. This result is most probably related to the age of the treated animals (21 weeks). In this period, chickens are characterised by a developed gastrointestinal system. However, as reported by Vispute et al. (2019), in the early stages of the production cycle (0-3 weeks), it would be better to avoid the use of hemp-based matrices, as at this stage the chickens have a poorly formed mucosa, which is not able to produce enzymes to degrade cellulose. This was also observed by Gakhar et al. (2012) in laying hens. A key parameter in the laying hen sector is certainly hen-day egg production (Lanzoni et al., 2023a). Regarding this, as previously reported, the 9% inclusion resulted in better results than T_3 and T_6, but similar to the control. For this reason, it can be deduced that the effect observed with the inclusion of the co-product at 3% and 6% is transitory and probably not specifically related to the dietary treatment. Indeed, it is important to emphasise that results regarding hen-day egg production are often discordant in the literature. Although, Skrivan et al. (2019) observed that the inclusion of 3% HSs in the diets of laying hens significantly improved the laying rate (93.6%), compared to the control (88.7%) and the inclusion of 6% (86.40%) and 9% (89.40%), Gakhar et al. (2012) observed no difference up to an inclusion of HSs of 20%. This was confirmed also by Neijat et al. (2016) with an HSs inclusion of 30%. This trend was also registered with the use of hemp co-products. In particular, the inclusion of 20% and 30% HSs in the diets of laying hens showed no change as demonstrated by Silversides and

Lefrancois (2005) and Rajasekhar et al. (2021), respectively. Finally, T_9 had a significantly higher egg mass production (g/hen/day) than the control. However, the latter was greater than T_3 and T_6. Again, it is possible that the effect observed for the inclusion of T_3 and T_6 is not related to dietary treatment. Indeed, as reported by Halle and Schone (2013), the inclusion of 15% HSs cake in the diet of hens did not increase egg mass production. This was also confirmed by Gakhar et al. (2012) with up to 20% HSs. For this reason, although better production performances was obtained in this study with the inclusion of 9% hemp co-product, further investigation is needed to understand the correlation between dietary treatment, deposition hen/day egg production and egg mass production.

As previously reported, interesting results were also obtained for egg physical parameters and nutritional/functional profile of egg yolks. As observed, the 9% inclusion of the hemp co-product resulted in significantly higher egg surface and weight than the other treatments, partially confirming what was described by Mierlita (2019). More specifically, the authors observed how the inclusion of 8% HSs in the diet of laying hens resulted in increased egg weight compared to the control (based on soybean meal). However, this trend was not reported by Konca et al. (2019) and Silversides and Lefrancois (2005) with an inclusion of 15% HSs and up to 20% HSs cake, respectively. Although, Johnston and Gous (2007) reported an increase in weight to be correlated with an increase in yolk weight, in this study the only increment was found to be egg albumen, confirming the findings of Halle and Schone (2013). Albeit shell thickness was negatively affected by the increase in hemp co-products inclusions, other parameters such as deformity, strength and weight were not altered. The latter plays a key role. This result is in agreement with the similar values of calcium and phosphorus in the different dietary treatments observed in Table 2. As reported by Keshavarz (1994), shell formation is influenced by the content of these two minerals in the diets, which are able to regulate the pH of the blood, consequently affecting its acid-base balance. More specifically, maintaining this equilibrium plays a key role in bicarbonate homeostasis and subsequent shell formation (Keshavarz, 1994). However, as shown by Neijat et al. (2014), it is difficult to find a direct link between dietary treatment and the content of these minerals in the blood; in fact, as reported in the literature, the inclusion of hemp products in the diets of laying hens has yielded discordant results. While Mierlita et al. (2024) and Park et al. (2014) observed no difference, Skřivan et al. (2019) showed a negative correlation as HSs inclusion increased. Finally, another parameter investigated in the literature concerns the colour of the yolks, although it is difficult to identify it as a quality parameter, as it depends a lot on individual preferences (Lanzoni et al., 2023a). As reported in Table 5, increasing inclusions of hemp co-product resulted in increasing redness and yellowness values, confirming what was observed by Goldberg et al. (2012), Park et al. (2014), Skrivan et al. (2019) and Konca et al. (2019). As argued by Goldberg et al. (2012), colour intensity is influenced by the presence of carotenoids. More specifically, in this study, observing the values reported in Table 7, it is most likely, that the differences observed are due to the lutein content, rather than β-carotene and zeaxanthin, confirming what Kasula et al. (2021) reported.

From a nutritional point of view, it is interesting to observe how the inclusion of the hemp co-product modulated the lipid profile of egg yolks. More specifically, although there would seem to be no correlation between different dietary treatments and total lipid content, the different fractions (SFAs, MUFAs, PUFAs) were highly modified. In particular, T_9 showed a significantly reduced SFAs content compared to the control, T_3 and T_6, mainly due to the reduction of myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0). This result is most likely also related to the digestive physiology of chickens. In fact, as reported by Ravindran et al. (2016), fatty acids released during digestion differ in their ability to form micelles and then be absorbed, with unsaturated ones characterised by a greater ease of formation than SFAs, especially long-chain ones. As argued by the authors, it is believed that the presence of even just one double bond is sufficient to increase the ease of micelle formation, due to a greater bending of the three-dimensional structure. In addition, unsaturated fatty acids are able to function as natural emulsifiers favouring this process. These would favour a higher absorption and consequently a higher presence in the egg yolks (Ravindran et al., 2016).

This trend, was also observed for the MUFAs content, confirming the values of Shahid et al. (2015), Konca et al. (2019), and Mierlita et al. (2024). In general, and also in this study, these results are due to the fact that hemp-based products are characterised by higher values of PUFAs than SFAs and MUFAs (Shahid et al., 2015). In particular, for n-3 and n-6 PUFAs in egg yolks, better results were observed with the inclusion of 6% and 9% of the co-product, partly reflecting the dietary content. Within the n-3 and n-6 PUFAs, it is important to assess the content of linoleic acid (C18:2, n-6) and α-linolenic acid (C18:3, n-3), the main fatty acids of the hemp co-product. More specifically, although no differences were shown for the content of linoleic acid, T_9 showed a significantly higher α-linolenic acid content than the control, T_3 and T_6. This result plays a key role as these two fatty acids are considered essential since they cannot be synthesised by the human metabolism but have to be introduced through the diet (Farinon et al., 2020).In parallel, they are the precursors of biologically active n-6 and n-3 PUFAs in humans. These include long-chain arachidonic acid (20:4, n-6) derived from the conversion of linoleic acid, docosahexaenoic acid (22:6-n3) and eicosapentaenoic acid (20:5, n-3) both obtained from the precursor α-linolenic acid (Lanzoni et al., 2023a). Indeed, in this study, arachidonic acid levels were significantly increased in eggs compared to dietary treatments, with better results for T_6. However, for eicosapentaenoic acid this trend was not observed. More specifically, although the inclusion of 6% allowed differences to be observed compared to the other dietary treatments, the values for T_6 and T_9 in eggs were lower than those in diets. This result may be explained because α-linolenic acid and linoleic acid compete for the same enzyme (Δ6 desaturase) that converts α-linolenic acid to eicosapentaenoic acid and linoleic acid to arachidonic acid, leading to a reduction in the synthesis of one of the two fatty acids (Shahid et al., 2015). At the same time, T_6 and T_9 reported a significantly higher value for docosahexaenoic acid than the control and T_3, confirming what has been reported above. The increase of these biologically active forms in egg yolks is of great importance, as they play major roles in the

maintenance of cell membrane structures, cardiovascular health, development of neurological functions, obesity and inflammatory processes (Farinon et al., 2020).

In addition to the evaluation of PUFAs, as reported by Simopoulos (2008), it is important to analyse the n-6/n-3 ratio, as this is a fundamental index involved in maintaining an optimal state of health, particularly in preventing the onset of chronic degenerative diseases. In this study, as noted earlier, the increased inclusion of the hemp co-product reduced the n-6/n-3 ratio, leading to a better result for T_9. Indeed, as pointed out by the European Food and Safety Authority (EFSA, 2009), the ideal n-6/n-3 ratio should be approximately 3.5; a value typical of diets, including the Mediterranean diet, that are poorly associated with coronary heart disease (Callaway, 2004).

In parallel, the evaluation of the ratio of hypocholestorolaemic/hypercholesterolaemic (h/H) fatty acids showed significantly better results with increasing inclusion of the hemp co-product. As reported by Chen and Liu (2020), for human health, higher values are considered more beneficial due to the effects of hypocholestorolaemic fatty acids (PUFAs and C18:1) on cholesterol metabolism. This trend was also confirmed for the atherogenic and thrombogenic indices. They describe the relationship between pro-atherogenic (saturated) and anti-atherogenic (unsaturated) fatty acids and the tendency of fatty acids to form clots within blood vessels, respectively (Chen and Liu, 2020). Although all groups reported values below the upper limits (0.5 and 1, respectively), increasing inclusions of the hemp co-product were found to be positively associated with improvements in these indices, confirming what was observed by Mierlita et al. (2024). As expected, the peroxidation index of the eggs showed higher values as the co-product inclusion levels increased, confirming a greater risk of oxidation. This effect is mainly due to the high content of n-3 PUFAs, which are highly susceptible to oxidation, confirming the above. Despite this, although not investigated in this study, products obtained after treatment with hemp-based products showed a lower content of Malondialdehyde, a marker of lipid peroxidation, than the control (Mierlita et al., 2024; Mierlita 2019). As explained by the authors, this effect is due to the high levels of antioxidant molecules, in particular tocopherols, that are able to prevent lipid peroxidation, a result also obtained in this study, as later discussed.

Finally, increasing concentrations of hemp co-product reduced cholesterol levels respectively. Although Mierlita (2019) reported no difference in cholesterol content in the egg yolks of hens fed HSs (80 g/kg and 200 g/kg), Shahid et al. (2015) observed a different trend. Specifically, the authors showed how increasing inclusions of HSs (150, 200 and 250 g/kg) significantly reduced the cholesterol content (16.91 ± 0.01 mg/g; 14.29 ± 0.01 mg/g; 11.65 ± 0.01 mg/g) compared to the control (19.27 ± 0.01 mg/g), respectively. Identical trend observed by Mahmoudi et al. (2015), Skrivan et al. (2019) and Vispute et al. (2019). This positive result can be related both to the presence of phytosterols in hemp-based products and to the peculiarities of the chickens' digestive process. In the first case, as argued by Shahid et al. (2015), the presence of phytosterols in HSs, in particular β-sitosterol, is able to reduce hypercholesterolaemia by absorbing cholesterol through processes such as co-

precipitation and crystallisation. At the same time, as phytosterols are less water-soluble than cholesterol, they are able to replace cholesterol from intestinal micelles. In the second case, as reported by Ravindran et al. (2016), within chicken enterocytes, fatty acids are re-esterified and combined with multiple molecules (lipoproteins and phospholipids) including cholesterol to form chylomicrons. From here, the chylomicrons are secreted directly into the portal circulation and transported to various tissues, in particular to the liver, where the lipids are used for the synthesis of various compounds needed by the organism, such as lipoproteins and phospholipids, metabolised as an energy source or stored in the tissues as fat stores. This phenomenon suggests that a higher concentration of fatty acids (characteristic of diets with increasing levels of hemp co-product) allows for a greater interaction with cholesterol leading to its physiological reduction both in the body and then in the yolk of the eggs produced. Reducing cholesterol content is an important factor, as it is responsible for chronic or degenerative disorders including cardiovascular disease, the leading cause of mortality and morbidity globally (Carrillo et al, 2012; Miranda et al, 2015).

In general, from a functional point of view, egg yolks are an important source of α-tocopherol (Skrivan et al., 2019). Although, as reported by Hansen et al. (2015), to increase α-tocopherol levels in egg yolks, it is necessary to act only on the dietary level, such an effect was not observed in this study. However, γ-tocopherol values were significantly higher for T_3, T_6 and T_9, confirming the values reported by Skrivan et al. (2019) and Mierlita et al. (2024). This result plays a key role, since as shown by Guinaz et al. (2009), egg yolks are generally characterised by a reduced γ-tocopherol value, below detection limits. Furthermore, as reported by Wagner et al. (2004), although all isoforms of tocopherol are absorbed in the intestinal mucosa, excessive enrichment of α-tocopherol in the human diet results in a lower absorption rate. This effect, however, is not reported for the γ-isoform, making it available for important functional activities in human health (Wagner et al., 2004). Among these, in addition to its antioxidant properties, γ-tocopherol is characterised by anti-inflammatory properties (reduction of prostaglandin synthesis), anti-diabetic properties (supports the viability of β-pancreatic cells and insulin production during inflammation stages), prevents the onset of diseases such as atherosclerosis and hypertension, reduces the occurrence of tumours and finally, is positively involved in the prevention of Alzheimer's disease by protecting the brain against reactive nitrogen species (Saldeen and Saldeen, 2005). In parallel, carotenoid values showed discordant results. More specifically, β-carotene was found to be inversely proportional to co-product inclusion levels. Although Skrivan et al. (2019) and Mierlita et al. (2024) observed different trends, this finding was confirmed by Taaifi et al. (2023). More specifically, the authors demonstrated how increasing inclusions of HSs (10%, 20% and 30%) reduced the β-carotene content, respectively. At the same time, lutein increased significantly with each inclusion of the hemp co-product, with an increment of 182.8 %, 275.9 % and 370.7 % for T_3, T_6 and T_9 respectively, compared to the control. This effect, as reported by Gao et al. (2020) is most likely due to the high deposition efficiency of lutein in yolk compared to β-carotene. Furthermore, as described by Donsou et al. (2023), carotenoids such as β-carotene are precursors of vitamin A. After absorption, β,β-

carotene 15,15′-dioxygenase oxidatively cleaves this carotenoid at the central 15,15′ double bond to produce one or two molecules of retinal (vitamin A aldehyde). Although this process increases the concentration of vitamin A in eggs, it decreases the β-carotene content, explaining the results obtained.

To study the availability of functional compounds, as previously reported, egg yolks were chemically extracted and digested using an *in vitro* protocol. In the first case, the binary extraction (H_2O:EtOH; 50:50) observed a significant increase in TPC in all dietary treatments that received the inclusion of the hemp co-product. This trend, although not statistically significant, was also observed following the digestion process. To our knowledge, there are no studies in the literature quantifying TPC in eggs following dietary treatment with hemp-based matrices. Despite this, Benakmoum et al. (2013) and Untea et al. (2020) confirmed these findings, observing an increase in TPC following inclusion of dried tomato peel and bilberry and walnut leaves respectively. As reported by the authors, the increase in TPC is caused by the deposition in the yolk of those compounds characterised more by a lipophilic nature; compounds highly present in hemp-based products (Untea et al., 2020; Farinon et al., 2020). This effect is of paramount importance because. As previously described, phenols, in addition to having important antioxidant, anti-inflammatory and gut-health enhancing activity (Mahfuz et al., 2021), are characterised by atheroprotective activity, affecting cholesterol metabolism positively (Zanotti et al., 2015). The above, coupled with the reduced cholesterol levels shown previously, demonstrate the potential of this co-product to improve the healthiness of eggs for human consumption. This trend was also recorded for antioxidant activity measured with the ABTS assay following both the chemical extraction and *in vitro* digestion. This result, in addition to proving the involvement of phenolic compounds in the modulation of antioxidant activity, may support previous evidence which reported that molecules such as α, γ-tocopherol, isoforms of vitamin E, are the main antioxidant compounds capable of providing primary protection against lipid peroxidation caused by free radicals (Traber and Atkinson, 2007). In parallel, Kim et al. (2011) attributed to molecules such as zeaxanthin, but especially lutein (significantly higher in the yolks of eggs from hens that received the hemp co-product), the ability to scavenge hydroxyl and superoxide radicals by means of a bond that results in their stabilisation. These molecules, as demonstrated by Panasenko et al. (2000), are also capable of eliminating peroxynitrite, a molecule characterised by a high pro-oxidant activity against nucleic acids. An increase in antioxidant activity was also recorded with the FRAP assay, although it is complicated to find a close correlation with the dietary treatment administered.

CONCLUSIONS

In conclusion, the inclusion of the hemp co-product did not alter the dry matter and protein digestibility of the diet, compared to the control, while improving its functional profile, with better results obtained for 9%. These results were confirmed for production performance. Although no differences were observed in the body weight of the treated animals at the end

of the trial compared to the control group for each % inclusion, egg mass production (g/hen day) showed better trends with 9% of hemp co-product inclusion. In parallel, for the nutritional profile of the egg yolks, although T_3 and T_6 showed no differences compared to the control for the content of saturated and monounsaturated fatty acids, T_9 showed significant differences, also increasing the content of polyunsaturated fatty acids with a better n-6/n-3 ratio. This trend was also confirmed for atherogenic, thrombogenic and peroxidation indices, as well as for cholesterol; factors negatively involved in the genesis of cardiovascular pathologies. Finally, following *in vitro* digestion, all inclusion levels of the co-product increased the antioxidant activity (ABTS) of the yolks of eggs capable of providing a healthier product for human consumption. Although these results support the valorisation of hemp co-products in the diet of laying hens, with better results with 9% inclusion, it is crucial to emphasise that the high variability of the co-products due to the genotype considered, environmental factors, agro-ecological conditions, harvesting techniques, but above all transformation processes represent a critical point in their utilisation, which may affect the repeatability of the results.

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Manuscript V

Antioxidant capacity and peptidomic analysis of *in vitro* digested *Camelina sativa L.* Crantz and *Cynara cardunculus* co-products

Davide Lanzoni, Francesca Grassi Scalvini, Elena Petrosillo, Simona Nonnis, Gabriella Tedeschi, Giovanni Savoini, Arianna Buccioni, Guido Invernizzi, Antonella Baldi and Carlotta Giromini.

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Antioxidant capacity and peptidomic analysis of *in vitro* digested *Camelina sativa L.* Crantz and *Cynara cardunculus* co-products

D. Lanzoni[a], F. Grassi Scalvini[a], E. Petrosillo[a], S. Nonnis[a,b], G. Tedeschi[a,b], G. Savoini[a], A. Buccioni[c,d], G. Invernizzi[a], A. Baldi[a] and and C. Giromini[a,e]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy; [c]Dipartimento di Scienze e Tecnologie Agrarie, Alimentari, Ambientali e Forestali. University of Florence, Piazzale delle Cascine 18, 50144 Firenze, Italy; [d]Centro Interdipartimentale di Ricerca e Valorizzazione degli Alimenti, University of Florence, viale Pieraccini 6, 50139 Firenze, Italy; [e]Institute for Food, Nutrition and Health, University of Reading, Reading RG6 5EU, UK.

ABSTRACT: In recent decades, the food system has been faced with the significant problem of increasing food waste. Therefore, the feed industry, supported by scientific research, is attempting to valorise the use of discarded biomass as co-products for the livestock sector, in line with EU objectives. In parallel, the search for functional products that can ensure animal health and performances is a common fundamental goal for both animal husbandry and feeding. In this context, camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM), due valuable nutritional profile, represent prospective alternatives. Therefore, the aim of this work was to investigate the antioxidant activity of CAMC, CC and CM following *in vitro* digestion using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Total phenolic content (TPC) and angiotensin converting enzyme (ACE) inhibitory activity, actively involved in modulating antioxidant properties, were also studied. Further, a peptidomic analysis was adopted to substantiate the presence of bioactive peptides after *in vitro* digestion. The results obtained confirmed an interesting nutritional profile of CAMC, CC and CM and relevant antioxidant and ACE inhibitory activities. In particular, considering antioxidant profile, CM and CC revealed a significantly higher (10969.80 ± 18.93 mg TE/100 g and 10451.40 ± 149.17 mg TE/100 g, respectively; $p < 0.05$) ABTS value than CAMC (9511.18 ± 315.29 mg TE/100 g); a trend also confirmed with the FRAP assay (306.74 ± 5.68 mg $FeSO_4$/100 g; 272.84 ± 11.02 mg $FeSO_4$/100 g; 103.84 ± 3.27 mg $FeSO_4$/100 g, for CC, CM and CAMC, respectively). Similar results were obtained for TPC, demonstrating the involvement of phenols in modulating antioxidant activity. Finally, CAMC was found to have a higher ACE inhibitory activity (40.34 ± 10.11%) than the other matrices. Furthermore, potentially bioactive peptides associated with ACE inhibitory, anti-hypertensive, anti-cancer, antimicrobial, antiviral, antithrombotic, DPP-IV inhibitory and PEP-inhibitory activities were identified in CAMC. This profile was broader than that of CC and CM. The presence of such peptides corroborates the antioxidant and ACE profile of the sample. Although the data obtained report the important antioxidant profile of CAMC, CC, and CM and support their possible use, future investigations, particularly *in vivo* trials will be critical to evaluate and further investigate their effects on the health and performance of farm animals.

Keywords: Antioxidant activity, Bioactive peptides, Camelina, Cardoon, NanoLC-MS/MS, Peptidomics.

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INTRODUCTION

In recent decades, the food system has been faced with the significant problem of increasing food waste. Indeed, as reported by the Food and Agriculture Organisation of the United Nations (FAO), approximately 1.3 billion tonnes of food are lost or wasted globally each year (FAO, 2011). For this reason, the European Union has implemented multiple strategies to ensure the sustainability of the food and feed sector. In this context, the Agenda 2030, later supported by the Green Deal, plays a major role, whose goal is to ensure a competitive, clean and circular economy (Fetting, 2020; Vastolo et al., 2022). For this reason, scientific research is investigating the use of non-edible biomass produced along the food chain as co-products (any product obtained from different agro-industrial processes) for livestock (Rakita et al., 2021). As shown by Govoni et al. (2023), the introduction of co-products (11-16%) to replace energy-rich food crops (such as cereals) would conserve 15.4-27.8 Mha of land, 3-19.6 km^3 of blue water and 74.2-137.8 km^3 of green water, representing an important strategy to ensure environmental sustainability. To date, although many co-products have already been tested (pomace, beet pulp, hemp seed cakes), new ones need to be investigated, meeting EU objectives (Garcia-Rodriguez et al., 2019; Muhlack et al., 2018; Lanzoni et al., 2023).

As reported by Turco et al. (2019) and Singh et al. (2023), the co-products of camelina (*Camelina Sativa* L. Crantz) and cardoon (*Cynara cardunculus*) are attracting the interest of scientific research thanks to their low environmental impact and interesting nutritional profile. More specifically, camelina and cardoon seeds are characterised by a protein content of 25.9% and 16.7% on dry matter (DM) and by a lipid percentage of 38.9±1.26% and 25-30% on DM, respectively (Genovese et al., 2015; Turco et al., 2019; Singh et al., 2023). As described by Singh et al. (2023) and Petropoulos et al. (2018), the fatty acid profile of camelina and cardoon seeds, rich in polyunsaturated (55.6% and 65.43±0.08%, respectively) and low in saturated fatty acids (9.04% and 13.23±0.07%, respectively), prompted the food and feed industry to isolate the lipid fraction for nutritional and nutraceutical purposes, creating in parallel scrap products. The main scrap matrices of this processing are definitely, camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM), which, due to their important nutritional profile, can find application in animal nutrition, as reported by Giromini et al. (2021), Lolli et al. (2020) and Serrapica et al. (2019).

However, the characterisation of the functional profile is still at an early stage. As reported by Corino and Rossi (2021), investigating the medical/functional characteristics of plant matrices plays a key role in the feed and livestock sector. Among the most important, the

antioxidant profile is certainly one of the main players involved in protecting welfare and ensuring high animal performance.

Therefore, in the light of the above, the aim of this work was to investigate the functional characteristics, particularly the antioxidant profile, of CAMC, CC and CM during the *in vitro* digestion process, with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. At the same time, total phenolic content (TPC) and angiotensin converting enzyme (ACE) inhibitory activity, parameters actively involved in modulating the antioxidant profile, were investigated. Finally, the peptidomic approach was adopted to identify the main bioactive peptides resulting from the digestive process.

RESULTS

Determination of nutritional profile and digestibility

The nutritional profile is shown in Table 1. As reported in Table 1, CAMC, CC and CM are characterised by an interesting nutritional profile. More specifically, although ashes were highly comparable, this trend was not observed for DM content. In fact, the DM recorded for CAMC (92.08 ± 0.16%) showed statistically significant differences ($p < 0.05$) compared to CC (93.23 ± 0.27%) and CM (93.70 ± 0.19%). In parallel, CAMC (31.42 ± 0.41%) presented a significantly higher crude protein (CP) content ($p < 0.05$) than CC (16.88 ± 0.98%) and CM (16.58 ± 0.84%). The same trend was also confirmed for lipid concentration (7.86 ± 0.19, 6.61 ± 0.09, 0.53 ± 0.05%, for CAMC, CC and CM, respectively). As shown in Table 1, the fibrous fractions reported similar values. More precisely, CAMC showed no difference for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) compared to CM. Although CC had a significantly lower NDF content than other matrices, this was not observed for both ADF (37.00 ± 0.44%) with statistically higher values ($p < 0.05$) than CAMC (32.88 ± 0.53%) and CM (33.76 ± 2.29%), and ADL (13.13 ± 0.65%) with a highly comparable content compared to CAMC and CM (15.50 ± 0.49% and 12.05 ± 0.93%, respectively).

Table 1. Nutritional profile of CAMC, CC and CM. Nutritional profile of camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM) (% W/W on DM basis). Data are presented as mean ± standard error of mean (SEM). (n=3). DM = dry matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin. Different superscript letters in columns indicate statistically significant differences (p<0.05).

Sample	DM	ASHES	CP	FATS	NDF	ADF	ADL
CAMC	92.08±0.16^{a}	4.76±0.43^{a}	31.42±0.41^{a}	7.86±0.19^{a}	67.40±0.04^{a}	32.88±0.53^{a}	15.50±0.49^{a}
CC	93.23±0.27^{b}	4.49±0.48^{a}	16.88±0.98^{b}	6.61±0.09^{b}	50.81±2.67^{b}	37.00±0.44^{b}	13.13±0.65^{a}
CM	93.70±0.19^{b}	6.11±0.25^{a}	16.58±0.84^{b}	0.53±0.05^{c}	67.12±0.55^{a}	33.76±2.29^{a}	12.05±0.93^{a}

At the same time, as can be noted in Figure 1, CAMC, CC and CM are also characterised by an interesting DM/protein digestibility. CAMC (64.08±0.77%) had a significantly higher (*p<0.05*) DM digestibility than CC (49.30±0.96%) and CM (39.14±0.74%), with also significant differences between the latter two. However, this trend was not observed for the protein one. More specifically, in this case, CC (77.24±0.24%) showed significantly higher digestibility (*p<0.05*) than CAMC and CC (68.69±1.83%, 51.24±0.93%, respectively).

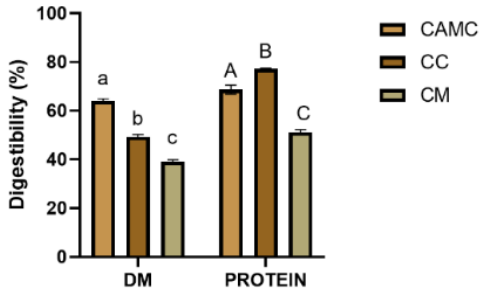

Figure 1. Dry matter (DM) and protein digestibility of camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM). Values in % are presented as mean ± standard error of mean (SEM) (n = 3). Small letters indicate statistically significant differences for DM digestibility, capital letters for protein digestibility (*p<0.05*).

Total phenolic content and antioxidant activity

Figure 2 shows the TPC of CAMC, CC, CM at the end of the oral phase and following filtration (10 kDa). As reported, at the end of the oral phase, the samples had similar values (621.19±88.98 mg TAE/100 g; 304.13±42.43 mg TAE/100 g; 581.64±95.15 mg TAE/100 g, for CAMC, CC and CM, respectively). In parallel, filtration resulted in a significantly higher TPC than that just described, with significant differences (*p<0.05*) between each matrix analysed (1636.67±78.89 mg TAE/100 g; 3357.60±79.82 mg TAE/100 g; 2947.98±8.15 mg TAE/100 g, for CAMC, CC and CM, accordingly).

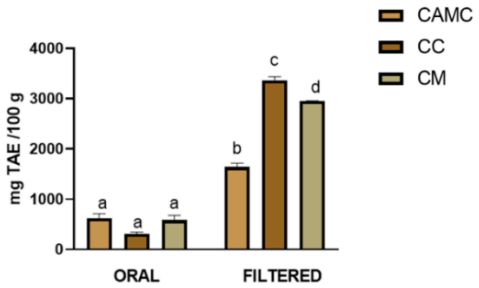

Figure 2. Total Phenolic Content of camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM). Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant different data (*p<0.05*).

At the same time, as shown in Figure 3, CAMC, CC and CM are also characterised by high antioxidant activity.

As can be noted, ABTS and FRAP trends correlated with that of TPC. More specifically, for the ABTS assay, although at the end of the oral phase, CAMC (1354.38±189.30 mg TE/100 g) showed significantly greater antioxidant activity ($p<0.05$) than CC (590.83±154.46 mg TE/100 g) and comparable to CM (764.64±135.32 mg TE/100 g), this was not observed after filtration, with significant ($p<0.05$) lower values (9511.18±315.29 mg TE/100 g) than the similar ones of CC (10451.40±149.17 mg TE/100 g) and CM (10969.80±18.93 mg TE/100 g). Even with the FRAP method, better results were obtained for CC and CM (103.78±12.98 mg $FeSO_4$/100 g; 96.10±8.42 mg $FeSO_4$/100 g, respectively) with significant differences ($p<0.05$) compared to CAMC (44.10±2.54 mg $FeSO_4$/100g) at the end of the oral phase. This trend was also observed following the filtration phase (306.74±5.68 mg $FeSO_4$/100 g; 272.84±11.02 mg $FeSO_4$/100 g; 103.84±3.27 mg $FeSO_4$/100 g, for CC, CM and CAMC, respectively). However, as shown in Figure 3, ORAC presented an opposite trend to that observed for ABTS and FRAP, with significantly higher values ($p<0.05$) for the oral phase (154.49±3.91 mg TE/100 g; 149.09±4.23 mg TE/100 g; 145.79±6.60 mg TE/100 g, for CAMC, CC and CM, respectively) than those observed following filtration (75.99±6.82 mg TE/100 g; 83.96±1.24 mg TE/100g; 83.29±0.90 mg TE/100 g for CAMC, CC and CM, respectively), with no statistically significant difference between the matrices at any stage of the digestive process.

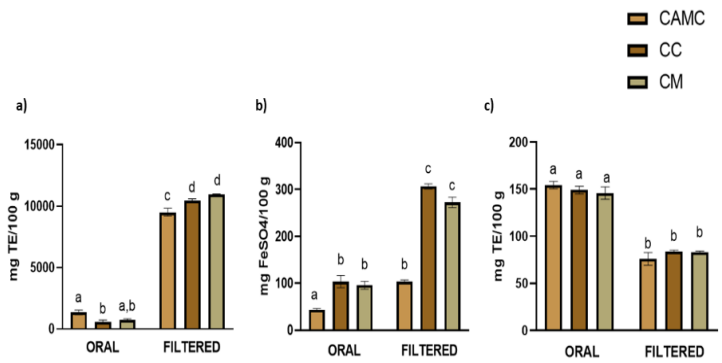

Figure 3. Antioxidant activity of camelina cake (CAMC), cardoon cake (CC) and cardoon meal (CM). Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant different data ($p<0.05$). a) ABTS assay; b) FRAP assay; C) ORAC assay.

ACE inhibitory activity

Interesting results were observed in the ACE inhibition assay. The highest inhibitory effect of ACE occurred in CAMC sample (40.34±11.11%) after *in vitro* digestion, which was

significantly higher ($p<0.05$) compared to the effect observed before digestion. This trend, although not statistically significant, was reported for CC, with an ACE inhibitor rate of 10.88±9.05% following the digestive process. No effect were detected in CM at the end of intestinal phase.

CAMC, CC and CM peptidomic profile

The peptidomic analysis was carried out as schematized in Figure 4A and allowed to identify all the peptides present in CAMC, as well as, those that were exclusively present or were common between CC and CM samples. In particular, 382 peptides were identified in CAMC (Supplementary Table S1), whose 188 are unique peptides and are produced by the proteolysis of 180 proteins. Well in accordance with the lower protein content described above in cardoon, less peptides were identified in CC and CM (6 and 13 respectively), of which 5 and 7 are unique peptides (Supplementary Table S2). A specific comparison identifies 5 peptides common in CC and CM samples (4 are unique peptides) and 1 and 8 exclusively present in CC and CM, respectively (Figure 4B). All these peptides were generated from 6 and 8 proteins in CC and CM (Figure 4C), respectively, of which 5 common to all samples: clpP-like protease, 30S ribosomal protein S3, photosystem II Q(b) protein (D1), NADH-plastoquinone oxidoreductase subunit 2 and the hypothetical chloroplast RF21 protein. In parallel, the search for potential bioactive peptides resulted in the identification of 30 peptides in CAMC, as shown in Table 2 (SATPdb database) and 3 (DFBP database), and one in CM (SATPdb and DFBP databases), as reported in Table 4.

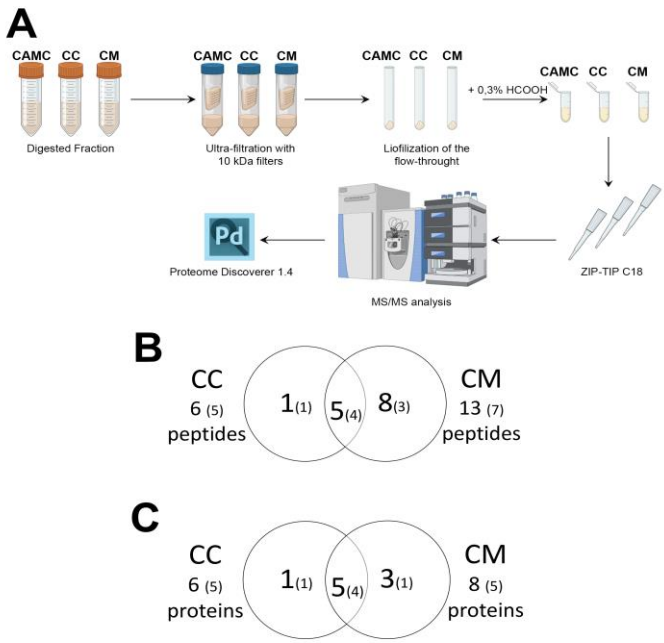

Figure 4. Schematic representation of the shotgun label-free peptidomic analysis. A) Overview of the workflow applied on CAMC, CC and CM specimens. B) Venn diagram of all peptides identified from the comparison CC vs CM. Unique peptides are indicated in brackets. C) Venn diagram of the proteins that generated all peptides from the comparison CC vs CM. Proteins that generated unique peptides are indicated in brackets.

Table 2. List of potentially bioactive peptides found in CAMC specimens in SATPdb database. In bold the sequence stretches corresponding to potential bioactive activity. Peptide sequence, database sequence, GI number, gene name and protein name are indicated for each peptide. Post-translational modifications are highlighted with lowercase letters: m=oxidated methionine, n= deamidated asparagine, q=deamidated glutamine.

	CAMC SATPdb				
Bioactivity	Peptides identified	Database SATPdb	Protein origin	Gene	Protein name
Ace Inhibitors (7)	EFCGGTDTKRVIKPTEDKRFEEmT nTVGMIEHYMnInHWVC	VIKP	1109068111	LOC 104746755	eukaryotic translation initiation factor 3 subunit C
	GAGAGPGLGGGIGPDNTLVFFmH DILGGSNP	GAGP	727457383	LOC 104786832	dirigent protein 10-like
	GQDHVQPSNMDSPAKKqKTSSqG PDVqIDSGEETLRNPSmERPYLC	RPYL	1109077495	LOC 104750981	probable inactive tRNA-specific adenosine deaminase-like protein 3
	GQGGqLLSPYqGSYNqGQGTPLP GQGQE	YQGS	727647923	LOC 104771657	multiple organellar RNA editing factor 4, mitochondrial-like
	IKDLNNYNYTPSYNHYNINNQNMm mNLPYVSGPSTYNAnMI	VSGP	727534450	LOC 104724745	probable WRKY transcription factor 8
	nISDGSKSFLPVDISEESEVSGSDK EDSSWISWFCnLRGNKFLC	SDGS	727423875	LOC 104730545	casein kinase II subunit beta-2-like isoform X1
	QGYVAGSPESSGFHLG	AGSP	727560928	LOC 104734746	protein MEI2-like 4
Antihypertensive (6)	EFCGGTDTKRVIKPTEDKRFEEmT nTVGMIEHYMnInHWVC	VIKP	1109068111	LOC 104746755	eukaryotic translation initiation factor 3 subunitC
	GQDHVQPSNMDSPAKKqKTSSqG PDVqIDSGEETLRNPSmERPYLC	RPYL	1109077495	LOC 104750981	probable inactive tRNA-specific adenosine deaminase-like protein 3
	GQGGqLLSPYqGSYNq GQGTPLPGQGQE	LSPY	727647923	LOC 104771657	multiple organellar RNA editing factor 4, mitochondrial-like
	RQGYVAGSPESSGFHLG	AGSP	727560928	LOC 104734746	protein MEI2-like 4
	mAmQSQMQLPqFPVMNRSAPQNH	AMQS	727446624	LOC 104781817	transcription factor PIF5 isoform X2
	SDQIRLNFLPqMSDYEAqqHLKMKS DYHQQALGYLPENTNKEMMGLNP	LGYL	727588840	LOC 104747487	transcription factor ABORTED MICROSPORES
Anti-cancer (1)	DLLAYERQLAMSKMVGMNPLMHH PHGQHALKHAAmGATGSSQGMYDGGFQNA	HHPHG	727573477	LOC 104740431	oligouridylate-binding protein 1B
Anti Microbial (2)	GLAGqLHMnSnKWKWFSSGDEVLHSGEGPI	KWKW	727605090	LOC 104754999	vacuolar protein sorting-associated protein 41 homolog
	KACCGSGPLRINTCGNRmGPSQS YELCENVTDYLFFDSSHLTEKAHRqI	SYEL	727617266	LOC 104758748	GDSL esterase/lipase 3-like
Anti Viral (1)	KACCGSGPLRINTCGNRmGPSQS YELCENVTDYLFFDSSHLTEKAHRqI	SYEL	727617266	LOC 104758748	GDSL esterase/lipase 3-like

Table 3. **List of potentially bioactive peptides found in CAMC specimens in DFBP database.** In bold the sequence stretches corresponding to potential bioactive activity. Peptide sequence, database sequence, GI number, gene name and protein name are indicated for each peptide. Post-translational modifications are highlighted with lowercase letters: m=oxidated methionine, n= deamidated asparagine, q=deamidated glutamine.

	CAMC DFBP				
Bioactivity	Peptides identified	Database SATPdb	Protein origin	Gene	Protein name
Ace Inhibitors (13)	EFCGGTDTKRVIKPTEDKRFEEmTnTVGMIEHYMnInHWVC	VIKP	1109068111	LOC 104746755	eukaryotic translation initiation factor 3 subunit C
	GAGAGPGLGGGIGPDNTLVFFmHDILGGSNP	GAGP	727457383	LOC 104786832	dirigent protein 10-like
	GQDHVQPSNMDSPAKKqKTSSqGPDVqIDSGEETLRNPSmERPYC	RPYL	1109077495	LOC 104750981	probable inactive tRNA-specific adenosine deaminase-like protein 3
	GQGGqLLSPYqGSYNqGQGTPLPGQGQE	YQGS	727647923	LOC 104771657	multiple organellar RNA editing factor 4, mitochondrial-like
	HqSFSYGQESnEFVGSFGASSSYVAAATIG	AAAT	727528607	LOC 104722956	dof zinc finger protein DOF4.3-like
	nGCGDGGGVTAAATNMQEPSIEDK	AAAT	727470599	LOC 104792737	patatin-like protein 6
	IKDLNNYNYTPSYNHYNINNQNMmmNLPYVSGPSTYNAnMI	VSGP	727534450	LOC 104724745	probable WRKY transcription factor 8
	KTFLEqVEILSERYRPDIAEDREDFDNRPYDPED	RYRP	727556889	LOC 104732697	RNA polymerase I termination factor-like
	LSLCDnFPqGPGTVVEVVSLVLQRICEDLEA	EDLE	1109046314	LOC 104730197	small subunit processome component 20 homolog
	nISDGSKSFLPVDISEESEVSGSDKEDSSWISWFCnLRGNKFLC	SDGS	727423875	LOC 104730545	casein kinase II subunit beta-2-like isoform X1
	RQGYVAGSPESSGFHLG	AGSP	727560928	LOC 104734746	protein MEI2-like 4
	TRESPLTqGSENNmGDSVmNADESV	GSEN	727450232	LOC 104783311	DUF724 domain-containing protein 6-like isoform X2
	VSGMNYYACLSMmSLLIVTPFAIAVEGPqMWTAGWqNAVSQI	LIVT	727432264	LOC 104770334	phosphate/phosphate translocator 2, chloroplastic-like
Anti-hypertensive (2)	GAGAGPGLGGGIGPDNTLVFFmHDILGGSNP	GAGP	727457383	LOC 10478683	dirigent protein 10-like
	GQDHVQPSNMDSPAKKqKTSSqGPDVqIDSGEETLRNPSmERPYC	RPYL	1109077495	LOC 104750981	probable inactive tRNA-specific adenosine deaminase-like protein 3
Anti cancer (1)	SESEEEIRASDDVLAHDEDDDE	DEDDD	727425466	LOC 104737174	B3 domain-containing protein At5g60130-like
Antithrombotic(6)	MGGPmGmGGPMGNIPAVQGLPATGPGGVPPGYFqGAGSDPMQQQQYM	GPGG	727630269	LOC 104764299	neurogenic protein mastermind-like
	NLGGGPAKNGGKGAPGGGGGGGKGGPGGGGENQNQGGGKNGGKnGP	GPGG	727630269	LOC 104764299	neurogenic protein mastermind-like
	nnKPmDDFDSPGPGGGRGSSSPVSKGQGL	GPGG	727551462	LOC 104730762	WPP domain-interacting protein 1-like
	QAVQGLPAmGPGGGGGGGASGGAPPGYFqGqVPGS	GPGG	727563725	LOC 104735970	S-antigen protein-like
	NAVSQLTnmGPPmPQAPRNMGSGGRFSGRGDSGPGHVSSF	GRGDSRGDS	727412897	LOC 104776219	zinc finger CCCH domain-containing protein 36
	TTFmnCLCRnGqIDEALKLLGEMKA	CLCR	1109054035	LOC 104735908	pentatricopeptide repeat-containing protein At5g18475-like
DPP IV inhibitory (9)	HqSFSYGQESnEFVGSFGASSSYVAAATIG	VAAA	727528607	LOC 104722956	dof zinc finger protein DOF4.3-like
	VSGMNYYACLSMmSLLIVTPFAIAVEGPqMWTAGWqNAVSQI	AIAV	727432264	LOC 104770334	glucose-6-phosphate/phosphate translocator 2, chloroplastic-like
	MGGPmGmGGPMGNIPAVQGLPATGPGGVPPGYFqGAGSDPMQQQQYLAAM	GPGG	727630269	LOC 104764299	neurogenic protein mastermind-like

	NLGGGPAKNGGKGAPGGGGGG GKGGPGGGGENQNQGGGKNGG KnGGGP	GPGG	727630269	LOC 104764299	neurogenic protein mastermind-like
	nnKPmDDFDSPGPGGGRGSSSPV SKGQGL	GPGG	727551462	LOC 104730762	WPP domain-interacting protein 1-like
	QAVQGLPAmGPGGGGGGGASG GAPPGYFqGqVPGS	GPGG	727563725	LOC 104735970	S-antigen protein-like
	QMLnAHKnGGGGPGPAGGK	GPAG	727563725	LOC 104735970	S-antigen protein-like
	AKNGPAGGRGGGRGGGNGRGR GGn	GPAG	727516553	LOC 104718584	plasminogen activator inhibitor 1 RNA-binding protein-like
	nPVGGILGSQnPGFVQNSMLPGG	LPGG	1109025921	LOC 104781161	VHS domain-containing protein At3g16270-like
PEP-inhibitory (2)	LAGHAEPVPRPPPVPPQLEE	PPPV	1109086438	LOC 104755904	factor of DNA methylation 1-like
	LPEFnNSYTYLPPVSGQAMmPVD ERPMLYGSNPN	LPPV	727504473	LOC 104712991	ETHYLENE INSENSITIVE 3-like 3 protein

Table 4. **List of potentially bioactive peptides found in CM specimens in SATPdb and DFBP databases**. In bold the sequence stretches corresponding to potential bioactive activity. Peptide sequence, database sequence, GI number, gene name and protein name are indicated for each peptide. Post-translational modifications are highlighted with lowercase letters: m=oxidated methionine, n= deamidated asparagine, q=deamidated glutamine.

CM SATPdb					
Bioactivity	Peptides identified	Database SATPdb	Protein origin	Gene	Protein name
Ace Inhibitors (1)	IQEESQQFLnPnEVVPP ESnEQQR	VVPP	916445796	atp4	ATPase subunit 4 (mitochondrion)
Antihypertensive (1)	IQEESQQFLnPnEVVPP ESnEQQR	VVPP	916445796	atp4	ATPase subunit 4 (mitochondrion)
CM DFBP					
Ace Inhibitors (1)	IQEESQQFLnPnEVVPP ESnEQQR	VVPP	916445796	atp4	ATPase subunit 4 (mitochondrion)

DISCUSSION

The results reported in Table 1 revealed an interesting nutritional profile. The DM content of CAMC, CC and CM partially confirmed what has been reported in the literature by Lolli et al. (2020), Serrapica et al. (2019) Nannucci et al. (2021) and Steppa et al. (2017). It is possible that the differences observed between the same matrices can be attributed to multiple factors, including plant genotype, agrological and meteorological conditions, and harvest type (Vastolo et al., 2022). Furthermore, as reported by Vastolo et al. (2022), great variability between co-products is caused by the processing techniques by which they are obtained. The higher protein content of CAMC is most likely due to the protein value of seeds, the original matrix from which these products are obtained. In fact, as reported earlier, camelina seeds are characterised by a higher protein content than cardoon, which reflects in a greater value in the co-product (Singh et al., 2023; Genovese et al., 2015). In general, oil extraction results in the concentration of the protein fraction (30% to 50%), as demonstrated by House et al. (2010) and Ely and Fike (2022) on hemp-based products. This was confirmed for CAMC, however, for CC and CM this did not occur, suggesting that the cardoon seeds

used to form the co-products in this study were most probably characterised by a lower protein content than that reported by Genovese et al. (2015) (16.7%).

Nevertheless, as shown in Figure 1, CC was characterised by a higher protein digestibility than CM, confirming the results of Serrapica et al. (2019). This observation is of fundamental importance as it shows how different oil extraction methods can alter the chemical-nutritional properties of co-products. In fact, as reported by Arrutia et al. (2020), Ancuța and Sonia (2020), the "cake" is the result of the oil extraction directly from the pressing, while the "meal" is obtained by adding another step in the de-oiling process, generally using an organic solvent and high temperatures (>105ºC), capable of extracting a higher percentage of oil. However, heat treatments, as reported by Teodorowicz et al. (2017), could cause the *Maillard reaction* between amino acids and reducing sugars, which leads not only to a decrease in nutritional value, but also to less digestion by gastric and intestinal enzymes, confirming what was observed in Figure 1. In parallel, CAMC showed an interesting protein digestibility, even if lower than CC. Although this parameter is not adequately described in the literature, it can be assumed that antinutritional factors such as phytic acid, condensed tannins and erucic acids, present in camelina, are able to decrease protein digestibility by altering the activity of digestive enzymes or forming insoluble complexes with proteins (Russo et al., 2012). Interesting results were also reported for lipid content. CM showed significantly higher content than CC. These results are a direct consequence of the extraction technique adopted, confirming what has been reported by Arrutia et al. (2020). In parallel, higher values observed for CAMC are most probably due to a higher lipid concentration in camelina seed than in cardoon seed, as demonstrated by Genovese et al. (2015), Turco et al. (2019), and Singh et al. (2023), the NDF, ADF, and ADL values observed for CAMC and CM showed differences from those reported by Nannucci et al. (2021) and Singh et al. (2023). Again, as highlighted before, genotype, harvest, agrological and environmental conditions and processing techniques may have influenced the total fibre content (Vastolo et al., 2022). In parallel, CC had a fibre profile highly comparable to that observed by Serrapica et al. (2019). More specifically, the authors reported a content of 46.8%, 36.0% and 6.43% for NDF, ADF, and ADL, respectively. Although, as demonstrated by Farinon et al. (2020), adequate fibre consumption may have a functional, mainly prebiotic role at the gastrointestinal level, high levels may affect total digestibility, especially for monogastrics.

Despite this, as shown in Figure 1, CAMC showed an interesting DM digestibility, confirming the value reported by Moloney et al. (1998) (60.0±3.01%). This result is highly comparable to the DM digestibility of soy protein extract, one of the main feed matrices used in the livestock sector, as demonstrated in our previous work (Lanzoni et al., 2023). At the same time, different methods for oil extraction observed differences in DM digestibility between CC and CM, suggesting that the use of high temperatures combined with solvents could affect the nutritional aspect of the food/feed matrix. In light of the above, CAMC, CC, CM are characterised by an interesting nutritional profile and digestibility, although the high level of fibre suggests a greater use for ruminants. Nevertheless, Zumbo et al. (2022) and Buccioni et al. (2020) investigated their use in poultry and pig sector. As reported by the

authors, although no difference in growth performance was found, the acid profile of meat and eggs was improved, suggesting a potential use in monogastric farming as an alternative to soy-protein. Specifically in this study, the results obtained by CAMC and CC were higher than those of CM, identifying cake as a preferred product compared to meal. This data enhances the use of pressed cake as a protein-rich ingredient in animal feed, which is regulated by the Commission Directive 2008/76/EC (European Commission, 2008).

In parallel, as reported in Figure 2, for TPC, no statistically significant difference was observed between the samples at the end of the oral phase. These results are related to the behaviour of phenolic compounds during the digestive process. More specifically, the oral phase, as reported by Hur et al. (2011), due to the short contact period between saliva and the food matrix, does not affect the availability of phenolic compounds in this stage, resulting in no high values in the TPC. The increase in TPC following filtration is related to the changes undergone by phenols during the digestive process. In fact, during the gastric phase, the acidic pH leads to the breaking of protein chains and polysaccharide compounds, mainly fibres, causing the release of phenolic compounds (Goulas and Hadjisolomou, 2019,; Lanzoni et al., 2024). Later, although at the intestinal level these values tend to decrease, as the alkaline pH and the action of pancreatin transform phenols into compounds with different structures and bioactivity (Wojtunik-Kulesza et al., 2020), as reported by Tarko et al. (2013), about 48% of phenolic compounds are digested in the small intestine, and only a small fraction (about 10%) remains bound to the food matrix, explaining the results observed for Figure 2. Considering individual matrices, as reported by Terpinc et al. (2012), CAMC is characterised by the presence of several phenolic compounds (rutin, catechin, quercetin, quercetin-3-O-glucoside, protocatechuic acid, p-hydroxybenzoic acid, ellagic acid, sinapic acid, salicylic acid and 4-vinyl derivatives of hydroxycinnamic acids), found to be equal to or greater than those contained in the seed, suggesting that oil extraction results in their concentration in the cake. However, TPC following *in vitro* are most probably preserved and concentrated during the production of the cake, as previously reported for CAMC. digestion is not properly described in the literature. Despite this, it is presumable that the values observed in Figure 2 are influenced by the presence of non-starch polysaccharides, compounds that can increase intestinal viscosity (a situation also observed in this experiment), thus trapping phenols and preventing their correct detection (Saeed et al., 2021; Singh et al., 2023). At the same time, the cardoon seed, as reported by Piluzza et al. (2020), is also characterised by the high presence of phenolic compounds, which Although, as reported by Juániz et al. (2017), only a few phenolic compounds of CC were observed following *in vitro* digestion, this is not confirmed in this study. Most probably, as reported by Petropoulos et al. (2018), genotypic differences and cultivation conditions may play an important role in the profile of phenolic compounds and could justify the conflicting results of the different studies. Finally, the significant differences observed between CC and CM are most probably due to the high temperatures adopted to obtain CM, able to cause a reduction in TPC, as reported by Ghafoor et al. (2019).

As shown in Figure 3, FRAP and ABTS assays are characterised by the same trend of TPC, suggesting that phenolic compounds are actively involved in the determination of antioxidant activity, confirming what was described by Wojtunik-Kulesza et al. (2020). Again, in addition to the food matrix, it is crucial to consider the conditions of digestion. The limited duration of the oral phase (2 min) results in a brief interaction between the oral enzymes and the food bolus, without major changes in phenolic compounds and consequently in relative antioxidant activity (Hur et al., 2011). However, as reported by Ginsburg et al. (2012), the saliva, but more specifically salivary albumin, mucins and proline-rich proteins, enable the solubilisation of phenols by increasing their availability, digestibility, absorption and antioxidant activity in subsequent digestion steps. This would explain the higher values obtained following filtration at the end of digestion. At the same time, the increase in antioxidant activity, as reported by Wojtunik-Kulesza et al. (202), is the result of the action of the acidic pH at stomach level, which increases the antioxidant power of phenolic compounds by mainly strengthening their ability to donate electrons, the principle of action of the ABTS assay. The latter plays a key role as it explains why higher values were recorded for ABTS than FRAP.

In parallel, as reported by Ngo et al. (2023), one of the major forms of oxidative stress is caused by hydroxyl radicals, as they are capable of interacting involving molecules such as DNA, proteins, lipid membranes and amino acids, consequently causing multiple cellular damages. For this reason, antioxidant activity has also been assayed using the ORAC method, an assay that quantifies compounds capable of breaking peroxyl radical chains (Ketnawa et al., 2022). However, as shown in Figure 3, ORAC reported an opposite trend to that observed for ABTS and FRAP. Although several studies have shown a positive correlation between TPC and ORAC in various fruits and vegetables, a high TPC does not always correspond to a high ORAC (Zhou et al., 2016). In parallel, these discrepancies can also be observed between antioxidant assays, especially when the samples analysed contain a high profile of phenolic compounds and antioxidant molecules with different mechanisms of action, as demonstrated by Zhou et al. (2016). This difference could also occur in different species of the same plant, as reported by Gutiérrez-Grijalva et al. (2017). More precisely, the authors observed how in three different species of oregano (*Hedeoma patens, Lippia graveolens, Lippia palmeri*), although antioxidant activity increased between the oral and gastric phases with ABTS, with the ORAC assay, the trend was the opposite, suggesting how plant matrices are characterised by multiple antioxidant compounds that can operate independently and differently (Gutierrez-Grijalva et al., 2017). Furthermore, as previously reported, during the digestive process, phenolic compounds, but in general antioxidant compounds, undergo profound changes. In this case, as shown in Figure 3, molecules with peroxyl chain-breaking activity appear to remain stable during the oral phase and are degraded at the end of digestion.

In spite of this, CAMC, CC and CM proved to be co-products characterised by a phenolic profile and antioxidant activity comparable or superior to that of soy protein extract (1841.11±23.02 mg TAE/100 g; 2968.49±93.87 mg TE/100 g; 48.14±16.40 mg $FeSO_4$/100

g, for TPC, ABTS and FRAP, respectively) (Lanzoni et al., 2023), suggesting their possible use as functional substitute matrices.

As reported by Ahmad et al. (2017), the study of ACE inhibitory activity in plant matrices plays a key role in the food/feed sector, although this has not yet been fully investigated. No detectable values were reported by the assay for CAMC and CC at the beginning of the oral phase, while increasing level were observed after *in vitro* digestion, confirming the findings of Vermeirssen et al. (2003). The authors reported how the *in vitro* digestive process of plant and animal proteins increased their ACE inhibitory activity. Most probably, this is the result of the action of enzymatic proteolysis during the digestion, which results in the release of bioactive peptides (Singh et al., 2016). At the same time, the CC showed an interesting trend, with values already present at the end of the oral phase (18.03±0.33%), partly confirming what Akillioglu and Karakaya described (Akillioglu and Karaya, 2009). Indeed, the authors reported how ACE inhibitory peptides may be present in intact dietary proteins.

As stated above, the search for potentially bioactive peptides was performed on all CAMC, CC and CM sample datasets using the Structurally Annotated Therapeutic Peptides database SATPdb (Singh et al., 2016) and the Food-Derived Bioactive Peptides database, DFBP (Qin et al., 2022). The main activity is related to ACE inhibitor and antioxidant activity, in accordance with previous reports (Liu et al.,2021; Chaudry et al., 2020). Oxidation is an important contributor to many human and animal diseases, including those cardiovascular that are associated with generation of increased amount of reactive oxygen species (ROS) (Zhang et al., 2022). Therefore, the protection against free radicals induced by oxidation is a key factor in preventing such diseases (Mirzaei et al., 2015). An important role in the regulation of blood pressure is played by ACE, that catalyses the hydrolysis of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II (Khurana et al., 2022). For this reason, ACE inhibitors with antioxidant activity are widely used in hypertension treatments (Zhang et al., 2022). For more than ten years, there has been some evidence that vegetables proteins and their hydrolysates are potentially excellent sources of antioxidants and antihypertensive peptides (Xia et al., 2012). In particular, proteins isolated from flax seed (Udenigwe and Aluko, 2010), rapeseed (Yu et al., 2013), pumpkin oil cake (Vastag et al., 2011) and wheat gluten (Khan et al., 2018) have been reported to show both ACE inhibitory and antioxidant activities. In keeping, the peptidomic results identified three peptides that contain a sequence stretch corresponding to ACE inhibitors activity (Table 2, Table 3), in CAMC. They originated from the dirigent protein 10-like, patatin-like protein 6 and WRKY transcription factor (TF) 8, respectively, whose involvement in oxidative stress response has already been described in literature. The dirigent protein 10-like is involved in the synthesis of lignin that plays pivotal roles in plant defence responses, both against biotic and abiotic stresses, exerting a variety of functions such as antimicrobial, antiviral, antioxidant and anti-cytotoxic (Khan et al., 2018). Patatin is the trivial name given to a family of glycoproteins that make up > 40% of the total soluble protein in potato (*Solanum tuberosum*) tubers that serves as storage protein, which globular structure offers versatile bioactive sites for numerous bifunctionalities, such as antioxidant (Elahi and Mu, 2017). A

previous study conducted by Fu et al. (2020) proved that patatin could capture radical in a concentration-dependent manner exhibited antioxidant activities. The probable WRKY TF 8 belongs to one of the major plant protein superfamilies that plays an important role in the regulation of transcriptional reprogramming associated with plants stress responses (Khoso et al., 2022). Recent studies have shown that WRKY-TF is induces through ROS and contributed to the ROS elimination transformation pathway (El-Esawi et al., 2019). Also plasminogen activator inhibitor 1 RNA-binding protein like generates a peptide involved in oxidative stress response, in addition to dipeptidyl-peptidase IV (DPP IV)-inhibitory activity (Table 2, Table 3). DPP-IV plays an essential role in glucose metabolism. In this context, food-protein-derived DPP-IV inhibitors are promising glycemic regulators which may act by preventing the onset of type 2 diabetes and the related oxidative stress (Fleury et al., 2022).

The peptidomics results on camelina are, therefore, well in accordance with the characterisation of its antioxidant profile described above. The same can be described for CM specimen. In this case the analysis of bioactive peptides identified one peptide, exclusively present in CM samples, containing a sequence stretch corresponding to one peptide with ACE inhibitors and antihypertensive potential activity (Table 4). Altogether, peptidomic results show the presence of several bioactive peptides with ACE inhibitors and antioxidant activities, according with the characterization of the antioxidant profile. These results, combined with the high content of phenolic compounds, perfectly explain the significant antioxidant activity measured in camelina and cardoon samples.

In conclusion, CAMC, CC and CM showed an interesting nutritional profile and high digestibility. In parallel, CAMC, CC and CM revealed a high antioxidant profile, superior to that of soy protein extract, a matrix highly used in the feed industry. The antioxidant capacity and ACE inhibitory activity seem to be positively influenced by presence of phenols and bioactive peptides. Although, the samples analysed showed relevant functional profile, the high fibre content, as well as, the possible presence of anti-nutritional factors and the high variability between co-products batches need to be considered for their application in the feed sector. These insights, coupled with future *in vivo* trials, will be crucial to evaluate the efficacy on the health and performance of farm animals.

METHODS

Nutritional profile determination

The determination of nutritional profile of commercially available CAMC (Panghea s.p.a., Milan, Italy), CC and CM (Novamont s.p.a., Novara, Italy) was performed following the official methods of analysis according to AOAC (2005). Specifically, DM was obtained by drying the samples at 65ºC for 24 h (AOAC method 942.05). Ashes were determined by incinerating the samples at 550ºC for 3 h (AOAC method 942.05). Crude protein content was assessed by the Kjeldahl method (AOAC method 2001.11), and ether extract by ether

extraction with the Soxtec system (DM 21/12/1998). Finally, the fibrous fractions were identified using the protocol developed by Van Sost et al. (1991). The procedure described above was performed at least in triplicate.

In vitro digestion and digestibility

The *in vitro* digestion protocol was performed according to Regmi et al. (2009). Aliquots (1 mL), corresponding to the soluble fraction, were taken at the beginning (oral phase) and at the end of digestive process (following filtration with 10 kDa filters), and immediately frozen at -20ºC to measure TPC and antioxidant activity. At the end of digestion, different replicates were vacuum filtered using a porcelain funnel covered with filter paper (Whatman 54, Florham Park, NJ). The undigested fraction (UF) was subsequently dried overnight at 65ºC and quantified to measure DM digestibility, using the following formula:

$$\text{Digestibility (\% DM)} = (\text{Sample DM} - \text{UF DM}) / \text{sample (DM)} \times 100$$

Subsequently, protein digestibility was also measured. Specifically, UF on the filters was analysed according to the Kjeldahl method for CP content quantification (AOAC method 2001.11). The procedures described above were analysed in biological triplicate (n=3). For each biological replicate, the technical duplicate was considered.

Total phenolic content

Reagents (tannic acid, methanol, Folin-Ciocalteu reagent (FC) and sodium carbonate (Na_2CO_3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The TPC was determined following the protocol of Attard (2013) with minor modifications (Lanzoni et al., 2024). More precisely, tannic acid was prepared in five (1:2) dilutions in distilled water (0 µg/mL to 480 µg/mL). FC reagent was diluted (1:10) with distilled water, while Na_2CO_3 was prepared at a concentration of 1 M. Subsequently, 100 µL of sample was incubated for 20 min in the dark at room temperature (RT) with 500 µL of FC and 400 µL of Na_2CO_3. At the end of the incubation, the samples were read using a spectrophotometer at a wavelength of 630 nm. Each analysis was performed including appropriate solvent blanks. Data were expressed in terms of tannic acid equivalent (mg TAE/100 g). Total phenolic content was analysed for each replicate of the digestion. For each biological replicate, at least the technical duplicate was considered

ABTS assay

Reagents (ABTS, 6-hydroxy-2,5,7,8-tetramethochroman-2-carboxylic acid (Trolox), and potassium persulphate ($K_2S_2O_8$)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The ABTS method was tested following the protocol of Re et al. (1999). Specifically, Trolox (2.5 M) was used as the antioxidant standard and prepared in six dilutions (0 µM/mL to 2000 µM/mL). Subsequently, 88 µL of $K_2S_2O_8$ (2.45 mM) was added to 5 mL of ABTS (7 mM) to form the ABTS radical cation ($ABTS^+$) and incubated for 16 h in the dark at RT

before use. For the quantification of antioxidant activity, the $ABTS^+$ solution was diluted with ethanol to an absorbance value of 0.7±0.02 at 734 nm. Then, 10 µL of sample was added to 1.0 mL of diluted $ABTS^+$ solution (0.7±0.02) and incubated at RT in the dark for 6 min. At the end of the incubation, the samples were read using a spectrophotometer at a wavelength of 734 nm. Each analysis was performed including appropriate solvent blanks. Data were expressed in terms of Trolox equivalent (mg TE/100 g). The ABTS assay was analysed for each replicate of the digestion. For each biological replicate, at least the technical duplicate was considered.

FRAP assay

The reagents (ferric chloride hexahydrate [$FeCl_3$ (6 H_2O)], ferrous sulphate ($FeSO_4$), sodium acetate trihydrate, glacial acetic acid, and 2, 4, 6-tripyridyl-s- triazine (TPTZ)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The FRAP assay was performed following the protocol of Lanzoni et al. (2024). The reagents were constituted as follows. a) Buffer acetate (300 mM, pH 3.6): 2.69 g sodium acetate trihydrate were dissolved in 16 ml glacial acetic acid and diluted to 1 L with distilled water; b) TPTZ solution (10 mM): 31.2 mg of TPTZ were dissolved in 10 mL of HCl (40 mM); c) [$FeCl_3$ (6 H_2O)] solution (20 mM): 0.054 g of [$FeCl_3$ (6 H_2O)] were dissolved in 10 mL of distilled water. While, $FeSO_4$ was used as an antioxidant standard and prepared in six dilutions from 0 µM/L to 1500 µM/L. Subsequently, the working solution (FRAP reagent) was prepared by combining 2.5 mL of TPTZ solution + 2.5 mL of [$FeCl_3$ (6 H_2O)] solution + 25 mL of acetate buffer. For quantification of antioxidant activity, 10 µL of each sample was added to 300 µL of FRAP reagent and incubated at RT for 20 min in the dark and read at 595 nm. Each analysis was performed including appropriate solvent blanks. Data were expressed in mg $FeSO_4$/100 g. FRAP was analysed for each replicate of the digestion. For each biological replicate, at least the technical triplicate was considered.

ORAC assay

Antioxidant capacity was determined using the ORAC kit (ab233473, Abcam, Cambridge, MA, USA). Fluorescein was used as substrate for the reaction, while Trolox (0.2 mM) was used as standard for calibration curve (0 µM/mL to 50 µM/mL). More precisely, 25 µL of digested sample were incubated with 150 µL of fluorescein in a 96-well plate and incubated at 37ºC for 30 min. At the end of the incubation, 25 µL of Free Radical Initiator solution was added to each well to complete the reaction. Fluorescence was measured every 5 min for one h at 37ºC at Ex/Em = 480/520 nm. All determinations were performed at least in triplicate. Each analysis was performed including appropriate solvent blanks. Data were expressed in mg TE/100 g.The ORAC assay was analysed for each replicate of the digestion. For each biological replicate, at least the technical duplicate was considered.

ACE inhibition assay

The ACE inhibition assay was performed as reported by Giromini et al. (2017) with minor modifications (2006), using furanacroloyl-Phe-Glu-Glu (FAPGG) as a synthetic substrate

for the ACE-1 enzyme. More specifically, 150 µL of FAPGG was incubated at 37ºC for one min. At the end of the incubation, 10 µL of each sample and 10 µL of ACE-1 (15 mU) were added to the substrate, thus initiating the kinetic reaction. The kinetic reaction was measured with Synergy KTX at a wavelength of 340 nm for each minute (30 min in total), using captopryl as a positive control. Hydrolysis of FAPGG by the enzyme ACE-1 caused a decrease in absorbance at 340 nm. A 100% ACE-1 activity would indicate complete inhibition of the enzyme.

$$\%\text{ACE-1 inhibition} = ((\text{Absno sample} - \text{Abssample}) / \text{Absno sample}) \times 100$$

Absno sample is the absorbance of the enzyme-substrate mixture in the absence of sample while Abssample is the absorbance of the enzyme-substrate mixture in the presence of the matrices under study. The experiment was performed at least in duplicate.

MS/MS peptidomic methods

To identify endogenous peptides, the permeate samples were analysed by peptidomic strategy by LC-nano ESI tandem mass spectroscopy, using a shotgun-label free approach, without any digestion prior to MS/MS (Giromini et al., 2013; Aletti et al., 2016). Following the digestion process, the samples were initially filtered using paper filters (Whatman 54, Florham Park, NJ), as previously reported (Regmi et al., 2009). Subsequently, the filtered component, corresponding to the digested fraction, was ultra-filtered using centrifuge filters (10 kDa) (Pierce^{TM}, Protein Concentrator PES, Thermo Fisher Scientific, UK), for high weight protein depletion (Tavares et al., 2011). Finally, the obtained fraction was lyophilised prior to mass spectrometric (MS) analysis. More precisely, before MS, freeze-dried supernatant of CAMC, CC, CM, containing peptides and low molecular weight proteins, was dissolved in 0.3% (v/v) formic acid and desalted (Zip-Tip C18, Millipore, Billerica, MA, USA) (Aletti et al., 2016; Tavares et al., 2011). Nano-HPLC coupled to MS/MS analysis was performed on Dionex Ultimate 3000 HPLC system with an EASY-SprayTM 2 µm 15 cm x 150 µm capillary column filled with 2 µm C18 100 Å particles, connected to a Q-Exactive Orbitrap (Thermo Fisher Scientific, San Jose, CA, USA) using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile 20/80, v/v) at a flow rate of 0.300 µL/min. The temperature was set to 35ºC. The acquired raw files were subjected to data analysis using Proteome Discoverer software (version 1.4). The searches were performed against the NCBI reference *Carduus* proteome (updated on November 2023; 867 sequences) for CC and CM samples, and against the NCBI reference *Camelina* proteome (updated on November 2023; 116195 sequences) for CAMC. The enzyme specificity was set as unspecific and methionine oxidation, and asparagine/glutamine deamidation were set as variable modifications (Tavares et al., 2011; Coccetti et al., 2008). Only peptides with high confidence were included for positive identification. All peptides were searched in SATPdb (Singh et al., 2016), a database of structurally annotated therapeutic peptides, and in DFBP, a database of food-derived bioactive peptides (Qin et al., 2022). To consider possible further proteolysis, the search was performed keeping a minimum sequence length

of four amino acids and applying an “IF” nested function to a matrix which compared the sequence of each peptide found with the ones of the database (Micrososft Excel 2023, version 16.80). The mass spectrometry raw data have been deposited in the ProteomeXchangeConsortium (http://proteomecentral.proteomexchange.org) via the PRiDE partner depository with the dataset identifier PXD049333.

Statistical Analysis

Chemical analysis, DM and protein digestibility were analysed using one-way Anova followed by Tukey's multiple comparison test. TPC and antioxidant activities following *in vitro* digestion were analysed by two-way Anova (*Time x Treatment*) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are reported as mean ± standard error of the mean (SEM) of at least three independent experiments. Values are considered statistically significant for a 95% confidence interval (p-value = 0.05).

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Chapter II

Enhancing and challenging microalgal profiles: studies on nutrients and nanoplastic contaminants

This chapter explores the potential and challenges associated with the cultivation of microalgae for food purposes, with a focus on *E. gracilis*, *C. vulgaris* and *H. pluvialis*. In particular, it was studied how supplementation of the culture medium with microelements and amino acids can modulate the nutritional and functional profile of *E. gracilis*, offering new opportunities to improve the quality of this microalgae. In parallel, the risk of contamination by nanoplastics (NPs) and microplastics (MPs) was analysed, an emerging problem in cultivation systems, especially under heterotrophic conditions, where the use of bioreactors may favour the introduction of plastic particles. The effect of these contaminations was evaluated on *C. vulgaris* and *H. pluvialis*, considering single and mixed exposures to polystyrene (PS) and polyethylene (PE) nanoplastics. The analyses included simulated *in vitro* and *ex vivo* digestion processes to study the functional changes of the microalgae, while the properties of the NPs (hydrodynamic size, polydispersity index and surface charge) were characterised by Dynamic Light Scattering (DLS). This integrated approach balances biotechnological optimisation with a critical assessment of food safety risks.

Manuscript VI

Functional characterisation of *Euglena gracilis* following growth medium enrichment

Davide Lanzoni, Raffaella Rebucci, Federica Cheli, Raffaele Cavaliere, Giacomo Ghilardi, Luca Marchetti, Antonio Crotti, Antonella Baldi and Carlotta Giromini.

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Functional characterisation of *Euglena gracilis* following growth medium enrichment

D. Lanzoni[a], R. Rebucci[a], F. Cheli[a,b], R. Cavaliere[c], G. Ghilardi[c], L. Marchetti[a], A. Crotti[a], A. Baldi[a] and C. Giromini[a,c]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy; [c]BGreen Technologies S.r.l., Via Stezzano 87, 24126 Bergamo, Italy.

ABSTRACT: In recent years, microalgae, particularly *Euglena gracilis*, have been candidates for the food/feed industry thanks to their nutritional and functional properties. However, the inclusion of E. gracilis in the diet of livestock animals is little studied, especially regarding its antioxidant activity. Furthermore, microalgae are known for their variability in nutritional quality and functional properties, mainly due to cultivation conditions. For this reason, the aim of the present work was to investigate the nutritional and functional aspect (total phenolic content TPC, Folin-Ciocalteu assay); antioxidant activity (ABTS and FRAP assays)) of E. gracilis, grown in three different media, following chemical extraction (H2O:EtOH) and *ex vivo* digestion method. The microalgae growth media were characterised as follows: EgM (standard medium); ETX (standard medium + aminoacidic-extract); DOE-ETX (ETX + microelements). The results showed an interesting nutritional profile for all the microalgae analysed, although the values were modulated by media nutrients. Although EgM (6.94±0.25 g/L) was characterised by a significant higher growth yield than ETX (5.75±0.14 g/L) and DOE-ETX (4.72±0.17 g/L), results also confirmed by the paramylon content (4.35±0.13; 3.16±0.08; 2.25±0.05 g/L, respectively) ($p<0.05$), it did not show a high functional profile. More specifically, DOE-ETX showed higher values for TPC, ABTS and FRAP, following chemical extraction, in particular 50% and 75% EtOH and *ex vivo* digestion. These results confirmed the potential of *E. gracilis* as a valuable source of functional feed ingredients. Further investigations will be crucial to optimise the formulation of the culture medium to obtain a high yield of algae with improved functional characteristics.

Keywords: Antioxidant activities; *Ex vivo* digestion ; Medium enrichment; Microalgae; Phenols.

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HIGHLIGHTS

- Constant population growth has prompted scientific research to investigate new protein alternatives. *Euglena gracilis* represents a valid candidate.
- The type of growth medium influences the nutritional and functional profile (total phenolic content and antioxidant activity) of *E. gracilis*.
- The addition of microelements to the *E. gracilis* medium resulted in a higher phenolic content and more marked antioxidant activity.

INTRODUCTION

In recent years, microalgae and cyanobacteria, part of the broader group of algae, thanks to the wide range of bioactive compounds with functional properties they possess, have emerged as attractive candidates for research applications and industrial purposes, such as pharmaceutical, cosmetic, industrial, agricultural, and nutritional ones (Alam et al., 2020). In particular, food and feed research is investigating the role of microalgae as valid alternatives to protein sources, given the forecasted growing demand. More specifically, according to the United Nations report, the global population will reach approximately 8.5 billion by 2030, 9.7 billion by 2050 and will peak at 10.4 billion by 2100 (United Nations Department of Economic and Social Affairs, 2022), a phenomenon that is expected to cause increasing difficulties in meeting demand for food by negatively impacting the environmental health (Röös et al., 2017). As the highest demand products will be of animal origin, mainly meat and dairy products, the increase in feed requirements for farm animals is significant (Bellet and Rushton, 2019).

In addition to the most studied and commercialize algae, such as *Chlorella vulgaris* and *Spirulina platensis* (Frazzini et al., 2022), *Euglena gracilis* has drawn a great deal of interest thanks to its potential several types of applications. *E. gracilis* is an ubiquitous and unicellular photosynthetic eukaryotic flagellate of the Discoba supergroup, phylum Euglenozoa, often included in the eukaryotic microalgae's group, commonly found in freshwater habitats (Zoltner and Field, 2022). It is characterized by the absence of cell walls, thus making the nutrients stored inside the cells easily available for absorption (Zeng et al., 2016). Due to this peculiar morphology, the green protozoan can grow under photoautotrophic (using direct sunlight), heterotrophic (using an external carbon source) or mixotrophic (using both kind of sources) conditions (Blum and Buetow, 1963; Šantek et al., 2010).Furthermore, due to its nutritional and functional composition, *E. gracilis* represents a very promising source of food and feed. Specifically, Aemiro et al. (2019) studied the chemical composition of *E. gracilis*, reporting the presence of up to 30.9% dry matter (DM) of crude protein (CP) (containing all 20 amino acids (AAs)), 20% DM of lipid content (rich of polyunsaturated fatty acids (PUFAs)), 5.8% DM of ash and 0.1 % of neutral detergent fibre (NDF). In parallel, several scientific studies have reported that *E. gracilis* is able to produce functional compounds such as pro-vitamin A (β-carotene), vitamin C (ascorbate), vitamin E (α-Tocopherol) and, in particular, paramylon. The latter is a high-molecular-weight polysaccharide (β-1,3-glucan), found exclusively in euglenoids and more specifically in *Euglena* species, whose role is to store energy and carbon (Gissibl et al., 2019). In particular, paramylon is characterized by immunomodulatory properties (Watanabe et al., 2017; Barsanti et al., 2022; Skoy et al., 2012), finding its own market space in the nutraceutical sector, especially after Food and Drug Administration (FDA) released the certification as a food additive in 2017 (GRAS Notice No. 513, FDA, 2014). For this reason, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) approved *E. gracilis* dried whole cells as a novel food (EFSA, 2020).

It has been widely reported that the composition of *E. gracilis* varies depending on several factors, mainly environmental (temperature, CO2, presence of stress-inducing elements, presence of light and aerobic or anaerobic conditions) (Constantopoulos and Bloch, 1967; Wang et al., 2018). Furthermore, as stated in the literature, the nutritional profile of *E. gracilis* is easily modulated by acting directly on the composition of the culture medium, particularly on the sources of nitrogen and carbon, the main nutrients for this microalgae (Regnault et al., 1990; Schwarzhans et al., 2015; Ivusic and Santek, 2015). At the same time, modulation of culture conditions, as a strategy to increase the functional value of *E. gracilis*, is only described to optimise paramylon production, but without considering secondary compounds such as phenols and antioxidant molecules.

Therefore, in light of the above, the aim of this study was to characterise not only the nutritional but also the functional profile of *E. gracilis* in order to assess its potential as an animal feed ingredient. Specifically, the total phenolic content (TPC) and antioxidant activity of *E. gracilis* were analysed by 2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays, after chemical extraction and *ex vivo* digestion (a faster digestion method), in standard medium and after supplementation with AAs extract and microelements.

MATERIAL and METHODS

Materials

Axenic *E. gracilis* (UTEX 753) was purchased from the Culture Collection of Algae at the University of Texas at Austin (UTEX). Cells were maintained in defined organic medium (EgM) at a predetermined temperature (22±2°C) in unshaken flasks (500 mL) in the dark and sub-cultured weekly.

The three media tested, whose formulations were based on preliminary data for the modulation of growth rate and nutritional and functional profile, are listed below.

- EgM medium (1 L): 2 g of trypticasein soy broth, 3 g of yeast extract, 0.01 g of calcium chloride ($CaCl_2$), 20 g of glucose, 0.1 mg of vitamin B1, and 0.05 mg of vitamin B12.
- Aminoacidic-extract (ETX) medium (1 L): 5 g aminoacidic extract (ETX, characterised by the presence of all AAs), 0.01 g of $CaCl_2$, 20 g of glucose, 0.1 mg of vitamin B1, and 0.05 mg of vitamin B12.
- DOE-ETX medium (1 L): same constituent of ETX, with the addition of 0.3 g of magnesium sulphate ($MgSO_4$), 1.5 g of potassium-dihydrogen-phosphate (KH_2PO_4), 0.05 g of tetrasodium-ethylenediaminetetraacetic acid (Na_4EDTA), and 0.45 mg of microelement mix consisting of 10% of iron (Fe), 5% of cupper (Cu), 5% of manganese (Mn), 1% of zinc (Zn), 0.5% of boron (B), and 0.5% of molybdenum (Mo).

All media were sterilised at 121°C for 20 min and then cooled before inoculation. Vitamins and glucose were sterilised separately and added before inoculation to avoid degradation or unwanted reactions.

Methods

Growth yield and paramylon content

The growth period lasted 7 days. Every day for one week, 4 ml of each sample were taken under sterile conditions to monitor growth yield and paramylon content (n=3). More specifically, the cell biomass was measured gravimetrically, whereby 2 ml of the sample were centrifuged, washed with distilled water (to remove salts and impurities), dried in an oven overnight, and finally measured with an analytical balance. Paramylon was quantified following the method of Barsanti et al. (2001). Specifically, 2 mL of the sample were centrifuged and resuspended in a solution containing 1% (w/v) of Sodium Dodecyl Sulphate (SDS) and 5% (w/v) of disodium-EDTA (Na_2EDTA) and then incubated at 37°C for 30 min. The resulting paramylon granules were recovered by centrifugation (10 min at 1000 g). After centrifugation, treatment with SDS-NA_2EDTA was repeated and the paramylon was washed (n=2) with hot distilled water (70°C). After the second washing, the granules were dried overnight at 60°C for total weight determination.

Chemical Analysis

The chemical analysis was performed following the Official Methods of Analysis according to AOAC (2005). In particular, DM was obtained by drying the samples in a forced-air oven at 65°C for 24h (AOAC method 942.05). Ashes were determined by placing the samples in a muffle at 550°C for 3h (AOAC method 942.05). CP content was evaluated by the Kjeldahl method (AOAC method 2001.11), while ethereal extract (EE) by ether extraction in Soxtec system (DM 21/12/1998). Finally, crude fibre (CF) was determined following AOACs (2009) (method Ba 6a-05).

Chemical Extractions

Different solvent concentrations of water:ethanol (H_2O:EtOH) were prepared as follows: 0% EtOH; 25% EtOH; 50% EtOH; 75% EtOH; 100% EtOH. Subsequently, the samples were extracted for three times (n=3), following the protocol of Brighenti et al. (2017) with minor adaptations. More precisely, (0.150±0.05 g) of samples, previously ground (diameter of 1 mm, rotor mill Retsch Mod. zm 200, Hann, Germany), were combined with 5 ml of solvent (H_2O:EtOH) and incubated for 1 h, at room temperature (RT), in the dark and under shaking conditions. At the end of the incubation, the samples were centrifuged at 4000 rpm for 5 min. The supernatant was removed and stored at 4°C, while the residual compound was used to repeat the extraction process two more times. Finally, the extracts obtained (15 ml for each sample) were stored at -20°C until further analyses (TPC, ABTS and FRAP).

Ex vivo digestion

Gastric and intestinal fluids were collected at the slaughterhouse from pigs (n=20) aged 50-110 days. After collection, the fluids were centrifuged at 4000 rpm for 10 minutes to remove undigested component (particulate fraction) and used to form gastric and intestinal pools to reduce variability. Gastric and intestinal fluids were frozen at -20°C for up to 48 h before digestion. Subsequently, pH and enzyme activity were checked to match the gastric and intestinal phase, and corrected if necessary. For the digestive process, 5 mL of gastric fluids were added to the previously ground and weighed samples (0.500±0.05 g) and incubated at 39°C, for 2 h, under agitation. At the end of the first incubation, 5 mL of intestinal fluids were added to the samples and incubated under the same conditions. Different aliquots were taken at each digestive phase (beginning of gastric phase (0h), end of gastric phase (2h), end of intestinal phase (4h)), frozen at -20°C and used to monitor TPC and antioxidant activities (ABTS and FRAP) trends during *ex vivo* digestion. The digestive process was replicated three times by taking two technical replicates for each digestion phase.

Total Phenolic Content

For TPC, the protocol of Attard (2013) was used with minor adaptations as reported by Lanzoni et al. (2023). Values were expressed in terms of tannic acid equivalent (mg TAE/100 g of dried algal material).

ABTS Assay

The antioxidant activity was assessed using the ABTS method according to the protocol of Re et al. (1999) with minor adaptation as reported by Lanzoni et al. (2023). Values were expressed in terms of Trolox equivalent (mg TE/100 g of dried algal material).

FRAP assay

The FRAP assay was performed following the protocol of Abdelaleem and Elbassiony (2021), with minor modifications (Lanzoni et al., 2023), with the only exception that ferrous sulphate ($FeSO_4$) was used as the standard for monitoring antioxidant activity. Values were expressed as mg $FeSO_4$/100 g of dried algal material.

Statistical Analysis

The chemical analysis, growth yield, paramylon content and TPC, ABTS, FRAP data of the chemical extracts were analysed by one-way Anova followed by Tukey's multiple comparison test, while TPC, ABTS and FRAP data of *ex vivo* digestion were analysed by two-way Anova (*Time x Treatment*) followed by Tukey's multiple comparison test, using GraphPad Prism 9 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All data are expressed as means ± standard error of the mean (SEM) of at least three independent experiments (biological replicates). Values are considered statistically significant for a 95% confidence interval (*p-value* = 0.05).

RESULTS and DISCUSSION

Growth yield and paramylon content

In Figure 1, the growth yield and paramylon content values, measured after one week (7 days), are shown.

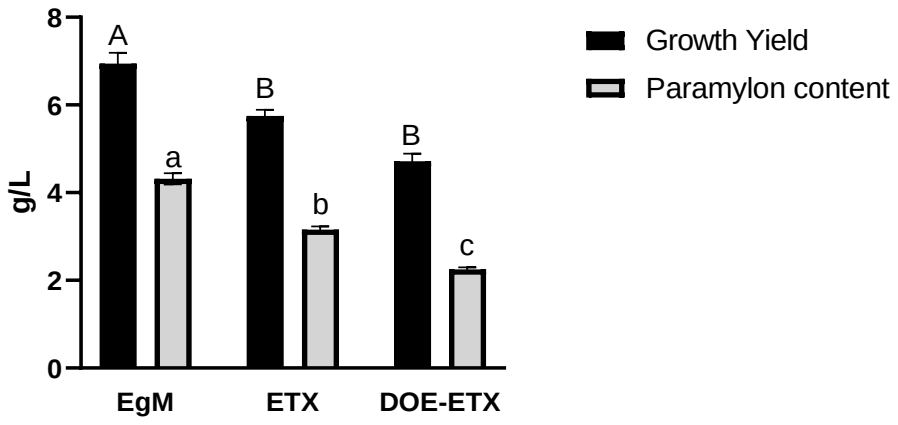

Figure 1. Growth yield and paramylon content in EgM, ETX and DOET-ETX after one week (7 days). Values are expressed in g/L. Capital letters indicate statistically significant differences in mass growth, small letters in paramylon content ($p < 0.05$).

As observed in Figure 1, EgM showed a higher growth yield (6.94±0.25 g/L), with statistically significant differences ($p<0.05$) compared to ETX (5.75±0.14 g/L), and DOE-ETX (4.72±0.17 g/L). The same trend was observed for paramylon content, with statistically significant differences ($p<0.05$) between EgM (4.35±0.13 g/L), ETX (3.16±0.08 g/L), and DOE-ETX (2.25±0.05 g/L).

As reported in the literature, the growth rates of *E. gracilis* are strongly influenced by the components in the culture medium (Schwarzhans et al., 2015). Optimisation of medium composition is highly important in any bioprocess. It must be aimed at reducing time and cost, thereby increasing the recovery of the desired product. In particular, the media to be optimised must support the growth of targeted cells or the production of specific metabolites, while maintaining cell viability (Ivusic and Santek, 2015). It is known that in order to proliferate, *E. gracilis* requires the supply of vitamins such as B1 and B12, a source of carbon and nitrogen (Oda et al., 1982; Ivusic and Santek, 2015). Vitamins B1 and B12, present in all the media conditions studied, play a key role in growth yield. The former, also known as thiamine, although not universally required among *Euglena* species, is essential in *E. gracilis* for its growth. More specifically, as reported by Cook (1968), the addition of at least 30 nM of vitamin B1 is required to support maximum growth. In parallel, *E. gracilis* shows a high requirement for vitamin B12. As demonstrated by Watanabe et al. (2017), *E. gracilis* requires at least 22.000 molecules of vitamin B12 per cell to ensure normal growth. In particular, *Euglena* has the ability to take up and accumulate this vitamin in an exogenous form due to the presence of numerous non-enzymatic proteins in the cytosolic, mitochondrial, chloroplast, and microsomal fractions that can bind and internalise it (Watanabe et al., 2017).

The carbon source in the present study is provided by the addition of glucose in all media (20 g/L). As reported by Ivusic and Santek (2015), the addition of 20 g/L maximised growth and paramylon content in *E. gracilis*. As demonstrated by the authors, glucose as well as fructose are the most efficient carbon sources under heterotrophic culture conditions, as they are easily metabolisable by the cell.

The differences found in the growth yield of the samples under study are due, most probably, to the type of nitrogen source, which differed in the microalgae tested. In EgM, the nitrogen source is yeast extract (3 g/L), which is widely used in the literature (Bhattad et al., 2021). More specifically, Bhattad et al. (2021) demonstrated how the addition of yeas extract (5 g/L) stimulated the growth yield, reaching biomass values of 6.4 g/L (Bhattad et al., 2021), which are highly comparable with those obtained in the present study (6.9 g/L). The microalgae ETX and DOE-ETX, on the other hand, were grown in media enriched with an AAs extract (containing all AAs) (5g/L). As reported in the literature by Oda et al. (1982), not all AAs are required for growth stimulation. More precisely, as claimed by the authors, AAs such as glutamine, asparagine, alanine and serine are only good sources of nitrogen when glucose is present in the medium as a carbon source. Branched-chain AAs, glycine, threonine, and proline are only utilised by cells in the presence of light, while basic, aromatic, and sulphur-containing AAs are unable to stimulate the growth of *E. gracilis* (Oda et al., 1982). Specifically, in the study conducted by Oda et al. (1982), AAs such as methionine, cysteine, threonine, leucine, and phenylalanine proved to be growth inhibitors, most likely due to their competition for cellular transport systems. So it is possible that all the AAs in the extract did not allow differences in cell growth and proliferation to be observed.

Finally, although microelements such as iron, copper, manganese, zinc as well as KH_2PO_4 are known to promote growth in *E. gracilis* (Hilt et al, 1987; Marcenko, 1972), no statistically significant difference on growth yield was observed between ETX and DOE-ETX, as shown in Figure 1. As reported in the literature, it is complex to identify the exact concentration of these compounds in the culture medium, due to their toxicity at a high inclusion level that would arrest their growth and affect cell morphology (Chen et al., 2019).

As previously reported *E. gracilis* is known to produce high amounts of paramylon, β-1,3-glucan, which is characterised by high functional and immunomodulatory properties (Bhattad et al., 2021). This ability is also typical of yeasts such as *Saccharomyces cerevisiae*. But whereas the latter is capable of producing paramylon up to 15% of its dry weight, *E. gracilis* reaches the values of 20-75 % (Bhattad et al., 2021). This was confirmed in our study, where EgM, ETX and DOE-ETX produced 62.7%, 54.9% and 47.7% of their final weight, respectively. These differences are in line with those found with the growth yield, again underlining the importance of the culture medium also in the final production of paramylon.

Chemical analysis

The nutritional profile of EgM, ETX and DOE-ETX is shown in Table 1.

Table 1. Chemical composition of EgM, ETX and DOE-ETX (% w/w on DM basis). Data are presented as mean ± standard error of mean (SEM). (n = 3). DM = dry matter; CP = crude protein; EE = ether extract; CF=Crude Fibre. Different superscript letters in columns indicate statistically significant differences ($p<0.05$).

Sample	DM	ASHES	CP	EE	CF
EgM	98.24 ± 0.04^{a}	1.71 ± 0.01^{a}	15.60 ± 0.30^{a}	30.37 ± 0.27^{a}	6.17 ± 1.33^{a}
ETX	$95.71\pm0.17^{a,b}$	2.14 ± 0.19^{a}	18.61 ± 0.30^{b}	15.16 ± 0.07^{b}	6.95 ± 0.58^{a}
DOE-ETX	94.52 ± 0.05^{b}	3.63 ± 0.07^{b}	25.11 ± 0.35^{c}	11.26 ± 0.06^{c}	6.18 ± 0.97^{a}

As reported, *E. gracilis* is characterised by a high nutritional profile.

DM recorded is in line with studies reported in the literature (Aemiro et al., 2019). In fact, as demonstrated by the authors, DM in *E. gracilis* is around 96%, a value that is a function of multiple factors, including the characteristics of the culture medium. More precisely, EgM (98.24±0.04%) shows statistically significant differences ($p<0.05$) compared to ETX (95.71±0.17%) and DOE-ETX (94.52±0.05%), the same trend in the growth yield recorded and shown in Figure 1.

The opposite trend was observed for the ashes content. As reported by Aemiro et al. (2019), their value is variable and can reach 3.5%, confirming what we observed for DOE-ETX (3.63±0.07%). The latter shows statistically significant differences to ETX (1.4±0.19%) and EgM (1.71±0.01%). This difference is most likely due to the addition of microelements in the DOE-ETX medium, which led to the uptake by the *E. gracilis*, appearing in the total ash content. However, the nutritional profile of a food/feed matrix is primarily a function of protein, fat and fibre content.

The protein content of *E. gracilis*, as reported in the literature, can reach up to 30.9% (Aemiro et al., 2019). In fact, microalgae can accumulate large amounts of protein at the intracellular level, characterised both by the presence of all 20 proteinogenic AAs and a high digestibility as shown by *in vitro* and *in vivo* studies, thus representing an interesting alternative to more traditional sources of dietary protein such as meat and fish (Ritala et al., 2017; Gissibl et al., 2019). However, as previously reported, cultivation methods can affect the total protein content, especially when comparing photoautotrophic, heterotrophic and mixotrophic growth conditions, with maximum levels for microalgae growing under heterotrophic conditions (Gissibl et al., 2019). These differences are also observed depending on the type of nitrogen that is added to the growth medium. As can be observed in Table 1, DOE-ETX (25.11±0.35%) showed statistically significant differences compared to ETX (18.61±0.30%) and EgM (15.60±0.30%). These differences are most likely due to both the source of nitrogen (increasing trends were also observed for ETX compared to EgM, although not statistically significant) and the addition of microelements in the culture medium. In the first case, as shown by Oda et al. (1982), AAs, added to the culture medium,

are easily metabolised by cells and consequently used for protein synthesis. In the second case, as reported in the literature, microelements play an important role in the protein synthesis process of plants (Kozera et al., 2013). More specifically, the application of zinc, copper, molybdenum, manganese, and boron (all microelements present in the DOE-ETX culture medium) resulted in an increase in the total protein content in bean. These microelements are essential parts of living tissue and perform many specific essential functions as components of many enzymes that control the metabolic processes of organisms. Among these, manganese is not only responsible for increasing the intensity of photosynthesis, but also stimulates nitrogen assimilation and protein biosynthesis; a function similar to that of boron (Kozera et al., 2013; Cakmak 2000). However, regardless of the cultivation method, *E. gracilis* would be a viable alternative for the feed industry due to the high protein content outlined above.

At the same time, *E. gracilis* is characterised by a high lipid content. Lipids play a key role in microalgae cells as they ensure their survival during night periods and provide energy for vital biological processes such as cell division, in particular DNA replication, and nuclear division (Kottuparambil et al., 2019). The lipid content of *E. gracilis* is characterised by the presence of 13 different types of fatty acids (50% unsaturated fatty acids), the production of which increases under photosynthetic, heterotrophic and anaerobic conditions (Kottuparambil et al., 2019). As reported in the literature, the lipid content can exceed 20% of dry weight (Kottuparambil et al., 2019), confirming our data. More precisely, EgM (30.37±0.27%) showed a higher lipid content, with statistically significant differences, than ETX (15.16±0.07%) and DOE-ETX (11.26±0.06%). The modulation of lipids under heterotrophic culture conditions has led to conflicting results in the literature. While Kottuparambil et al. (2019) reported that lipid production is only a function of the type and concentration of the carbon source and independent of the nitrogen source, Jung et al. (2021) observed how the latter influenced the total lipid content in *E. gracilis*. The data obtained in this work would corroborate the findings of Jung et al. (2021). More specifically, the authors observed that lipid production was highest in the groups treated with the highest nitrogen concentrations compared to the microalgae grown under starvation conditions. For this reason, as the samples in this study were grown under the same carbon source, it is very likely that the lipid content of EgM, ETX and DOE-ETX was also modulated in this case by the nitrogen source, and that the addition of the amino acid extract, although it increased the total protein value, was in this case a limiting factor, a result previously confirmed in the growth yield and total paramylon production. These results would confirm the yeast extract as an important source of nitrogen in *E. gracilis*, previously reported. Finally, the addition of microelements in DOE-ETX resulted in a further reduction in lipid content. As noted by Abou-Shanab et al. (2012), the addition of zinc, cobalt, copper and manganese did not allow dose-response effects in lipid production to be observed in *Micractinium pusillum*, a freshwater alga. More precisely, the enrichment of culture medium observed a decrease in their total production, underlining the need for further investigation.

With regard to crude fibre content, no differences were observed between the EgM (6.17±1.33%), ETX (6.95±0.58%) and DOE-ETX (6.18±0.97%) groups, suggesting that differences in culture media in this case did not affect the final total content. In the literature, few studies focus on the determination of fibre in *E. gracilis*, being a negligible component of its nutritional profile. In fact, as reported in the literature, the maximum value observable in crude fibre content can reach a maximum of 10-11% (Metsoviti et al., 2019), confirming the data we obtained.

In light of the above*, E. gracilis* represents a food/feed matrix characterised by a high nutritional profile, especially in terms of protein and lipid, also confirmed by *in vivo* trials conducted on broilers (Choi et al., 2004; Pieniazekl et al., 2016). However, the variability in their yields, which can be observed following modulation of cultivation methods, requires further study and investigation in order to delineate the best characteristics of culture media that guarantee high nutritional profiles.

Total phenolic content of chemical extractions

Figure 2 shows the TPC of EgM, ETX and DOE-ETX following chemical extractions (H2O:EtOH).

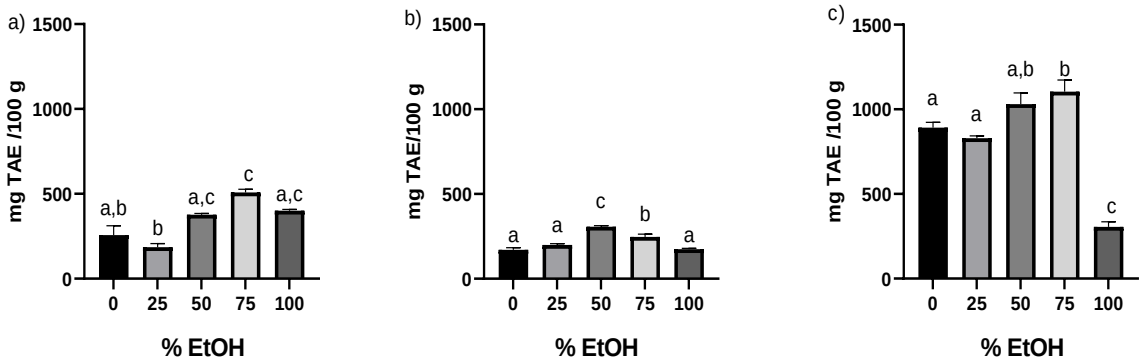

Figure 5. Total phenolic content of chemical extractions. a) EgM. b) ETX. C) DOE-ETX. TAE: Tannic Acid Equivalent. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant different data ($p < 0.05$).

As can be seen in Figure 2, the highest TPC in each of the microalgae tested was obtained following chemical extractions with 50% EtOH and 75% EtOH. More specifically, although ETX algae showed a higher TPC following 50% EtOH (307.11±5.40 mg TAE/100g) with statistically significant differences compared to the other concentrations tested ($p<0.05$), EgM and DOE-ETX showed a better trend with 75% EtOH (507.54±19.68 mg TAE/100g; 1104.40±68.18 mg TAE/100g, respectively), not statistically significant compared to extraction with equal concentrations of H_2O and EtOH (377.02±6.77 mg TAE/100g; 1030.33±66.43 mg TAE/100g, respectively). These results are confirmed in the study conducted by Čagalj et al. (2021). More specifically, the authors demonstrated how the 50%

EtOH extraction tested on *Padina pavonica* (brown alga) resulted in a higher TPC. This occurs due to the *'like dissolves like'* principle where water dissolves polar compounds while ethanol, an organic solvent, dissolves less polar ones (Lim et al., 2019). This result is also confirmed by Tierney et al. (2013). As reported by the authors, the best phenolic extraction in different macroalgae was found in aqueous organic mixtures. However, although the results obtained following extraction with 0% EtOH were in line with those observed by Čagalj et al. (2021), higher concentrations of 50% EtOH reported different result. More specifically, the authors reported how extraction with 70% EtOH resulted in a decrease in TPC, a trend different from what was observed in this study. Phenols, are secondary metabolites produced by plants as defensive weapons against abiotic and biotic stresses. They are therefore recognised for their intrinsic antioxidant effect that protects cells against oxidative stress (Sorrentino, 2021). For this reason, most studies in the literature characterise phenols following heavy metal contamination to see modulation in TPC. However, few studies characterise phenolic compounds under normal conditions (Bernard and Gueguen, 2022). As reported by Bernard and Gueguen, phenols production is stimulated by the carbon source. In particular, glucose is not only used by cells to produce energy, but also for the production of biological compounds including phenolic compounds via the phosphoenolpyruvate pathway (Bernard and Gueguen, 2022). However, in our study, glucose represents the common carbon source for all treated groups. For this reason, it is conceivable how the microelements added in the medium of DOE-ETX resulted in high TPC values compared to EgM and ETX. No TPC values are reported in the literature following the addition of these microelements in the culture medium of *E. gracilis.* However, Klimek-Szcykuwicz et al. (2019) demonstrated how the addition of copper, iron and zinc stimulated the production of phenolic compounds in *Nasturtium officinale* R. Br., an aquatic or semi-aquatic plant. As reported, besides not observing a dose-dependent effect, the concentrations tested were higher than those added in DOE-ETX. For this reason, although these data suggest an involvement of these microelements in the stimulation of phenolic compounds, they require further investigation in order to better characterise this phenomenon.

At the same time, the involvement of microelements in the production of phenolic compounds is reported by Sampaio et al. (2011). The authors demonstrated how copper, iron, manganese and zinc stimulate phenol production in the leaves of *Lafoensia pacari*, a plant recognised for its antipyretic and anti-inflammatory properties (Sampaio et al., 2011). The relationship between these microelements and phenol production can be explained by several factors. Firstly, these microelements, particularly iron, represent nutrients associated with the process of photosynthesis, with the synthesis of phenolic compounds, such as simple phenolic acids (Sanpaio et., 2011). Furthermore, as reported by KovaCik and Klejdus (2008), copper is directly involved in phenol production by acting as an activator of the phenylalanine ammonia-lyase (PAL) pathway, an important enzyme involved in the biogenesis of phenolic compounds. A further explanation for the accumulation of phenols may be associated with the copper tolerance mechanism, as copper is a catalyst for redox reactions that can lead to the production of damaging free radicals. Consequently, the

increase in TPC could have two meanings: decreasing the concentration of free copper through its reaction with phenols and reducing the damaging effects of the free radicals formed, thanks to the antioxidant action of phenolic compounds (Sanpaio et al. 2011). Finally, manganese and zinc are involved in phenols synthesis and conversion processes as enzyme cofactors (metalloproteins) (Sanpaio et al., 2011).

Antioxidant activity of chemical extractions

The next step was to assess the antioxidant activity of each microalgae by ABTS and FRAP assay. In Figure 3, only the values for DOE-ETX are shown, having obtained for EgM and ETX non-detectable values in both assays, in almost all the concentrations tested.

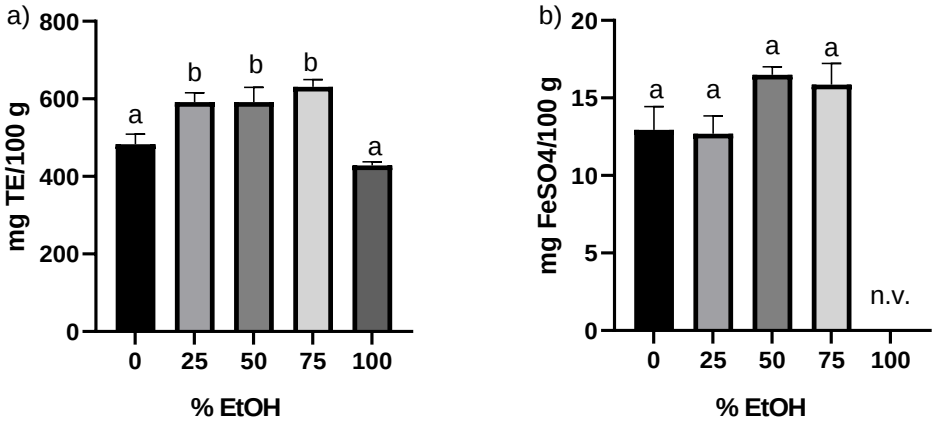

Figure 3. Antioxidant activity of DOE-ETX. a) ABTS assay. b) FRAP assay. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant different data ($p < 0.05$). n.v.:not valuable.

More specifically, EgM and ETX showed no detectable values for FRAP in all concentrations tested.

For the ABTS assay, the only positive value for EgM was recorded following 75% EtOH extraction (21.50±2.32 mg TE/100g), whereas for ETX following 50% EtOH (17.92±0.42 mg TE/100g) and 75% EtOH (35.94±5.04 mg TE/100g) extraction, with statistically significant differences. This trend was also confirmed for DOE-ETX, although showing no significance between the two concentrations, confirming the results obtained in TPC content. In general, it was observed that the values recorded by the ABTS assay are higher than those of the FRAP assay.

This disparity in antioxidant activities at different H_2O and EtOH concentrations indicates that the type and amount of recoverable antioxidant compounds in *E. gracilis* are influenced by EtOH concentrations, as confirmed by Lim et al. (2019). In fact, as can be seen in Figure 3, varying the polarity of the solvent from very polar (0% EtOH) to less polar (100% EtOH), also varies the solvent's ability to dissolve selected groups of antioxidant molecules by modifying their activity (Abbasi et al., 2015; Turkmen et al., 2006). This observation is

consistent with the results observed in this study, in which the antioxidant capacities of the extracts were sensitive to the polarity of the solvent, which may also be related to TPC.

As previously reported, the values recorded using the ABTS assay are much higher than those observed using the FRAP method. As reported in the literature, *E. gracilis* is characterised by the presence of compounds characterised more by the antioxidant action of free radical scavenging rather than the metal chelation action, the principle of action of the ABTS and FRAP assays, respectively (Gissibl et al., 2019).

Of these, vitamins E and A are the most important. Vitamin E includes several isoforms of tocopherol (α, β, γ, δ). These isoforms in addition to being powerful antioxidants, are a very important source in *E. gracilis*. More specifically, although the α-tocopherol is considered to be the most important for animal physiology, γ-isoform is the main consumed in countries where the vegetable oils consumed are predominantly derived from soybean and maize, leading to an insufficient intake of α-tocopherol. However, although *E. gracilis* possesses a plant-like α-tocopherol pathway, it almost exclusively produces the α-tocopherol isoform, thanks to the enzyme γ-tocopherol methyltransferase that converts γ-tocopherol to α-tocopherol using S-adenosyl methionine (Gissibl et al., 2019).

In parallel, vitamin A plays a key role in the antioxidant action of *E. gracilis*. Vitamin A comprises a group of compounds such as retinol, retinoic acid and various retinoic esters (Gissibl et al., 2019). It is only found in products of animal origin and human supply is currently supplemented with provitamin A of plant origin (β-carotene). Like other photosynthetic microorganisms, *E. gracilis* produces β-carotene as a protective pigment to avoid photo-oxidative damage to chloroplasts, which is why beta-carotene titres may be higher under phototrophic conditions. However, as shown in the literature (Gissibl et al., 2019), β-carotene levels in *E. gracilis* can be easily increased by changing cultivation conditions.

However, although the values reported for FRAP are lower than those observed for ABTS, they are, especially following 50% and 75% EtOH extractions, comparable to other matrices widely used in the feed industry due to their high nutritional and functional profile, such as flaxseed and soya protein extract, as reported by Lanzoni et al. (2023). Most likely, microelements also seem to be involved in this case. Indeed, as explained by Jin et al. (2007), phenolic compounds can complex with Fe ($Fe3^{+}$) and be transported to other tissues, facilitating the mobilisation of this mineral between different tissue types and participating in the reduction of $Fe3^{+}$ to $Fe2^{+}$, the principle of action of the FRAP assay.

Total phenolic content and antioxidant activity of DOE-ETX ex vivo digested

In light of the interesting results obtained with DOE-ETX, it was decided to test the TPC and antioxidant activity following the *ex vivo* digestion process, as show in Figure 4.

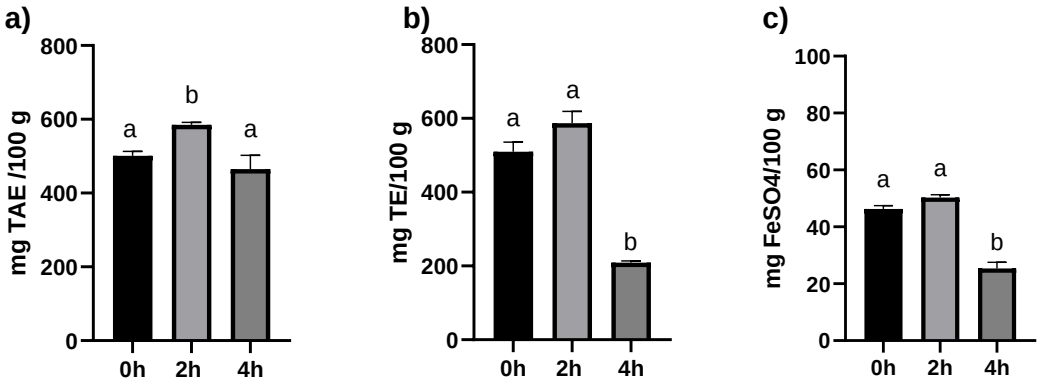

Figure 4. Total Phenolic Content and Antioxidant activity (ABTS and FRAP) of *ex vivo* digestion of DOE-ETX. TAE: Tannic Acid Equivalent; TE: Trolox Equivalent. Data are presented as mean ± standard error of mean (SEM), (n = 3). Different superscript letters in columns indicate significant different data ($p < 0.05$). (a) Total Phenolic content; (b) ABTS assay; (c) FRAP assay. 0h: start of gastric phase, 2h: end of gastric phase, 4h end of intestinal phase.

The TPC trend shows a peak at the end of the gastric phase (584.98±6.77 mg TAE/100g) with statistically significant differences to the other digestion phases (beginning of the gastric phase (0h) (501.01±12.05 mg TAE/100g) and end of the intestinal phase (4h) (465.24±37.36 mg TAE/100g). This trend is confirmed in the literature on different matrices such as hemp-based products, bamboo leaves, Butia and Carob fruits, digested with *in vitro* digestion protocols (Lanzoni et al., 2023; Ma et al., 2020; Goulas and Hadjisolomou, 2019). The peak observed at the end of the gastric phase, as argued in the literature, is due to the low pH value (2±0.05), which allows the release of phenols following the breaking of bonds within the matrices, including proteins and polysaccharides (Goulas and Hadjisolomou, 2019). Subsequently, as anticipated, a decrease with statistically significant differences is observed at the end of the intestinal phase. This result, as shown by Lanzoni et al. (2023) and Friedman and Jurgens (2000), could be a direct consequence of the instability of phenolic compounds at high pH values (6.8±0.05).

The antioxidant activity, assayed by the ABTS and FRAP methods, shows the same trend as that observed for TPC. As reported in the literature, the peak in the gastric phase (586.86±32.10 mg TE/100 g for the ABTS and 50.30±1.02 mg FeSO4/100 g for the FRAP assay) is probably due to the release of phenolic compounds. Subsequently, a significant decrease is observe following intestinal digestion (208.78±4.67 mg TE/100 g for ABTS and 25.38±2.17 mg FeSO4/100 g for the FRAP assay). As discussed by Ma et al. (2020), this result is due to the degradation of almost all phenolic compounds in the sample as a consequence of the basic pH.

As demonstrated in figure 4, the values recorded by the ABTS assay are higher than those of the FRAP assay. As previously reported, the explanation lies in the multiple compounds characterised by scavenging action as opposed to metal chelation. Finally, the *ex vivo* digestion process proved to be a reliable model for characterising the TPC and antioxidant activity of *E. gracilis*.

In light of the above, *E. gracilis* has great potential for the food/feed industry due to its high growth rate and ability to store high quality lipids, proteins, carbohydrates, and functional compounds (Klinthong et al., 2014). In parallel, as reported in the literature by Klinthong and colleagues (2014), *E. gracilis* plays a key role in environmental protection due to its ability to store CO_2 through the process of chlorophyll photosynthesis to directly fix carbon in cells. This role is also crucial in the energy sector. Indeed, the pollution-free conversion of CO_2 into chemicals and fuels through this approach is a promising way to reduce environmental CO_2 (Klinthong et al., 2014).

CONCLUSIONS

In conclusion, the modulation of the culture conditions of *E. gracilis* plays a key role in the growth yield, in paramylon content, and in determining the nutritional and functional profile of this microalgae. In particular, although DOE-ETX showed lower growth yield and paramylon content than EgM and ETX, it demonstrated a greater functional profile. More precisely, DOE-ETX presented a higher TPC and antioxidant activity following chemical extraction, for each condition tested (H_2O:EtOH), deepening the role of nitrogen sources and trace elements in the modulation of the functional aspect. However, to be able to confirm the large-scale use of *E. gracilis* in the feed industry, further studies and in-depth investigations are required to characterise the best cultivation methods for this microalgae, in order to obtain a homogeneous, constant and non-variable matrix; fundamental requirements to ensure animal welfare and guarantee high standards of production performance in farm animals. Furthermore, given the high nutritional, functional profile and the important role in environmental protection of *E. gracilis*, a possible application in the food sector should be considered.

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Manuscript VII

Impact of Nanoplastics on the Functional Profile of Microalgae Species Used as Food Supplements: Insights from Comparative *In Vitro* and *Ex Vivo* Digestion Studies

Davide Lanzoni, Marisa Sarria Pereira de Passos, Dora Mehn, Sabrina Gioria, Antonio Vicente and Carlotta Giromini.

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Impact of Nanoplastics on the Functional Profile of Microalgae Species Used as Food Supplements: Insights from Comparative *In Vitro* and *Ex Vivo* Digestion Studies

Davide Lanzoni[a+], Marisa Sárria Pereira de Passos[b+†], Dora Mehn[b], Sabrina Gioria[b*], António A. Vicente[c], Carlotta Giromini[a,d].

[a] Department of Veterinary Medicine and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy. [b] European Commission, Joint Research Centre (JRC), 20127 Ispra, Italy. [c] CEB – Centre of Biological Engineering, University of Minho, 4710-057, Braga, Portugal. [d] Institute for Food, Nutrition and Health, University of Reading, Reading, RG6 5EU, UK. [+]These authors contributed equally to this work. † Current address: Institute of Biotechnology, RWTH Aachen University, Worringerweg 3, 52074 Aachen, Germany.

ABSTRACT: The widespread use of plastics in the food industry raises concerns about plastic migration and health risks. The degradation of primary polymers like polystyrene (PS) and polyethylene (PE) can generate nanoplastics (NPs), increasing food biohazard. This study assessed the impact of PS, PE, and PS+PE NPs on *Chlorella vulgaris* (CV) and *Haematococcus pluvialis* (HP) before and after *in vitro* and *ex vivo* digestion, focusing on particle size, polydispersity index, and surface charge. The modulation of total phenolic content (TPC) induced by NPs contamination was also evaluated. Results demonstrated that NPs behaviour varied with the microalgae medium and persisted post-digestion, posing health risks. Significant size increases were noted for PS+PE in CV and HP. TPC increased significantly with NPs exposure, especially PS+PE. These findings underline the need for regulatory measures to ensure food safety in cases of plastic contamination and to address the behaviour and toxicity of NPs.

Keywords: *Chlorella vulgaris*; Food supplements; *Haematococcus pluvialis*; nanoplastics; Polyethylene; Polystyrene.

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INTRODUCTION

Plastic is a polymer commonly used throughout the world, characterised by versatility, strength and cost-effectiveness (North and Halden, 2013). In 2020 alone, the global production of plastic products reached nearly 320 million tonnes, a number that is anticipated to increase dramatically by 2050 (Sana et al., 2020), with an estimated production of 1.1 billion of new products (Taylor, 2017). The widespread use of plastic products generates a huge amount of plastic waste, of which only 9% is properly recycled (Mazzoleni et al., 2023). Within all uses of plastics, special attention has been given to the food sector, due to safety concerns that are fundamentally associated to the plastic materials propensity to migrate into foodstuffs, and consequently, to their potential human and animal health impact (Mazzoleni et al., 2023; Wen et al., 2024; Arvanitoyannis and Bosnea, 2004). Among the most used plastic polymers are polystyrene (PS) and polyethylene (PE) (Plastics Europe, 2019). Although plastic polymers are generally recognized as chemically inert materials, microplastic (MP, <5 mm) and nanoplastic (NP, <1000 nm) particles, resulting from degradation of primary plastic products, may however "carry" a masked biohazard (EFSA,

2016). The small size of MPs and NPs is a critical factor in their interaction with the human organism. Specifically, they can enter the human body through three main routes: skin contact, inhalation and ingestion (Yee et al., 2021). Of these, the latter deserves particular attention, as it is estimated that the annual intake of plastic particles per person is approximately 39.000-52.000 (Cox et al., 2019). Contact between MPs and NPs and the food matrix can occur in multiple ways: (a) environmental contamination; (b) during transport and storage; (c) during food processing; (d) distribution; (e) food packaging (Nerin, 2016; Jadhav et al., 2021). However, data gaps exist in the understanding of the effects associated to mixtures of plastics on food matrices, and the implications of plastics transformations once undergoing gastrointestinal digestion (Stock et al., 2020; Fournier et al., 2021). In accordance with the recommendations of the European Food Safety Authority (EFSA, 2016), a comprehensive understanding of these dynamics is of high priority, not only because fluids in the digestive tract can change the surface properties of plastics, leading to the formation of a protein corona that can alter the bioavailability of food nutrients, but also because of the high specific surface area of NPs, which makes them more easily at risk of toxicity due to the impact of their greater reactivity (given their smaller size) on their fate, more easily translocating cellular barriers, and consequently highly susceptible to being absorbed from the gut.

In a rapidly changing world, food supplements are becoming increasingly relevant to cope with the altering dietary habits, the growing health awareness, and the aging of global population, as due to their concentrated nutrient content, these play a crucial role in supporting overall health and well-being alongside regular diets (Cencic and Chingwaru, 2010). In this context, microalgae are enjoying great success in food and feed industry for the production of functional foods (Frazzini et al., 2022; Lanzoni et al., 2024). *Chlorella vulgaris* (CV) and *Haematococcus pluvialis* (HP) are two microalgae species gaining prominence as food supplements due to their rich nutritional profiles and bioactive compounds. While the first is certainly the most well-known due to its important nutritional profile (55-67 % protein, 7-15 % lipids, and 9-18 % dietary fibre on a dry matter basis) and important bioactivity (Matos et al., 2017; Bito et al., 2020), the last is attracting particular interest due to the production (around 5 % of dry matter) of astaxanthin (3,3′-dihydroxy-β, β-1-carotene-4,4′-dione), a potent antioxidant contributing to its wide recognition for anti-tumour, anti-aging and anti-inflammatory benefits (Oslan et al., 2021), being therefore acknowledged by the European Regulation 2015/2283 on novel Foods as a food supplement (European Parliament, 2015). Among the functional components, microalgae are recognised for their high phenolic content, an important parameter known to have interesting beneficial effects on human health.

This plays a key role in the food sector, especially if one considers that cultivation conditions can influence the total content (Frazzini et al., 2022; Lanzoni et al., 2024). Nevertheless, important data gaps are yet to be addressed for a comprehensive safety profiling of microalgae as food supplements, *e.g.* studies are still needed to understand how plastic mixtures affect the functional profile of microalgae at the different stages of the digestive

process. On the other hand, micro-size plastic particles, but not NPs, have received extensive research attention regarding their presence in food matrices and potential impacts on food safety and quality. Studies have documented their accumulation in various food items and assessed their interactions with food components, highlighting concerns over contamination and potential health risks (van Raamsdonk et al., 2020). Adding to this, analytical techniques for detecting and quantifying MPs are well-established (Ye et al., 2022), facilitating a deeper understanding of their behaviour and effects in food systems. In contrast, NPs represent a newer area of research with limited available data on their specific impacts on food matrices. A growing recognition of NPs as a significant human health concern, alongside the increasingly exploration of microalgae as sustainable high-added value sources for food supplements, highlights the need of studies targeting the interactions and transformations of NPs, especially as mixtures of polymers, once in contact to food matrices, not only before, but particularly during the digestive process, to more accurately inform the regulatory measures aiming to safeguard food quality and ensure consumer safety regarding plastics use for food sector.

To date, the study of the toxic effects of plastic contaminants on microalgae has focused on growth inhibition, morphological changes and modulation of essential pigments (Khoshnamvand et al., 2021). On the other hand, no studies so far have investigated the impact of NPs on the production of phenols by microalgae. These secondary compounds serve as a defence mechanism for microalgae against biotic and abiotic stresses (Lanzoni et al., 2023). Understanding phenols production is paramount for ensuring the safety and efficacy of microalgae-based food supplements for consumers, as these may influence their bioactivity and potential health benefits. Phenols possess antioxidant properties that can contribute to human health by protecting cells from oxidative damage and inflammation (Lanzoni et al., 2023). Although, to the best of our knowledge, there are no live microalgae in food applications, live microalgae can be exposed to plastic materials during industrial culture systems, where microalgae are commonly cultivated in large-scale plastic bioreactors, especially PE bags or photobioreactors (Dogaris et al., 2015). During the cultivation phase, contamination by plastic nanoparticles can occur, especially if the plastic material degrades or infiltrates the culture. This step in the supply chain can lead to the live microalgae being exposed to plastic contaminants prior to processing, creating a potential contamination pathway with plastic nanoparticles, which can be transferred into downstream products, even if the algae are subsequently processed into non-living forms.

By testing live microalgae, we are modelling the highest-risk scenario: if there is plastic contamination during the cultivation phase, when the algae are still alive, it is possible to understand and mitigate the likelihood of contamination at later stages of the production process.

Taking above mentioned, in this work we aimed to understand the effects on the functional profile of two microalgae species (CV and HP) relevant as food supplements, upon single (PS, PE) and NPs mixture (PS+PE) exposure (the latter poorly investigated in the literature),

before and after *in vitro* and *ex vivo* digestion. Variations on the microalgae total phenolic content (TPC) was assessed using the Folin-Ciocalteu method. To investigate NPs transformations before and after the two digestion approaches, different analytics were considered. More precisely, particles size (hydrodynamic diameter) and polydispersity index (PdI) were determined by Dynamic Light Scattering (DLS), while modifications on NPs surface charge were measured by zeta (ζ) potential analysis.

MATERIAL and METHODS

The experimental design is depicted schematically in Figure 1.

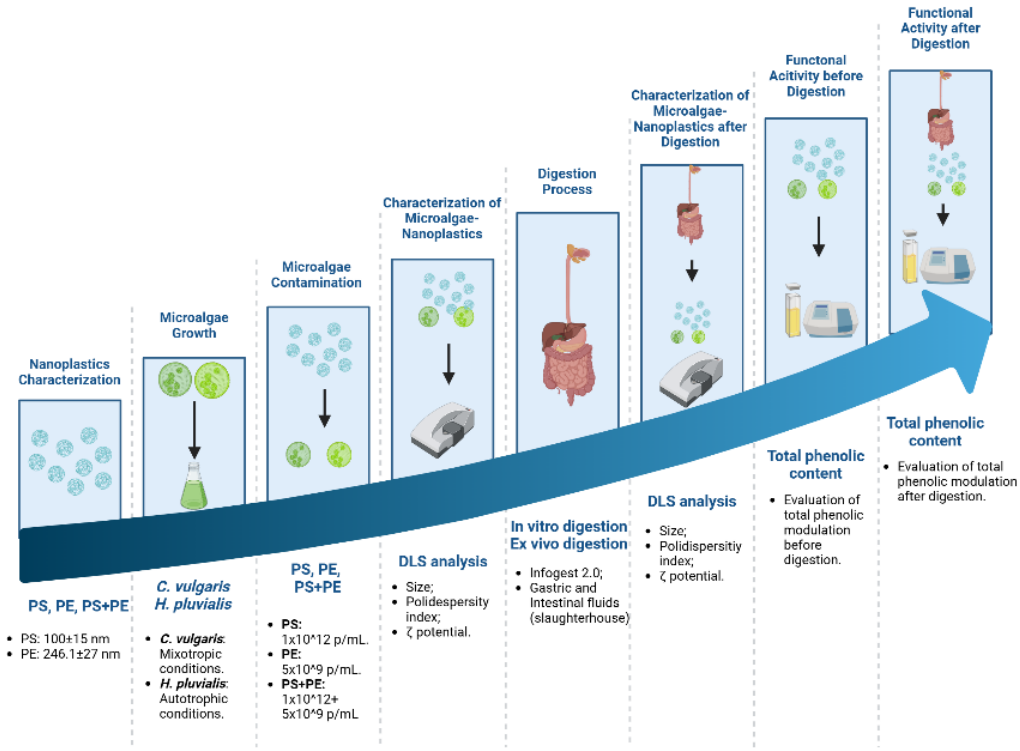

Figure 1. Experimental design. Figure created with Biorender.com

Nanoplastics

Size analytical standard (spherical, monodisperse) plain (not functionalized) PS particles of physical diameter 100.0 (± 15) nm (CV, Coefficient of Variance of 15 %) at 2.5 % (w/v) solids in aqueous dispersant were acquired from Polysciences (Polybead® Microspheres, 00876-15). Particles average diameters are in agreement to calibration and traceability procedures from National Institute of Standards and Technology (NIST, USA). Polyethylene NPs were *in-house* synthetised following an oil-in-water emulsion precipitation protocol

(Cassano et al., 2021). Raman spectroscopy was conducted to characterize the colloidal PE plastic particles chemical composition, as to ensure that the polymer was not chemically modified or degraded during the synthesis (unpublished data). Polyethylene NPs size distribution and particles density were determined using a centrifugal sedimentation method (Supplementary material, Figure S1). Gathering data on these particular physical-chemical characteristics is key to provide insights into the behaviour of the PE NPs under physiological conditions, particularly during *in vitro* and *ex vivo* digestion (as intent). By knowing these parameters, the stability, the aggregation tendencies and potential bioavailability of the PE NPs can be inferred. Factors that are essential to elucidate how particles interact to the food matrices, as these can influence their transformations, transport and toxicity in the gastrointestinal tract.

Microalgae growth and nanoplastics contamination

Mother strain (axenic) cultures of the two microalgae species used as food matrices to NPs exposure experiments were acquired from BMCC - Basque Microalgae Culture Collection: [BMCC127] CV (Trebouxiophyceae) and [BMCC673] HP (Chlorophyceae). Once at controlled (light intensity, photoperiod, temperature, humidity) laboratory conditions under an environmental chamber (CLR Srl, Z01-S-029), the estuarine (Santurce, Spain) microalgae species (the first) was cultured in mixotrophic conditions using industrial dairy waste (hydrolysed cheese whey) as organic carbon source (Abreu et al., 2012), while the freshwater (Amurrio, Spain) microalgae species (the latter) was cultured in autotrophic conditions using the Blue Green 11 (BG-11) medium, a widely reported mainstream medium for microalgal biomass and lipid production (Jin et al., 2024). To determine the appropriate microalgae cell density to use for the experiments, changes in the number of cells mL^{-1} were monitored in the corresponding growth media overtime by regular sampling and counting of the cells using a Neubauer chamber, under an ECLIPSE Ts2 inverted microscope coupled to a DS-Fi3 digital camera. Specific growth curves were then established for the two microalgae species (Supplementary material, Figure S2) that permitted to define the optimal nominal concentrations (cell density) for the contact tests, and therefore ensure that the cells were at the exponential growth phase during exposure to NPs. For a constant growth rate at T_0, pre-cultures derived on corresponding microalgae mother strain cultures were prepared 48 h prior the contact tests to NPs in respective growth media. *Chlorella vulgaris* at 3.80 x 10^7 cells mL^{-1} and HP at 1.75 x 10^7 cells Ml^{-1} were then exposed to 1 x 10^{12} part mL^{-1} of standard PS and 5 x 10^9 part mL^{-1} of *in-house* synthetized PE, either as individual (single) exposure or combined (mixture), under stirring at the same environmental chamber as the microalgae mother strain cultures, for 24 h.

As plastic nanoparticles size and density significantly drives their behaviour and interaction to microalgae cells as food matrices on *in vitro* and *ex vivo* digestion, NPs test (nominal) concentrations were selected considering the following rationale: 1) PS NPs (100.0 ± 15 nm) have a higher density (1.050 g cm^{-3}) and a larger surface area-to-volume ratio corresponding to a greater number of particles per unit mass diffusing faster, however, these are particularly

prone to higher sedimentation rates towards the bottom of the exposure vessel, therefore decreasing the possibility of encounters to microalgae cells that are largely suspended in the exposure media - a higher test (nominal) concentration (1 x 10^{12} particles mL^{-1}) was therefore set to ensure sufficient exposure, despite the major propensity of aggregation and (or) agglomeration events; 2) as larger particles, PE NPs (246.1 ± 27 nm, Supplementary material, Figure S1) tend to exhibit a different exposure profile due to their lower density (0.882 g cm^{-3}, Supplementary material, Figure S1) and smaller surface area-to-volume ratio resulting in fewer particles per unit mass interacting to microalgae cells at lower diffusion rates, but being more likely to remain suspended in the exposure media due to slower settling - therefore, despite the lower (as compared to PS NPs) test (nominal) concentration set (5 x 10^9 particles mL^{-1}), their reduced tendency to rapidly sediment or aggregate and (or) agglomerate can still ensure that an adequate number of particles are accessible for effective encounters to microalgae cells. Ultimately, NPs test (nominal) concentrations were set above detection limits of the analytics considered on this study (DLS) (de Bruin et al., 2022) (that aims to comprehensively assess how different particles characteristics (as above-mentioned) can impact the functional profile of microalgae as food supplements, as to understand NPs behaviour and the various transformations that might occur upon interacting to microalgae as food matrices during a digestive process, thereby providing valuable insights into the potential risks associated to the use of NPs in food sector.

Dynamic Light Scattering (DLS) analysis

Batch Mode DLS was used to characterize the size (intensity-weighted mean hydrodynamic diameter, Z-average) and PdI of standard PS and PE NPs in the different microalgae growth media, pre- and post-digestion. Moreover, given the significant (nanoscale) size difference expected between the particles size populations (modes), Batch Mode DLS was further considered to resolve multimodal NPs size distribution once in a (NPs) binary mixture (pre- and post-digestion studies); as to investigate the NPs post-digestion changes in size (hydrodynamic diameter), PdI and size distribution once in mixture to microalgae cells debris (lysate). Time-resolved DLS was used to characterize the NPs behaviour (e.g. particles sedimentation, aggregation and (or) agglomeration events) in the different microalgae growth media during the same exposure time (24 h) as the contact tests (pre-digestion). Electrophoretic DLS Mode was used to measure, pre-digestion only (residual enzymes or by-products of post-digestion can modify the electrical properties of the sample, as interfering on NPs particles, interfere with the measurement process, influencing the time required for stabilization and the overall accuracy of the results.), the NPs electrophoretic mobility in the different microalgae growth media, with and without microalgae cells, for ζ potential assessment and particles surface charge analysis. Dynamic light scattering measurements were conducted using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at 25 ± 1 °C for a backscattering angle of 173°. Nanoplastics hydrodynamic diameters (Z-average) and the dispersity from cumulative analysis were determined according ISO 22412:2017 (ISO, 2017). ζ-potential analysis for NPs surface charge assessment was conducted in agreement to ISO 13099-1:2012 (ISO, 2012).

In vitro digestion

In vitro digestion studies were performed following the standard static INFOGEST (Brodkorb et al., 2019; Minekus et al., 2014). The reagents and enzymes used were purchased from Sigma Chemical Co. (*St. Louis, MO, USA*). Simulated digestion fluids for oral (SOF), gastric (SGF) and intestinal (SIF) phases were prepared in agreement to Brodkorb et al. (2019) and Minekus et al. (2014) . For the oral digestion phase, microalgae cells pre-contaminated (for 24 h) to NPs were diluted to the final (nominal) cell density of 9.5 x 10^6 cells mL^{-1} and 4.4 x 10^6 cells mL^{-1}, respectively, for CV and HP in SOF, and incubated for 2 min at 37 ºC to α-amylase (75 U mL^{-1}, pH 7.0), under stirring. Subsequently, the oral bolus was diluted with SGF and pepsin (2000 U mL^{-1}, pH 3.0), and incubated at 37 ºC for 2 h, under stirring. At the end, the gastric chyme was diluted with SIF and incubated with bile salts (10 mM, pH 7.0) and the pancreatic enzymes (100 U mL^{-1}, pH 7.0) for 2 h at 37 ºC, under stirring. Once the digestion process was completed, the samples were centrifuged following the protocol of Gonçalves et al. (2021), with the aim of separating the digested fraction (supernatant) from the undigested fraction (pellet). Subsequently, the samples were stored at -20 ºC until further analysis, to characterize and investigate changes on the NPs primary features and how these affect their interactions with the microalgae as food matrices, the behaviour modifications that can occur once these are in contact to the digestive fluids and discuss whether these can impact nutrients bioavailability and absorption upon the digestive process, but also to provide insight of the NPs-exposure associated variations on the microalgae TPC that can affect their nutritional value as food matrices.

Ex vivo digestion

The *ex vivo* digestion process represents an innovative and as yet unexplored method in the literature for replicating digestion conditions. *Ex vivo* digestion was performed using gastric and intestinal fluids collected from slaughter housed (Lodi, Italy) pigs (n=24), aged between 50 and 110 days, as reported by Lanzoni et al. (2024). Microalgae cells pre-contaminated (for 24 h) to NPs were diluted in the gastric and intestinal pig fluids to the final (nominal) cell density of 9.5 x 10^6 cells mL^{-1} and 4.4 x 10^6 cells mL^{-1}, respectively for CV and HP. At the end of the digestion process, samples were centrifuged at 18.700 RCF for 30 min at RT, thus separating the digested fraction (supernatant) from the undigested fraction (pellet) (Goncalves et al., 2021). The fractions obtained were frozen at -20 ºC until further analysis, as above-mentioned.

Total phenolic content

For quantification of microalgae TPC, the protocol reported by Attard (2013) was performed with minor modifications (Lanzoni et al., 2023). The reagents used (Tannic acid, Folin-Ciocalteu and sodium carbonate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Briefly, 0.100 mL of each sample was incubated to 0.500 mL of Folin-Ciocalteu (diluted 1:10 with distilled H_2O) and 0.400 mL of sodium carbonate (10.589 g in 100 mL of distilled H_2O) for 20 min in the dark, at RT. At the end of the incubation period, the

absorbance of the resulting blue colour was measured on the samples using a spectrophotometer at λ = 630 nm. Appropriate blanks were included in the analysis. Values for TPC (expressed in %) were normalized towards the control (microalgae only).

Statical analysis

The size (Z-average, nm), PdI and ζ-potential (mV) of the NPs and the TPC of the microalgae pre- and post-digestion (*in vitro* and *ex vivo*) was analysed using an one-way ANOVA followed by Tukey's multiple comparison, using GraphPad Prism (9) 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). All parametric assumptions were met. Data was expressed as average (arithmetic mean) ± SEM of at least three independent experiments. Values were considered statistically significant for a 95 % confidence interval (*P value* = 0.05).

RESULTS and DISCUSSION

Pre-digestion characterization of nanoplastics

To better understand the biological impact of NPs, in addition to the nominal values, it is necessary to perform an in-depth characterization of their physicochemical properties in the exposure media (Sendra et al., 2019). As accordingly, the size (Z-average), PdI and ζ-potential of PS, PE and PS+PE in the different microalgae culture media was investigated (Table 1).

Table 1. Size (Z-average), PdI and ζ-potential of PS, PE and PS + PE nanoplastics in microalgae growth media. Different superscript letters indicate statistically significant differences among groups (P < 0.05). Results are reported mean ± SEM.

Sample	Z-average (d.nm)	PdI	ζ-potential (mV)
PS in *CV* growth medium	85.32 ± 0.32[a]	0.05 ± 0.01[a]	-10.60 ± 0.26[a], pH 7.65
PS in *HP* growth medium	87.86 ± 0.09[a]	0.10 ± 0.01[a]	-11.93 ± 1.09[a], pH 7.36
PE in *CV* growth medium	207.50 ± 1.18[b]	0.13 ± 0.01[b]	-15.57 ± 0.29[b], pH 7.68
PE in *HP* growth medium	205.30 ± 1.45[b]	0.13 ± 0.00[b]	-24.60 ± 0.95[c], pH 7.17
PS + PE in *CV* growth medium	267.20 ± 4.84[c]	0.33 ± 0.02[c]	-19.03 ± 0.17[b], pH 7.73
PS + PE in *HP* growth medium	204.67 ± 1.77[b]	0.17 ± 0.00[b]	-17.23 ± 0.98[b], pH 7.13

PdI = Polydispersity index; PS = Polystyrene; PE = Polyethylene; *CV* = *Chlorella vulgaris*; *HP* = *Haematococcus pluvialis*. *CV* growth media (only, no NPs) pH = 7.69 ± 0.03. *HP* growth media (only, no NPs) pH = 7.22 ± 0.10.

All these parameters are crucial in determining the colloidal stability of NPs, which in turn influences their reactivity (Sing et al., 2019; Xiang et al., 2023).

Although, PS NPs showed a lower intensity-weighted mean hydrodynamic diameter in both CV (85.32 ± 0.32 nm) and HP (87.86 ± 0.09 nm) growth media, these values are still within the range of the expected standard particles nominal size (100 ± 15 nm). Similarly, PE NPs Z-average did not differ from the expected determined particles nominal size (Supplementary material, Figure S1), as also no differences in intensity-weighted mean hydrodynamic diameters were recorded among CV (207.50 ± 1.18 nm) and HP (205.30 ± 1.45 nm) growth media. Once in a binary mixture (PS + PE), though, different Z-average results were recorded in the two microalgae growth media. While in HP growth medium, PS + PE showed a in intensity-weighted mean hydrodynamic diameter (204.67 ± 1.77 nm) highly comparable to single PE (independently of the microalgae growth media), in CV growth medium a significantly higher Z-average ($P < 0.05$) was recorded (267.20 ± 4.84 nm).

By DLS analysis, the intensity of the light scattered by a particle is proportional to the sixth power of its radius (Rayleigh scattering theory), therefore, larger particles are detected more readily than smaller ones (Rodriguez-Loya et al., 2023). Larger particles also diffuse more slowly due to Brownian (random) motion that permits easier-to-measure fluctuations in the scattered light, leading to a higher signal-to-noise ratio, and making the detection of larger particles more prominent (Caputo et al., 2021). It is therefore understandable that in the NPs binary mixture, the data analysis algorithms used in DLS could not deconvolute the signal of PS (smaller particles) over the strong scattering of PE (larger particles) that dominates, overshadowing and masking the detection of the (weaker) signal of PS. It was further interesting to notice that aside to the higher Z-average values obtained for PS + PE in the CV growth media, a higher PdI value (0.33 ± 0.02) was detected, suggesting the occurrence of agglomeration and (or) aggregation events, as in accordance to Seoane et al. (2019) that demonstrated PdI values greater than 0.20 to represent an agglomeration and (or) aggregation factor among NPs. Additionally, once investigating the count rate overtime (Figure 2), it could be observed that not only the T_0 count rate of the NPs binary mixture in the HP growth media was higher (~ 4000 kcps) as compared to the one in CV growth media (~ 3000 kcps), but also constant, remaining stable along the measurement time (24 h). On the contrary, an erratic count rate was recorded overtime for the PS + PE NPs in CV growth media, showing a significant decrease in the number of particles in the detection volume (count rate ~ 2000 kcps) for the first 12 h (Figure 2), therefore corroborating the hypothesis of particles clustering overtime, becoming larger than the expected nominal size and being more prone to settle out, thus lowering the count rate because of the fewer particles contributing to the scattering process. ζ-potential is key to understand the stability of colloidal dispersions, influencing cell permeability, protein interactions and toxicity (Martin et al., 2022). It measures the magnitude of the electrostatic forces at the slipping plane of a particle or the degree of repulsion among adjacent and similarly charged particles in a colloidal system. ζ-potential threshold values of absolute 30 (negative or positive) mV are

often cited as the boundaries for colloidal stability (Xiang et al., 2023; Sendra et al., 2019; Wu et al., 2021). When having a ζ-potential outside this range, the repulsive forces among particles are higher, making these less prone to agglomerate and (or) aggregate, being thus considered more stable. As shown in Table 1, the ζ-potential values are outside the boundaries for colloidal stability for the NPs tested. Polystyrene NPs showed similar values of ζ-potential in CV (-10.60 ± 0.26 mV) and in HP (-11.93 ± 1.09 mV) growth media, but for PE NPs a more negative charge close to - 30 mV was recorded in HP growth media (-24.60 ± 0.95 mV), therefore suggesting that particles stability is higher than in CV growth media, for which a ζ-potential similar to PS NPs was detected (Table 1). In agreement, it was also in HP growth media that once in a binary mixture, the NPs showed less propensity to agglomerate and (or) aggregate overtime (Figure 2).

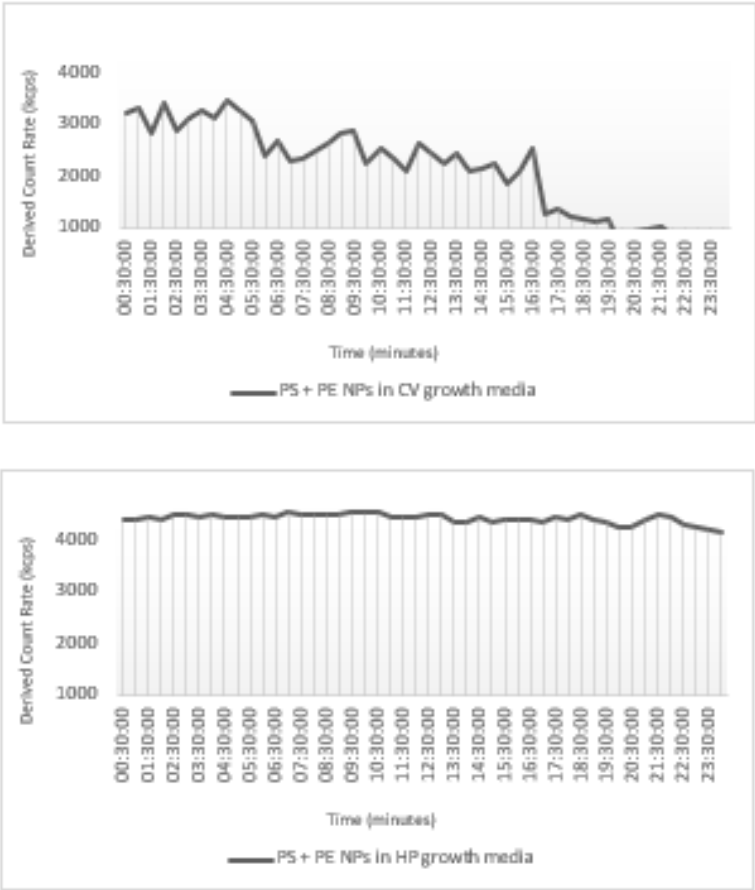

Figure 2. Derived count rate of PS + PE NPs in microalgae growth media overtime. PS = Polystyrene; PE = Polyethylene; NPs = Nanoplastics; CV = *Chlorella vulgaris*; HP = *Haematococcus pluvialis*; Kcps = kilo counts per second.

Given the different behaviour of PS+PE NPs in the two microalgae growth media, their stability overtime was investigated using DLS analysis to collect real-time data of NPs size distribution and monitor the changes on particles size that might suggest agglomeration and (or) aggregation events. Measurements were conducted for 24 consecutive h, to simulate the same exposure time of the microalgae pre-contamination tests, as reported in Figure 3 and Figure 4.

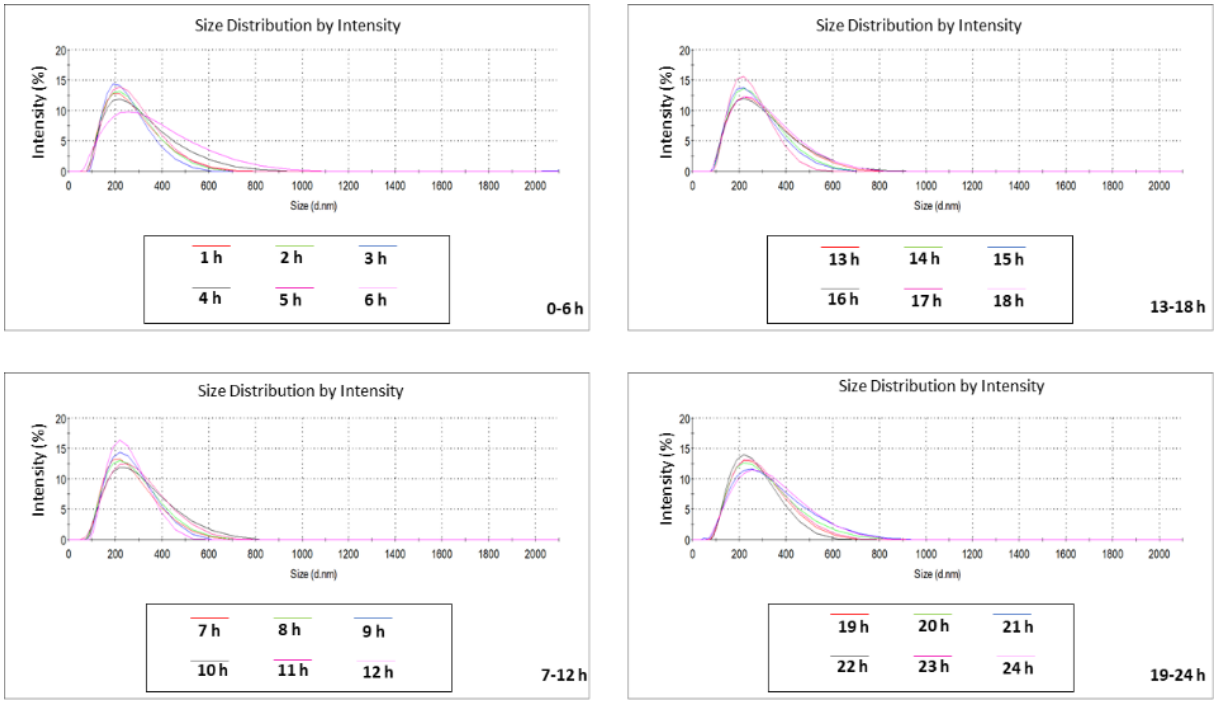

Figure 3. Analytical characterisation of the size distribution of PS+PE nanoplastics in *Haematococcus pluvialis* growth media for 24 h. Results are expressed in d.nm and reported every 6 h.

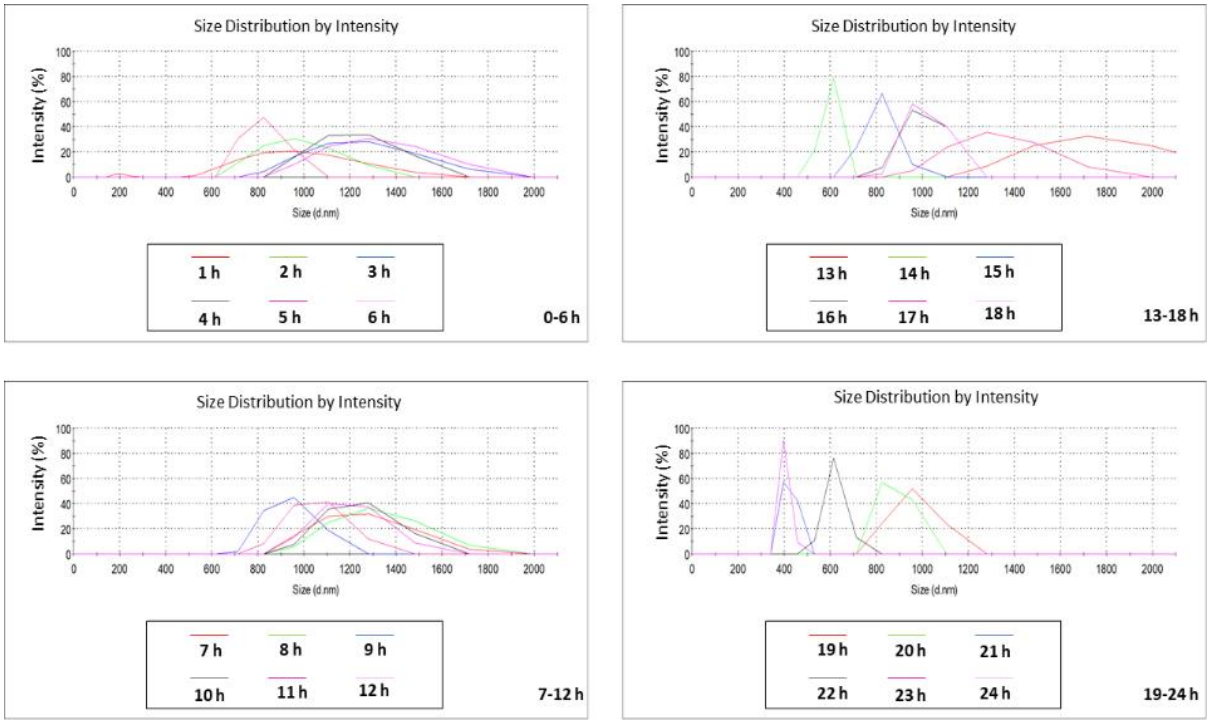

Figure 4. **Analytical characterisation of the size distribution of PS+PE nanoplastics in *Chlorella vulgaris* media for 24 h**. Results are expressed in d.nm and reported every 6 h.

Size distribution results of PS + PE NPs in HP growth medium (Figure 3) corroborated the previously anticipated stability.

More precisely, along the 24 h analysis, DLS recorded identical (overlapping) peaks showing a maximum intensity (%) at ~200 nm, confirming not only that PE NPs indeed masks the scattering signal of PS NPs, but also permitting to pre-empt that no agglomeration and (or) aggregation events will tend to occur during the microalgae pre-contamination contact tests. In contrast, an inconsistent and reversible overlapping of the intensity peaks overtime was recorded for PS + PE NPs in the CV growth medium (Figure 4), demonstrating that the particles tend to highly agglomerate, rather than aggregate, since a wider size distribution can be observed with intensity peaks that might suggest the presence of both single particles and loosely bound clusters, indicating therefore a highly heterogeneous mixture as according to the increased (> 0.20) PdI values previously detected (Table 1). Indeed, distinct from aggregates, agglomerates are formed by two or more particles held together by weak physical-chemical interactions in a reversible process (Sokolov et al., 2015).

This reversibility is clearly noticeable in Figure 4 with the alternation of random major and minor intensity peaks recorded every h. Above-mentioned differences are to some extent probably due to the distinct salt composition of the two microalgae growth media that can influence their ionic strength and the electrostatic interactions among NPs. However, several other factors can influence the agglomeration of NPs. Among these, pH and the presence of additives and dispersants are particularly relevant (Hazeem et al., 2020; Shupe et al., 2021). In fact, sodium cholate was used in PE NPs *in-house* synthesis as an anionic surfactant for particles steric stabilization. It can adsorb onto the surface of PE NPs, imparting a significantly negative surface charge, as actually it was recorded in HP growth media that is mostly neutral (Table 1). At this pH, due to the deprotonation of surface groups or the adsorption of ions from the HP growth medium, PS NPs are just slightly negatively charge (Table 1). ζ-potential results suggest that once in a binary mixture, while PE NPs might contribute moderately to stabilize the (closed) system, the PS NPs' tendency towards instability could still trigger aggregation and (or) agglomeration events. However, the (estimated) ionic strength of HP growth media at neutral pH is relatively low (~ 0.03 M). A thicker electrical double layer can then be formed around the particles that imposes important electrostatic repulsion forces, therefore leading to greater stability (Figure 3). Inversely, at a slightly basic pH, the higher ionic strength estimated for CV growth media (~ 0.15 M), can compress the electrical double layer around the particles, reducing the range of electrostatic repulsion, thus permitting these to approximate enough to agglomerate, as it seemed to occur (Figure 4).

Knowledge gathered on the behaviour of PS and PE NPs in a closed system is key to understand their dynamics once in an open system, as it is the case of the human gastrointestinal tract and the associated digestive process. Upon ingestion of food supplements - as microalgae - contaminated to NPs that migrated from the (plastic) bioreactors or package, particles that come into contact to biological fluids (saliva, gastric

juice or intestinal fluids) encounter a variety of biomolecules (proteins, lipids, enzymes) that can adsorb onto their surface forming a "corona" coating. As a result, hetero-agglomerates (combination of polymer types or bonds with other naturally occurring particles) can then occur that cause changes on NPs density, impacting their buoyancy and propensity to deposit (Summers et al., 2018), which ultimately can modify the bioavailability of nutrients derived from digested food.

Interaction of nanoplastics and microalgae on pre- and post- in vitro and ex vivo digestions

Prior digestion studies, microalgae cells of the two species were contaminated to NPs on a contact test. For a better understanding of the multiplicity of interactions that can occur among NPs and the food biomatrix, a pre-digestion analytical characterization of the microalgae suspensions of NPs was conducted (Figure 5). Polydispersity index values of PS, PE and PS + PE increased in both microalgae growth media (Figure 5), as compared to those observed in absence of microalgae cells (Table 1).

At the same time, although minimal changes were reported for the ζ-potential, values were still outside of the boundaries for colloidal stability as previously referred. Moreover, the size (Z-average) of the NPs was significantly affected, but still easily distinguishable from those measured for the microalgae (only) cells suspensions (Figure 5).

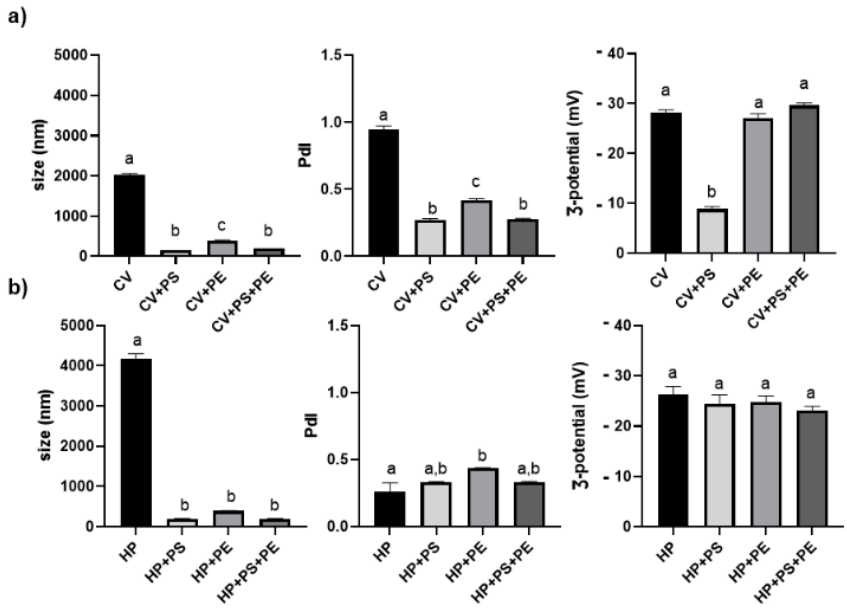

Figure 5. Size (Z-average) detected for microalgae suspensions of NPs post (a) *in vitro* and (b) *ex vivo* digestion. Different superscript letters indicate statistically significant differences among groups ($P < 0.05$). PS = Polystyrene; PE = Polyethylene; CV = *Chlorella vulgaris*; HP = *Haematococcus pluvialis*.

More precisely, a size of 2040 ± 27.43 nm was recorded for CV, confirming the values reported in the literature (Safi et al., 2014). For HP, the cells size range was 4169 ± 133.67

nm. As documented in the literature, HP can reach 30 µm, although this value is dependent on the replicative stage (Niizawa et al., 2018). Specifically, this size can be reached during the haematocyst phase, also known as the *'red nonmotile astaxanthin accumulated encysted phase'*, the last step of the life cycle (Shah et al., 2016). In the case of our study, the smaller size observed is correlated with the proliferation (exponential) phase (*green vegetative palmella*), the phase selected for the microalgae contact tests to NPs. As it can be observed in Figure 5, the intensity-weighted mean hydrodynamic diameter of single NPs, but not in mixture, showed a significant increase once in co-culture to the microalgae cells. For PS NPs in co-culture to CV, a smaller value was recorded (146.17 ± 0.32 nm) than that obtained in co-culture to HP (197.30 ± 5.18 nm). A trend that was not observed though for PE NPs, for which no relevant particles size differences were recorded between the microalgae co-cultures (Figure 5).

Interaction among PS NPs and the negatively charged surface of CV cells might not be strong enough to overcome the hydrophobic repulsion forces, resulting in the less pronounced agglomeration as compared to PE NPs. At neutral pH, despite electrostatic interactions are weaker, repulsion forces prevent larger-scale aggregation, but are not enough to impede the formation of stable two-particle agglomerates (dimers). An outcome that can be due to a combination of reduced electrostatic repulsion, dynamic equilibrium in the system that prevents larger aggregate formation and specific molecular interactions facilitated by microalgae exudates. In fact, both CV and HP are microalgae species know to produce extracellular polymeric substances, which are natural biopolymers secreted in response to stress that act as a protective layer against external agents (Xiao and Zheng, 2016). Extracellular polymeric substances consist of polysaccharides, enzymes and structural proteins, among other biomolecules, which once released to the intracellular media can coat NPs surface, leading to the formation of a bio-corona that consequently can influence their physical-chemical properties (as affecting particles size and density), diffusion, sedimentation, aging, propensity to cellular membranes translocation or other target interactions (Cao et al., 2022; Neagu et al., 2017).

For PS + PE in co-culture to *CV*, PS particles may preferentially adhere to PE NPs or be sterically hindered by these, leading to a size distribution that stabilizes around the size of the larger particles (PE), rather than interacting to microalgae exudates. In fact, the size and surface curvature of NPs are critical factors in determining their affinity towards microalgae extracellular polymerics substances [54]. For this reason, it is plausible to assume that an increased curvature due to PS + PE NPs preferable agglomeration, diminished the contact area and interaction strength between NPs and the biomolecules, reducing but not entirely preventing bio-corona formation (Yu et al., 2022). Moreover, the formation of bio-corona can counteract ionic effects and stabilise NPs, thus reducing agglomeration through steric interactions, explaining the lower values recorded (Martin et al., 2022).

A similar result was obtained for PS + PE in co-culture to HP (199.20 ± 2.81 nm). In neutral microalgae growth media, weaker electrostatic interactions lead to less aggregation overall.

PE NPs being larger again dominate the size distribution, stabilizing the mixture around their weighted mean hydrodynamic diameter (Figure 5).

Given the complexity of the NPs-associated interactions to microalgae, as discussed, changes on the NPs size (Figure 6) and on the microalgae functional profile (Figures 7 and 8) were investigated pre- and post- human digestion simulations, to anticipate the scenarios that might occur during cultivation of microalgae in bioreactors.

Obtained results (Figure 6) suggest the ability of NPs to persist in the digested fraction, making thus these available for absorption by intestinal cells, corroborating data as reported by Paul et al. (2024). Once digested, NPs can: (I) remain in the intestinal lumen causing local tissue irritation; (II) be absorbed by intestinal cells and released into the lumen following cell death (approximately 72 h later); (III) cross the intestinal epithelium by paracellular pathways (through tight junctions) or by per-sorption (that is, through intracellular spaces), or by cells of the intestinal epithelium therefore reaching the basal side (Paul et al., 2024; Paul et al., 2020). However, these outcomes are strongly influenced by digestion. In fact, digested NPs are particularly more prone to be absorbed due to the presence of organic matter that facilitates translocation to the intestinal epithelium (Paul et al., 2024). For this reason, it is of great relevance to investigate the individual changes that might occur on NPs characteristics and behaviour during digestion.

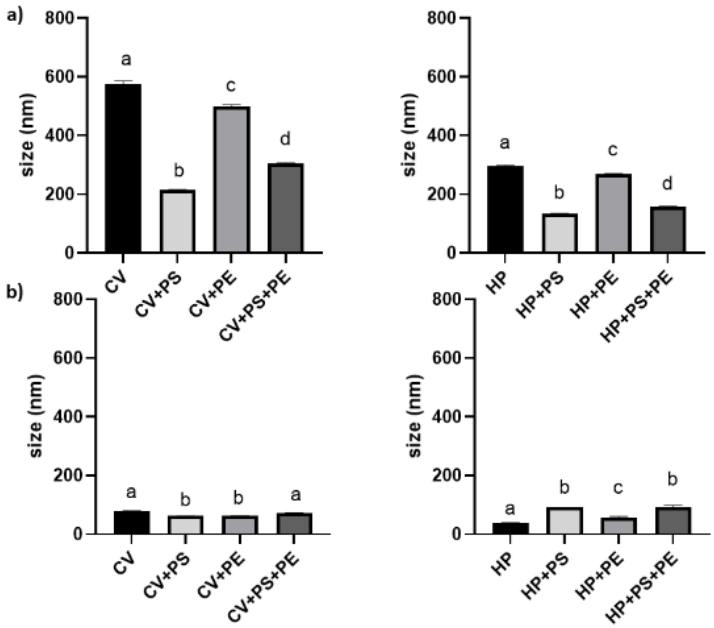

Figure 6. Size (Z-average) detected for microalgae suspensions of NPs post (a) *in vitro* and (b) *ex vivo* digestion. Different superscript letters indicate statistically significant differences among groups ($P < 0.05$). PS = Polystyrene; PE = Polyethylene; CV = *Chlorella vulgaris*; HP = *Haematococcus pluvialis*.

Post *in vitro* digestion, the size of the microalgae cell debris allows easy distinction from PS, PE and PS + PE NPs (Figure 6). Interestingly, the size detected for cell debris related to HP (296.77 ± 2.48 nm) was smaller than for those related to CV (574.83 ± 11.04 nm), although,

as previously reported, the first was characterised by a larger diameter of the cells (Figure 5). These differences are most probably due to the structure of the cell wall of these microalgae. More precisely, although at the beginning of the growth phase, CV is distinguished by a single microfibrillar layer, the cells rapidly develop a three-layer structure, with a very thick outer layer and a thinner one forming the daughter cell wall (Weber et al., 2022). For this reason, CV cells walls are often classified into a soluble and a rigid fraction, the latter consisting of complex resistant biopolymers, and therefore, more sensitive to the action of "harsher" enzymes (e.g. chitinases) that are not present in the digestion protocol used (Weber et al., 2022).

At the same time, although HP in the palmella stage (the one considered for NPs contact testing) is characterised by a complex structure of the cells wall that presents a double membrane layer, one of which is thick and gelatinous, only in the final stage (*non-motile red astaxanthin-accumulating incyst phase*) these cells increase dramatically in volume becoming surrounded by three thick and tough resistant layers that are difficult to degrade (Kim et al., 2022; Boussiba, 2000). Furthermore, HP cells wall are sensitive to treatment with hydrochloric acid, which is highly present during the gastric phase of the digestion protocol used (Ye et al., 2020). On regards NPs size characterization post *in vitro* digestion, while in co-culture to HP, NPs showed a reduced weighted mean hydrodynamic diameter (Figure 6) as compared to pre-digestion data (Figure 5), in co-culture to CV a modest increase of the NPs size was recorded.

This trend was also confirmed for PS+PE in CV and HP medium with an incremented diameter of 303.53±4.33 nm and 262.0±0.89 nm, respectively. Bio-corona therefore reducing agglomeration through steric interactions, explaining the lower values recorded as compared to NPs in co-culture to CV as most likely fewer and larger cell debris were produced as final product.

Obtained results are partially confirmed by the literature, but unique comparisons are difficult due to the numerous factors varying among studies. Krasucka et al. (2022) reported that despite PS NPs showed no change in primary features, PE NPs were particularly distinguished by a rough and heterogeneous appearance showing deep surface cracks, suggesting propensity to partial degradation for this plastic polymer. In another study, Paul et al. (2024) demonstrated that organic residues derived from microalgae digestion are involved in the increase of NPs size, confirming that the higher occurrence of microalgae cell debris can indeed influence the NPs propensity to agglomerate, as further corroborated by Li et al. (2023). In support of this, Fournier et al. (2021) emphasised the importance of considering the formation of a bio-corona on the surface of the NPs due to the adsorption of organic matter as proteins, carbohydrates and lipids released as a result of the digestive process.

Post *ex vivo* digestion seemed to result in a more pronounced digestion of the biomatrix, as weighted mean hydrodynamic diameters of 76.89 ± 3.12 nm and 38.74 ± 1.23 nm were detected for CV and HP, respectively, besides confirming the structural cell wall differences

among the two microalgae species. A significant higher number of smaller microalgae cell debris is therefore expected interacting to the NPs surface for *ex vivo* than for *in vitro* digestion, increasing the repealing forces among particles due to the formation of an eventually thicker bio-corona on the NPs surface.

These differences were also confirmed by PdI analysis, as in the *in vitro* digestion, the PdI of the NPs were just slightly modified compared to pre digestion values (for PS NPs, PdI values of 0.34 ± 0.02 and 0.20 ± 0.01; for PE NPs, PdI values of 0.42 ± 0.02 and 0.40 ± 0.01; for PS + PE NPs, PdI values of 0.45 ± 0.01 and 0.29 ± 0.01 were recorded in co-culture to *CV* and *HP*, respectively), while for *ex vivo* the PdI values reported were > 0.90, indicating a more polydisperse final digested product due to the more competent digestion of the gastric fluids. Although there are studies in the literature analysing the behaviour of NPs digested using *in vitro* systems, to our knowledge this is the first one using gastric and intestinal juices of animal origin. For this reason, a direct comparison is also difficult due to the nature of the fluids used.

Total Phenolic Content of microalgae before and after in vitro and ex vivo digestion

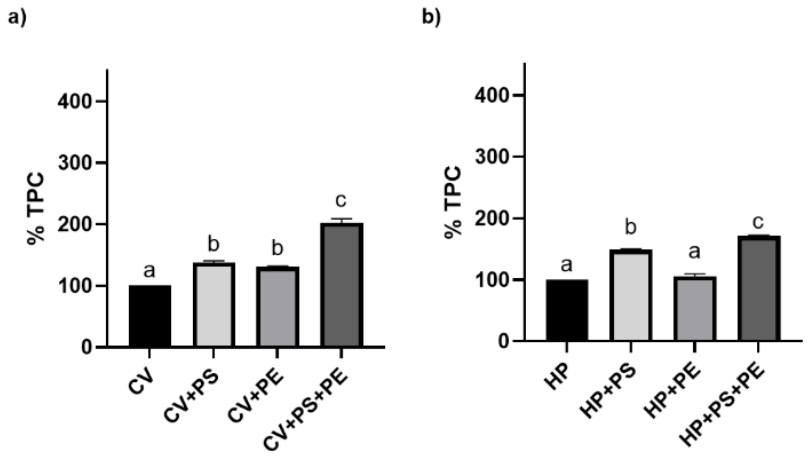

Figure 7. Pre-digestion analysis of microalgae total phenolic content: a) *Chlorella vulgaris*; b) *Haematococcus pluvialis*. Data expressed in %, are reported as mean ± standard error of the mean (SEM) (n = 3), and are standardised towards experimental control group (microalgae in growth media only; no NPs co-exposure). Different superscript letters indicate statistically significant differences among groups ($P < 0.05$). PS = Polystyrene; PE = Polyethylene; CV = *Chlorella vulgaris*; HP = *Haematococcus pluvialis*.

Overall, pre-contamination of microalgae to NPs significantly increased the TPC (Figure 7). Specifically, exposure of CV to PS NPs (137.86 ± 2.44 %) and PE NPs (130.43 ± 1.29 %) resulted in a significant ($P < 0.05$) higher phenol content compared to the control group, with no differences being recorded though among the two NPs.

However, once exposed to PS + PE NPs (202.10 ± 6.97 %), a significantly ($P < 0.05$) higher TPC was recorded, than that obtained once CV was exposed to the single NPs. The same trend was registered for HP, although contamination to PE NPs led to a statistically lower TPC (104.72 ± 5.13%) than that recorded in co-culture to PS NPs (148.22 ± 2.14%), and highly comparable to the control group. Similarly to CV, also for the co-culture of HP to PS + PE NPs, the highest TPC value was recorded (170.84 ± 1.53 %). To the best of our knowledge, microalgae phenolic content assessment following contamination with plastic polymers is not highly investigated in the literature.

The majority of the studies focus on the effect of MPs and NPs in modulating growth rates, morphology, chlorophyll content and photosynthesis process in microalgae (Li et al., 2023; Ye et al., 2022). This trend was confirmed by Menicagli et al. (2022). In particular, the authors observed how NPs led to an increase in phenols in the shoots of *Cymodocea nodosa*, a seagrass, highly described in literature.

Post *in vitro* and *ex vivo* digestions, interesting results were obtain for microalgae TPC (Figure 8).

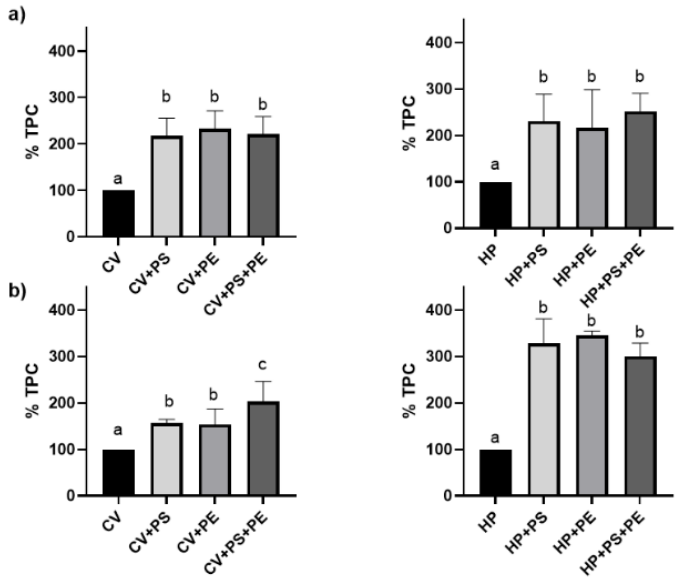

Figura 8. Post-digestion analysis of microalgae total phenolic content: a) *in vitro;* b) *ex vivo*. Data expressed in %, are reported as mean ± standard error of the mean (SEM) (n = 3), and are standardised towards experimental control group (microalgae in growth media only; no NPs co-exposure). Different superscript letters indicate statistically significant differences among groups ($P < 0.05$). PS = Polystyrene; PE = Polyethylene; CV = *Chlorella vulgaris*; HP = *Haematococcus pluvialis*.

Tarko et al. (2013) demonstrated that the absorption and metabolism of phenols in the digestive tract are responsible for their biological properties. Phenolic compounds behaviour during simulated digestion is adequately described in the literature (Tarko et al., 2023; Ginsburg et al., 2012; Goulas and Hadjisolomou, 2020). More precisely, it is estimated that about 48 % of phenols are digested in the small intestine, 42 % in the large intestine, while

only a small part corresponding to circa 10 %, remains bound to the source matrix. As confirmed by Ginsburg et al. (2012), although the partial digestion of phenols begins in the oral cavity, it is only in the gastric cavity that the action of the acid pH allows their release (Goulas et al., 2019; Lanzoni et al., 2024). The reported release of phenols following the digestive process would also explain the higher TPC values obtained, compared to those observed in pre-digestion, although the instability of phenols in an alkaline environment, such as that typical of the small intestine, and in particular during pancreatic action, leads to the transformation of these compounds into unknown secondary structures with different bioactivity and bioaccessibility (Wojtunik-Kulesza et al., 2020). In case of microalgae, the production of TPC is most likely associated with a NPs-induced stress response, however it is difficult to ascertain with the current data whether phenols released as a result of the digestive process play a beneficial role in animal and human health. As reported by Halliwell (2008), no *in vivo* data are available in literature on the ability of phenols to act as antioxidants or pro-oxidants in the stomach, intestine and colon, sites where these may be present at higher concentrations in the organism. However, no evidence of systemic pro-oxidant effects by phenolic compounds has yet emerged after absorption (Halliwell, 2008). In light of the above, although the high phenol content in the treated microalgae suggests that CV and HP are able to cope with the restrictive conditions induced by NPs, the effect of these compounds on animal and human health needs to be further investigated.

In conclusion, the obtained results permitted to demonstrate that NPs behaviour and the various transformations occurring whether as single particles on in a binary mixture, or upon interacting to microalgae as food matrices (pre-digestion) was highly influenced by the microalgae growth media. Post-digestion studies revealed that NPs were indeed detected in the digested fraction, indicating a potential risk to human and animal intestinal health. Finally, the increase recorded in the phenolic content in NPs pre-contaminated microalgae used as food matrices upon *in vitro* and *ex vivo* digestion suggests a complex interplay between the polymer particles and the biological components (microalgae cells and cells debris, fluids, enzymes and other biomolecules derived from the digestive process). While enhanced phenolic content could offer some antioxidant benefits, the presence of NPs in the digested fraction might introduce higher potential risks that need to be thoroughly investigated. Understanding these dynamics is key to understand the impact of NPs exposure on the nutritional value of microalgae as food matrices. This includes examining the variations that trigger changes in the bioavailability and absorption of derived nutrients, and on the synthesis of bioactive compounds (e.g. phenols), after a digestive process. Addressing these factors comprehensively is essential for ensuring the quality and safety of using plastics as contact materials of microalgae as food supplements.

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SUPPLEMENTARY MATERIALS

Characterization of size distribution and particles density of synthesized polyethylene (PE) nanoplastics (NPs) using analytical ultracentrifugation (AUC)

A centrifugal sedimentation method - analytical ultracentrifugation (AUC) - was used to characterize the size distribution and density of the *in-house* synthesized PE NPs. It measures the velocity at which particles move through a liquid medium under centrifugal force. Sedimentation velocity experiments can provide data on polydispersity and the presence of different size populations of NPs. The sedimentation coefficients depend on the particle size and density. In case both of these parameters are unknown, multiple experiments at different liquid medium densities can be performed to determine both the effective density and size distribution of nanoparticles (ISO 18747-2_2019). A Proteomelab XL-I analytical ultracentrifuge (Beckman Coulter) equipped with interference and absorbance optics was therefore used to register the sedimentation of the particles in water and in 10 % water 90 % deuterated water (D_2O) mixture at 1000 rpm rotation speed in two sector sapphire window cells using the interference optics. The sample sector was loaded with 390 µL 10x diluted sample in water or in D_2O, while the reference cell contained 400 µL of the corresponding liquid medium. Interference data were fit using the ls-g*(s) model of the software sedfit with a linear grid in the -20000-0 S range at 100 resolution for water and in the -30000-0 S range at 150 resolution for D_2O. The modes of the resulting sedimentation coefficient distributions were inserted as sedimentation speed values in the equation suggested by the multi velocity approach ISO standard (ISO 18747-2:2019). For 10 % water 90 % D_2O mixture, liquid medium density and viscosity were determined by linear interpolation and values of 1.095 g cm^{-3} and 1.226 mPas were respectively applied. Particle density determined in this way (0.882 g cm^{-3}) was applied to transform the sedimentation coefficient distribution obtained in water to the particle size distribution shown in Figure S1. Considering that the interference signal is directly proportional to the mass concentration in a wide particle size range, the distribution generated can be considered as a mass based differential size distribution.

Figure S1. **Mass based size distribution of synthesized PE NPs using AUC**.

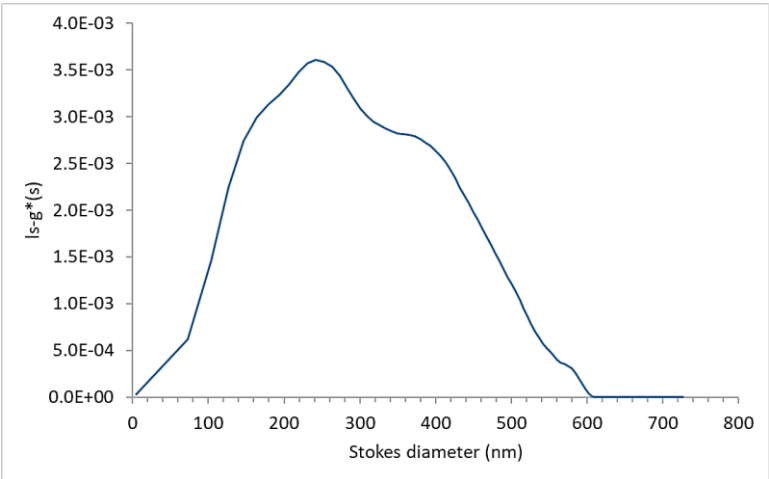
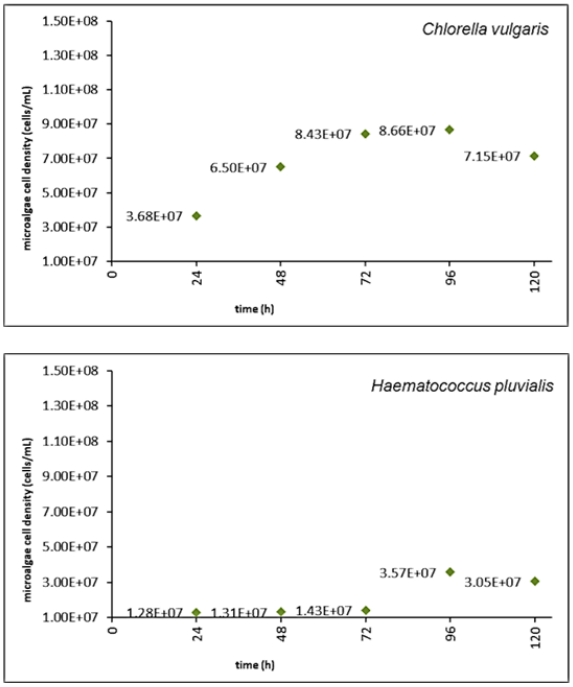


Figure S2. **Microalgae growth curves**: the number of cells mL^{-1} (cell density) was monitored in the corresponding growth media overtime by regular sampling and counting of the cells using a Neubauer chamber, under an ECLIPSE Ts2 inverted microscope coupled to a DS-Fi3 digital camera.



Chapter III

Cultured meat development: exploring biological advances, regulatory hurdles, and alternative growth media

This chapter explores the main challenges and opportunities related to the development of cultured meat, an emerging technology that promises to revolutionise the food industry through the production of meat without animal husbandry. The main biological and technical strategies aimed at overcoming current obstacles are analysed, such as replacing foetal bovine serum (FBS) with more sustainable alternatives, optimising cell-biomaterial interactions and developing scalable biofabrication methods. Despite the progress, many of these solutions are still in the experimental stage, requiring further research to improve their efficiency and make industrial production of cultured meat a viable reality on a large scale. In addition to technical aspects, the chapter addresses regulatory and food safety challenges, with a particular focus on the legislative framework of the European Union, where cultured meat is beginning to enter the regulatory debate. It also examines the need to ensure that the organoleptic and nutritional properties of cultured meat are comparable to those of the conventional product, a key objective for consumer acceptance. Finally, the economic implications of this technology are discussed, from initial production difficulties to potential future markets, highlighting how social acceptance and affordability may influence the commercial success of cultured meat. Furthermore, developments related to the use waste products, such as whey proteins, to promote cell proliferation and differentiation without FBS are explored, paving the way for more ethical and sustainable culture methods. This approach represents an important step in improving cell culture techniques and overcoming some of the major technical and economic limitations that currently hinder large-scale production.

Manuscript VIII

Biotechnological and Technical Challenges Related to Cultured Meat Production

Davide Lanzoni, Filippo Bracco, Federica Cheli, Bianca Maria Colosimo, Davide Moscatelli, Antonella Baldi, Raffaella Rebucci and Carlotta Giromini.

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Biotechnological and Technical Challenges Related to Cultured Meat Production

D. Lanzoni[a], F. Bracco[b], F. Cheli[a,c], B.M. Colosimo[b], D. Moscatelli[d], A. Baldi[a], R. Rebucci[a] and C. Giromini[a,c]

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b] Department of Mechanical Engineering, Politecnico di Milano, Via La Masa 1, 20156 Milano, Italy; [c]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122 Milano, Italy; [d] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Piazza Leonardo da Vinci 32, 20133, Milano.

ABSTRACT: The constant growth of the population has pushed researchers to find novel protein sources. A possible solution to this problem has been found in cellular agriculture, specifically in the production of cultured meat. In the following review, the key steps for the production of *in vitro* meat are identified, as well as the most important challenges. The main biological and technical approaches are taken into account and discussed, such as the choice of animal, animal-free alternatives to fetal bovine serum (FBS), cell biomaterial interactions, and the implementation of scalable and sustainable biofabrication and culturing systems. In the light of the findings, as promising as cultured meat production is, most of the discussed challenges are in an initial stage. Hence, research must overcome these challenges to ensure efficient large-scale production.

Keywords: cultivated meat, cellular agriculture, FBS alternative, 3D scaffolding, 3D bioprinting, edible bioink, sustainability, *in vitro* meat.

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INTRODUCTION

The current global population is approximately 8 billion, a number that is set to grow rapidly by 2050, when it is estimated that the number of people on Earth will reach 9-11 billion (Roos et al., 2017). Such a significant population increase will lead to an exponential growth in the demand for food products, reaching 50% by 2030 and, doubling by 2050, at which point the demand will be difficult to meet without negatively impacting environmental health (Bellet and Rushton, 2019; Rischer et al., 2020). Products subject to high demand are those of animal origin, especially meat and dairy products. In 2012, the Food and Agriculture Organization (FAO) estimated that the global demand for meat is expected to reach 455 million tons by 2050, a 76% increase from 2005. This trend also applies to fish farming, where the amount of fish obtained from aquaculture has increased from 4.7 to 66.6 million tons in just 32 years, and the global demand is expected to reach 140 million by 2054 (Rubio et al., 2020; Bellet and Rushton, 2019). Consequently, to meet the massive demand for animal products, intensive farming has been largely applied. Despite their success after the industrialization process of the 1960s, animal production systems have proven to be extremely fragile. Current industrial-scale breeding systems have been the subject of debate regarding animal welfare, environmental protection, and public health. Since the 1980s,

intensive livestock farming has been the source of numerous public health crises, such as mad cow disease and later the contamination of farmed chicken, beef, pork, milk and salmon products with dioxin, and more recently, swine flu and poultry flu (Bellet and Rushton, 2019).

Another problem related to intensive livestock farming is the environmental impact. The intensification of livestock and agricultural production is involved in greenhouse gas (GHG) emissions, particularly methane (CH_4) and nitrous oxide (N_2O). As reported by Guerci et al. (2013), agriculture contributes 10%-12% of all total emissions, while livestock production, especially ruminants, contributes a total of 14,5% (Guerci et al., 2013; FAO, 2022). However, this statement is at odds with what Chriki and Hocquette (2020) reported. According to these authors, while it is true that livestock has an environmental impact, especially in terms of CH_4, N_2O and carbon dioxide (CO_2) emissions, it is also true that the latter is the main source of emissions of *in vitro* meat, due to the fossil energy consumed to ensure cell growth and proliferation (Chriki and Hocquette, 2020). In support of this contention, Lynch and Pierrehumbert (2019), confirmed how the impact of cultured meat will be minor only in the short term (within 20 years), but not in the long term (beyond 100 years), since CO_2, unlike CH_4, accumulates for long periods within the atmosphere (Lynch and Pierrehumbert, 2019). Moreover, although livestock, mostly ruminants, consume food not intended for humans, they occupy about 70% of global agricultural land and consume about 35% of agricultural crops, directly competing with the production of crops for human consumption and with potential alternative land uses, including nature conservation (Stehfest et al., 2009). The agricultural sector is extremely resource-rich and continues to transform with population growth. Global food production is the largest user of freshwater and uses about 38% of the land. The remaining 62% of the global land area is estimated to be unsuitable for cultivation due to climate, topography, poor soil quality, urban development, or because it is covered by natural lands such as forests. Therefore, little arable land remains for agricultural expansion without a negative impact on environmental health (Rizvi et al., 2018).

Cellular agriculture and Cultured meat

Industrial biotechnology holds a possible key to providing humanity with nutritious, safe, and healthy food while minimizing the use of resources such as energy, water, and land (Rischer et al., 2020). This solution is called cellular agriculture and involves producing food products such as meat or fish from single cells rather than whole organisms such as animals, whose priority is to manufacture products that are similar at the molecular level to those made by traditional techniques (Eibl et al., 2021). With cellular agriculture methods is it possible to produce artificial meat, also called cultured or clean meat, through the differentiation of muscle satellite cells *in vitro* (Warner, 2019). Cultured meat represents the *in vitro* production of meat without the sacrifice of animals. More specifically, it is produced from cells using tissue engineering techniques. Production primarily involves the generation of skeletal muscle tissue. However, it often includes adipocytes, for fat production,

fibroblasts and/or chondrocytes, for connective tissue generation, and endothelial cells, to provide vascularization (Djisalov et al., 2021). To date, cultured meat production is a hotly debated topic with conflicting opinions on its actual feasibility and sustainability. For this reason, the following review aims to discuss the *in vitro* meat production process by highlighting its main advantages and challenges from both a biotechnological and technical perspective.

Cultured meat production process

Cultured meat production follows a precise workflow, which is briefly summarized in Figure 1.

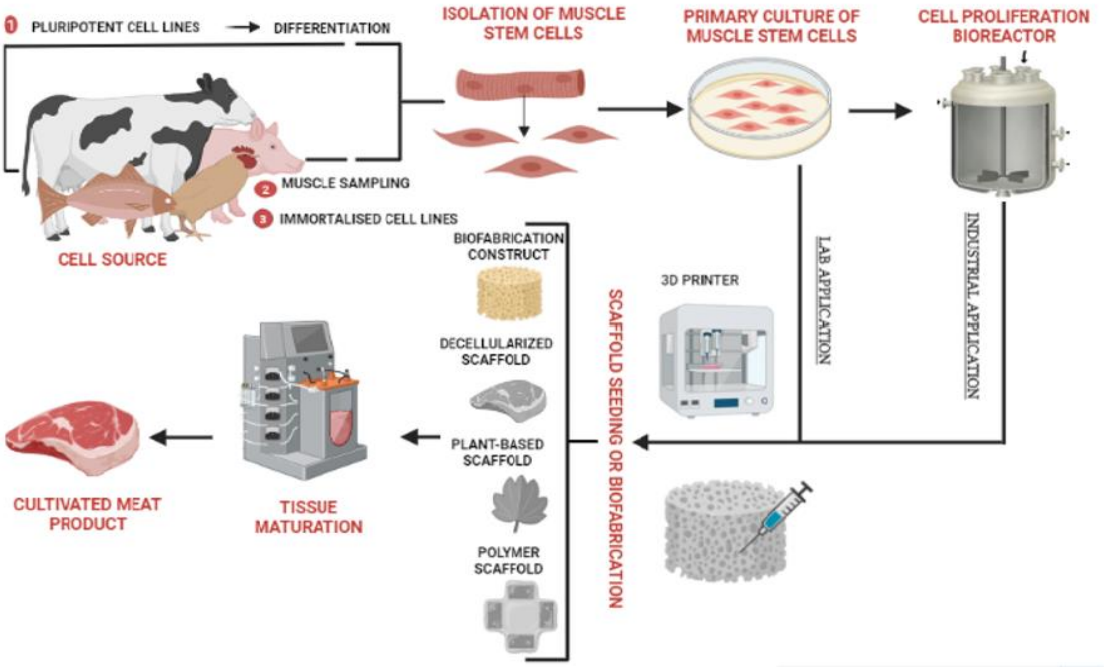

Figure 1. **Cultured meat production process** (Source Biorender).

Cell harvesting. The fundamental step in the production of cultured meat is cell procurement, for which three methods are used. (1) A cell or tissue biopsy is acquired from a living animal or recovered post mortem. The cells acquired in this way are called primary lines. (2) Pluripotent cells, such as embryonic stem cells or induced pluripotent stem cells, are used (Reiss et al., 2021). (3) Immortal cell lines. Primary cell cultures can be used to study the mechanisms of cultured meat production on short time scales, however they only undergo a finite number of divisions before undergoing senescence. Immortal or continuous cell lines, on the other hand, do not undergo senescence and can therefore undergo infinite divisions allowing for safer and more consistent cultured meats without the need for continuous animal biopsies. However, there are currently no immortalised cell lines suitable for cultured meat available to researchers and developers. The closest existing cell lines are the myoblasts of model species commonly used in research, such as mice, rats, hamsters and Japanese quails.

Such cell lines would lack the taste, nutrition and texture of traditional meat. Working with tissue biopsies involves an additional step. It is necessary to isolate individual muscle stem cells from the rest of the fibers, achieved using proteases including trypsin, pronase, dyspase and collagenase. After enzymatic dissociation it is crucial to remove fiber fragments, tissue debris and connective tissues in order to ensure an efficient selection process (Choi et al., 2021). The next step is to maintain the harvested cells in culture in order to first achieve cell proliferation and then cell differentiation and/or maturation with the ultimate goal of increasing biomass.

Proliferation. The cells are initially allowed to proliferate small flasks in which they can grow in two-dimensional sheets, anchored to a surface. This method represents the standard for laboratory cell culturing methods, mainly devoted to research activities. However, for large scale industrial cell production suspension cultures in bioreactors are required to increase the efficiency of the process: these systems require to generate a great number of cells with minimal energy and resource consumption (i.e., culture medium, consumables materials), time and handling steps (Moritz et al., 2015). The current cells culturing method involves the use of specific growth media, which contain all those substances necessary to ensure cell proliferation such as glucose, inorganic salts, amino acids, vitamins, growth factors, antibiotics and antifungals. Other substances can be added to confer organoleptic properties such as proteins or pigments that give a meat-like colour (Ong et al., 2021). However, to date, viable alternatives to classical culture media are being sought, where the goal is to introduce into the medium edible by-products to ensure rapid cell growth. The same principle should be applied to fetal bovine serum (FBS), which is essential for cell proliferation, but not at all ethical and sustainable.

Scaffolding. Tissue fabrication and maturation processes can be accomplished only the cells are provided the correct environment, in which they can adhere, proliferate and differentiate (O'Brien, 2011; Seah et al., 2021). Scaffolds are three-dimensional structures characterized by correct porosity, texture architecture, mechanical and chemical properties suitable for specific cell type maturation. Moreover, considering the food engineering purposes, scaffolds must be biodegradable or edible or both, and their structure is also involved also in the final organoleptic properties of the final product (Seah et al., 2021). The traditional approach, based on tissue engineering, involves scaffold fabrication and subsequent cell seeding in a 3D culture. Scaffolds can be obtained through tissue/organ decellularization or can be fabricated. There are several different techniques for scaffold production. The most used are solvent casting and porogen leaching (SCPL), phase separation, gas foaming, sintering, electrospinning, self-assembly and 3D printing (3DP) techniques (e.g., powder-bed 3D printing, fused deposition modelling, and stereolithography) (O'Brien, 2011).

Biofabrication. Recently, another approach is gaining importance: the biofabrication (O'Brien, 2011). This process enables the production of cell-laden constructs using materials containing mixed cells and biological molecules. The main advantage is the possibility to fabricate thicker structures and to control the spatial arrangement of cells, even of multiple

types, which are the major limitations of the conventional scaffolding methods (Moroni et al., 2018). 3D bioprinting (3DBP) is biofabrication technology based on additive manufacturing (AM) allows precise cell positioning, density deposition, and precise control of the structure, and the ratio amongst different populations in case of multicellular constructs. Cell-materials mixtures used in the process are called bioinks (Handral et al., 2020). 3DBP represent a possible game-changer in the field of the *in vitro* meat manufacturing, assuring high scalability, complexity free process, minimal energy expenditure, and lower N20 emissions (Handral et al., 2020). The major bioprinting methods are extrusion-based, inkjet, stereolithography, and laser- and light-based methods (Derakhshanfar et al., 2018).

Tissue differentiation – bioreactors: Whatever the construct fabrication, cells inside the 3D scaffold must be placed in a bioreactor for maturation. Mechanical, chemical and eventually electrical stimuli are required to complete this stage through dedicated culturing systems (Martin et al., 2004; Rosser and Thomas-Vazquez, 2018). Bioreactors are closed, automated systems in which cells can proliferate, differentiate and maturate within the construct forming the tissue. Bioreactors provide precise control over relevant variables such as temperature, oxygen concentrations, pH, and cell density (Ong et al., 2021). Several types of bioreactors are used, the most common being static culture, spinner flask, and perfusion bioreactors (e.g., stirred tank or rotating wall) (Rosser and Thomas-Vazquez, 2018). Inside the bioreactors, cells go through proliferation and differentiation. The last process is triggered by changing the scaffold and culture media and through the addition of elements such as proteins, amino acids, and minerals (Martin et al., 2004). The lack of cell adhesion and the creation of structures suitable for cell growth are the critical points of this complex procedure.

From laboratory to industrial scale-up: The number of cells required to produce 1 kg of protein from muscle cells is in the range of 2.9×10^{11} to 8×10^{12} (Rosser and Thomas-Vazquez, 2018). To achieve these huge numbers, it is necessary for a cell proliferation stage to occur in large-scale bioreactors (Zidaric et al., 2020), such that cells can grow and replicate in the order of 5000 liters (Post et al., 2020). According to Zidaric et al. (2020), bioreactors will be essential in the industrial production process of cultured meat. Tissue development would require at least two different stages: (i) the cell proliferation phase to provide a sufficient amount of cells for construct fabrication (e.g., using a stirred tank bioreactor), and (ii) the tissue differentiation to a stage (e.g., a porous scaffold placed inside a perfusion bioreactor) which will lead to the final cut of meat providing the proper chemical, mechanical, electrical stimuli (Zidaric et al., 2020; Post et al., 2020). Existing products and processes in the chemical and biomedical industry do not meet requirements for large-scale cell and tissue cultures, mainly in terms of cost, sustainability. According to Specht et al., there is the perspective to develop methods and technologies to achieve the goal of clean *in vitro* meat in the near future (Specht et al., 2018). However, beyond industrial requirements, small-sized laboratory meat cultures too face several common challenges. Cells used for cultured meat production require anchor sites in order to grow and proliferate. A solution to

this problem, especially within 3D printing and bioprinting, is the optimal formulation of biomaterials and bioinks, and mixing products such as polystyrene, gelatine, collagen, or protein-based additives to provide adhesion points for the cells preventing them from remaining suspended within the construct (McKee and Chaudry, 2017). Such anchoring sites are critical to allow cells to grow on scaffolds and structures specifically textured to ensure muscle cell differentiation. Such scaffolds are responsible for ensuring cultured meat products with multiple structural properties, including cell shape and arrangement (Reiss et al., 2021). Scaffolds can be created from many materials, which may be of natural origin (cellulose, decellularized plants, alginate, chitosan, collagen, and gelatine) or synthetic (polyethylene glycol or polyacrylamide) (Ong et al., 2021). To date, a great concern is that animal-derived products are the most suitable and efficient for cell affinity and attachment (McKee and Chaudhry, 2017), but they are inadequate in the view of an animal-free method for the cultured meat process. Moreover, since the final product must be edible, tissue scaffolds, if not edible, must be at least biodegradable and non-toxic or alternatively designed to be removable before consumption (Reiss et al., 2021; Ong et al., 2021). Once it has reached maturity, the final product must be harvested, which adds a step in the process, increasing the complexity of the procedure. This step can be performed using enzymatically, chemically, or manually, with the first two being preferred (Nienow, 2014). Hence, the cultured meat and its industrial process scale-up for commercial purposes is a complex and multidisciplinary matter, that require a synergic effort from the biological, chemical, technical, and industrial field. The main research focus must be the development and improvement of cell lines available to set up cell culture and culture media, bioreactors, cell lines, scaffolding and biofabrication.

Challenges related to cultured meat production

The benefits of cultured meat production are widely discussed in the literature. Cultured meat production would lead to an 89% reduction in water used, a 99% reduction in land used and a 96% reduction in greenhouse gases (GHGs) as a result of moving away from intensive livestock production (Reiss et al., 2021; Melzener et al., 2021). While these aspects are interesting but still controversial, it is also true that there are multiple aspects about *in vitro* meat production that need to be considered, both from a biotechnological and technological points of view (Table 1).

Biotechnological Challenges	Technical Challenges
Choice of animal for cell harvesting	
Choice of site of collection	Scaffold fabrication
Methods for cell harvesting	Alternatives to scaffold fabrication
FBS: ethical challenges	Biofabrication and 3D bioprinting
High cell proliferation and genetic instability	Bioreactors
Nutritional and functional properties of cultured meat	Industrial process scale-up
FBS alternatives	
Food control system for cultured meat	

Table 1. The main biotechnological and technical challenges for cultured meat production.

Biotechnological challenges

Choice of animal for cell harvesting

The first challenge facing in cultured meat production is the choice of animal from which to perform the cell biopsy. The choice should not be random, but must take into account multiple variables including age, sex, and rearing conditions, as they affect the presence or absence of satellite cells (adult skeletal muscle stem cells). As the animal ages, the concentration of satellite cells within the muscle decreases. The fastest decrease in the number of satellite cells occurs most during the first few months post birth. In addition, because satellite cells in younger animals have undergone fewer mitotic cell divisions, they might retain their differentiation capacity for a longer proliferative period (Reiss et al., 2021; Melzener et al., 2021). The sex of the animals is another factor involved in the proliferation of muscle stem cells. Sex hormones such as estrogen and testosterone are able to influence cell growth. Compared with female animals, male animals are characterized by a higher content and activity of satellite cells, thanks to the positive influence of testosterone (Choi et al., 2021). This beneficial action is also underlined by Mulvaney et al. (1988), who demonstrated that castrated animals presented a lower concentration of activity of satellite cells than non-castrated ones, a trend reversed with the administration of testosterone propionate. Animal rearing conditions also play an important role in the composition of muscle fibers. Vestergaard et al. showed that extensively reared animals had a higher number of type I muscle fibers but also a reduction in type II muscle fibers compared to intensively reared animals. This difference, according to the authors, is due to the different diets to which the animals are subjected; more precisely, to the amount of roughage consumed, which is higher in animals reared under extensive conditions (Vestergaard et al., 2000).

Choice of site of collection

Another fundamental parameter to consider concerns the biopsy site. The concentration of satellite cells varies between muscles or muscle groups. It has been shown that type I, slow-twitch fibers are characterized by a higher number of satellite cells in contrast to type II fast-twitch, muscle fibers, in which the concentration is lower [30]. More specifically, in cattle, muscles belonging to the chuck contain primarily type I fibers, while those of the round contain predominantly type II (Melzener et al., 2021).

Methods for cell harvesting

Another factor to be considered is the method of taking the cell biopsy. Satellite cells can be harvested either at the time of slaughter of the animal, which is not an accepted route for cellular agriculture, or through biopsies of muscle tissue. Tissue biopsy, a procedure widely used within veterinary medicine, involves the use of needle biopsy. Although this technique is quick and causes little stress to the animal, it allows a limited sample to be taken, around 0.5 g, and is not very precise due to the blind nature of the sample (Melzener et al., 2021). A second option involves a small incision at the sampling site, allowing more sampling, around 15 g, and greater success, although it is characterised by greater invasiveness for the animal

(Melzener et al., 2021). To reduce the number of donors required for cultured meat production, it is desirable to maximise the number of biopsies taken from each animal, considering the levels of stress and discomfort caused. As reported by Melzener et al. (2021), a possible approach to harvesting muscle biopsies for cultured meat production could involve taking multiple biopsies (up to four) from each donor animal in one session every three months, using the needle biopsy technique, thus ensuring appropriate recovery times for animal welfare (Melzener et al., 2021).

Fetal Bovine Serum: ethical challenges

One of the cornerstones of cellular agriculture is to ensure the sustainability of the production process. Today, almost all cell cultures involve the addition of FBS to the culture media to ensure optimal growth. Fetal Bovine Serum is an extremely complex mixture that, in addition to providing a large number of constituents such as fatty acids, lipids, vitamins, carbohydrates, inorganic salts, proteins (more than 1800) and more than 400 metabolites, provides essential hormonal factors for cell growth and proliferation. In parallel with the nutritive function, FBS ensures adhesion and diffusion factors that act as germination points for cell attachment. In addition, it allows minimizing physical damage caused by pipette handling and agitation (Brunner et al., 2010; Subbiahanadar et al., 2021). Although the positive aspects of the use of FBS are widely demonstrated and discussed in the literature, it is also true that it presents multiple problems related to sustainability and ethicality by clashing with the basic principle of cellular agriculture. The collection of FBS has always caused a stir. When a pregnant cow is slaughtered, the fetus is removed and a cardiac puncture is made in the still-beating heart to collect serum under the most aseptic conditions possible, causing first enormous suffering and then death (Brunner et al., 2010). It is essential that foetuses are at least 3 months old to ensure anatomical formation of the heart to ensure perfect serum collection (Jochems et al., 2002). The exact amount of FBS produced and sold in the world is unknown. However, it is estimated that approximately 800 thousand liters of FBS is sold annually, which translates into approximately 2 million foetuses sacrificed (Subbiahanadar et al., 2021). However, these numbers are expected to increase due to an increasing use of cell cultures for drug discovery, recombinant proteins, vaccines, and therapeutic diagnostic treatments (Paradkar et al., 2019). From a process sustainability perspective, the FBS market is highly dynamic, leading to continuous price fluctuation making it unsustainable on a large scale (Kolkmann et al., 2020). Price and availability fluctuate due to changes in the number and cost of cattle raised worldwide, import regulations, beef and dairy prices, costs, and weather conditions (Brunner et al., 2010).

FBS being an animal derivative is characterized by qualitative and quantitative differences dependent on the batch to which it belongs and therefore on the animal used for sampling, which makes it necessary to test the product before its use (Honn et al., 1975). In addition to issues regarding variability, serum may contain varying amounts of endotoxins, hemoglobins, and other factors adverse to cell growth, as well as be a potential sources of microbial contaminants such as fungi, bacteria, mycoplasma, viruses, or prions introduces

during the sampling phase (Brunner et al., 2010). In addition, many other substances within FBS are still unknown, which prevents us from studying and knowing the possible effects on cell viability (Anderson, 1980). Therefore, it is necessary to find viable alternatives to FBS to support the large-scale production of cultured meat.

High cell proliferation and genetic instability

Cultured meat production involves the processing of cells characterized by a high proliferative capacity. However, there is always a possibility of genetic instability that can lead to the formation of cancerous cells within the culture without being clearly identified. These cells, although harmless, as they are dead at the time of meat consumption and therefore not incorporated alive inside the body, represent a great challenge of acceptance for the consumer (because the cells are subsequently digested inside the stomach), which is why this process must be further investigated and studied to ensure the total absence of risks (Hocquette, 2016).

Nutritional and Functional properties of cultured meat

One of the greatest challenges for cultured meat is matching the nutritional, functional, and organoleptic properties typical of conventional meat. The ultimate goal of cultured meat production is to create a product that is as close as possible to the original one. However to date, we are far from achieving this. As far as the consistency of the final product is concerned, it cannot be similar to that of the original meat, which become tender only after the animal is slaughtered, when the supply of oxygen ceases, triggering multiple biochemical changes that lead to the formation of lactic acid, responsible for the reduction of pH, which activates different families of enzymes necessary for the breakdown of muscle proteins and the subsequent tenderization of the meat. This process is referred to as maturation and to date, it is a less considered problem in literature but not less important to be deepened with further studies (Hocquette, 2016; Balasubramanian et al., 2021). Another discordant characteristic is the color of the final product. Meat color varies according to two basic parameters: myoglobin and iron concentration. The color of artificially produced fibers is yellow, distant from the pink/red color of the original product (Hocquette, 2016). This discordance occurs because myoglobin is repressed by cultured cells in the presence of oxygen and because commonly used culture media such as IMDM, RPMI1640 and DMEM contain minimal iron content. This problem can be addressed if the media is supplemented with iron, but this supplementation remains limited (Hocquette, 2016; Balasubramanian et al., 2021; Rubio et al., 2019). A further problem relates to taste. Many of the biochemical metabolites present in conventional meat are net products of food intake and biological metabolism, but are not derived from the muscle itself (Balasubramanian et al., 2021). Furthermore, animal meat is the result of complex interaction of proteins, carbohydrates, flavors of the lipid fraction, nerves, and blood vessels that give the product its characteristic final taste (Hocquette, 2016). Research groups are developing co-cultures with fat cells in order to achieve this goal, as well as to provide micronutrients such as vitamin B12, essential for human health and is easily introduced within the diet through meat consumption, but

would risk being lost with the production of cultured meat (Hocquette, 2016). Over the years, intensive breeding has undergone profound changes, helping obtain a safe, nutritious, quality products for the consumer. Red meat, in fact, is considered a high source of protein which provides about 20-24 g of protein per 100 g. This value, together with the fat content, guarantees a high energy intake. The latter is the main source of energy in the human diet and its content varies according to the type of meat considered. The profile of fatty acids in red meat varies according to the proportions of lean meat and fat present. The former is richer in polyunsaturated fatty acids (PUFA), whereas fat is characterized by a high saturated fatty acids (SFA) content, containing about 37 g of SFA per 100 g of meat. Overall, lean red meat contains similar proportions of monounsaturated fatty acids (MUFAs) and SFAs, although the exact proportions vary depending on the type of meat. The main SFAs found in red meat are palmitic acid (C16:0) (about half) and stearic acid (C18:0) (about one-third). While the former appears to increase blood cholesterol levels, the latter has a neutral effect on total and LDL cholesterol. Red meat also contains smaller amounts of myristic acid (C14:0) and lauric acid (C12:0), which are thought to raise blood cholesterol more potently than palmitic acid. In addition, although it contains low levels of PUFAs, red meat forms a substantial part of the diet providing 18% of n-6 PUFAs (linoleic acid) and 17% of n-3 PUFAs (α-linoleic acid), contributing about 23% of total fat intake (Calder, 2019). Therefore, cultured meat must include these characteristics in order to be a nutritionally competitive product. Finally, it is difficult to think that in the near future there may be a supply of *in vitro* meat such that consumers are offered a variety of muscles or cuts of the animals. In fact, the sensory quality of meat differs between species, breeds, genera and types of animals, as well as the conditions under which they are raised (Chriki and Hocquette, 2020).

FBS alternatives

As previously reported, to comply with the principles of cellular agriculture, it is necessary to find reliable alternatives to FBS for cell cultivation that guarantee sustainability and ethical development. Several studies have been performed to meet the demand for edible FBS alternative. However, most of the studies in the literature seems to be in conflict with the principles of cellular agriculture, as they applied animal-based alternatives to FBS such as foetal serum from other species (e.g., goat) or other animal by-products (e.g., bovine ocular fluid), which, although very efficient, are characterised by the same problems as FBS (Subbiahanadar et al., 2021; Paranjape, 2004). Similarly, human platelet lysate has also been considered, due to its ability to promote the proliferation of stem cells derived from human adipose tissue, but being human-derived it is not suitable for consumption (Brunner et al., 2010; Guiotto et al., 2020). When working with cell culture the use of chemically defined media (recombinant protein and growth factors) is a common practice. These, although ethical and suitable for human consumption, are characterised by a high cost that makes them unsuitable for large-scale application. It is therefore essential to study innovative matrices that, when added to the culture medium, can sustain cell proliferation and viability in both the short and long term, thus ensuring the sustainability and ethicality of the production process. Possible matrices tested on the proliferation of different cell culture are

those reported by Ho et al. (2021) (Table 2). However, although most of them may be good candidates as alternative to FBS due to their high proliferative capacity on cell, they need to be discussed in terms of sustainability and ethics.

Table 2. Applications and analysis of different matrices in cell cultures. Modified by Ho et al. (2021).

Matrices	Cell type	Effects	Ref
Plant peptone	CHO-320 (CHO K1 clone)	Improved cultivation and productivity of human interferon gamma	Burteau et al. (2003)
Yeast hydrolysate	CHO, rCHO	Higher productivity of human beta interferon; higher cell growth	Spearman et al. (2014); Sung et al. (2004)
Rice protein hydrolysate	CHO-320, Human HepG cells	Protection against oxidation stress from hydrogen peroxide	Mols et al. (2004) Wang et al. (2016)
Soy peptone	CHO DG44	Improved cell production	Davami et al. (2015)
Wheat hydrolysates	CHO	Improved cell viability	Ho et al. (2016)
Marine cyanobacterium S.M.	Human Lung Carcinoma	Improved cell viability and proliferation	Jeong et al (2016)
Chlorella vulgaris extract	CHO-K1 and MSC	Promoted cell growth	Ng et al. (2020)
Rapeseed cake	CHO-C5	Promoted cell growth	Chabanon et al. (2007)
Silk sericin hydrolysate	CHO and Hela cells	Improved cell growth and proliferation	Zhang et al. (2019)
Whey protein	CHO K1, JURKAT E6.1	Improved cell viability and proliferation	Pradkar et al. (2019)

The matrices shown in Table 2 are those that achieved promising results in cell proliferation and viability. Most of these are of plant origin (plant peptones, rice, soy wheat, Marine cyanobacterium Spirulina *maxima*, Chlorella vulgaris, and rapeseed), in light with the principle of cellular agriculture. Particular attention should be focused on hydrolysates. Their relatively low cost makes them very attractive as FBS replacement components. However, as hydrolysate products are not fully characterised, further understanding of their components and the mechanism by which they influence cell growth and maintenance is crucial to their large scale application (Ho et al., 2021).

Sericin is a macromolecular, globular, biodegradable and biocompatible protein produced within the central gland of silkworms. It is obtained by degumming the cocoon of *Bombyx mori.* For a long time, it was considered a waste products of silk processing, until researchers explored its potential within pharmaceutical, biomedical and cosmetic application (Subbiahanadar et al., 2021; Suryawanshi et al., 2020). In addition to these fields, sericin finds application as an edible coating material within food products due to its ability to retard oxidative activity to polyphenols damage, opening an interesting debate on its possible use as an alternative to FBS in the maintenance of cell culture (Thongsook et al., 2011). Being an animal derived product, its use would not fully satisfy the requirements of ethics and

edibility. However, it must be reiterated that this is a waste product of the silk-processing industry and its use within a different sector can meet the circular economy principle.

Whey protein, is one of the main components of milk. Specifically, it is a by-product of the dairy industry characterized by a high biological value thanks to its antioxidant, anti-inflammatory, antiviral and antitumor properties, expressed both when consumed individually and as an additive in other foods (Veskoukis et al., 2020; Smither, 2008; Kerasioti et al., 2012). These functions can be attributed to its excellent nutritional composition; it consists mainly of α-lactalbumin, albumin, β-galactoglobulin, and immunoglobulin (Veskoukis et al., 2020). For these reasons, it has been investigated as a substitute for FBS within culture mediums. Again, this is a product of animal origin. However, it is a waste product with a high environmental pollution load as reported by Veskoukis et al. (2020). Indeed, it has been calculated that its polluting potential is equal to a biochemical oxygen demand approximately 175-fold higher than the sewage system of modern cities. Therefore, it causes serious environmental problems when discarded (Veskoukis et al., 2020). For this reason, although it is of animal origin, its use as an alternative to FBS might be considered. Moreover, its production is high; this would make it possible to meet the high market demand typical of FBS. Overall, it is of paramount importance to find suitable alternative of FBS, also considering the combined use of plant-based products (e.g., those reported in table 2). However, due to the high variability in their chemical composition and mechanism of action of these matrices, it is necessary to investigate the effects of their combination on cell cultures.

Food control system for cultured meat

A fundamental aspect within any production is monitoring along the entire production chain. As reported by Chriki and Hocquette (2020), there has been much discussion about the safety standards of cultured meat. Proponents of *in vitro* meat consider it a much safer product than conventional one, due to the fact that it is produced in a closed and controlled environment with no possible contact with external pathogens.

This aspect plays a key role, especially during the slaughter process of the animal, where pathogenic intestinal bacteria such as E. coli, Salmonella or Campylobacter can contaminate meat, that is subsequently marketed. However, the objective of completely eliminating possible risks throughout the production chain is difficult to achieve, and so it is necessary to adopt appropriate controls to identify these risks before marketing the product. At the same time, it is indisputable that, although food disease episodes occur, every year the surveillance of the meat production chain increases its quality standards, ensuring increasingly safe products. Therefore, production in a closed and controlled environment, combined with surveillance along the entire supply chain, characteristic of conventional production, would make cultured meat production a possible safe product.

Technical challenges in cultured meat production

The main technical challenge in producing cultured meat is the replicating the 3D environment of real muscles, in which cells can maturate in a laboratory or a factory to mimic the tissue. This complex process involves a great number of tasks and unsolved problems, which can be globally aggregated at a higher hierarchical level into three main categories: scaffolds, biomaterials or bio-inks and their interaction with cells, fabrication procedures, and culture processes for cell proliferation and differentiation techniques (Stephens et al., 2018; Post, 2014). From a technical point of view, well-established methods and processes from tissue engineering and regenerative medicine are used as after adapted to the specific purpose (Post, 2014). In the following sections, conventional scaffolding to cutting-edge technologies, such as bioprinting, are presented, in terms of their current state and perspectives. Moreover, the most common bioreactors as culture systems are described.

Scaffold fabrication

A scaffold can have a porous, tubular, or tissue-like structure. The most important parameters are porosity and material composition. The type and structure of the scaffold depend on the specific application for which it is designed. However, the general requirements to be fulfilled are essentially two: allow cells to adhere and allow material transport through its structure. The following sections present several methods for fabricating porous scaffold, divided into conventional (Figure 2) and non-conventional fabrication techniques.

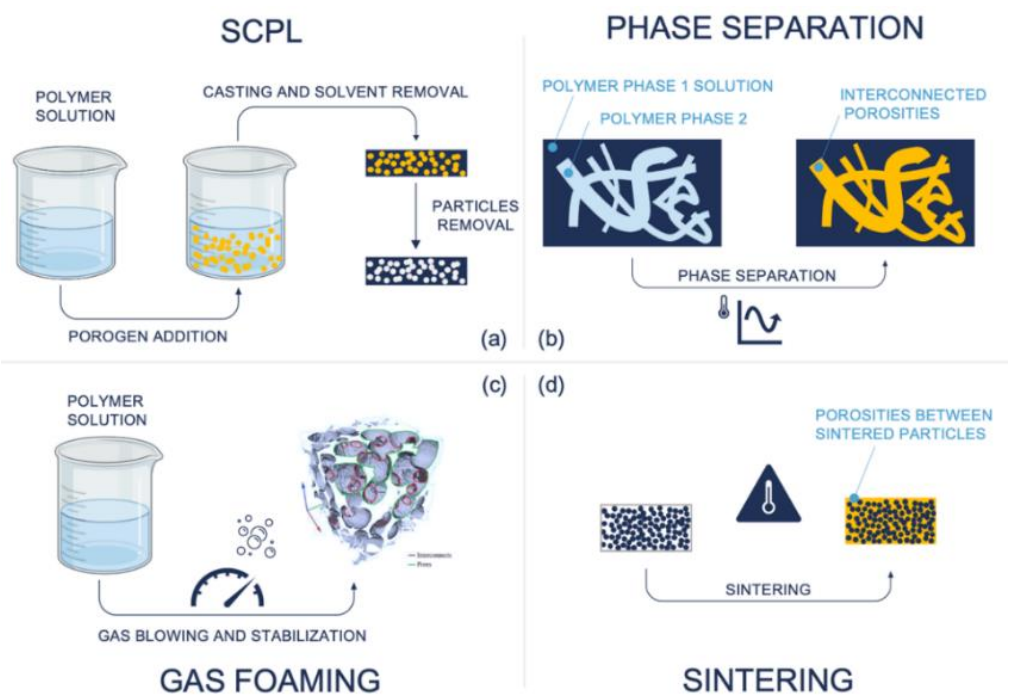

Figure 2. **Conventional porous scaffold fabrication technologies process.** Representation and example of scaffold, respectively (a) SCPL, (b) phase separation, (c) gas foaming, (d) sintering.

Conventional porous scaffold fabrication technologies

- **Solvent casting and porogen leaching (SCPL**) (Mikos et al., 1994): This process involves mixing a polymer solution dissolved in an organic solvent composed of insoluble particulates (porogen). The mixture is then cast into a mold or a membrane and the solvent is evaporated. Finally, the structure is immersed in an aqueous solution to leach out particulates in the structure. Porosity, in terms of shape, size and uniformity depend on the particulate choice of particulates, typically salt particles. The main drawbacks are the lack of control of internal architecture and uniformity, reduced reproducibility, formation of a skin layer due to polymer thickening that can limit access to internal porous, limited thickness (2-3 mm) (Prasad et al., 2017), weak mechanical properties, possible cytotoxicity due to residual solvent and porogen (Mikos et al., 1992).
- **Phase separation** (Martinez-Perez et al., 2011): The technique is used to produce a scaffold through the separation of a mixture into two phases, a polymer-rich one, and a polymer-poor. This is achieved under thermodynamically unstable conditions. For example, cooling the solution below the freezing point of the solver, induces crystal nucleation inside the solution; after that, the solid material is sublimed, ensuring that the structure is composed of only the polymer-poor region with porosity, because the solvent and the polymer-rich phase are evacuated from the scaffold. This technique leads to highly interconnected porosity which can be used to reproduce channel-like structures by applying a directional temperature gradient. Nevertheless, control and optimization of process parameters (e.g., temperature, polymer concentration, surfactants use, crystal nucleation) are the main problems in managing pore size and distribution (Akbarzadeh and Yousefi, 2011). Moreover, the typical pore size achievable is often smaller than the typical dimension in tissue engineering applications (< 200 μm).
- **Gas foaming** (Mooney et al., 1996): This is a class of techniques for scaffold fabrication exploiting a blowing agent to generate gas inside the material which acts as a porogen agent. The main advantage is the absence of solvents or porogen materials, which can induce cytotoxicity due to possible residuals. The Gas formation can be induced chemically or thermally or by pressure change. The main drawbacks of the technique are: low control over pore size and interconnectivity, low reproducibility and structural uniformity, and difficulty in incorporating biological molecules in thermally induced processes (Costantini and Barbetta, 2018; Dehghani and Annabi, 2011).
- **Sintering** (Gibson and Ashby, 1999): The technique is used to produce cohesive porous scaffolds through bonding of a polymeric phase and ceramic particles or fibers. The usual procedures involve a bed of randomly packed particles bonded through heating up to a temperature above the glass transition temperature of the base material, but lower than its melting point, creating a local fuse area only in the contact surfaces, leading to a porous microstructure. Alternative sintering modes are mild solvent treatment and pressure. Sintered scaffolds are characterized by lower porosity, small pore size with difficulty in precise control and distribution, and higher mechanical properties and it is used mainly in dental and bone repairing applications (Pilliar et al., 2001).

Non-conventional porous scaffold fabrication technologies

- **Electrospinning** (Braghirolli et al., 2014): The method is based on an electric field generated between a polymer solution delivery system at a controlled flow rate and a collector, drawing the solution into a fiber. The result is a membrane of non-woven fibers. The textile-based technique has been created to reproduce fiber-based materials, such as those similar to the extracellular matrix (ECM). The resulting porosity is interconnected and the achievable pore size is lower than that by other scaffolding techniques, achieving fibers with diameter up to a few nanometres (Braghirolli et al., 2014), which can be an advantage for specific applications (e.g., vascular (Hobson et al., 2015)), but tends to limits the cell migration to a point where its applicability in tissue engineering becomes a problem. Several process parameters can be controlled to tune fiber diameter and alignment, adapting textural properties to the specific cell type to be seeded.

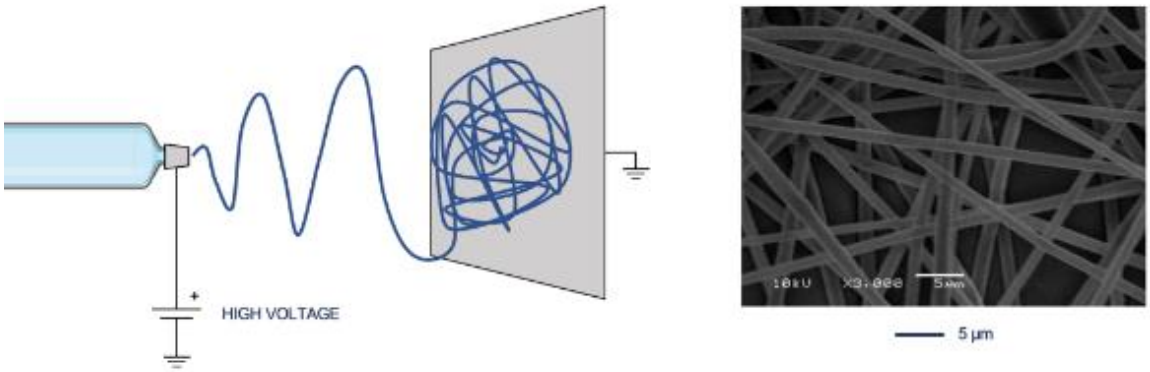

Figure 3. Illustrative example of the electrospinning method for scaffold fabrication and image of construct. Adapted from Sola et al. (2018).

- **Self-assembly** (Hartgerink et al., 2002): The technique involves specifically designed amphiphilic peptides with the capacity to spontaneously organize into ordered structures, including nanofibers. The method allows a great process control starting from the building blocks in a bottom-up design approach for tissue engineering application.
- **Hybrid scaffolds:** With the aim of controlling structural and composition features, mainly porosity, at different length-scales, several approaches with mixed techniques are used, such as, SCPL and electrospinning combination (Steele et al., 2014), multilayer electrospun composites with different parameters (McCullen et al., 2012), and a combination of more than two fabrication techniques (Vaquette and Cooper-White, 2013; Salerno, 2011).
- **Additive manufacturing (AM):** The conventional techniques of porous scaffolds fabrication, as well as other emerging alternatives, are implemented to produce scaffolds to recreate the complex micro and macrostructures of biological tissues. However, all of them have limitations and allow narrow control over important textural parameters such as pore shape, dimensions, and interconnectivity (Rey and Pierre, 2019). An emerging family of technologies, based on additive AM techniques, has proved to enable the

manufacture and control of complex shapes. AM, popularly called 3D printing, is a generic definition and represents a large group of processes that can be classified in several ways (Badylak et al., 1995). Within the porous scaffold fabrication context, according to Rey and Pierre (2019), some of the AM technologies could be used to produce scaffold with a high spatial resolution, the structural complexity, and control over the internal pore architecture. The most promising techniques are: powder-bed based 3DP, such as selective laser sintering (SLS), and liquid raw-material-based 3DP, such as stereolithography (SLA) (Rey and Pierre, 2019).

Alternatives to scaffold fabrication

Tissue Decellularization*:* An alternative approach to engineered scaffold is plant or tissue/organ decellularization. This approach is based on the removal of resident cells and a large proportion of the major histocompatibility complex from a tissue, in order to obtain a natural scaffold to be seeded. In this way, the ECM structure is preserved (Badylak et al., 1995). There are several successful examples in tissue engineering regeneration that follow the so-called "like-to-like" strategy, in which donor and regenerated tissues are of the same type (Rey and Pierre, 2019). Nevertheless, in case of tissue/organ decellularization, the tissue procurement would require the use of animal-derived tissues, completely in contrast with the fundamental prerequisite of cultured meat.

Microcarrier cultures: Microcarriers are beads with typical dimensions between 100 and 200 µm, and they represent a possible solution because mammalian cells require a surface on which they can grow (Moritz et al., 2015). For tissue engineering applications, especially for food products, microcarrier-based systems are retained as the major culturing system to achieve a high volume of cells because they can provide a large surface area per unit volume of medium (Verbruggen et al., 2018). Verbruggen et al. (2018) proposed a myoblast cell production system based on microcarriers suspended within cells and medium inside a stirred tank bioreactor, achieving promising results in terms of cell growth for efficient and cost-effective development of cultured meat. Bodiou et al. (2020), provided three scenarios based on cultures with microcarriers: temporary microcarriers culture for proliferation, non-edible but degradable microcarriers, and edible microcarriers embedded in the final product. According to the author, the third is the most promising for cultured meat production (Bodiou et al., 2020). Beyond the great possibilities, the main drawback of the use of microcarriers or aggregates is that the cells may form clusters do not proliferate in the correct manner, because of which, if not modified, the cell proliferation phase would be difficult to control (Moritz et al., 2015).

Biofabrication and 3D Bioprinting

Biofabrication refers to the production of complex biological products combining cells, matrixes, biomaterials, and biomolecules, especially for tissue engineering, regenerative medicine, and food engineering. This emerging field has been highly stimulated by the development of AM-based technologies (Tenenhaus et al., 2016). Direct 3D printing of

biological material, including cells, is defined as 3D bioprinting. The main challenge is to adapt technologies developed to molten plastics and metals to work with sensitive, soft, and biological materials (bioinks). The central objective is to reproduce the complex micro-architecture of the ECM better than other methods and have higher control on cell density and deposition (Murphy and Atala, 2014). The main drawback of the scaffolding techniques biofabrication aims to overcome is the limited cell migration inside porous scaffolds. According to Sachlos and Czernuszka (2003), cells do not necessarily recognise the scaffold surface and, most importantly, they do not migrate more than 500 µm from the surface. There are several 3DBP strategies, characterized by different features, used to biofabricate 3D cell-laden structures. The most diffused methods are extrusion, inkjet and stereolithogaphy-based bioprinting, while hydrogels are commonly used as bioink base materials (Zhu et al., 2016).

3D Bioprinting strategies

There are different bioprinting strategies, each with its own pros and cons (provided in Table 3¸Figure 4 provides a graphical representation of the most common methods or those of higher research). According to Vijayavenkataraman et al. (2018), no one method could be excursively used to achieve the goal of biofabrication of complex tissue, the current trend being research in the development of hybrid methods.

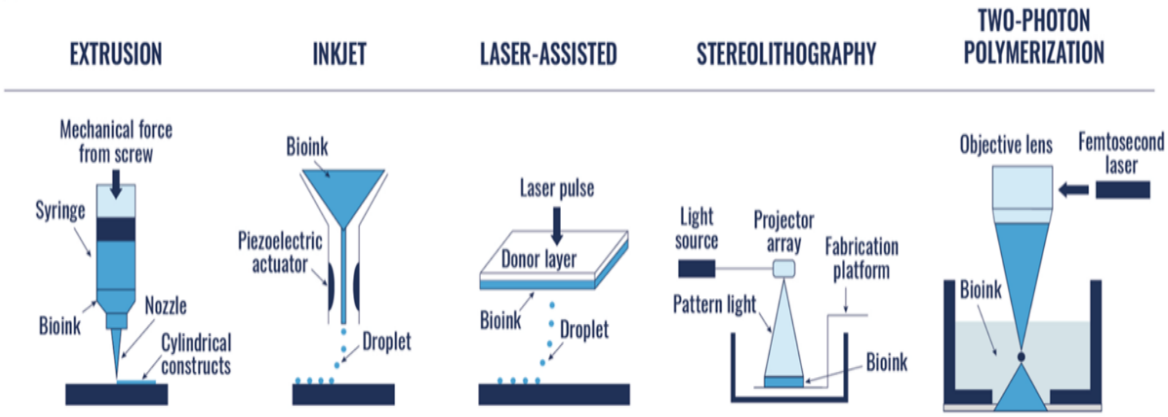

Figure 4. Different 3D bioprinting techniques, adapted from Santoni et al. (2021).

<u>Extrusion bioprinting:</u> The most common 3DBP method is the extrusion-based one, mainly because it is versatile, and affordable and several entry-level bioprinters are available on the market [94]. Kang et al. for example, successfully used it to bioprint meat like constructs (Kang et al., 2021). Extrusion bioprinting usually relies on a dispensing system, such as a syringe with an appropriate nozzle or needle, installed on a print-head used to precisely deposit bioinks on a print bed. The dispensing system can be pressure, piston, or screw driven: the first allows better cell viability, but with low control on material flow rate and shape fidelity (Murphy and Atala, 2014; Zhu et al., 2016). The main advantages of the extrusion-based method are scalability, printability of a wide range of materials of high viscosity, and high cell concentration (Vijayavenkataraman et al., 2018).

Nevertheless, the resolution is the lowest if compared with the other methods and it is related to the nozzle diameter, in that reduction is limited by the consequent cell viability drop (Vijayavenkataraman et al., 2018). Moreover, the post-printing cell viability depends on the bioink viscosity and cell concentration (Chang et al., 2008). Further drawbacks are nozzle-clogging and the limitation due to material rheology: only bioinks with shear-thinning property can be used (Vijayavenkataraman et al., 2018).

Table 3. **Comparison of major bioprinting methods considering several properties, advantages and drawbacks, and main applications**. Table adapted from Derakhshanfar et al. (2018); Vijayavenkataraman et al. (2018); Santoni et al. (2021).

Properties	Extrusion	Inkjet	Laser-Assisted	Stereolithography	Two-Photon
Speed	Slow	Fast	Medium	Fast	Very fast
Cost	Moderate	Low	High	Low	Very high
Cell viability	85-95%	80-95%	<85%	25-90%	>80%
Cell density	High	Low	Medium	Medium	Medium
Scalability	High	High	Low	Medium-high	-
Resolution	100-500 µm	100-500 µm	20-100 µm	20-100 µm	0.1-10 µm
Bioink viscosity	6-30x10^7 mPa·s	< 10 mPa·s	1-300 mPa·s	No limitation	No limitation
Advantages	Simple, capable of printing various biomaterials, high cell densities	Ability to print low viscosity biomaterials, fast fabrication speed, low cost, and a high resolution	Has a high resolution, is nozzle-free, and can deposit biomaterials in solid or liquid phase	Is nozzle-free, has high complexity, and has a high resolution	Is nozzle-free, has a high complexity, has the highest resolution, and has high cell viability
Drawbacks	Only for viscous liquids, resolution	Limited to low viscous fluids, resolution, cell density	High cost, thermal damage due to nanosecond/femtosecond laser irritation, scalability, cost	Lack of printing multi-cells, cell damage during photo-curing	Lack of printing multi-cells, cost
Applications	Tissue models for cell research, drug testing and regenerative medicine, meat-analogue constructs	Supplementary to other technologies	Precise cells deposition	Scaffolds and complex structures with channels	Vascularised and high precision models

Inkjet bioprinting: A liquid droplet dispensing system, based on temperature or piezoelectric driving technology is used. Due to the nature of the process, inkjet bioprinting is characterized by high speed and resolution, and associated to lower costs. However, it can be used only with low viscosity materials, and it is strongly limited by the nozzle-clogging mechanism. Moreover, to enhance and facilitate droplet formation, low cell concentration is allowed (Murphy and Atala. 2014; Vijayavenkataraman et al., 2018).

Stereolithography and two-photon polymerization-based bioprinting: Stereolithography is based on the polymerization of light-sensitive polymers; UV or visible lights can be used to

photo-cure the material in a layer-by-layer manner (Derakhshanfar et al., 2018). It is a nozzle-free method, and so does not face the problem of nozzle clogging, which represents a great limitation in the previously explained methods. Moreover, it allows obtaining very high resolution and high printing velocity. However, only light-sensitive polymers can be used and the UV lights, as well as UV-activated photo-initiators, can damage cells and consistently reduce post-printing viability (Derakhshanfar et al., 2018; Vijayavenkataraman et al., 2018; Chang et al., 2008; Bejoy et al., 2021). On the basis of this technology, digital light processing (DLP) has been developed to photo-cure polymers. The method uses a projector emitting visible light, to overcome problems due to UV irradiation (Bejoy et al., 2021). With the advent of two-photon polymerization-based stereolithography nanoscale resolution is achieved, the highest spatial resolution among biofabrication techniques but with costly systems (Vijayavenkataraman et al., 2018; Lee et al., 2008).

Laser-assisted bioprinting: The method is based on the laser-induced transfer principle (Barron et al., 2004): it uses a pulsed laser beam that acts, through a focusing system, on a ribbon composed by a donor transport support material covered by a laser-energy-absorbing and a biological material layer. The focused laser pulses on the absorbing layer generates high-pressure bubbles that propel the material against a collector substrate facing the ribbon. LAB is a nozzle-free technology capable of high speed and high cell density bioprinting. However, it is less common than inkjet and extrusion, mainly due to the costs of the technology and the difficulty to produce ribbons. Moreover, it is difficult to scale-up for high-volume production (Murphy and Atala, 2014).

Bioink formulation

Bioink formulation is one of the most important branches in bioprinting research, with about 25% of the overall publications in the field (Santoni et al., 2021). These materials are typically based on cytocompatible hydrogels and they must have several key properties both from a mechanical (e.g., viscosity, printability, and stiffness) and biological (e.g., cytocompatibility, and cell-material interaction) point of view (Holzl et al., 2016).

Hydrogels are a class of hydrophilic polymers that can be cross-linked forming a 3D network capable of absorbing and holding a large quantity of water (even upward of 100 times their dry weight). This property allows the polymers to reach the hydration levels found in most tissues. Moreover, the porous network allows for a great degree of diffusion of nutrients and wastes out of the material. These hydrogels can be chemically stable or they may degrade and dissolve (Hoffman, 2002). Hence, they offer the opportunity to recreate engineered microenvironments suitable for cells, mimicking the natural ECM properties and native cellular niche, important for tissue regeneration (Rey and Pierre, 2019). Hydrogels can be formed using natural biopolymers, synthetic biopolymers or combinations of the two. The most common have been formed using proteins (such as collagen, elastin and fibrin), polysaccharides (alginate, agarose, chitin/chitosan, etc.), and synthetic polymers (such as polyethylene glycol, polyvinyl alcohol, polyacrylamide, and polylactic acid) (Rey and

Pierre, 2019). A more complete framework of materials used for hydrogel fabrication is provided in Table 4.

Table 4. **Hydrophilic polymers used to synthesize hydrogel matrices**. Adapted from Hoffman et al. (2002).

Natural Polymers	Synthetic Polymers	Combination of Natural and Synthetic Polymers
Anionic polymers: HA, alginic acid, pectin, carrageenan, chondroitin sulfate, dextran sulfate *Cationic polymers:* chitosan, polylysine *Amphipathic polymers:* collagen (and gelatin), carboxymethyl chitin, fibrin *Neutral polymers:* dextran, agarose, pullulan	*Polyesters*: PEG-PLA-PEG, PEG-PLGA-PEG, PEG-PCL-PEG, PLA-PEG-PLA, PHB, P(PF-co-EG) ± acrylates P(PEG/PBO terephthalate) *Other polymers:* PEG-bis-(PLA-acrylate), PEG±CDs, PEG-g-P(AAm-co-Vamine), PAAm, P(NIPAAm-co-AAc), P(NIPAAm-co-EMA), PVAc/PVA, PNVP, P(MMA-co-HEMA), P(AN-co-allyl sulfo- nate), P(biscarboxy-phenoxy phosphazene), P(GEMA-sulfate)	P(PEG-co-peptides), alginate-g-(PEO-PPO-PEO), P(PLGA-co-serine), collagen-acrylate, alginate-acrylate, P(HPMA-g-peptide), P(HEMA/Matrigel®), HA-g-NIPAAm, GelMA

The main advantage of natural polymers is the higher cytocompatibility and presence of recognizable biological moieties (typically only from animal sources), that can act as signals and modulate cellular responses such as attachment, proliferation, and differentiation. However, they are affected by batch-to-batch variability, they often require stringent extraction and purification protocols and they face procurement problems related to sustainability and availability (Post et al., 2020; Rey and Pierre, 2019). Moreover, those from animal-derived source are useful just for research activities, but they are inadequate for large-scale cultured meat production due to their source, synthetic polymers potentially can reach higher reproducibility and uniformity in mechanical and rheological behaviours, with high controllability of physical properties. In addition to these advantages, they have the worst biological behaviour, lacking in moieties to interact with cells and create an appropriate environment. Thus, several combinations have been formulated to combine the properties of the two classes. Alternatively, functionalization processes are applied to synthetic polymers in order to enhance cell adhesion (Hoffman et al., 2002; Rey and Pierre, 2019). As an example, Chaudhuri et al. (2015) show the beneficial effect of alginate modification with the peptide motif RGD (arginine-glycin-aspartic acid), an integrin-binding ligand. Integrins are transmembrane receptors of cells and turned out to be fundamental in tissue engineering because they activate signal transduction and regulate the cell cycle, including cell spreading, migration, guidance, proliferation, and apoptosis (Chaudhuri et al., 2015; Humphries et al., 2007). The network of the hydrogel is formed via cross-linking (fixation or gelation) with hydrogel precursor polymers. This can be carried out before, during, or

after the 3D printing and it is fundamental to preserving shape and structural integrity and avoiding collapse. The gelation mechanisms can be divided into two main categories: chemical and physical. Typically, physical cross-linking is a reversible process but associated with poor mechanical stability, while chemical reagents are able to increase the mechanical stability by creating covalent crosslinks (Holzl et al., 2016). Crosslinking can be stimulated by light (i.e., UV or visible), heat, or crosslinker bath (i.e., ionic crosslinking). A combination of mechanisms or steps may be pursued to improve the process. For example, Colosi et al., proposed a bioink blend composed of alginate and gelatin methacroyl (GelMA) at low concentrations (<5% w/v) characterized by low viscosity, which positively impacts on cell viability during the extrusion process. The ink is crosslinked in two steps: CaCl2 during (using a coaxial nozzle) and after bioprinting and then the construct is further stabilised by UV-crosslinking (Colosi et al., 2016). The formulation and preparation of a bioink and its bioprinting is complicated because of the presence of cells and their strict requirements for sterility and viability. Concerning printability, the most important physio-chemical parameters of a hydrogel include the rheological behaviour, swelling properties, surface tension, gelation properties, and kinetics. These properties must be tuned taking into consideration the bioprinting technique (e.g., extrusion or inkjet) and the type of cell to be used. Thus, the characteristics of the bioink should meet the mechanical requirements from the process point of view and at the same time ensure cell survival after the bioprinting and within the constructs (Holzl et al., 2016). Most of the recent papers outline the necessity to find the best compromise between printability and specialization for the specific cell type or tissue under analysis (Santoni et al., 2021). According to Rutz et al. (2017), the optimal formulation of a bioink must take into account the overall process. The author refers to the extrusion bioprinting, which is to date the most common; lists the major factors responsible for achieving an optimal bioink design capable of reaching high cell viability and evaluates their impact (Figure 5).

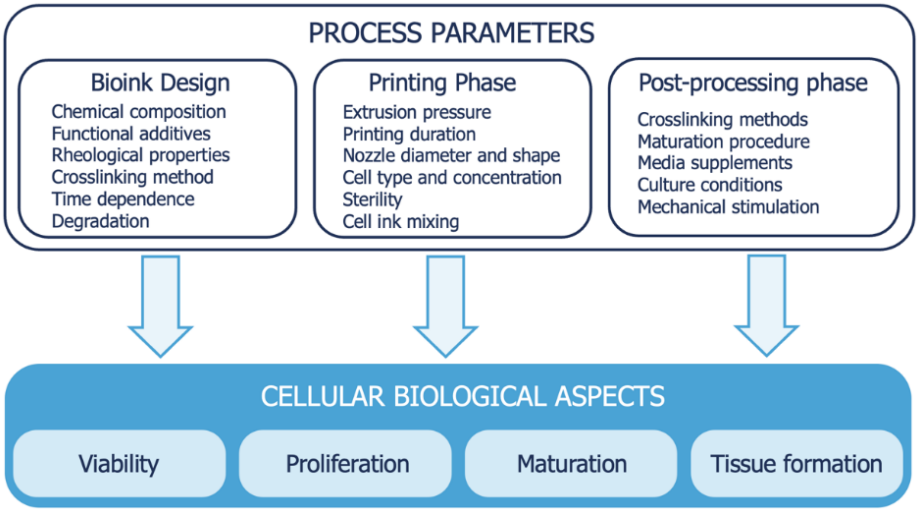

Figure 5. Schematic representation of major process parameters, grouped by bioinks, printing and post-printing design phases, and the biological behaviour of cells on which parameters can act. Adapted from Rutz et al. (2017).

Viability is considered the key challenge because it impacts the subsequent cellular events, such as proliferation, differentiation, and tissue formation, even if many factors impacting cell stress and their severity are not yet completely understood. Additionally, cellular density, cellular projections and network formation are retained of fundamental importance (Rutz et al., 2017).

According to Rutz et al. (2017) the major factors in bioink design affecting cell viability and network formation are the followings. Their effects are schematically displayed in Table 5.

Table 5. Relationship between bioink material properties and cellular viability and behaviour, adapted from Rutz et al. (2017).

Bioink	Cells
Printing pressure ↑	↓ Viability
Nozzle diameter ↓	↓ Viability
Printing time ↑	↓ Viability
Degree of crosslinking ↓	↑ Density in bioink
Viscosity ↓	↑ Density in bioink

- There is a circular relationship between cells and bioink rheology: the firsts impact the rheology – and thus process parameters – and vice versa. For example, Billiet et al. found a twofold lowering in the viscosity of a GelMA-based bioink when prepared with 0.5 and 1.5 million cells/mL and a fourfold lowering when prepared with 2.5 million cells/ml (Billiet et al., 2013). Hence, there is the need to predict or test the rheological properties of ink with cells inside.
- Mechanical stresses must be minimized, reducing printing pressure and increasing nozzle diameter because cells are mechanical sensing and suffer higher mechanical stresses.
- The modulus of the gel-phase highly impacts cell viability, and probably also molecular weight, polydispersity. The mechanical properties of the material surrounding the cells is a crucial aspect poorly understood yet.
- Post-printing crosslinking can affect cell viability as well. Frequently, bioprinted constructs are UV-crosslinked and it is not clear if the amount of irradiation cells can tolerate, probably between 30 seconds and a few minutes. Moreover, the final degree of cross-linking can interfere with cell projections and network formation, an important mechanism to ensure tissue formation. This can become important when polymer concentration is increased to increase printability. According to the author, the concentration of the polymer must be between 5 and 10%, but it is obviously a polymer-dependent quantity.

Another important matter of focus these days is the assessment techniques to standardise the evaluation of printability (Paxton et al., 2017; Ouyang et al., 2016). Paxton et al. (2017) propose a printability assessment method to evaluate the rheological properties of bioinks for extrusion processes. It is divided in two steps: (1) a qualitative screening of fiber

formation and layer stacking capabilities, and (2) a rheological evaluation focused on flow initiation, shear-thinning and post-printing recovery properties.

Through image analysis during bioprinting, other parameters related to the shape fidelity can be calculated, such as the printability index (Pr) based on the circularity calculation of a grid-like structure (Ouyang et al., 2016). Research for novel solutions in bioink development for 3D cell culture and bioprinting purposes can push emerging technologies in tissue engineering and food bioprinting. The challenge is to accomplish the correct balance between a material's chemical, morphological and structural features that have positive effect on cellular cycle processes. Moreover, non-replicative materials should be avoided for the standardization of the production process. In the case of re-usable materials after maturation, also animal-derived material can also be used (Stephens et al., 2018; Verbruggen et al., 2018). Specifically for the food bioprinting field, further considerations must be made. According to Post et al., the most important new requirements for edible bioinks are related to biological and environmental concerns: sustainability (i.e., water and land consumption, and energy and carbon footprint), source of raw materials (consistent, animal-free and scalable), taste and safety for human consumption (Post et al., 2020).

Bioreactors

In the context of tissue engineering, bioreactors are used to apply and control environmental parameters and conditions to constructs or cell cultures. The most important parameters in culture are temperature, pH, CO_2, and other biological, biochemical (such as oxygen, nutrients transfer, or waste removal), and physical (mechanical stimuli) conditions. The specific requirements and the architecture of a bioreactor depend on the cell type or tissue culture. Thus, they must be designed and manufactured for tissue-specific purposes (Rosser and Thomas-Vazquez, 2018).

- **Static culture systems:** They are the simplest and provide the required nutrient in a static fluid environment. Thus, the media must be changed often and it perfuses by passive fluid diffusion (Rosser and Thomas-Vazquez, 2018). These systems can be easily coupled with load-bearing mechanisms, for example, to provide a compressive load to the engineered tissues (Waldman et al., 2006; Correia et al., 2015).
- **Spinner flasks:** Spinner-flask-based systems are used to apply fluid-induced shear stresses to constructs submerged within a re-circulating and nutrient-rich medium solution (Rosser and Thomas-Vazquez, 2018). Although this system provides a better environment to construct with respect to the static culture, spinner flasks may not be optimal due to turbulent flow and the related higher shear stress generated (Chen and Hu, 2006).
- **Perfusion systems:** The poor diffusion condition of static culture can be improved by perfusion bioreactors, especially in the internal parts of porous scaffolds (Martin, 2000). These systems are characterised by a culture encasing bioreactor, vessels for the medium (nutrient-rich and oxygenated), and a pump generating the flow (Post et al., 2020). Moreover, perfusion systems allow for automatic media circulation, waste removal, and

also provide shear stress due to the flow which is beneficial in specific tissue cultures such as for dermis and cartilaginous tissues (Derakhshanfar et al., 2018; Chen and Hu, 2006).

- **Rotating wall vessel:** An alternative approach for reducing diffusional limitations of nutrients and waste with limited shear stress is the use of rotating wall vessel bioreactors (Martin et al., 2004). Although shear stress is important for cell maturation, an excessive force will lead to damages or to the formation of undesired capsules surrounding the tissue (Chen and Hu, 2006). This method uses a dynamic laminar flow induced by the rotating fluid inside the bioreactor, and it has been proved to be effective for cell cultures, especially chondrocytes and cardiac cells (Martin et al., 2004). The main drawback is the non-uniform tissue growth, due to the force field. Moreover, the centrifugal force can cause collision between scaffolds and the walls of the bioreactor (Chen and Hu, 2006).
- **Pulsatile flow:** For cardiovascular cell cultures that require a pulsatile stimulation, bioreactors exploiting pulsatile flow are used to mimic *in vivo* conditions. Typically, vascular cells are cultured into tubular scaffolds (Chen and Hu, 2006).

With technological and design tools, increasingly powerful, bioreactors designed for specific application can be fabricated, with high specialization and effectiveness (Martin et al., 2004). The main assumption in bioreactor design is that the same factors and stimuli that determine phenotypic nature and functionalities to tissue and cells *in vivo*, also determine the progression of cells *in vitro* (Tandon et al., 2013). Several tissues are cultured providing mechanical, electrical, chemical, and mixed systems mimicking the environment of *in vivo* conditions. Skin and cartilaginous tissues are one of the most successfully cultured – the last mainly due to its avascular nature – providing mechanical stimulation within static or dynamic cultures (Mauck et al., 2000; Derakhshanfar et al., 2018). Additionally, high shear-induced flow is used to culture bone tissue (Grayson et al., 2009). Zimmermann et al. (2002) implemented a system capable of applying passive cyclic mechanical stretch inside the culturing system to constructs for cardiac tissue engineering (Zimmermann et al., 2002; Zimmermann et al., 2006). For cardiac tissue, perfusion bioreactors capable of also providing electrical stimulation are specifically designed and tested (Radisic et al., 2008; Tandon et al., 2009; Maidhof et al., 2011). Other specific bioreactors are implemented for heart valves and blood vessels (Tandon et al., 2009; Chen and Hu, 2006).

Industrial process scaling-up

In cultured meat production, there are several technological challenges to reach an appropriate scaling-up. Within this context, the key issue is related to large-scale bioreactors for a high volume of cell production and tissue maturation. According to Post et al., the research will test other configurations and bioreactor types, to achieve higher cell densities by minimizing bioresources utilization and costs to make cultured meat a commodity (Post et al., 2020). According to the same author, the initial cell production and tissue maturation will be two separate and different stages with different problems involved. The first is related to cell proliferation by a suitable multiplier factor – not lower than $x10^9$ – and aims to

maintain cells in an exponential growth state and prevent them from undergoing precocious differentiation. The second is related to providing correct stimuli and providing nutrients in an efficient way (Verbruggen et al., 2018). The industry standard for mammalian cell bioreactors is stirred tanks, in which cells are in suspension, aggregated, or attached to microcarriers (Melzener et l., 2021; Moroni et al., 2018). To establish efficient and cost-effective microcarriers-based culture systems, several challenges must be faced, the first being, the surface and physical properties of the microcarriers: charge, coating, surface, and size (Verbruggen et al., 2018). Moreover, according to the outlook provided by Bodiou et al. (2020), the relationship between the microcarriers and the final product must be addressed, considering technological possibilities. The temporary microcarriers scenario presents the unsolved problem of cell separation and recovery, while for the other two scenarios (non-edible but degradable microcarriers and edible microcarriers embedded in the final product), the edible or biodegradable materials to be used and production technologies are the primary issues (Bodiou et al., 2020). In the case of scaffolds or cell-laden constructs in the maturation process, the main challenges are related to the correct mechanical stimulation cells require correct alignment and, eventually, mechanical tension, and an increase in material transport for efficient medium utilization, introducing also recycling techniques (Martin et al., 2004; Badylak et al., 1995; Verbruggen et al., 2018) According to Martin et al. (2004), the transition from a laboratory batch to an industrial bioreactor will require the transition from flexible bioreactors to highly specialized systems, optimized and standardized from the bioprocess point of view.

The industrial scaling up represents also an essential step to obtain a competitive product on the market. The first example of a cultivated burger was presented in 2013 in the Netherlands and it required a total fabrication cost of 300.000 $ (Balasubramanian et al., 2021). After that presentation, several companies and research groups sought to address this complex challenge. According to Guan et al., the current (2020) estimated cost for cultivated meat or fish products ranges from 66.4 $/kg to 2200.5 $/kg, compared to a few dollars per kilogram for the conventional meat and most of the cost is attributed to the cell and tissue culturing (Guan et al., 2021).

From the abovementioned discussion, the main goal of achieving cultivated meat could be accomplished only if new approaches for affordable, scalable, and sustainable culturing systems will be found. These must be implemented by exploiting several design methods, including also in silico models for the bioreactor production process (Sachlos and Czernuska, 2003). The process must be cost-effective, hence materials used must come from abundant and animal-free sources, and the production process must be scalable, economical, and sustainable, with minimal waste production (Post et al., 2020).

Consumer acceptance

Although the main purpose of this review was to consider and investigate the technological and biotechnological challenges, it is necessary to emphasise that consumer acceptance plays

a key role in the spread of cultured meat. Bryant at al. (2018) conducted a systematic review of several surveys on this topic. This work highlighted the complexity in formulating a complete picture in people's perception of cultured meat. The different surveys reported different results. The average acceptance rate of cultured meat reported by Wilks and Philip was 63,5%, while the same parameter, identified by Hocquette varied between 5% and 11% (Wilks and Phillips, 2017; Hocquette et al., 2015). These results are discordant due to the population and sample considered as well as the structure of the questions. The most common objections relate to the unnaturalness of the product, a personal perception that is less safe and healthy than conventional meat, and an anticipated impression that the product has an inferior taste, texture and appearance, accompanied by a higher price. In contrast, the positive arguments are related to animal welfare and environmental benefits, however accompanied at the same time by doubts feasibility, ethical status (Hocquette et al., 2015). As suggested by Mark Post, a leading author in the field of cultured meat, customer acceptance of this product will remain speculative until this product is actually on the market (Post, 2014).

CONCLUSIONS

The constant and rapid increase in the global population has led to the research to find novel protein sources to meet the increasing demand. In this scenario, cellular agriculture, and specifically cultured meat, is arousing increasingly interest. Cultured meat has opened an intense debate between those who see it as an innovative, ethical, and sustainable product and those who are sceptical. The full realisation of this product will face multiple challenges, both from a biotechnological and technological point of view. In the former case, the choice of animal and method for cell harvesting represent a crucial step in the large-scale production of cultured meat accompanied by the identification of FBS substitutes capable of sustaining cell viability and proliferation in both the short and long term. Although, as reported before, first steps have been taken in this direction, a completely animal-free alternative, that can match the performance characteristics of FBS is still a long way from being identified. The biotechnological approach will also be essential to create a product that is not only safe, but also reflects traditional meat. Albeit it is true that the changes undergone by the livestock sector have had an impact on the environment, it is equally true that these have made it possible to bring to our tables products characterized by high nutritional and functional quality. This second aspect, in addition to playing an important role in terms of consumer acceptance, represents one of the most difficult challenges to overcome. It is therefore necessary that all those organoleptic and functional characteristics, which in traditional meat are a direct consequence of animal feeding and wellbeing, are reproduced in cultured products. From a technical point of view the challenge is related to the implementation of a reliable and scalable process chain. The overall challenges are both related to the production and culturing systems. Concerning the production, in literature several approaches are shown to be very promising: from scaffolding, that is an older but well-known technology, to its alternatives, through finally biofabrication and 3D bioprinting. This latter could potentially

represent a game changer, even if several specific challenges must be overcome, such as for example the correct choice of materials as balance of chemical, mechanical and biological features optimized for both processability and cell cycle process compatibility. Moreover, 3DBP can lead to a closed-system and process, designed to reduce the contamination risk (Vestergaard et al., 2000), in a scalable and modular way. Another big deal is related to the differentiation of cells within constructs trough bioreactors, that must be accomplished for the elevated size request that is different from the laboratory scale typically adopted. In conclusion, several biotechnological and technical challenges need to be further investigated to meet quality, safety and consumer acceptance goals. In this scenario, it is of paramount importance to promote research initiatives with an open access character to disseminate research studies, results and solutions between public and private partners involved in the production of cultured meat.

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Manuscript IX

Cultured meat in the European Union: Legislative context and food safety issues

Davide Lanzoni, Raffaella Rebucci, Giulia Formici, Federica Cheli, Giada Ragone, Antonella Baldi, Luciana Violini, Tamil Selvi Sundaram and Carlotta Giromini.

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Cultured meat in the European Union: Legislative context and food safety issues

D. Lanzoni[a], R. Rebucci[a], G. Formici[b], F. Cheli[a,d], G. Ragone[c,d], A. Baldi[a], L. Violini[c,d], T.S. Sundaram[a] and C. Giromini[a,d].

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Department of Law, Politics and International Studies, Department of Excellence 2023-2027, Financed Through Funds of the Italian Ministry of University and Research, University of Parma, Via Università 12, 43121, Parma Italy; [c]Department of Italian and Supernational Public Law, University of Milan, Via Festa del Perdono 7, 20122, Milan, Italy; [d]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122, Milano, Italy.

ABSTRACT: The current food system, which is responsible for about one third of all global gas emissions, is considered one of the main causes of resource depletion. For this reason, scientific research is investigating new alternatives capable of feeding an ever-growing population that is set to reach 9-11 billion by 2050. Among these, cell-based meat, also called cultured meat, is one possible solution. It is part of a larger branch of science called cellular agriculture, whose goal is to produce food from individual cells rather than whole organisms, tracing their molecular profile. To date, however, cultured meat aroused conflicting opinions. For this reason, the aim of this review was to take an in-depth look at the current European legislative framework, which reflects a 'precautionary approach' based on the assumption that these innovative foods require careful prior risk assessment to safeguard consumer health. In this context, the assessment of possible risks made it possible not only to identify the main critical points during each stage of the production chain (proliferation, differentiation, scaffolding, ripening and marketing), but also to identify solutions in accordance with the recommendations of the European Food Safety Authority (EFSA). At the same time, the main criticalities in the modulation of organoleptic and nutritional properties, fundamental aspects in the creation of a product that must follow the traditional one, were investigated. Finally, possible future markets were studied, which would complement that of traditional meat, implementing the offer for the consumer, who is still sceptical about the acceptance of this new product. Although further investigation is needed, the growing demand for market diversification and the food security opportunities associated with food shortages, as well as justifying the commercialisation of cultured meat, would present an opportunity to position cultured meat as beneficial.

Keywords: Novel Food; Cultured Meat; EU regulation; Food Market; Food Safety.

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IMPLICATIONS

The current food system is characterised by a high environmental impact. For this reason, scientific research is investigating new alternatives capable of feeding a constantly and rapidly growing population. Among these, cultured meat could be a viable alternative. However, given the lack of knowledge about this new technology and its recent introduction

on the market, it is necessary to investigate not only the legislative aspects, but also the possible challenges in guaranteeing a similar and safe product as traditional meat, investigating what the possible future markets will be.

INTRODUCTION

The high impact of the food system on the environment is attracting increasing attention from the scientific community. The food system is a major cause of resource depletion and negative ecological footprint, being responsible not only for high land consumption, but also for the global eutrophication of oceans and fresh waters (Vermeulen et al., 2012; Lindgren et al., 2018). At the same time, as reported by Benton et al. (2021) and Dalin and Iturbe (2016), over the decade (2006-2017), greenhouse gases (GHGs) production by the food system accounted for 28.9% (20.4-37.3%) of total global anthropogenic GHGs (52.0±0.45%). More specifically, agriculture and land use were responsible for about 4.9±2.5% of all GHGs, methane from ruminants and soil for about 4.0±1.2%, fertilisers and manure for about 2.2±0.7%, while transport, manufacturing and cooking were liable for 2.4±4.8% (Benton et al., 2021). This scenario is expected to worsen, especially considering the steady growth of the world population, which is set to reach 9-11 billion people by 2050 (Röös et al., 2017). In parallel, there will be a dramatic growth in the demand for food, especially of animal origin, due to the fact that the Western diet, characterised by a high content of meat, fish and dairy products, has become a worldwide symbol of prosperity and economic growth, as well as an aspiration for newly urbanised countries (Bellet and Rushton, 2019). More precisely, as reported in the literature, food global demand will increase by 50% by 2030 and double by 2050, at which point it will be difficult to supply the demand without further worsening environmental health (Bellet and Rushton, 2019; Lanzoni et al., 2023). Therefore, considering the goal of feeding future generations, the promotion of socio-economic and environmental sustainability in the agri-food sector should be accompanied by the guarantee of a high level of food safety and consumer protection.

For this reason, traditional breeding is trying to move towards a more sustainable system, adopting strategies and technologies to achieve this goal. Among these, the use of feed matrices with a low environmental impact is a valid solution (Lanzoni et al., 2023a; Lanzoni et al., 2024). In parallel, precision livestock farming is attracting great interest. It is, as reported by Tullo et al. (2019), defined as *'the application of process engineering principles and techniques to livestock farming to automatically monitor, model and manage animal production*', the primary objective of which is to make livestock farming more economically, socially, and environmentally sustainable, through observation and, where possible, individual animal control. As demonstrated by Tullo et al. (2019), precision livestock farming has made it possible to reduce production risks and environmental side effects, such as the emission of pollutants into the air, soil and water, thus ensuring more sustainable livestock farming, safeguarding good health and high animal production.

Innovation and new technologies can therefore be considered valuable allies (Mouat and Prince, 2018; Siegrist and Hartmann, 2020; Martini et al., 2021; Treich, 2021). In this context, novel foods represent a great opportunity (Sforza, 2022).

Among the various alternatives, cultured meat is one possible solution. To date, it is known by several names, including cell based meat, *in vitro*, clean, synthetic, artificial meat, and lab- or factory-grown meat, although there is still no consensus on the correct terminology (Verbeke et al., 2015). Cultured meat, part of the broader branch of cellular agriculture science, represents the *in vitro* production of meat without the sacrifice of animals. More specifically cellular agriculture uses tissue engineering techniques, the aim of which is to produce food products (e.g. meat, fish, milk) tracing the molecular profile of traditional ones (Mouat and Prince, 2017; Eibl et al., 2021; Lanzoni et al., 2022).

Although cultured meat is of recent interest, the original idea has ancient roots. It first appeared in 1897 in a scientific novel entitled *Auf Zwei Planetem*, and then appeared in other accounts in the last century, as reported by Treich (2021). Later, in 1931, Winston Churchill criticised farming methods by introducing the subject of cultured meat with the following sentence: "*We shall escape the absurdity of growing a whole chicken in order to eat its breast or wing, by growing these parts separately in suitable soil. In the future, of course, synthetic food will also be used*" (Churchill, 1932; Ford, 2011). However, the development of cultured meat did not get much interest until the end of the 20th century. Starting in these years, before with the first patenting of the method of cultured meat production by Van Eelen et al. (1998), and then with the cultivation of goldfish meat by the National Aeronautics and Space Administration (NASA), cultured meat began to receive increasing interest (Benjaminson et al., 2002). The popularity of cultured meat, however, was only consolidated in 2013 with the presentation on live television of the first synthetic hamburger by Dutch researcher Mark Post (Post, 2014). From 2013 onwards, as reported by Chriki and Hocquette (2020), the number of scientific publications on cellular agriculture began to increase exponentially until the first marketing of the first cultured meat products in December 2020 in Singapore (Treich, 2021). To date, most of the research is conducted within startups located mostly in the USA and Europe, with a few others in Asia and Israel, financed by private investors (Treich, 2021; Cameron et al., 2019; Ye at al., 2022).

Given the rapid and growing interest, but above all, the possible future introduction of cultured meat in the European Union (EU) food market, it is necessary for scientific research to continue studying its possible critical points. While the production process has been described in the literature, it is incumbent upon us to investigate the critical points in the modulation of sensory and nutritional properties, deepening the issue of food safety along the entire production chain.

For this reason, the aim of the review is to provide an overview of the current legislative, food safety, technical, but also economic challenges of cultured meat. In particular, the paper intends to examine first of all the legislative regulations governing the marketing of this product, with a focus on the EU context. The pre-marketing authorisation procedure,

established by the Novel Foods Regulation (European Commission, 1997), shows a close link between, on the one hand, legislative, political and ethical considerations and, on the other hand, scientific assessments. For this reason, this paper promotes an in-depth examination of food safety issues and the need to provide a comprehensive and careful analysis on this point. At the same time, the main critical points in the modulation of organoleptic and nutritional properties that can guarantee a product similar to the conventional one. Finally, the paper aims to illustrate possible future markets for cultured meat, with a focus on consumer acceptance.

How to regulate the marketing of cultured meat: the EU Novel foods Regulation between open challenges and political considerations

In recent years, innovation in the agri-food sector brought delicate and unprecedented challenges to food regulation (Ni and Lin, 2022). As highlighted in the recent European Commission Communication "Safeguarding food security and reinforcing the resilience of food systems" (23.03.2022), new technologies - including New Genomic Techniques - are an indispensable tool for food security (European Commission, 2022). In this vast context, particular attention has been paid to the discipline of innovative foods, including both *per se* new foods, not existing before, and traditional food produced through innovative production procedures (Scaffardi and Formici, 2022). The entry into market of these so-called Novel Foods is usually subordinated to a prior authorisation based on a food safety risk assessment, delegated to scientific – generally independent – food authorities or agencies. This regulatory solution characterizes several countries, such as Canada, Australia, the EU, Israel, and the United Kingdom (FAO, 2022; Gross, 2021), where legislators have elaborated provisions specifically addressing the marketing of Novel Foods with the primary aim of ensuring a high standard of consumers' health protection.

Due to its innovative (non-traditional) production process, cultured meat is mostly considered a Novel Food and should therefore follow the general rules established for these food products (Post, 2020). That's the case of Singapore, where the chicken nuggets and processed shredded poultry products containing cell-based chicken have obtained the world's first approval in 2020 (Singapore Food Agency, 2021). The authorisation has been granted by the Singapore Food Agency (SFA) following the procedure established by the regulatory framework for Novel Foods and Novel Food Ingredients, introduced in 2019 and disciplining the marketing of foods not having a history of safe use (Singapore Food Agency, 2019). According to this discipline, "substances with a history of safe use are those that have been consumed as an ongoing part of the diet by a significant human population (e.g. the population of a country), for a period of at least 20 years and without reported adverse human health effects" (SFA, 2023). Based on this definition, producers interested in placing on the market Novel Foods are required to submit safety assessments to the SFA, who is responsible for reviewing the studies. Precise documents, submitted by applicants, are periodically updated by the SFA as well as by the newly appointed Novel Food Safety Expert Working Group (Singapore Food Agency, 2023). Interestingly, in 2021 the SFA also promoted the

Novel Food Virtual Clinics, “where novel food companies are able to proactively engage SFA at early stages of their research. With a clearer understanding of SFA’s requirements at an early stage, companies can prioritise resources towards productive research directions which will minimise compliance costs and time” (Singapore Food Agency, 2022). Clear requirements and information, together with a cooperation and dialogue between SFA and private companies in an early phase, seem to have facilitated the authorisation procedure of cultured meat in Singapore: after the chicken nuggets, the SFA subsequently approved new formats of cultured poultry in 2021 and, more recently, in 2023, the use of serum-free media for the production of cultured meat, which represent a key advancement towards a completely slaughtering-free production (Good Food Institute, 2022). As affirmed by the SFA in several documents, with specific reference to cultured meat, the safety of the product is reviewed at three different levels, focusing on the I) production process (cell lines, culture media, reagents, toxicology etc.), the II) process and controls ensured (e.g. contaminants, adherence to good safety and hygiene practices) and, finally, on the III) final product which must meet the standards established by the national food regulation (e.g. presence of allergenic proteins) (Singapore Food Agency, 2022).

Similarly to Singapore, in the EU cultured meat falls under the Novel Foods Regulation (Reg. EU 2015/2283). Although, at the time of writing, no authorisation procedures concerning cultured meat have been submitted to the EU Commission, the latter could undoubtedly be considered as a food "which has not been used for human consumption to a significant degree within the Union before 15 May 1997 (when the first EU novel food legislation entered into force), regardless of the dates of Member States' accession to the Union" (Art. 3, EU Reg. 2015/2283). The legislation also requires the new food to fit at least one of the ten categories listed in Art. 3, paragraph 2, letter a). The category n. VI, which refers to “food consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, micro-organisms, fungi or algae”, clearly includes cultured meat, that therefore necessitates to obtain a pre-market approval in order to be marketed in the EU territory.

The current Reg. EU 2015/2283, entered into force in 2018, vastly reformed the previous authorisation procedure established by the outdated Reg. EC 258/97 (Pisanello and Caruso, 2018): now the procedure is entirely centralized both in the risk assessment and in the risk management phases (Volpato, 2022). The applicant is asked to submit a scientific dossier – including food safety studies – to the EU Commission that should provide a first formal check before appointing the European Food Safety Authority (EFSA) for the centralized and unique scientific risk assessment phase (maximum time length: 9 months, extensible for specific reasons) (Dall’Asta, 2022). On the basis of the EFSA opinion – which, by the way, is not binding – on the food safety of the product, the Commission is then in charge of the risk management phase, by preparing a draft implementing decision establishing the acceptance or the denial of the authorisation request and determining the possible marketing conditions – for example those concerning the labelling –. This draft decision needs the final approval of the Standing Committee on Plants, Animals, Food and Feed, where Member

States' representatives are seated (European Commission, 2023). Even if until now the decisions of the Commission have usually followed the assessment provided by EFSA, this last phase could be influenced by political and ethical considerations, differing from scientific evaluations focusing on food safety.

If the product obtains the authorisation, it is included in the so-called Novel Foods Union List (European Commission, 2023a) with a generic effect, meaning that all food business operators other than the applicant, interested in marketing the approved Novel Food, could place it on the market without submitting another application, unless a specific data protection and "secrecy" is granted (according to Article 26 of the Reg. EU 2015/2283) (La Porta, 2021).

As clearly appears, the current legislative framework reflects a "precautionary approach" (Scaffardi, 2020) based on the assumption that innovative foods need a prior careful risk assessment in order to safeguard the highest standard of consumers' health protection. EFSA consequently plays a significant and key role in the food safety procedure (Martini et al., 2020); for this reason, it comes with no surprise that this Authority is currently preparing to face possible authorisation requests concerning cultured meat: what emerges from initiatives such as the 2023 EFSA's Scientific Colloquium on "Cell culture-derived foods and food ingredients" is that guaranteeing a clear and fruitful communication with interested food business operators, institutions as well as consumers reveals extremely important when innovative foods are concerned (EFSA, 2023).

Cultured meat, in particular, seems to be a highly debated Novel Food in the EU territory, not only by the scientific community but also the civil society and, interestingly, by national policy makers and legislators. The case of Italy seems to be paradigmatic of the complex issues regulating Novel Foods entails: facing fears about the safety of cultured meat and its potential disruptive effect on traditional meat production systems (and cultural heritage). The Italian government has decided to propose, in March 2023, the adoption of a specific law banning food and feed made, isolated or produced from cell cultures or tissue cultures derived from animals, which clearly includes cultured meat (https://www.senato.it/leg/19/BGT/Schede/Ddliter/dossier/56933_dossier.htm). The legislative text, approved by the Senate (A.S. no. 651-A), was subsequently debated and approved by the Chamber of Deputies of the Italian Parliament on 1 December 2023 with law no. 172 (Official Gazette of Italian Republic, 2023). In the currently approved version, the production, use, sale, import, distribution and promotion of cultured meat (defined by the Government as 'synthetic meat') will be banned. Recalling the precautionary principle recognised by Article 7 of EC Reg. 178/2002 and the possible risks not only for the health of consumers but also for the livelihood of the Italian agricultural sector, the Government's decision has opened up a lively political and academic debate (Formici, 2023) that also includes the possible future relationship between this national legislation and the aforementioned EU Novel Foods Regulation. As previously pointed out, the European regulatory framework is directly enforced in each Member State, so any future authorisation

regarding cultured meat obtained at EU level would also have a binding effect in Italy, and the generic reference to the precautionary principle (already much debated in GMO cases) (Ragone, 2019) could prove insufficient to maintain the legitimacy of a national ban. Apart from the questions regarding the multi-level regulatory dimension, the Italian example shows how new foods – and in particular highly innovative products such as cultured meat – pose delicate legislative issues and prompt a regulatory discussion that is not entirely based on food safety considerations but reveals to be strictly interrelated with ethical, political and economic evaluations. Moreover, the Italian legislative proposal "comes as other governments are acknowledging the strategic importance of cultured meat towards both food security and global sustainability" (Bertero et al., 2023), thus underlining different regulatory and policy approaches to innovation in the agri-food sector. This situation, which could potentially lead to diverse political positions expressed by Member States within the EU Institutions, should prompt a renewed and careful debate, also concerning other aspects related to the marketing of innovative foods such as the information provided to the consumers and, therefore, the labelling of cultured meat: should this product be called "meat" and which information should be provided to consumers about its origin? These questions, which have already been at the centre of a complex political discussion with regards to vegetal or vegan products such as burger or milk, need to be thoroughly explored (Sirsi, 2020).

The need to boost this regulatory analysis and debate seems to be extremely urgent, also considering recent relevant advancements concerning cultured meat. In the United States of America in June 2023 the Good Meat's cultured chicken obtained approval from the US Department of Agriculture (USDA), after having received in March of the same year a "no questions" letter from the US Food and Drug Administration (FDA) (Congressional Research Service, 2023). This landmark decision represents the signal of an evolving scenario and shows, at the same time, a different regulatory solution: while in the USA there is not a specific legislation dedicated to the entry into market of Novel Foods, in 2019 – with an anticipatory move – the two most relevant federal Authorities in the agri-food sector, the USDA and the FDA, outlined the marketing path of cultured meat through a specific inter-agencies agreement (FDA, 2019; Grossman, 2019; Sollee, 2022). Under this document, the FDA is in charge of the controls and assessment of the initial stages of production while the USDA is responsible for the oversight of the processing, labelling and packaging. The interested food business operators should promote a pre-market consultation firstly with the FDA, who evaluates the food safety information the company submitted and poses possible questions if doubts arise during the reviewing process. Moreover, the pre-market consultation process "allows developers to work with the FDA on a product-by-product basis and informs them of issues they must consider to produce safe food that does not violate the Federal Food, Drug and Cosmetic Act's requirements" (FDA, 2023). Notwithstanding the absence of a comprehensive legislation, the federal agencies' agreement covered the procedural issues by providing indications on the consultation phase, on the information required as well as on the role attributed to the two interested federal authorities, in order to

prompt a coherent and clear cooperation. Once again, particular attention has been paid to the cooperation between private actors and public agencies since the early development and research stage. The USA case, in which the marketing of cultured meat seems to be in an already well advanced phase, demonstrates the importance to provide regulatory answers and *ad hoc* procedural solutions to specific innovative foods, through the prior determination of rules and agreements. A similar approach could be identified also in Japan, for example, where the Association for Cellular Agriculture, a group of different stakeholders and institutions, guided by the Center for Rule-Making Strategies, has been founded with the final aim of "creating an industry guideline and a recommendation for new law to be implemented" (Miyake et al., 2023).

The different regulatory solutions promoted in several Countries as well as the political debate and the diverse approaches promoted in recent years (interesting are the cases of Israel and Chine, that boosted, also through public investments the research in alternative protein sources, FAO, 2022) demonstrate the importance not only of a comprehensive and accurate food safety assessment but also of an in-depth legislative debate on all the regulatory issues that innovative foods pose. In fact, we should consider that "the manner in which cellular meat is regulated will be a determining factor in the success of the product" (Sollee, 2022). The final aim of such debate is of fundamental importance: finding a correct and efficient balance between food security needs, environmental protection and food sustainability, economic interests, ethical considerations as well as consumers protection and food safety safeguard.

Cultured meat production: potential safety hazards

To date, cultured meat is one of the most hotly debated topics in science. It could be considered a more sustainable and safer product than traditional meat. However, as reported by Chriki and Hocquette (2020), this type of comparison is incomplete and sometimes biased, because nowadays there are no certain data, but only projections over the long term, which are difficult to compare with the data for traditional meat.

For sure, from an environmental point of view, the production of cultured meat will require less land and water use. More precisely, as reported by Haraguchi et al. (2022),1 kg of cultured meat (approximately $5x10^{10}$ cells), will require 50 L of water (used almost exclusively for the production of the culture medium), which is significantly less than the 550-700 L of traditional meat (Chriki and Hocquette, 2020; Santinello et al., 2023). Although this is well-established in the literature, it is also necessary to assess the quality of the water resulting from processing, the main waste product, the volumes of which are as yet unquantifiable. Indeed, as argued by Chriki and Hocquette (2020), waste media, containing growth factors, hormones and other chemicals, would represent a critical issue for environmental sustainability. However, scientific research is already investigating a green utilisation of such waste, promoting its use for the growth of microbial proteins for animal/human nutrition, as demonstrated by Haraguchi et al. (2022). In comparison with

conventional livestock farming, as reported by Lynch and Pierrehumbert (2019), it will also be important to consider the impact of CO_2, the main GHG produced in cultured meat production, which has a longer bioaccumulation period in the atmosphere than CH_4, although it will need to be monitored over the long term for accurate analysis. Although based on long-term projections, environmental sustainability has been widely described in the literature. At the same time, as reported by Chriki and Hocquette (2020), the issue of safety is still a topic that need to be investigated. Proponents of cultured meat consider it a safer product than traditional meat, as it is produced in a closed and controlled environment (Chriki and Hocquette, 2020). However, it must be emphasised, that on a large scale the product will not be produced in the laboratory but on an industrial level, where it is impossible to completely eliminate possible risks, especially those due to human error. This is a common problem with plant-based protein products. Indeed, as reported by Banach et al. (2023), processing can introduce microbiological hazards such as *Staphylococcus aureus*, mainly through food handling (skin contact), or *Listeria monocytogenes* during processing, as it can be found in the processing environment. As reported by Jahir et al. (2023) and Stephens et al. (2018), to prevent this possibility, new research courses and skills will be required that can provide high levels of knowledge beyond the more traditional roles, including chemists, cell biologists, materials scientists, chemical engineers, skeletal muscle scientists, technicians, and food technologists. However, before showing the possible critical points in the production chain of cultured meat (Figure 1), it is necessary to consider that traditional meat production is characterised by a high control system that must also be integrated for the cultured meat.

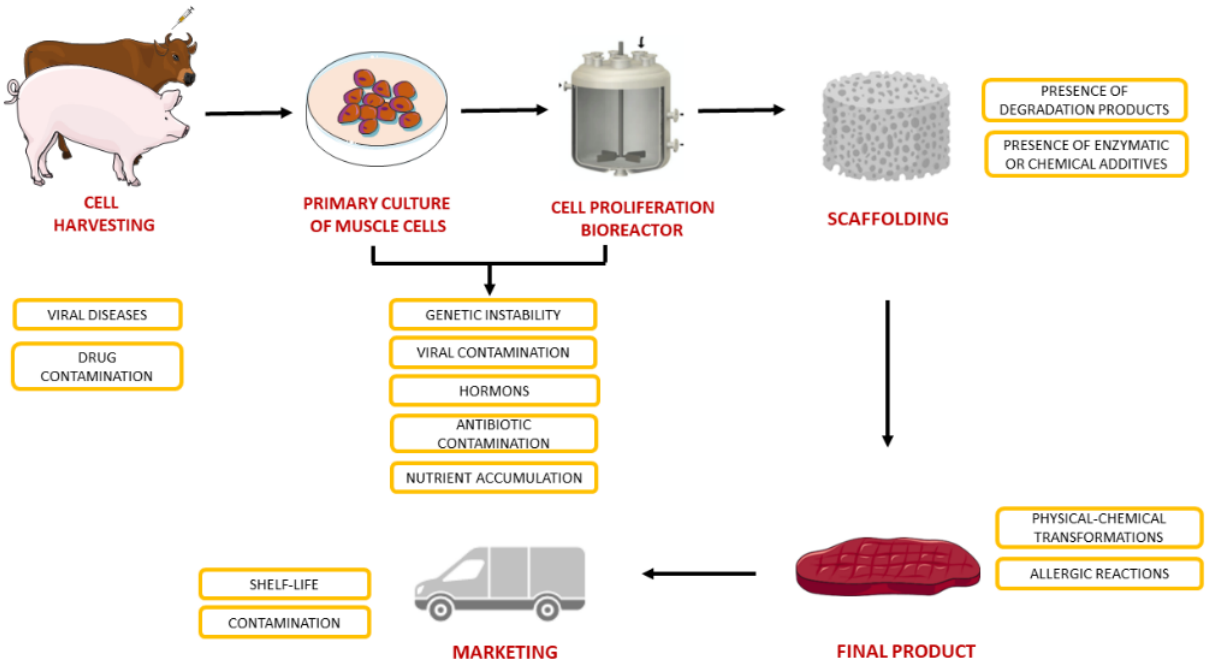

Figure 1. Description of all critical point on the production chain of cultured meat. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Cell Harvesting: It represents the first step in the production of cultured meat. It consists of a cell or tissue biopsy from a live animal or in the post-mortem period (Lanzoni et al., 2022). This step has been extensively studied in the literature with the aim of obtaining the greatest number of stem cells (satellite muscle cells) from a single animal (Post, 2012; Zhu et al., 2022; Guan et al., 2022). More precisely, the choice of cell sampling must not be random, but must take into account multiple variables, including age, sex and breeding conditions, in addition to genetic ones (Lanzoni et al., 2022). Indeed, during the animal's ageing, in addition to the decrease in the concentration of satellite cells in the muscle, the latter undergo a high number of mitotic divisions, thus maintaining their differentiation capacity for a much shorter period, compared to cells taken from young animals (Melzener et al., 2021). At the same time, as reported by Choi et al. (2021), male animals show a higher concentration of stem cells than females, due to the positive action of testosterone. Similar results are obtained with extensive compared to intensive housing, most probably due to the different type of diet (Lanzoni et al., 2022). In parallel, it is also essential to preserve animal welfare. For this reason, as argued by Melzener et al. (2021), cell harvesting should be done by needle biopsy, a less invasive technique than tissue harvesting. Furthermore, in order to reduce the number of donors, it is desirable to maximise the number of biopsies (maximum four in one session) from the same animal every three months, thus ensuring adequate recovery times for animal welfare (Melzener et al., 2021).

However, to date, the relationship between the health status of the animal on which the harvesting is performed and the potential introduction of biohazards into the cultured product has not yet been studied. As reported by Ong et al. (2021), the main food safety issues almost exclusively include the transmission and spread of infectious viral diseases. The latter can be transmitted in the following ways: I) In the form of free viral particles via faecal contamination of foodstuffs; obviously, this is not the case of cultured meat production; II) By transmission of infected cells to other hosts, e.g. hepatitis A, hepatitis E, bovine leukaemia virus (Ong et al., 2021). The latter mode of transmission represents a very delicate point, both because it is still unclear whether the cells of an infected animal undergoing biopsy are able to persist in culture, and also because of the risk of transmission of zoonotic diseases (e.g. bovine leukaemia virus) (Juliarena et al., 2017; Ong et al., 2021). Nevertheless, this risk can easily be circumscribed by a strict inspection of the source animals and biopsied cells/tissues for signs of infection. Another possible risk at this stage concerns contamination by veterinary drugs, including antibiotics. They may be present as contaminants in the tissue used for cell harvesting and potentially present in the final product, causing adverse effects on human health (FAO and WHO, 2023). However, for this to occur, the drug must first be present in the sampled tissue and then in the cell culture throughout the production cycle, thus reaching the final product at a concentration that exceeds the maximum safe level. Nevertheless, this risk can easily be monitored either by using tests for the quantification of veterinary drugs on both the cell line and the final product, but especially by consulting the health data of the source animals at the time of tissue biopsy (FAO and WHO, 2023).

At this stage, it is also crucial to further investigate the controls for chemical contaminants, e.g. cryoprotectants used to store cellular models. Indeed, as reported by Ong et al. (2021), some cryoprotectants could be toxic if present in the final product. However, as pointed out by Savini et al. (2010) and Ong et al. (2021), cryoprotectants such as inulin, sorbitol, and dimethyl sulfoxide are already used in food processing to date and have proven to be safe. Despite this, to ensure total safety of cultured meat, it is expected that cryoprotectants will either be eliminated or diluted to very low concentrations and combined with washing of the final product (Ong et al., 2021).

Proliferation and Differentiation: The next step after cell harvesting is the isolation of satellite muscle cells and their culturing to first promote proliferation and then differentiation within bioreactors. At this stage, there are several critical points relating to food safety. As reported by Rosser and Thomas-Vazquez (2018), the number of cells required to produce 1 kg of protein from muscle cells is in the range of 2.9×10^{11} to 8×10^{12}. To achieve these high numbers, the cells need to have a high proliferative capacity. However, this could lead to the formation of cancerous cells within the culture due to genetic instability, without being clearly identified within the cell cultures. Although such cells are harmless, as they are dead on consumption of the meat and therefore not incorporated viable within the body, they present a great challenges of acceptance for the consumer. For this reason, this needs to be further investigated to ensure the total absence of risk (Hocquette, 2016). As previously reported, cells proliferate and differentiate in bioreactors, closed and controlled systems capable of providing all the stimuli the cells need to ensure their viability. In particular, cells need a constant supply of nutrients (carbohydrates, lipids, vitamins, minerals and micronutrients) provided through culture media. To date, identifying all the critical points at this stage is very complex due to the many different source of nutrients needed for different species, cell types and production steps (Burton et al., 2000; Yao and Asayama, 2017). For this reason, it is necessary to make a general overview of the possible risks at this stage. Nutrients present in culture media are commonly found in conventional foods. However, in culture products, a potential food safety problem would occur if in the formulation of a specific culture medium, one or more of these substances were present in the final product at concentrations that would be hazardous to the consumer (FAO and WHO, 2023). This could occur if the nutrient is accumulated abnormally or through cellular internalisation, or aggregation on structural material. In both cases, cells are able to metabolise the substance completely (FAO and WHO, 2023). To prevent this possible risk, different controls exist such as: I) use of minimum levels of nutrients that still allow cell growth, II) constant monitoring of cellular parameters (e.g. viability and morphology) as indicators of cellular damage, III) chemical analysis of the final cellular product to identify the nutrients present, whereby the maximum safe levels related to intake are already known for traditional foods (FAO and WHO, 2023).

To proliferate, cells not only need nutrients, but also additional secondary components that are essentials to provide cells with signals for their viability, replication and differentiation. These include animal serum, proteins, peptides, nucleic acids (micro ribonucleic acid (RNA)

or miRNA, messenger RNA or mRNA), growth factors and hormones (FAO and WHO, 2023; Ong et al., 2021). For sure, to date, the greatest challenge in cultured meat production is to find a substitute for animal serum, in particular foetal bovine serum (FBS), that can replicate its characteristics while guaranteeing ethicality. Foetal bovine serum is a complex mixture of fatty acids, lipids, vitamins, carbohydrates, inorganic salts, growth factors, proteins, and more than 400 metabolites, which are essential for cell adhesion, growth, and proliferation (Lanzoni et al., 2022). Despite these many positive aspects, the production of FBS clashes with the ethicality promoted by cellular agriculture. In fact, it is taken by cardiac puncture from foetuses up to three months old from cows sent to slaughter, causing suffering and pain (Brunner et al., 2010). The exact amount of FBS produced and sold worldwide is unknown. However, it is estimated that about 800.000 L of FBS are sold annually, which corresponds to about two million foetuses sacrificed (Subbiahanadar et al., 2021). These data explain why its use for the production of cultured meat, besides being unethical, would in any case be unsustainable in the long run. Furthermore, FBS being an animal by-product could contain endotoxins, haemoglobins and other factors adverse to cell growth, as well as being a potential source of microbial contaminants (fungi, bacteria, mycoplasmas, viruses and prions) posing a health problem for the consumer (Brunner et al., 2010). Although, as reported by Chriki and Hocquette (2020), companies have already found a viable substitute to FBS (patent-protected), scientific research is investigating multiple substitutes. These include products of plant peptones, hydrolysates (yeast, rice protein, wheat and sericin), dairy by-products (whey proteins) and the use of extracts from microalgae (*Chlorella vulgaris* and *Spirulina maxima*) (Ho et al., 2021; Lanzoni et al., 2022). While the FBS problem is widely described in literature, the other components deserve further investigation. Indeed, the addition of proteins, peptides but also growth factors of animal origin, although essential to support cell growth, can introduce viral or prion contamination, as claimed by Jayme and Smith (2000). However, the same authors suggest how this problem can easily be curbed by using animal-free culture media, thus limiting the introduction of pathogenic organisms (Jayme and Smith, 2000). Possible substitutes may be plant-based products, as suggested by Chriki and Hocquette (2020). To date, companies are working hard to achieve this goal. One example may be BioBetter, an Israeli company founded in 2015, which has started to produce and market growth factors produced from tobacco plants for use in the production of cultured meat.

Particular attention must be paid to the use of hormones. Their excessive consumption can lead to imbalances and adverse human health outcomes, including pro-carcinogenic effects and reproductive toxicity, as argued by Jeong et al. (2010). For this reason, as early as 1996 (Council Directive 96/22 EC of April 1996), the European Union regulated the use of hormones in the traditional food chain, banning the use of certain hormonal substances such as testosterone, progesterone, zeranol, trenbolone acetate, melengestrol acetate, and oestradiol 17β, as they can remain as residues in the meat of treated animals following their slaughter (European Union, 1996; Ong et al., 2021). This ban plays a fundamental role in food safety, being implemented not only for Member States but also for imports from third

countries (European Union, 1996). Possible solutions, as suggested by FAO and WHO (2023), could be the use of these substances at minimum concentrations that still allow the desired effect to be achieved, the use of product washing steps, and finally the implementation of safety and quality control measures (FAO and WHO,2023).

Another problem related to cell proliferation phase concerns the use of antibiotics in the culture medium to prevent any contamination. Although the laboratory is a controlled environment with careful monitoring, it is difficult to stop any signs of infection, which is why they are added to the culture medium. However, it must be emphasised that these within the cell cultures will be added (when necessary) at lower concentrations than those used in traditional breeding and used almost exclusively in the early stages of production, where the cells will then be rinsed and purified, reducing the possibility of these being found in the final product, without the possibility of causing allergic reactions (Ong et al., 2021). At the same time, another possible problem concerns the development of drug resistance in the cells used. To prevent this phenomenon, as reported by Ramani et al. (2021), a possible solution could be the substitution of antibiotics with natural or synthetic antimicrobial peptides, lysins, bacteriocins, hydrolysed peptides, and biological extracts, which do not constitute a stress factor or create drug resistance. However, it is still necessary to document and record the use of antibiotics or a substitute, the type and concentration, increasing controls for human health safety (FAO and WHO, 2023; Ong et al., 2021).

At this stage it is also crucial to pay attention to chemical contaminants that are used in the medium, including antifoaming, pH buffers, culture media stabilisers as well as the accidental introduction of microplastics from water and the external environment (FAO and WHO, 2023). In this case, as suggested by FAO and WHO (2023) to safeguard consumer health, it is necessary to quantify the levels of these chemical contaminants at every stage, until the final product. In fact, such contaminants can occur at any stage of the production process, from harvesting to market.

Scaffolding: Tissue maturation only takes place if cells are provided with an environment in which they can first adhere and proliferate and subsequently differentiate. To enable this, scaffolds are used in the production of cultured meat, i.e. three-dimensional structures characterised by correct architecture, porosity, mechanical and chemical properties (Lanzoni et al., 2022; O'Brien, 2011; Seah et al., 2021). Considering the purposes of food engineering, they must be either biodegradable or edible or both, their structure being involved in the organoleptic properties of the final product (Lanzoni et al., 2022). Depending on the nature of the scaffold, different safety issues may arise for the end consumer. If the scaffold is designed to degrade, it is necessary that the material used and the degradation products are safe for human consumption, requiring a safety assessment typical of any food additive or ingredient (Ong et al., 2021). Where, on the other hand, the scaffold used is not designed to degrade and it is necessary to act via chemical or enzymatic dissociation, a characterisation of the additives used is required, as reported by Stephens et al. (2018), it is possible for them to persist within the final product.

Final Product: As a result of cell proliferation and differentiation, cultured meat is subject to the phenomenon of maturation before reaching the final stage. Although Olenic and Thorrez (2023) reported that cell lines do not undergo a true maturation process, Ramani et al. (2021) emphasised that maturation is influenced by electrical, mechanical factors, co-cultivation with other cell types, and growth factors. Despite this, at this stage, it is essential to implement controls to ensure quality and food safety. An important aspect to be assessed concerns the physical-chemical transformations that can be triggered in the final product. These types of transformations occur when the components present in the products interact with other substances leading to changes in the structure and/or sequence of the compound with the undesired appearance of reactive species harmful to human health (FAO and WHO,2023). They can be induced by food processing as heat/chemical treatment (pasteurization, extrusion, smoking, and freeze drying) or during sterilisation in production processes (irradiation). In the first case, it is important to emphasise that the high temperatures reached during the cooking of high-protein foods, including cultured meat, can lead to the production of harmful substances such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons and advanced glycation, end-products from the Maillard reaction (Zhang et al., 2023). However, although to date there is no confirmation that this can also occur in cultured meat, as reported by Zhang et al. (2023), scientific research has rarely reported the presence of chemically hazardous substances in meat analogues, the latter of which are structured to resemble the typical structure of conventional meat. In the second case, although food irradiation has been approved in more than 50 countries, including Australia, Belgium, Brazil, Canada, China, Russia, South Africa, Thailand, the USA and Vietnam, there is no universal list of irradiable products, but varies from country to country with its own national regulations for labelling irradiated products (Madureira et al., 2022). In this context, the EU would seem to be curbing such treatment, having allowed only dried aromatic herbs, spices and vegetable seasonings to be irradiated through Directive 1999/3/EC (European Union, 1999). For this reason, to ensure the total absence of risk, in addition to evaluating and testing the physico-chemical transformations of the ingredients included in the formation of the final product, as suggested by the Food and Agriculture Organisation of the United Nations (FAO), it is necessary to have universally applicable food processing regulations (FAO and WHO,2023).

One of the most important aspects to take into account in the final product concerns possible allergic reactions. Allergy to conventional meat is rare in adults and in most cases it is the alpha-gal syndrome, i.e. the immune system's reaction to a sugar molecule that could enter the bloodstream through a tick bite (Bryant, 2020). However, cultured meat, being molecularly similar to conventional meat, could trigger the same allergic reaction (Bryant, 2020). This doesn’t represent the only risk. Indeed, during the production process of cultured meat, ingredients such as structural materials, media nutrients and modulators of cell function, whose adverse reaction is not yet known, may be introduced. This is an aspect in common with plant-based proteins and meat analogues (fungi-based) (Banach et al., 2023; Zhang et al., 2023). In fact, as reported by Banach et al. (2023) and Zhang et al. (2023), the

increased prevalence of food allergies can occur in multiple ways: I) when proteins are removed from their natural matrix and incorporated in higher amounts into other constructs; II) by introducing proteins that are not normally consumed and cause primary sensitisation or show cross-reactivity to immunoglobulins of existing allergens; III) Triggered sensitisation towards new proteins can lead to cross-reactivity events towards foods that are currently not or rarely considered allergenic. For this, as reported by FAO and WHO (2023), it is necessary to increase controls at this stage including the selection of substances from non-allergic sources, use of minimum levels of these substances, quantification of potential residues in the final product and assessment of potential consumer exposure (FAO and WHO,2023). Finally, as reported by Bryant (2020), a key aspect concerns clear labelling of the final product.

Marketing: The last and next step concerns the marketing and preservation of the final product. While on traditional meat, scientific research has adequately investigated the best strategies to maximise shelf life, on cultured meat it is still in its early stages. However, as reported in the literature by Gasteratos (2019), cultured meat, being prepared in sterile conditions, could be characterised by a longer shelf life than traditional meat while simultaneously reducing the costs of transport, refrigeration, and waste products. These aspects could also be favored by the fact that the production sites could be located closer to the consumer, compared to the farms (Tuomisto and de Mattos, 2011). The marketing of the product must take into account multiple aspects such as taste, colour and texture of the meat for the structure of even the final packaging (Siddiqui et al., 2022). Indeed, as previously reported, although bacterial contamination is possible during the production stages of cultured meat, it is crucial to note that bacterial infection can occur predominantly during transport and distribution due to poor quality packaging materials (Siddiqui et al., 2022). In this regard, the quality of the packaging material plays a key role in prevention, safeguarding consumer health. For this reason, Siddiqui et al. (2022) made an overview of packaging that can extend the shelf life of cultured meat while safeguarding food safety. In particular, the following packaging methods are taken into consideration: I) Modified atmosphere packaging, II) Vacuum packaging, III) Active packaging; the characteristics of which are briefly listed below.

I)Modified atmosphere packaging: This type of packaging prevents the oxidation of eme-proteins such as myoglobin and thus colour changes during storage (Siddiqui et al., 2022). More precisely, modified atmosphere packaging allows the atmosphere within the packaging system to be modified by reducing and/or removing oxygen inside the package from the top of the pack by modifying the gaseous atmosphere with nitrogen and carbon dioxide (Esmer et al., 2011; Siddiqui et al., 2022). At the same time, as reported by Djordjevic et al. (2018), such packaging is able to reduce microbial growth; however, oxygen concentrations must be kept under control, as an absence of oxygen can lead to the development of anaerobic bacteria (Siddiqui et al., 2022).

II)Vacuum packaging: These packaging systems have been found to have positive effects on the shelf life of traditional meat (Lorenzo and Gomes, 2012; Devatkal et al., 2014; Brenesselová et al., 2015), which is why it can be assumed that they can also be used for cultured meat. Such packaging systems are effective in preventing colour change and the oxidation process by removing oxygen. The plastic material used for packaging the final product must ensure impermeability to prevent the absorption of oxygen from outside/inside the packaging system (Siddiqui et al., 2022).

III)Active packaging: These packages are of recent introduction to the market. They are defined as such because they are characterised by the presence of an active agent capable of interacting with the food contained in the packs, allowing them to increase their shelf life. Today, there are several types: I) The product to be consumed is coated with an edible material in such a way that the consumer can easily consume it while simultaneously ensuring a longer shelf life (Umaraw et al., 2020), II) The packages may contain an antioxidant agent or an oxygen scavenger inside them (Gvozdenko et al., 2022; Siddiqui et al., 2022), III) Introduction of an antimicrobial agent into the packaging system (Yildirim et al., 2017). Obviously, no reference is made to antibiotics, but natural compounds such as natural seeds to be integrated into the polymer. In this way, the packaging absorbs moisture from the meat and supports the release of antimicrobial compounds (Bahmid et al. 2021). An alternative solution could be the encapsulation of gases such as carbon dioxide and the incorporation of volatiles such as ethanol or essential oils that can inhibit bacterial growth (Siddiqui et al., 2022).

In the light of the above, it is clear that in order to prevent any form of contamination and ensure the safety of the final product for the consumer, it is necessary to follow the rules of good cell culture practice (GCCP) and good manufacturing practice (GMP). GCCP's primary objective is to promote the maintenance of high standards in the application of procedures and products for cell and tissue culture of animal/human origin and in parallel to encourage greater international harmonisation and standardisation of laboratory practices, quality control systems, safety procedures, recording and reporting, and compliance with laws, regulations and ethical principles (Bal-Price and Coecke, 2011). As just reported, among the main recommendations in addition to keeping a detailed record of all procedures carried out to identify possible contaminants in the final product, the GCCP recommends working under aseptic conditions, avoiding the use of antibiotics and controlling the quality of culture media (Bal-Price and Coecke, 2011; Ong et al., 2021). GMP refers to all those practices aimed at preventing the occurrence of hazards. More precisely, it involves widely applied food production practices that describe the sanitary operations and maintenance and related production and process controls that enable safe food production (Ong et al., 2021; Blanchfield, 2005). In parallel, alongside GMP, it is necessary to ensure Good Hygienic Practices, which are essential in the supply of food, applicable to industrial food production. In parallel, the Food Safety Management System must be applied to the future market for cultured meat. This system is not only responsible for food production, but also aims to transparently demonstrate how food safety has been planned and implemented throughout

the entire production chain (Kafetzopoulos et al., 2013). Within the Food safety management system, an important role is played by Hazard Analysis and Critical Control Points, which is the most widely used international system for ensuring product safety and identifying possible microbiological, chemical and physical hazards that may occur during the production and processing of cultured meat, including quality assurance monitoring at every stage (not only for the final product, but also for all starting materials, solutions/products used, contamination procedures applied, and waste disposal/recycling) (Kafetzopoulos et al., 2013; Bryant, 2020). In this context, as reported by Bryant (2020), alongside the European regulation for the approval of *in vitro* products, a system of inspections at national level will be applied to ensure the wholesomeness of the final product, all under the monitoring of EFSA.

Organoleptic properties and nutritional profile: major challenges for cultured meat

One of the main challenges of cultured meat is to replicate the organoleptic, techno-functional and nutritional properties of conventional meat. Although, in some cases (e.g. Israel), cultured products are currently available to be marketed by specific companies, scientific research has a duty to explain and investigate possible critical points. Organoleptic properties (texture, colour and taste) play a key role in consumer acceptance (Broucke et al., 2023).

Texture: The texture of conventional meat is guaranteed by the maturation process, namely the reaction triggered only after the death of the animal (Lanzoni et al., 2022). More precisely, the cessation of oxygen leads to the accumulation of lactic acid and a lowering of pH that can activate several families of enzymes, that are essential for the breakdown of proteins and the subsequent tenderization of meat (Hocquette, 2016; Balasubramanian et al., 2021). However, to date, it is difficult to confirm that the maturation process also occurs for cultured products, due to reduced information in this regard. Certainly, texture would not be a critical point in products such as hamburgers or sausages, where the use of thin sheets of cultured cells would be able to replicate this characteristic (Broucke et al., 2023). In contrast, the production of whole cuts, due to their thickness, absence of blood and limited diffusion of nutrients and oxygen would make it difficult to replicate conventional texture (Broucke et al., 2023). To achieve this, various solutions such as cell stimulation in culture and co-cultures of myoblasts-fibroblasts-adipocytes have been adopted (Fraeye et al., 2020). At the same time, as reported by Broucke et al. (2023), additives such as proline, hydroxyproline, ascorbic acid in the culture medium can also be considered to alter the mechanical properties of the tissue or through the use of scaffolds that are essential for creating connective tissue. As reported by Cheng and Sun (2008), the tenderness of traditional meat is also due to its important water-retaining property. This is influenced by the formation of the actin-myosin bond, which is only created after the death of the animal. Although, cultured muscle fibres are characterised by the presence of actin and myosin, they are embryonic or neonatal forms and therefore would not be able to guarantee this feature (Thorrez and Vandenburgh, 2019). For this reason, although further investigation is needed, inexpensive solutions such as

cellulose scaffolds or the use of water-retaining ingredients such as powdered egg white, fibre or starch may be applied to replicate this techno-functional property (Broucke et al., 2023).

Colour: The colour of the conventional product depends mainly on two basic parameters: myoglobin and iron concentration (Post and Hocquette, 2017). Laboratory-grown muscle fibres tend to be yellow, both because of the lack of myoglobin as it is repressed by cultured cells in the presence of oxygen, and because the main culture media contain minimal iron concentrations. To achieve the traditional meat colour, it is possible to stimulate myoglobin production by reducing oxygen levels, increasing the iron content in the culture medium, and adding natural dyes directly to the final product (Fraeye et al., 2020). Another possible solution, as reported by Zhang et al. (2020), could be to add haemoglobin directly into the culture. This solution, however, would involve extracting haemoglobin either from animal blood, plant tissue or produced by microbial cells, which are expensive, time-consuming and therefore not feasible on a large scale (Zhang et al., 2020).

Taste: As reported by Balasubramanian et al. (2021), most of the chemical metabolites present in conventional meat are not only derived from muscle, but are the result of the animal's food intake and biological metabolism. These, together with the interaction of proteins, lipids, carbohydrates, nerves and blood vessels are responsible for the unique taste of meat (Hocquette, 2016). At the same time, it is crucial to consider how flavour also depends strongly on alterations in sugars, organic acids, peptides, free amino acids and degradation products that occur exclusively post-mortem (Broucke et al., 2023). Considering cultured meat, it is difficult to understand how these changes could occur in the absence of the animal being slaughtered. Therefore, to replicate a sensory profile similar to the traditional one, it is necessary to intervene directly on the cultured cells, particularly the adipose cells. In fact, as reported by Khan et al. (2015), Fraeye et al. (2020) and Broucke et al. (2023), fat is crucial in the aroma, juiciness and tenderness of the final product. For this reason, it is possible to adopt solutions such as co-cultures of muscle cells and adipocytes, the use of pre-adipocytes to increase intramuscular fat (Fraeye et al., 2020; Kuppusamy et al., 2020), the addition of carotenoids that can prevent the oxidation of fatty acids by limiting their rancidity and preserving the final flavour (Stout et al., 2020; Broucke et al., 2023), and choosing a biomaterial that enables the differentiation of a particular cell type, such as adipocytes (Post et al., 2020). Finally, it is feasible to add aromas directly to the final product that take consumer preferences into account. As reported by Zhang et al. (2020), possible options such as hydrolysates of soy sauce, defatted soy or mushroom protein when heated produce flavour compounds similar to those in beef.

The aim of culturing meat is also to replicate and also improve the nutritional profile of traditional meat.

Micronutrients: Among the main micronutrients in traditional meat, minerals (iron, selenium, zinc) and vitamins (vitamin B12) play a key role in maintaining human health (Hocquette, 2016). However, cells in culture are not able to synthesise them independently.

For this, it is necessary to add these nutrients directly into the medium associated with binding and transport proteins to facilitate uptake by the cells (Broucke et al., 2023). Although such a practice is feasible, as argued by Chriki and Hocquette (2020), it needs to be investigated whether even in cultured products, these micronutrients provide the same positive effects for human health.

Lipid content: As previously reported, co-cultures with fat cells would allow the lipid fraction in cultured products. Although, traditional meat is characterised by a high lipid content, approximately 37 g per 100 g of meat are saturated fatty acids (Calder, 2018). For this reason, to increase the functionality of these new products, the production of particular polyunsaturated fatty acids (PUFAs) could be added to the disadvantage of saturated fatty acids, creating a functional and beneficial product for the consumer (Broucke et al., 2023).

Protein content: To date, characterising the protein profile of cultured products is complicated due to limited information. The primary goal remains to simulate the protein content of traditional meat (20-24 g per 100 g) (Calder, 2018). To achieve this objective, several strategies can be adopted. I) Use of electrical stimulation to encourage sarcomeres synthesis. This method, although very efficient, is characterised by a high cost and for this reason not applicable on a large scale (Thorrez and Vandenburgh, 2019); II) Optimisation of the culture medium by providing a higher content of free amino acids and resulting in a higher protein content. However, as argued by Broucke et al. (2023), although this approach would be more cost-effective, there is a need to further investigate the uptake of nutrients by cells and what changes they undergo once internalised. III) Use of edible or biodegradable protein scaffolds. This alternative, besides being economical and applicable on a large scale, would make it possible to modulate the amino acid profile of cultured products. More precisely, matrices rich in essential amino acids could be chosen for the formulation of these structures, opting for derivatives of plant origin or exploiting genetic engineering to produce transgenic organisms capable of synthesising desired amino acids (Stein et al., 2009; Broucke et al., 2023).

Cultured meat: potential perspective markets

The reasons that led to the discovery and development of the cultured meat sector are mainly related to sustainability and ethical reasons. In particular, as reported before, today's global population stands at 8 billion, a number that is set to grow dramatically by 2050, when the inhabitants on earth will reach 9-11 billion (Roos et al., 2017). At the same time, there will be an increase in demand for food, especially meat and dairy products. More precisely, in 2012, the FAO estimated that global demand for meat will reach 455 million tonnes by 2050, a 76% increase since 2005 (Bellet and Rushton, 2019; Lanzoni et al., 2022). All these reasons prompted the investigation of an as yet unknown market. As previously reported, enormous progress has been made in the production of cultured meat over the years. In 10 years alone, since the first cultured beef burger dated 2013, many start-ups (Table 1 and Figure 2) with different production goals have emerged, as shown in Figure 3.

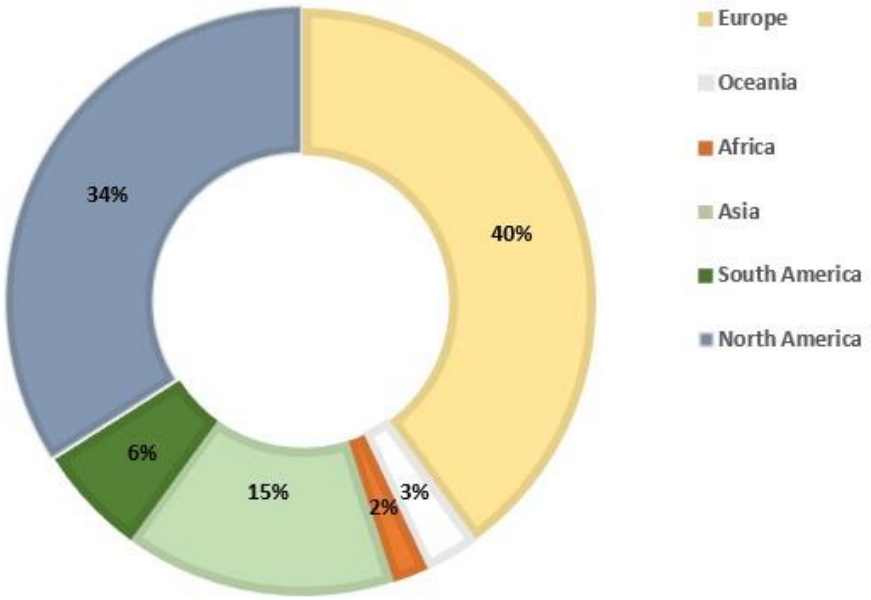

Figure 2. **The global distribution of the cultured meat companies by 2021**. Adapted by Ye et al. (2022).

Table 1. **Consolidated companies operating in the field of cellular agriculture from 2015 to 2021**.

Year	Company	State	Focus
2015	Integriculture	Japan	Cultured meat
	MosaMeat	Netherlands	Cultured meat
	SuperMeat	Israel	Chicken cultured meat
	Modern Meadow	New Jersey	Cultured meat
	Upside Foods	California	Beef, chicken, duck cultured meat
	BioBetter	Israel	Synthesis of growth factors for cultured meat
2016	Aleph Farms	Israel	Beef cultured meat
	Gelatex	Estonia	Scaffolding and microcarriers
	Because Animals	Canada	Cultured meat for petfood
2017	Nissin	Japan	Cultured meat
	Future Meat	Israel	Cultured meat
	BalleticFoods	California	Cultured meat
	Appleton Meats	Canada	Beef cultured meat
	Bio.Tech.Foods.	Spain	Cultured meat
	BlueNalu	Wales	Cultured sea-food
	Heuros	Australia	Cultured meat, synthesis of growth factors, media development, innovative packaging
2018	Fork&Good	New Jersey	Cultured meat
	denovoMATRIX	Germany	Production of microcarriers and scaffolds
	VitalMeat	France	Cultured meat
	Clear Meat	India	Cultured meat, FBS alternatives, synthesis of growth factors
	Meatable	Netherlands	Cultured meat
	New Age Meats	California	Pork cultured meat
	CubiQ food	Spain	Production of healthy fats
	Biftek.co	Turkey	Synthesis of growth factors
	Shiok Meats, Seafood, reinvented	Singapore	Cultured meat and sea-food
	Avant	Singapore	Cultured sea-food
	Innocent Meat	Germany	Synthesis of growth factors
	Higher Steaks	England	Cultured meat
	Cell Ag Tech	Canada	Development and production of sustainable cell-cultured sea-food
	Peace of Meat	Belgium	Chicken and duck cultured meat
	Orbillion	California	Beef Cultured meat
	Ivyfarm	England	Cultured meat
	LabFarm	Poland	Chicken cultured meat
	BioMilq	North Carolina	Cultured human milk
	MeaTech	Israel	Cultured meat

	Gaia Foods	Singapore	Cultured meat
	Brunocell	Italy	Cultured meat
2019	Artemys foods	California	Cultured meat
	TurtleTree Labs	Singapore	*In vitro* lactoferrin
	Vow	Australia	Cultured meat
	Mirai Foods	Switzerland	Cultured beef meat
	Matrix Meats	Ohio	Scaffolding
	OKPI	Russia	Cultured meat
	Joes Future Food	China	Pork cultured meat
	3DBT	England	Three-dimensional structures and serum-free medium
	Bluu Biosciences	Germany	Cultured seafood
	SiCell	China	Cultured meat
	BioMilk	Israel	Cultured milk
	Luyef	Chile	Cultured meat
	Novel Farms	California	Pork cultured meat
	Oxton Farms	England	Production of healthy fats
2020	Better Milk	Canada	Cultured milk
	Renaissance Farms	England	Cultured meat
	Umami Meats	Netherlands	Cultured meat
	MyoWorks	India	Scaffolding
	Mogale Meat CO	South-Africa	Cultured meat and Antelope cultured meat
	Meweri	Czech Republic	Pork cultured meat
	CellX	China	Cultured seafood, chicken and wagyu meat
	Another fish	Canada	Cultured seafood
	Meatafora	Israel	Cultured meat
	MicroMeat	Mexico	Cultured meat
	Bluefin Foods	California	Cultured bluefin tuna
2021	Quest Meat	England	FBS alternatives and primary cell lines
	Edge	USA	Synthesis of growth factors
	Anjy Meat	Croatia	Cultured meat
	JBS	Brazil	Cultured meat

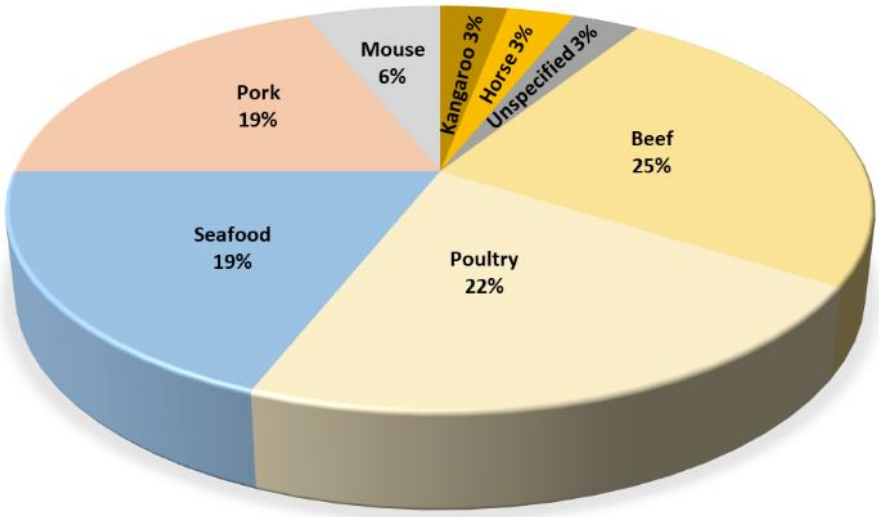

Figure 3. Primary meat focus for the cultured meat companies. Adapted from Choudhury et al. (2020).

More precisely, as Figure 2 shows, the main companies are located for 40% in Europe (Croatia, Czech Republic, Estonia, France, Germany, Israel, Italy, Netherlands, Russia, Spain, Switzerland, Turkey, England), 34% in North America (America and Canada), 15% in Asia (China, India, Japan, Singapore, South Korea), 6% in South America (Argentina,

Brazil, Chile, Mexico), 3% in Oceania (Australia) and 2% in Africa (South Africa) (Ye et al., 2022). Of these, as presented in Figure 3, about 25% focus on beef, 22% on poultry such as chicken and duck, and 19% on pork and seafood such as fish and shrimp. In addition, two companies are investigating mouse meat as an alternative food for pets (Choudhury et al., 2020). Between 2015 and the beginning of 2020, the amount of capital invested in cultured meat companies (publicly disclosed), reached approximately $320 million. Approximately $243 million was allocated for the production of cultured meat from pork and beef, $50 million for seafood following the *business-to-consumer* business model, the main one in this sector. Alongside this, other business models have begun to emerge, such as *business-to-business*, the aim of which is the production of cell culture media, cell line generation, growth factors or, more generally, ingredients to be added to the culture medium (Choudhury et al., 2020).

However, to date it is difficult to go and study what the possible markets for cultured meat might be. There are no studies in the literature to refer to. In our opinion, cultured meat will not replace a market as complex as the traditional meat market, but will open up new markets to flank it, as reported below.

Over the years, intensive animal husbandry has undergone many changes that have resulted in a safe, nutritious and quality product for the consumer. As reported before, red meat is characterised by a high protein source. This value, combined with the lipid content, ensures a high energy intake (Lanzoni et al., 2022). In particular, meat is rich in saturated fatty acids, more specifically palmitic acid (C16:0) (about half), stearic acid (C18:0) (about one third) and lower concentrations of myristic acid (C14:0) and lauric acid (C12:0). Although stearic acid does not promote any effect on cholesterol, palmitic, myristic and lauric acid are responsible for raising blood cholesterol concentrations (Calder, 2018). At the same time, concentrations of PUFAs, recognised for their fundamental activity in maintaining human health, are low (Calder, 2018). In light of the above, a possible market could be the development of a "functional products" with a better nutritional and functional profile. Such an avenue would be pursued by adding cell-metabolisable nutrients to the culture medium, which would perform a positive function for the consumer. Although these products are not intended for vegans or vegetarians, as the origin is still animal (Mancini and Antonioli, 2020), they might be intended for a particular type of consumer.

Cultured meat could also find a place within certain religious communities: Jewish, Muslim, Hindu and Buddhist. The Jewish religion is characterised by Kashrut, i.e. a set of religious dietary rules, which prohibits the consumption of certain foods and requires others to be prepared in a specific way. To date, several issues concerning the Kashrut status of foodstuffs are still being examined with regard to cell-based products. Firstly, if products derived from animals prohibited by religious laws and considered Tareif, or forbidden for consumption by Jews, are themselves Tareif. Secondly, it must be determined whether these cell-based products, specifically those derived from mammals, are not considered meat products and should be handled as Parve (not classified as meat or a dairy product) as defined by Kashrut

laws allowing them to be handled and consumed with dairy products. An example is the decision of the rabbinical organisation Thozar, which declared that meat products derived from embryonic stem cells taken from bovine blastocysts should be considered Parve, and as such eaten with dairy products. Such religious rulings play a crucial role in that they may substantially alter the diet of religious Jews (FAO and WHO,2023).

For Muslims, the relevant question is if cultured meat is halal. As reported by Hamdan et al. (2018), based on Qur'anic scriptures, cultured meat can be considered halal if the cells used are derived from a halal slaughtered animal and if no blood or serum of animal origin is used in the production process. For this reason, it is very improbable that halal meat from pigs and other haram (not permitted) species will be approved. In fact, as reported by Bryant et al. (2019) out of 193 Muslims, 68% would eat cultured lamb or goat meat, 58% cultured beef, 49% cultured chicken, while only 28% cultured pork. In parallel, many Hindus interpret the principle of non-violence (ahimsā) as a requirement for vegetarianism (Bryant, 2020). This principle ensures that vegetarian Hindus consider cultured meat as a solution to avoid animal suffering. However, it is still unlikely that cultured beef will be accepted by Hindus, due to the sacred nature of this animal (Bryant, 2020). In fact, a study by Bryant et al. (2019), reported that out of 730 Hindus, 68% would eat cultured chicken, 65% goat, but only 19% beef. Finally, for Buddhist, 81% would eat cultured beef, 73% would eat cultured pork, 66% would eat cultured goat and 61% would eat cultured chicken (Bryant, 2020).

Another possible market is pet-food, an ever-expanding market worth around USD 100 billion a year. Trends in pet-food production towards so-called ‘human grade’ meat (meat perceived as of a quality suitable for human consumption), as well as potential changes in human dietary practices leading to fewer waste animal products, risk exacerbating the impact of pet-food, requiring a parallel increase in breeding and slaughtering mainly for the production of pet-food (Oven et al., 2022). All this has prompted pet owners to question what might be more sustainable alternatives, as reported by Oven et al. (2020). At the same time, pet feeding practices can raise ethical issues. Vegetarians and vegans face what has been termed the *vegetarian's dilemma* or the *animal lover's paradox* when deciding what to feed their pets (Oven et al., 2022). While they want products that meet the nutritional requirements of their animals, they also consider it a mistake to slaughter animals for food production. For this reason, the need and possibility of producing pet food using cellular agriculture technologies has arisen. To date, one of the main challenges concerns the final cost, given the fact that food intended for animals must be cheaper than food intended for human consumption. However, the possibility of using meat for which donor animals are not required due to the presence of immortal cell lines or the use of animals for biopsy whose breeding is less costly (e.g. mice, fish or invertebrates) may solve this critical point (Oven et al., 2022). In parallel, the application of cultured meat in the pet-food market would also require less regulatory burden, as it is generally less regulated than the human food chain (Oven et al., 2022). All these factors, coupled with the fact that pet food does not need to faithfully replicate existing products and thus less technologically demanding production, can be a springboard for the cultured meat market.

Cultured meat: consumer acceptance

Although scientific research is actively working to ensure the safety of cultured meat, consumer acceptance still remains a major challenge to overcome. The acceptance or rejection of this new product is generating conflicting opinions, also due to personal factors, also referred to as demographic predictors, such as age, gender, education, socio-economic status, and political orientation (Bryant and Barnett, 2020; Pakseresht et al., 2022). More precisely, as reported by Dupont and Fiebelkorn (2020), due to a lower level of food disgust, the younger part of the population would be more likely to consume cultured products. This difference is also visible between the male and female population. Although, Tobler et al. (2011) had reported that women were more willing to adopt ecological food diets, as shown by Slade (2018), Bryant and Barnett (2018, 2020), Mancini and Antonioli (2019) and Pakseresht et al. (2022), it would be men who showed a higher level of acceptance for cultured meat. As argued by Pakseresht et al. (2022), Grasso et al. (2019), education also plays a key role in acceptance, where more educated individuals are in favour of this new product. In fact, in support of this, as reported by Gomez-Luciano et al. (2019), in higher-income countries, cultured meat found greater favour than in lower-income countries, where people attribute status to greater meat consumption. Finally, political orientation also showed a division between supporters, the political left, versus opponents, the political right, distinguished by a more conservative feeling for cultural traditions (Bryant et al., 2019; Wilks et al., 2019).

Despite these predictive factors, as reported below, there are many different reasons for the rejection or acceptance of cultured meat.

Opinions against accepting cultured meat

Food *neophobia* and unnaturalness: Food *neophobia* has been identified as a major predictor of novel food rejection in Europe, Asia and America (Pilner and Hobden, 1992; Bryant and Barnett, 2020). This can be attributed to *food fussiness*, the strong preference for food prepared and served in a specific and familiar way, over a product that is often considered unnatural (Grasso et al., 2019). This is coupled with the unnaturalness of these new products leading to the rejection of cultured meat (Bryant and Barnett, 2020).

Disgust: Linked to unnaturalness and *food neophobia* is certainly the perception of disgust, a much stronger feeling in Western cultures, as reported by Siegrist et al. (2018). However, it is interesting to note that several researches have reported that cultured meats elicit less disgust than GMOs and insects, but more disgust than plant-based products (Dupont and Fiebelkorn, 2020). This difference is probably due to the familiarity of these products. At the same time, the disgust is not only related to the sensory profile, but should also be understood as a moral one. This distinction plays an important role as such objections may be surmountable in the long run, when cultured meat is likely to be a more well-known product (Bryant and Barnett, 2020).

Safety: As reported by Siegrist and Sütterlin (2017), it is also common for a proportion of consumers to have doubts about safety, in particular due to uncertainty about the long-term health effects of cultured meat. However, although this attitude seems to decrease in the presence of additional information about the entire production process (Bryant and Barnett, 2020), to date, as also reported by Chriki and Hocquette (2020), it is impossible to know what the harmful effects on human health might be, as cultured meat is a newly developed product.

Nutritional aspects: As initially reported by Laestadius and Caldwell (2015) and subsequently confirmed by Bryant and Barnett (2018) and Mancini and Antonioli (2019), sceptical consumers consider cultured meat an unhealthy and nutritionally inferior product compared to traditional meat. This aspect, which is also common for plant-based products, is most probably to be related to the artificial aspect and thus the non-naturalness of these new technologies (Bryant and Barnett, 2020).

Opinions in favour accepting cultured meat

Sustainability: Sustainability is considered to be the first advantage in the acceptance of cultured meat. As reported by Tuomisto (2019), consumers keen to support cultured meat promote its benefits on research use, such as reduced land use, less water wastage and reduced GHGs. This is reinforced with additional information demonstrating the low environmental impact compared to conventional meat (Mancini and Antonioli, 2020).

Ethics and morality: Cultured products are considered to be more ethical and moral as they would avoid suffering (confinement in confined spaces, probable bad breeding conditions) and the slaughter of animals, an advantage considered crucial for these new products (Van der Weele and Driessen, 2019). This aspect also plays a key role in the vegetarian's dilemma, using cultured meat for pets unable to follow a vegetarian diet, as previously reported (Oven et al., 2022).

Healthiness and safety: The potential benefits of consuming cultured meat could be both a healthier product, including a reduction in saturated and monounsaturated fatty acids in favour of PUFAs (Laestadius and Caldwell, 2015; Bryant and Barnett, 2018), as previously reported, and a safer product (Bryant and Barnett, 2018; Bryant and Barnett, 2020). However, as shown by Bryant and Barnett (2020), such benefits tend to be less commonly perceived than ethical and environmental issues, and are only identified after being solicited. It is important to note that safety has previously been identified as a parameter for the rejection of cultured meat. It is likely that safety, as a factor in support of this new product, is associated with those countries where conventional meat production has been regularly marked by deficiencies and diseases, as reported by Zhang et al. (2020a).

World Hunger: In parallel, as reported by Laestadius (2015), cultivated products are seen as an important means of feeding the world's population. In support of this, in the survey

conducted by Mancini and Antonioli (2019), participants identified this benefit as one of the most common, only after sustainability and ethicality

While scientific research has focused so much on consumer perception, it is also important to consider the opinions of stakeholders. As reported by Freeman (1994), stakeholders are groups or individuals that can influence or are influenced by the achievement of specific economic goals. These groups may include employees, suppliers, shareholders but also public groups such as governments and communities that provide infrastructure and indirectly regulate market activities (Clarkson, 1995). Among the main positive aspects called for by stakeholders, as reported by Amato et al. (2023), animal welfare and environmental protection are certainly the most important. However, these are associated with the belief that the technology industry will bring drastic changes to traditional agriculture, negatively impacting biodiversity and agricultural landscapes where animals are no longer needed (Amato et al., 2023). Another important category concerns the economic aspect, which involves conflicting opinions. While the positive aspects relate to better efficiency in manufacturing, the diversification of production, the establishment of new sectors and the creation of new job opportunities, one of the main negative aspects, expressed by stakeholders, concerns the possibility of monopolisation of new markets by large companies at the expense of smaller ones, especially in the early stages of market development, where large investments would be required (Newton and Blaustein-Rejto, 2021; Bohm et al., 2018; Amato et al., 2023). In parallel, stakeholders consider cultured meat to be a healthier and more nutritious alternative, with less hormones, antibiotics, animal-derived bacteria and easily modulated, which would allow the creation of specific functional products for certain consumer classes, as previously reported (Woll and Bohm, 2018). At the same time, however, the issue of safety is still unclear, with a split in stakeholder opinion, suggesting a more thorough investigation of this delicate topic (Amato et al., 2023).

Although the above aspects are crucial in the acceptance or rejection of cultured meat, the still uncertain price plays perhaps the most important role in determining the long-term success of this product. To date, there is much contradiction with respect to the economic issue. In fact, although Bryant and Barnett (2018), and Laestadius and Caldwell (2015) identified a probable high cost as a major barrier to purchasing cultured meat, greater even than *food neophobia*, in the study conducted by Mancini and Antonioli (2019), about 23.2% of the interviewees were willing to pay more for this new product, about 20.8% were 'maybe' willing, while 26.7% were not willing to pay a premium (those who were not willing to try cultured meat). These percentages may increase if a sensory experience is associated, as reported by Rolland et al. (2020).

But what is the likely cost of cultured meat? According to the study reported by Garrison et al. (2022), cultured meat produced on a large scale could be produced at a cost of 63 $ for kg, where the major production costs are associated with the culture media (especially hormone production), bioreactors/equipment and labour, accounting for about 87% of the final cost (55 $ for kg). However, this cost estimate may never be reached as it will require

huge technological advances to be realised. For this reason, possible solutions must be found to lower prices. First of all, low-cost culture mediums need to be investigated, which would lead to a substantial reduction in the price; secondly, used equipment from the medical and pharmaceutical industry could be used (Garrison et al., 2022). Although, great progresses have been made since 2013, where the cost of production was 2.3 million $ per kg (Post, 2014), it is unthinkable that cultured meat could be considered an affordable product for everyone, but it could be considered a niche product, especially in those economically developed countries such as Western Europe and the United States, confirming earlier reports on parallel markets for this new technology (Garrison et al., 2022).

In general, it is important to emphasise how different surveys lead to different results. For example, in the work reported by Wilks and Philip (2017), the average acceptance rate for cultured meat was 63.5%, while the same parameter, identified by Hocquette et al. (2015), varied between 5 and 11%. These results, as pointed out in our previous review (Lanzoni et al., 2022) are discordant due to the population and sample considered, as well as the structure of the questions. Most probably, as also suggested by Post (2014), the acceptance of this product by future consumers will remain speculative until the product will be on the market.

Cultured meat: Future perspectives

The research of cultured meat is an ever-expanding field, the literature is growing rapidly and global escalation seems imminent, although there are still many doubts that need to be cleared in the future. In terms of environmental benefits, cultured meat will face the challenge of being the second most energy-intensive source of protein during its production; a challenge that can be overcome by scaling up its production, as reported by Deliza et al. (2023). Achieving this goal would allow this new product to be classified as environmentally friendly. As reported by FAO and WHO (2023), the issue of safety has already been extensively discussed, identifying all possible risks at every stage of production, up to the final product. This approach will have to be kept alongside the control systems typical of the traditional supply chain in order to guarantee total safety. Nevertheless, before cultured meat reaches consumers' tables, large-scale follow-up studies will be needed, identifying new possible critical points and solutions, which in a narrow market would not be identifiable (Zhang et al., 2023). This step will have to be implemented especially in those countries where cultured meat struggles to find favour with food safety authorities and policy leaders, taking Israel, the first country to regulate the human consumption of cultured meat, as a model. Clear regulation would certainly meet with a greater consensus of public opinion, some of which is currently unfavourable. For this reason, as reported by Berry et al. (2017), it is necessary to implement a multidisciplinary approach involving more diverse fields (scientists, designers, marketing experts, psychologists, sociologists) in order to better understand consumer concerns and significantly increase acceptance, while optimising the design of new products.

As previously reported, cultured meat will not replace a market as complex as the traditional meat one, but will open new commercial windows alongside it. However, the commercial starting point should replicate those of existing meats both for acculturation purposes and for initial market penetration, and then facilitate market segmentation at a later stage (Deliza et al., 2023). In parallel, the high and flexible technological nature of cultured meat would also allow for a greater focus on customer needs during product and packaging development, further customising flavour, nutritional and sensory properties. Ultimately, the growing demand for market diversification and the food security opportunities associated with food scarcity, as well as justifying the marketing of cultured meat, would present an opportunity to position cultured meat as beneficial.

CONCLUSION

In conclusion, cultured meat could represent a viable alternative to proteins of animal origin, whose future introduction into the market needs clarity, especially from a regulatory perspective. The current European legislative framework for cultured meat reflects a precautionary approach based on the assumption that such innovative foods require thorough prior risk assessment in order to safeguard consumer health. This assessment must be carried out at every stage of the production chain, more precisely from cell harvesting and related proliferation and differentiation, to the marketing of the final product, identifying possible solutions in accordance with EFSA warnings. A clear regulation, coupled with a safe and transparent production process, would allow both to increase the consensus of public opinion, still today divided on the positive and negative aspects, and the development of future markets, which will most likely parallel that of cultured meat. Although these aspects must continue to be investigated in order to ensure a safe product for the consumer, the growing demand for market diversification and the food security opportunities associated with food scarcity, in addition to justifying the commercialisation of cultured meat, would present an opportunity to position cultured meat as beneficial.

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Manuscript X

Milk whey as a sustainable alternative growth supplements to fetal bovine serum in muscle cell culture

Tamil Selvi Sundaram, Carlotta Giromini, Raffaella Rebucci, Davide Lanzoni, Elena Petrosillo, Antonella Cheli and Federica Cheli

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Milk whey as a sustainable growth supplement to fetal bovine serum in muscle cell culture

T.S. Sundaram, C. Giromini[a,b,c], R. Rebucci[a], D. Lanzoni[a], E. Petrosillo[a], A. Baldi[a] and F. Cheli[a,c].

[a]Department of Veterinary and Animal Science (DIVAS), Università degli Studi di Milano, Via dell'Università 6, 29600 Lodi, Italy; [b]Institute for Food, Nutrition and Health, University of Reading, Reading RG6 5EU, UK. [c]CRC, Innovation for Well-Being and Environment, Università degli Studi di Milano, 20122, Milano, Italy.

ABSTRACT: *In vitro* meat cultivation, a cutting-edge innovation in food science, may represent a more sustainable and ethical source of animal proteins compared to conventionally grown meat. An important challenge for meat cultivation lies in eliminating the use of fetal bovine serum (FBS) in cell culture media due to ethical concerns. Milk whey is a nutrient-rich liquid portion of the milk, derived as a by-product of dairy industry. Similar to FBS, whey contains proteins crucial for nutrition, cell adhesion, and biomolecular transport. In this study, we investigated whether whey proteins (WP) can replace FBS in supporting muscle cell cultivation, using the C2C12 myoblast model. Accordingly, under serum-free conditions, cells were treated with two WP mixtures, grouping high (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) and low (β-LG 0.07%, α-LA 0.15%, BSA 0.15%) selected concentrations of individual proteins that positively affected cell growth in a preceding dose-response study. Cells cultured in only basal DMEM medium, was included as a negative control, and 10% FBS as a positive control. Cells were maintained in the treatment media for 48 h (day 1 and 2) to support myoblast proliferation. Subsequently, all the treatments were replaced with a standard low mitogenic 2% horse serum (HS) medium until full differentiation (day 6). The treatment effects on morphology, viability, and lactate dehydrogenase release were assessed after days 1, 2, and 6, respectively. The results showed that WP stimulated cell proliferation under serum-free culture conditions, similar to the FBS control, and subsequently facilitated myotube formation when the WP or FBS treatments were switched to HS media. After differentiation, these cells also exhibited increased expression of cell differentiation markers such as creatine kinase and citrate synthase, and underwent morphological changes from spindle-shaped cells to fused elongated myotubes, in contrast to negative control. This study demonstrates that WP are a promising and sustainable alternative to FBS-based growth supplements for use in cultivated animal products.

Keywords: Whey proteins; Fetal Bovine Serum Alternative; Cultured Meat; Cellular Food

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INTRODUCTION

The global population is projected to increase by 2.3 billion people between 2009 to 2050 reaching an estimated world population of 9.1 billion people in 2050 (FAO, 2009). The Food and Agriculture Organization anticipates a surge in agricultural and livestock production to sustain the growing population (FAO, 2009, Keating et al., 2014, Makkar, 2018, OECD et

al., 2023). The continuous rise in global food demand and food production, alongside increasing greenhouse gas emissions, forecasts significant pressure on Earth's land and water resources (Keating et al., 2014, Makkar, 2018). Recent advancements in cellular agriculture and bioprocess technologies have introduced innovative methods for sustainably cultivating meat (Gaydhane et al., 2018, Lanzoni et al., 2022) and milk (Mendly-Zambo et al., 2021, Slade and Zollman Thomas, 2023). Specifically, cultivated meat is produced by stimulating myogenesis in muscle cells grown on edible 3D scaffolds (Sharma et al., 2015, Gaydhane et al., 2018, Lanzoni et al., 2022), while *in vitro* milk is derived from mammary gland cells or recombinant bacteria and yeast cells, cultured in nutrient-rich bioreactors (Mendly-Zambo et al., 2021). Cells required for *in vitro* cultivation can be obtained either through biopsy or freshly fertilized eggs, thus eliminating animal slaughter and the environmental impact of extensive farming (Melzener et al., 2021, Azhar et al., 2023). The nutrient media for cell culture contain basal media supplemented with fetal bovine serum (FBS), which provides glucose, amino acids, vitamins, lipids, carbohydrates, growth factors, and all other essential components for optimal cell growth (Gstraunthaler, 2003). However, the challenge is to replace FBS in cell culture media due to ethical concerns, its batch-to-batch variations, and high costs (Gstraunthaler, 2003, Karnieli et al., 2017, Lanzoni et al., 2022). Limitations of FBS application and the lack of suitable FBS substitutes impede the standardization of cell culture conditions for developing more sustainable and reliable methods for consistent production of cultivated foods. Recently, research has shown promising alternatives to FBS derived from plants (Girón-Calle et al., 2008), algae (Ng et al., 2020, Yamanaka et al., 2023), microbes (Venkatesan et al., 2022), industrial by-products (Christel Andreassen et al., 2020), and even food-waste (Christel Andreassen et al., 2020), which can partially or completely replace FBS in mammalian cell cultures. Notably, microalgae extracts was shown to promote the growth of epithelial cells (Ng et al., 2020, Yamanaka et al., 2023), myoblasts (Yamanaka et al., 2023), and mesenchymal stem cells (Ng et al., 2020). Interestingly, in another study, hydrolysates of various food industry by-products such as cod backbone, eggshell membrane, egg white powder, yeast extract, chicken carcass, and pork plasma, consisting of approximately 2-15 amino acids, was shown to enhance myoblast growth and metabolic activity (Christel Andreassen et al., 2020). This study offers valuable insights on transforming food waste and by-products into sustainable and cost-effective cell culture components for use in cellular agriculture (Christel Andreassen et al., 2020). Milk is a complex biological fluid, characterized by functional and molecular similarity to blood serum. For over three decades, studies have consistently shown that milk whey and whey proteins (WP) support the growth of various mammalian cells, including myoblasts (Belford et al., 1995, D'Souza et al., 2020), adipocytes (D'Souza et al., 2020), fibroblasts (Belford et al., 1995), lymphocytes (Hashizume et al., 1983), epithelial (Belford et al., 1995), and hybridoma cells (Capiaumont et al., 1994). Essentially, whey is a nutrient-rich liquid portion of the milk, derived as a by-product of cheese production in the dairy industry. Approximately 96% of the lactose, 25% of the proteins, and 8% of the fat in milk reside in whey (Olvera-Rosales et al., 2023). The WP fraction primarily comprises β-LG (35-65%), α-LA (12-25%), immunoglobulins (8%), serum albumins (5%), and lactoferrin (1%) (Patel,

2015, Mehra et al., 2021). Additionally, whey contains non-protein nitrogenous compounds, organic acids, essential minerals, and B vitamins (Olvera-Rosales et al., 2023). These whey components modulate diverse cellular functions including the regulation of cell signaling, structural organization, metabolism, immunomodulation, synthesis, and transport of biomolecules such as fatty acids and iron (Olvera-Rosales et al., 2023). Notably, a recent proteomic study has profiled over 1500 WP from bovine and donkey colostrum and mature milk, relevant to these cellular pathways (Li et al., 2022). WP also serve as a source of branched-chain amino acids, which regulate glucose uptake and glycogen synthesis in skeletal muscle cells (Morifuji et al., 2009). However, limited evidence exits regarding its impact on the myogenesis of muscle cells, especially under serum-deprived culture conditions. Due to challenges in establishing primary muscle cell cultures, the mouse C2C12 myoblast cell line is widely utilized as a preliminary experimental model to study muscle cell mechanisms, including the myogenesis process for modelling *in vitro* meat cultivation (Jaques et al., 2021). Provided appropriate culture conditions, C2C12 cells proliferation and differentiation into multinucleated myotubes, closely mimicking the characteristics of primary muscle cells. Presently, we assessed whether WP could support C2C12 myoblast proliferation under FBS-free conditions and later facilitate myotube formation with a standard low mitogen differentiation medium.

MATERIALS AND METHODS

Cell culture and maintenance

The murine skeletal muscle cell line C2C12 (IZSLER, Brescia, Italy) was cultured in complete growth medium (GM) for 15 to 16 passages in 75 cm^2 culture flasks. The GM consisted of high-glucose Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, Milan, Italy), supplemented with 10% FBS (Immunological Sciences, Rome, Italy), 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), and 1% penicillin-streptomycin (Immunological Sciences, Rome, Italy). Cells were detached at 60-70% confluency with 5 mL of 1X trypsin-EDTA (Immunological Sciences, Rome, Italy), and cell count was estimated using the trypan blue staining method (Sigma-Aldrich, Milan, Italy) with a haemocytometer. Cells were seeded in 96-well plates at a density of 7×10^3 cells/well in GM for cell viability and cytotoxicity assays. For cell morphological assessment, creatine kinase (CK), and citrate synthase (CS) assays, 2×10^5 cells were seeded per 75 cm^2 culture flask. Cell cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. The working volume was 150 µL/well in 96-well plates and 15 mL in 75 cm^2 flasks.

Cell proliferation and differentiation conditions

At confluency below 60%, C2C12 cells actively proliferate in mitogen-rich culture conditions provided by FBS in GM. On the other hand, at confluency above 70% under a reduced serum or low-mitogen conditions, C2C12 cells exit the cell cycle and eventually

differentiate to form myotubes by cell-cell fusion as reported earlier. In the present study, cells at 50-60% cell confluency were exposed to different treatment media composing FBS, individual WP, or a mixture of WP (Sigma-Aldrich, Milan, Italy) as given in Table 1, to investigate their proliferative effects. Briefly, for the dose-response study, cells were treated with increasing concentrations of β-LG, α-LA, BSA, or FBS (0-10%) in a basal medium containing high-glucose DMEM and 2 mM L-glutamine. Likewise, to assess the effects of WP mixture, cells were treated with either a high concentration (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) or a low concentration (β-LG 0.07%, α-LA 0.15%, BSA 0.15%) protein group in basal medium. The concentrations of individual proteins in each mixture were selected to be below the inhibitory range identified in the dose-response study. Cells cultivated in basal medium alone was considered as the negative control, while those cultivated in basal medium supplemented with FBS as the positive control. Cells were maintained in the treatment media for 48 h to support myoblast proliferation. The cell seeding density was optimized to achieve complete confluency at the end of proliferation stage. Subsequently, in all the experimental groups, the treatment media were replaced with a standard, low-mitogenic differentiation medium containing high-glucose DMEM, 2% horse serum (HS) (Immunological Sciences, Rome, Italy), 2 mM L-glutamine, and 1% penicillin-streptomycin. Cells were maintained in differentiation medium for 96 h to stimulate myotube formation. Fresh differentiation medium was replenished every 2 days. The treatment effects on cell viability and cytotoxicity were accessed after day 1 and 2, corresponding to the cell proliferation stage, and day 6, corresponding to the cell differentiation stage. Markers of oxidative metabolism and cell differentiation such as CK and CS, were assessed after day 6. Prior to cellular assays, time points for proliferation to myotube formation were confirmed through microscopic observations over the culture period.

Cell morphology

Cell morphology was monitored throughout the proliferation and differentiation stages using an inverted light microscope with a 10x objective lens (Optika, Bergamo, Italy). Images were captured after days 1, 2 (cell proliferation stage), and 6 (cell differentiation stage). Cell morphology images represent cells cultured in the presence and absence of FBS or WP mixtures. Four fields were analysed for each treatment group and the most representative images were shown in the results. Cells were rinsed with 1X PBS prior to imaging.

Table 7. Treatments tested during the proliferation phase

No.	Treatment groups	% applied
1	Serum supplement	FBS 0-10%
2	Individual WP	β-LG 0-10%
3		α-LA 0-10%
4		BSA 0-10%
5	WP high concentration mixture	β-LG 1.25%, α-LA 1.25%, BSA 1.25%
6	WP low concentration mixture	β-LG 0.07%, α-LA 0.15%, BSA 0.15%

Cell viability assay

Mitochondrial dehydrogenase activity was assessed as an indicator of cell viability and proliferation using the XTT-based colorimetric assay (Immunological Sciences, Rome, Italy). The assay buffer was prepared and applied to cells (25 µL/well) in a 96-well plate as per the manufacturer's instructions. Subsequently, cells were incubated at 37 ºC for 2 h and 30 min. Then, the absorbance was measured at 490 nm in a colorimetric plate reader (Bio-Rad, Milan, Italy) and normalized against the background buffer measured at 630 nm.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release in culture media was assessed as an indicator of cell membrane damage and cytotoxicity. For the assay, 50 µL of cell supernatant was mixed with an equal volume of CytoTox 96 LDH buffer (Promega, Madison, USA) in a 96-well plate and incubated at room temperature for 30 min in the dark. Subsequently, the absorbance was measured at 490 nm in a colorimetric plate reader (Bio-Rad, Italy) and normalized against the background buffer.

Citrate Synthase and Creatine Kinase activity

Mitochondrial enzymes such as CS and CK, which are key regulators of oxidative metabolism in differentiating muscle cells were assessed as indicators of myotube formation. After the treatments, cells (2.5×10^6) in culture flasks were rinsed twice and harvested in 5 mL of 1X PBS. Cell suspensions were centrifuged at 1000 g for 10 min to obtain the pellets. For the CK assay, cell pellets were homogenized in 150 µL of ice-cold potassium phosphate buffer (50 mM, pH 7.5) using an ultrasonic bath for 10 min. Likewise, for the CS assay, pellets were homogenized in a same volume of assay buffer provided in the kit (Sigma-Aldrich, Milan, Italy). Subsequently, cell lysates were centrifuged, supernatants obtained, followed by incubation with respective reaction buffers as per the manufacturer's instructions (Sigma-Aldrich, Milan, Italy). Absorbance was measured at 340 nm for the assessment of CK and at 412 nm for CS, respectively. Subsequently, the enzymatic activities were normalized against the total protein concentrations determined by BCA assay (Immunological Sciences, Rome, Italy).

Statistical Analysis

Treatment effects were assessed by 1- or 2-way ANOVA followed by Tukey post-hoc test using the GraphPad Prism software (Version 9). At least three independent experiments were performed with three technical replicates. All data are expressed as the mean ± SEM. A value of $P < 0.05$ was considered statistically significant difference.

RESULTS

Dose-response effects of FBS and WP on cell viability

The dose-response effects of FBS and individual WP on cell viability as determined by XTT assay are reported in Figures 1 and 2, respectively. Days 1 and 2 represent the proliferation stage of C2C12 cells cultured in medium containing 0-10% of FBS or WP for 48 h. The treatment medium was then replaced with differentiation medium for 96 h to support myotube formation, represented as day 6. In each dose-response study, cells cultured in DMEM media without any additives were considered as control (0%). As shown in Figure 1, FBS concentrations between 2.5-10% ($P \leq 0.02$) during the proliferation stage and only 10% ($P = 0.01$) during the differentiation stage, significantly enhanced cell viability compared to control. Similarly, α-LA significantly improved viability in the concentration range between 0.15-0.31% on day 2 ($P = 0.0002$) compared to control (Figure 2B). Also, BSA improved viability on day 2 between 0.15-1.25% ($P \leq 0.04$) (Figure 2D). While, concentrations above 2.5% of α-LA ($P < 0.02$) and BSA ($P < 0.0001$) negatively impacted cell growth (Figure 2A-C). Further, during the differentiation stage on day 6, except for inhibitory effect at 10% of α-LA ($P < 0.0001$), no significant effect of all other concentrations of α-LA, BSA, and β-LG on cell viability was found.

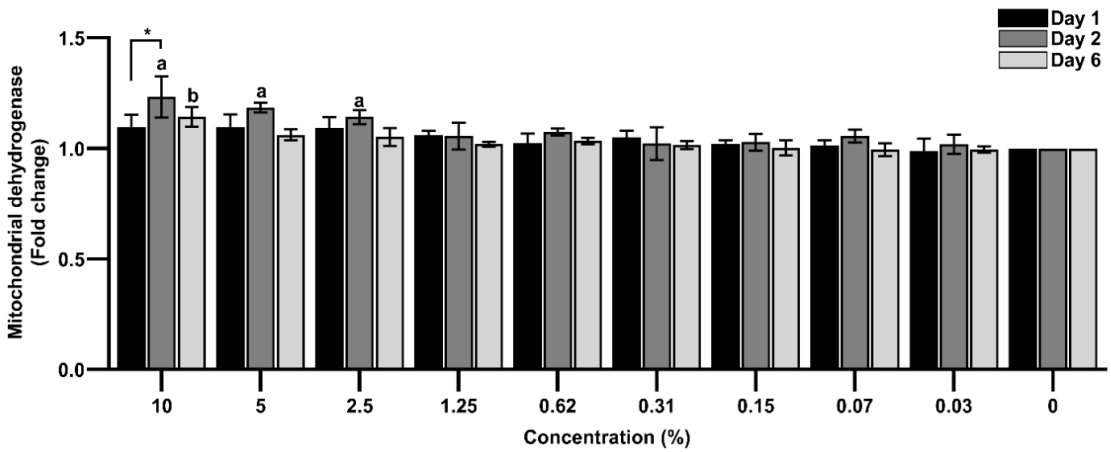


Figure 1. Dose–response effect of FBS from 0-10% on viability of C2C12 cells. Day 1 and 2 corresponds to cell proliferation with or without FBS and day 6 to cell differentiation with 2% HS medium. Data are normalized against the DMEM control and presented as mean ± SEM. Significant differences between treatments and the control within the same time points are indicated by different letters ('a' for day 2, 'b' for day 6), where [a] $P \leq 0.02$ and [b] $P = 0.01$. Significant differences between same treatment concentration at different time points are indicated by '*', where $P = 0.02$.

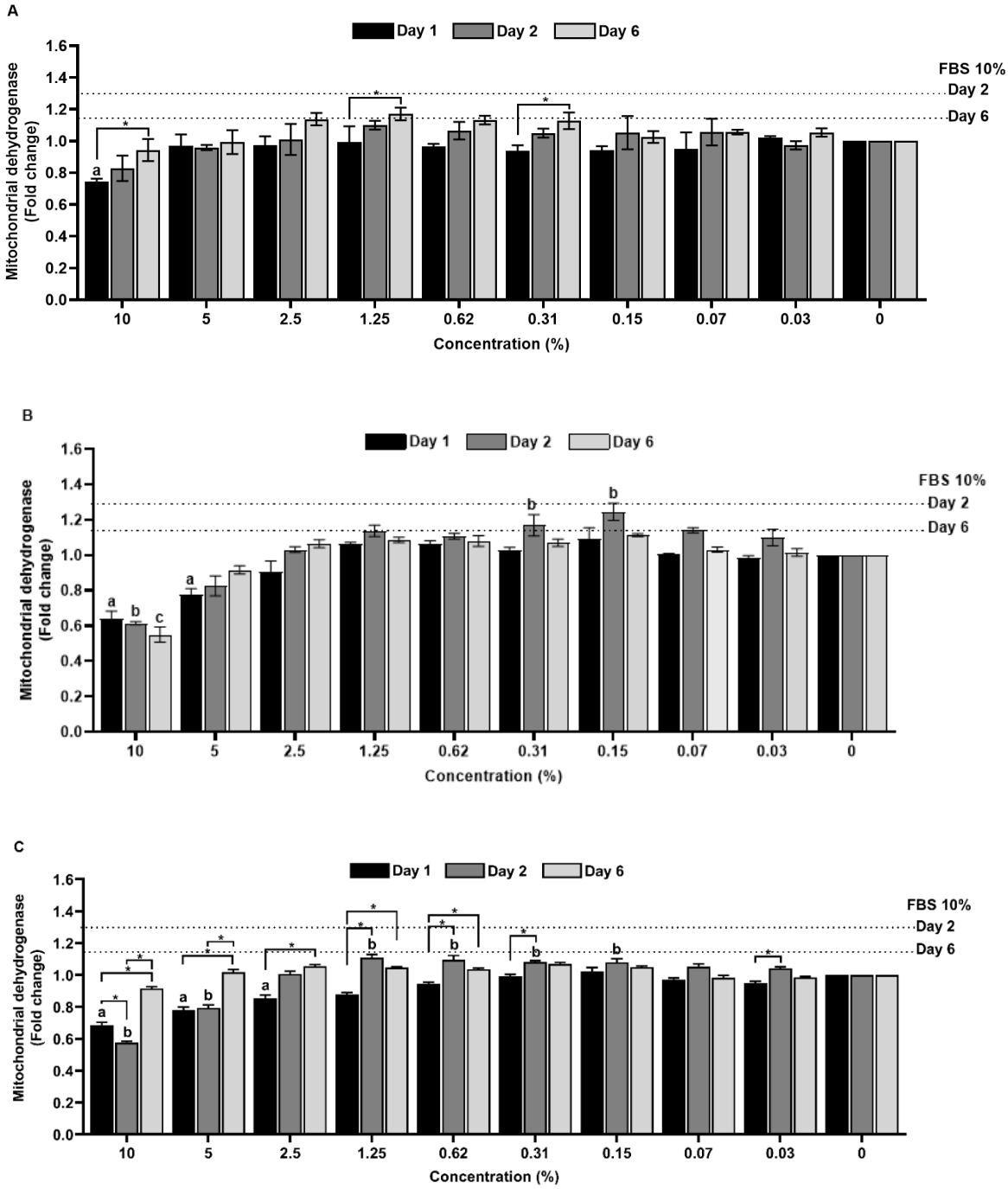
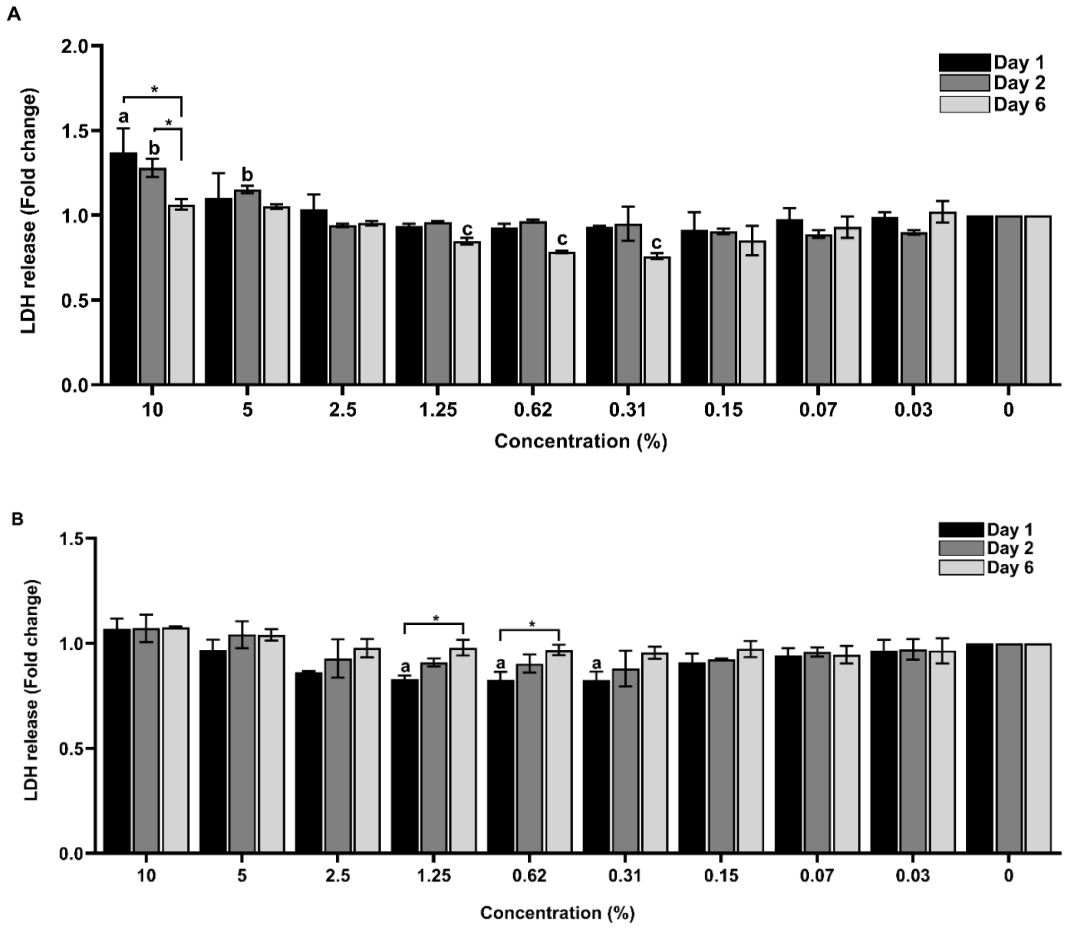


Figure 2. Dose–response effect of (A) β-LG; (B) α-LA; and (C) BSA from 0-10% on viability of C2C12 cells. Day 1 and 2 corresponds to cell proliferation stage with or without WP treatment and day 6 to cell differentiation stage with 2% HS medium. Data are normalized against the 0% FBS control and presented as mean ± SEM. 10% FBS that maximally improved cell viability is indicated in each graph with dashed lines for comparison. Significant differences between treatments and the control within the same time points are indicated by different letters ('a' for day 1, 'b' for day 2, 'c' for day 6), where [a] $P < 0.001$, [b] $P \leq 0.04$, and [c] $P < 0.0001$. Significant differences between same treatment concentration at different time points are indicated by * $P < 0.05$.

Dose-response effect of WP on lactate dehydrogenase release

The effect of individual WP on cell membrane integrity was assessed by detecting cytosolic LDH release into the culture media. Results are reported in Figure 3. During the proliferation stage, when treated with β-LG concentrations ranging from 0-10%, detrimental effect on membrane integrity was observed only at higher concentrations of 5-10% ($P < 0.0001$) compared to the DMEM control (0%) (Figure 3A). Conversely, during the differentiation stage, 0.31-1.25% of β-LG ($P < 0.006$) significantly reduced cellular LDH release. On the other hand, administration of α-LA between 0.31-1.25% ($P < 0.0001$) significantly reduced LDH release during the proliferation stage compared to the control (Figure 3B). A similar positive effect was observed for BSA at concentrations between 0.15-1.25% ($P < 0.04$) during proliferation. During differentiation, 0.62% of BSA ($P = 0.004$) significantly reduced LDH release, while concentrations of 5-10% ($P = 0.0006$) had a negative impact (Figure 3C).



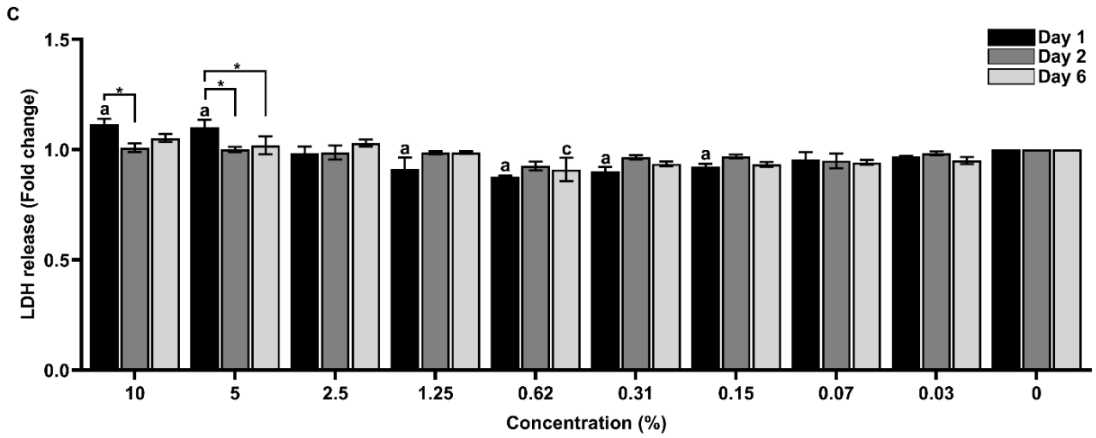


Figure 3. Dose–response effect of (A) β-LG; (B) α-LA; and (C) BSA from 0-10% on lactate dehydrogenase (LDH) release in C2C12 cells. Day 1 and 2 corresponds to cell proliferation stage with or without WP treatment and day 6 to cell differentiation stage with 2% HS medium. Data are normalized against the DMEM control and presented as mean ± SEM. Significant differences between treatments and the control within the same time points are indicated by different letters ('a' for day 1, 'b' for day 2, 'c' for day 6), where [a] $P \leq 0.04$, [b] $P < 0.0001$, and [c] $P \leq 0.005$. Significant differences between same treatment concentration at different time points are indicated by * $P < 0.05$.

WP mixture on cell morphology, mitochondrial activity, and lactate dehydrogenase release

Two WP mixtures were prepared as supplements for the culture media, and subsequently, cell growth and differentiation were assessed. The mixtures consisted of a WP high (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) and a WP low (β-LG 0.07%, α-LA 0.15%, BSA 0.15%) concentration group. The concentrations of individual proteins in each mixture were selected to be below the inhibitory range identified in the dose-response study.

As shown in Figure 4, cells cultivated in both WP mixtures showed significantly increased viability on day 2 of the proliferation stage compared to the DMEM control ($P < 0.002$). Notably, the WP low mixture exhibited the highest viability ($P = 0.0007$), indicating enhanced cell proliferation. Moreover, this growth-promoting effect of WP mixtures were comparable to 10% FBS group. Although the WP high mixture improved viability ($P = 0.002$) on day 2, it also significantly stimulated LDH release on days 2 ($P < 0.004$) and 6 ($P < 0.002$) compared to the control (Figure 5). Additionally, cell morphology analysis as shown in Figure 6, indicated WP mixtures to increase cell density comparable to 10% FBS between days 1 and 2 of the proliferation stage. When switched to HS media, all treatment groups stimulated myotube formation, as evidenced by the elongated and fused fibre-like cell structures observed under the light microscope. In contrast, control cells cultivated in DMEM media alone showed poor proliferation and differentiation (Figure 6).

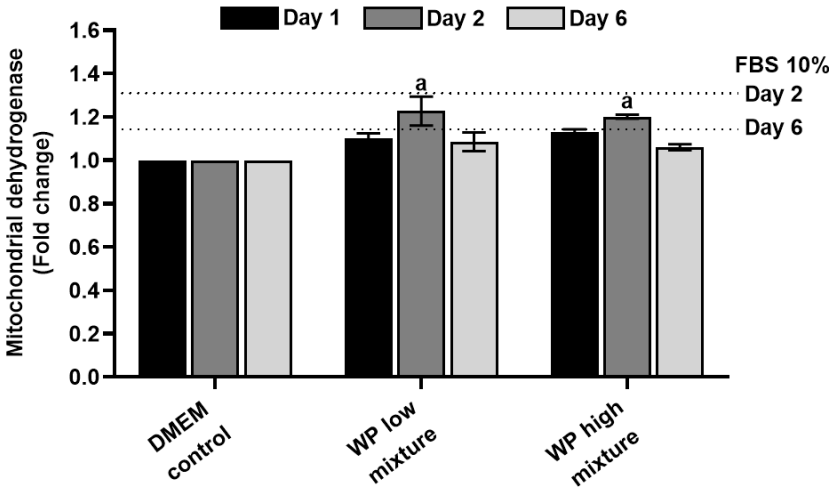


Figure 4. Viability of C2C12 cells cultured in media comprising WP low (β-LG 0.07%, α-LA 0.15%, BSA 0.15%) or WP high (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) mixture. Day 1 and 2 corresponds to cell proliferation stage with or without WP mixture treatment and day 6 to cell differentiation stage with 2% HS media. 10% FBS that maximally improved cell viability is indicated with dashed lines for comparison. Data are normalized against the DMEM control and presented as mean ± SEM. Significant differences between the experimental groups are denoted by different letters, where [a] $P < 0.002$.

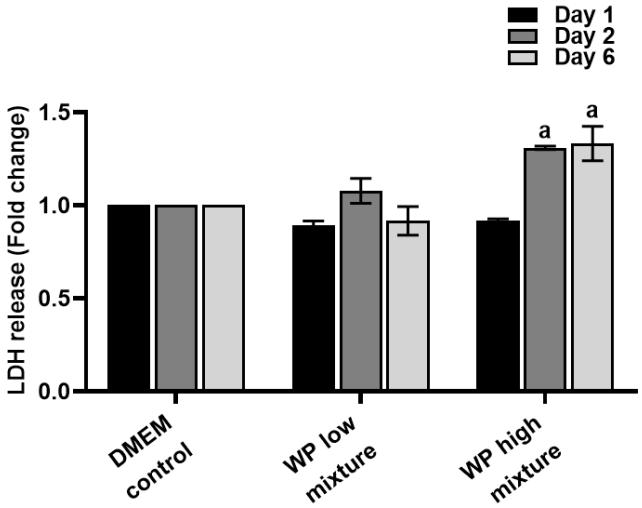


Figure 5. Lactate dehydrogenase (LDH) release of C2C12 cells cultured in media comprising WP low (β-LG 0.07%, α-LA 0.15%, BSA 0.15%) or WP high (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) mixture. Day 1 and 2 corresponds to cell proliferation stage with or without WP mixture treatment and day 6 to cell differentiation stage with 2% HS medium. Data are normalized against the DMEM control and presented as mean ± SEM. Significant differences between the experimental groups are denoted by different letters, where [a] $P < 0.005$.

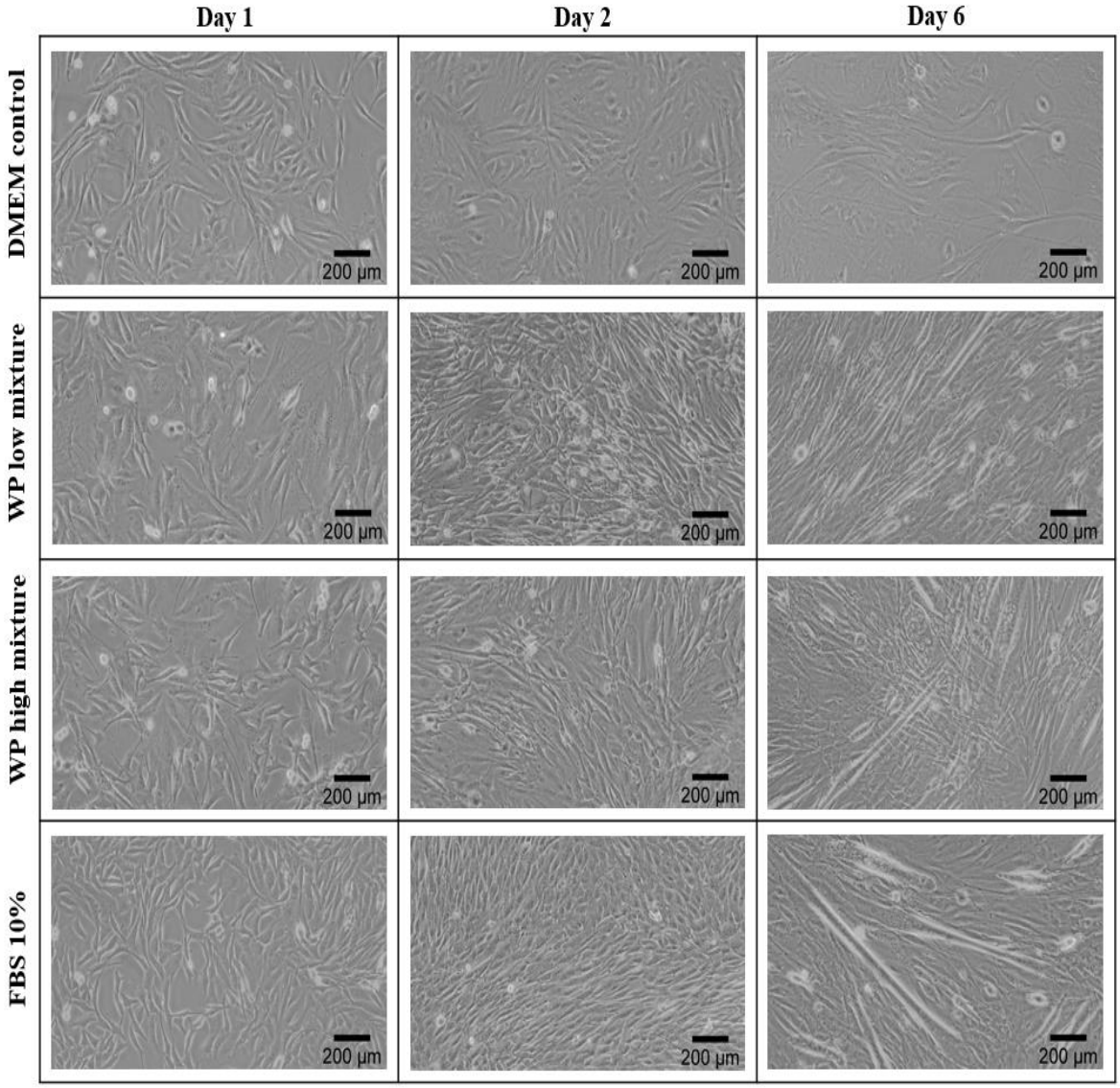


Figure 6. Morphology of C2C12 cells cultured in media supplemented with 10% FBS, WP low mixture (β-LG 0.07%, α-LA 0.15%, BSA 0.15%), WP high mixture (β-LG 1.25%, α-LA 1.25%, BSA 1.25%) mixture or without (DMEM control). Day 1 and 2 corresponds to cell proliferation stage with or without FBS or WP mixture treatment and day 6 to cell differentiation stage with 2% HS medium. Images were acquired at 10x magnification.

Citrate synthase and creatine kinase activities after cell differentiation

CS and CK activities were assessed as indicators of myogenic differentiation on day 6 after initial proliferation of C2C12 cells under different experimental conditions (10% FBS, DMEM control, WP high or WP low mixture), followed by differentiation in HS media. CK activity significantly increased in the cells cultivated in media containing WP low ($P < 0.0001$) and WP high ($P = 0.0004$) mixtures, compared to the control group (Table 2). Notably, 10% FBS exhibited the highest CK activity ($P < 0.0001$), followed by WP low and high mixtures, while CK activity was not detected in the control group. On the other hand,

WP high mixture showed the highest CS activity (P = 0.0001), followed by the WP low mixture (P = 0.0008) and 10% FBS (P = 0.0007) compared to the control group (Table 2).

Table 2. Citrate synthase and creatine kinase activities

Experimental groups	CK activity (U/L/mg protein)	CS activity (nmol/min/mg protein)
0% FBS	$0^{\$}$	0.012 ± 0.0002
10% FBS	20.24 ± 0.52^{a}	0.043 ± 0.001^{c}
WP low mixture (β-LG 0.07%, α-LA 0.15%, BSA 0.15%)	18.13 ± 1.23^{a}	0.042 ± 0.001^{c}
WP high mixture (β-LG 1.25%, α-LA 1.25%, BSA 1.25%)	10.79 ± 0.52^{b}	0.059 ± 0.001^{d}

Enzyme activities assessed post differentiation on day 6 and normalized against the total protein concentration. Data are presented as mean ± SEM. Within each assay, significant differences between the experimental groups are denoted by different letters, where $^{a}P < 0.0001$, $^{b}P < 0.001$, $^{c}P < 0.0008$, and $^{d}P < 0.008$. Groups that share the same letter do not differ significantly from one another. $^{\$}$no change in colorimetric reading.

DISCUSSIONS

Animal serum, particularly FBS, has long been an essential component of cell culture media for propagating most mammalian cell types. FBS is preferred in various cellular applications due to its rich abundance of mitogenic factors, transport proteins, and cell-adhesion components, along with relatively lower levels of antibodies compared to other animal sera (Gstraunthaler, 2003). Nearly 80% of regulatory submissions to the Food and Drug Administration reported the use of bovine serum in manufacturing cellular products for research and clinical applications (Minonzio and Linetsky, 2014). However, its inherent batch-to-batch variability has been associated with quantitative and qualitative discrepancies in *in vitro* cell productions. Additionally, previous studies have highlighted the potential presence of microbial contaminants in FBS, including mycoplasma, viruses, and prions (Gstraunthaler, 2003, Pecora et al., 2020, Paim et al., 2021). In particular, numerous known and emerging species of bovine viruses, containing either single- or double-stranded DNA or RNA genomes were frequently detected in commercial FBS batches from different geographical locations (Xia et al., 2011, Paim et al., 2021). Ethical challenges and concerns about the potential presence of non-human pathogenic factors in FBS raise safety issues (Gstraunthaler, 2003, Lanzoni et al., 2022), particularly for its use in cultivated food products intended for human consumption. Although *in vitro* meat cultivation has been introduced for at least a decade, the transition to serum-free cell cultivation seems to be slow. In recent years, proteins and cell growth factors derived from recombinant technology (Xu et al., 2020), food industry by-products (Christel Andreassen et al., 2020), or natural sources such as plants (Girón-Calle et al., 2008), algae (Ng et al., 2020, Yamanaka et al., 2023), and microbes (Venkatesan et al., 2022) are increasingly utilized as nutritious food supplements. Many of these derivatives were shown to promoted cell growth, especially myoblasts

(Knowles et al., 2019, Yamanaka et al., 2023). Notably, components of milk whey facilitate cell growth regardless of the presence of serum in the culture media (Hashizume et al., 1983, Capiaumont et al., 1994, Belford et al., 1995, Knowles et al., 2019). Above all, many of these whey components are commonly found in FBS.

As WP assist in muscle metabolism and development (Cava et al., 2024), we hypothesized it could substitute FBS in propagating muscle cells for use in cultivated meat production. As a starting point to this interest, we firstly assessed the effects of major WP (β-LG, α-LA, and BSA) on muscle cell growth through a dose-response analysis in comparison to FBS. We then determined the safe and inhibitory concentration ranges for these proteins. The maximal cell viability for the positive control, FBS, was observed at 10% concentration on day 2 of the proliferation stage. Our study confirms that concentrations below 5% for β-LG and α-LA, and below 2.5% for BSA, effectively promote cell proliferation and maintain cell membrane integrity, similar to the effects observed with FBS. While, treatments exceeding these ranges negatively impacted cell growth either by inhibiting mitochondrial activity or inducing cellular LDH release. Although LDH release indicated membrane damage, this effect was not evident at the microscopic level.

Serum albumins have long been a part of serum-free media as they are critical for transporting lipids, amino acids, hormones, peptides, metals, and other undefined low molecular weight biomolecules (Francis, 2010). Our study demonstrates that BSA stimulates cell proliferation at concentrations ranging from 0.15-1.25% on day 2, as indicated by enhanced cell viability compared to control cells. Consistent with our observations, previous research has shown that a chemically-defined medium comprising recombinant human albumin (BSA replacement), combined with commercial growth supplements (N2 and B27) having various components not limited to insulin, transferrin, sodium selenite, fibroblast growth factor (FGF), epidermal growth factor (EGF), and vitamin C, promoted the growth of human mesenchymal stem cells (Xu et al., 2020). More recently, BSA (75 µg/ml) with a mixture of different growth factors such as FGF2, fetuin, insulin, transferrin, and selenium, facilitated the proliferation of C2C12 cells and stem cells of bovine and porcine origin (Skrivergaard et al., 2023). Given that BSA is present both in FBS and whey, we propose that the later can be sourced as a potential BSA substitute.

The major component of the WP fraction is β-LG. Its bioactivity has been linked to GSH synthesis and binding of small hydrophobic ligands such as fatty acids, vitamins, and calcium (Madureira et al., 2007). While there is ample data on the growth-promoting activities of whey, information on the specific effects of pure β-LG on muscle cells is limited. Presently, we showed β-LG to enhance cell viability in a dose-dependent manner (0.31-1.25%), particularly on day 6. Earlier, peptides derived from β-LG, α-LA, and BSA having at least one branched-chain amino acid, was shown to cross the intestinal epithelial barrier and subsequently regulate free radical damage in undifferentiated C2C12 cells (Knowles et al., 2019). In their study, notably, only the peptide derived from β-LG promoted cell growth (Knowles et al., 2019), substantiating our present outcome. α-LA renders multiple biological activities either in the form of intact protein, peptides, or as multimers (Lönnerdal and Lien, 2003). Its role is mainly associated with provision of essential amino acids, passive

immunity, and cellular uptake of divalent cations such as Ca and Zn (Lönnerdal and Lien, 2003). Similar to the behaviour of FBS and other WP tested, the proliferative effects of α-LA was evident on day 2. Mitochondrial dehydrogenase enzyme acts as an indicator of both viability and proliferation. It is interesting to note that except β-LG, other compounds as BSA, α-LA, and FBS stimulated mitochondrial activity on the second day of proliferation stage, which then diminished post-differentiation with HS media. The decrease in this enzyme on day 6, coupled with increased cell density and changes in cell morphology from spindle-shape to fused elongated fibers, suggests a potential transition from proliferation to differentiation. Although β-LG stimulated proliferation and myotube formation, the enhanced activity of mitochondrial dehydrogenase during differentiation stage is unclear.

It is crucial to note that whey is as complex as FBS, which varies in molecular composition due to factors like animal diet, lactation stage, and production processes (Kuczynska et al., 2012, Minj and Anand, 2020, Hayes et al., 2023). To avoid discrepancies in cellular applications, we decided to establish and test WP mixtures as cell growth supplements based on selected concentrations of individual proteins that positively affected in the dose-response study. Through this approach, the essential characteristics of growth supplements needed for establishing well-balanced cell growth in *in vitro* meat culture systems could be achieved. During myogenesis, muscle stem cells or myogenic precursor cells undergo mitochondrial changes. This process involving the activation, proliferation, and differentiation of these cells into myotubes, is energy-intensive. Consequently, muscle stem cells increase the rate of oxidative phosphorylation in mitochondria to rapidly generate ATP for sustaining the high energy demand (Kunz, 2001, Kelley et al., 2002, Remels et al., 2010). The impact of WP mixture on two key regulatory enzymes involved in mitochondrial metabolism such as CK and CS, were assessed on day 6 post-differentiation. These enzymes were extensively evaluated in muscle cells as indicators of differentiation in muscle regeneration (Kelley et al., 2002, Remels et al., 2010, Xu et al., 2022). Our study clearly showed cells proliferated in media consisting WP mixtures or FBS and when differentiation in HS media, exhibited enhanced CK and CS levels compared to the control group. The control group cells, which was cultivated in basal media alone, exhibited poor proliferation and differentiation as evidenced by morphological observations and lower CK and CS levels. Consistent with our observations, earlier, the expression of CK, CS, and other important differentiation markers (myogenic factor 5, myoblast determination protein 1, and myogenin) from the family of myogenic regulatory factors (MRF), shown to increase in serum-starved C2C12 cells after 24 h supplementation with 80% whey concentrate in a dose-dependent manner (0.1 and 0.4 mg/ml) and then differentiated with HS media (Xu et al., 2022).

CONCLUSIONS

In our experimental conditions, WP, individually or as a mixture, substituted FBS in stimulating muscle cell proliferation under serum-free culture conditions. When subsequently switched to a low-mitogenic HS medium, these WP facilitated myotube formation. The WP mixture established in this study could serve as a sustainable cell growth

supplement not only for cultivated meat but also cultivated milk and other cellular productions requiring a defined alternative to FBS. Furthermore, individual proteins within the WP mixture can meet nutritional requirements, regulate biological functions, and provide essential characteristics of FBS, such as cell adhesion and biomolecular transport. While the C2C12 cell line is valuable for this study, possibilities of discrepancies in cellular responses compared to cells in *in vivo*, validation of current findings with primary muscle cell culture is essential. Besides, further studies are needed to explore the impact of WP on gene and protein expressions related to MRF and mitochondrial metabolism. Additionally, the valorisation of whole whey serum as a cell growth supplement is an interesting aspect for further study. It is also crucial to replace HS during differentiation, as with FBS. Therefore, characterization of other whey components, such as growth factors, is essential for use as HS substitutes.

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General conclusions

The escalating demand for food and feed, driven by rapid population growth and intensifying resource constraints, underscores the critical importance of identifying sustainable and innovative protein sources. This thesis embarks on a comprehensive examination of different promising alternatives: hemp-based products, oil-seed co-products, microalgae, and cultured meat. These systems were not only analysed for their potential to reshape food and feed production but also evaluated against the ambitious sustainability objectives outlined in EU policies, such as the Agenda 2030, the European Green Deal, and the Farm to Fork strategy.

Hemp-based products have shown considerable potential as a nutritionally rich and ecologically sustainable alternative in the feed industry. This research, in particular, has highlighted its high nutritional value and functional properties, corroborating this evidence through both *in vitro* approaches and *in vivo* trial on laying hens. Although the scientific literature reports numerous studies on the beneficial effects of hempseed supplementation in the diets of poultry and laying hens in terms of growth and production parameters, the present thesis reached similar results by enhancing a hemp co-product, integrated up to 9% in the diet. This strategy not only improved production performance, but also enriched the nutritional and functional profile of the eggs, offering a higher quality product, characterised by a reduced cholesterol content and an improved lipid profile. These data are of high importance if we consider that hemp plants contribute to environmental sustainability through carbon sequestration, soil phytoremediation and reduction of the use of synthetic agrochemicals; generally in line with the EU policy. Despite its nutritional and environmental benefits, the large-scale adoption of hemp in agricultural systems is hampered by several critical issues, including limited land availability, high production costs and stringent regulatory restrictions.

The present work has demonstrated the nutritional and functional quality of co-products from the food industry. The presence of multiple bioactive peptides and related activities in camelina and cardoon co-products suggests their potential use in animal diets, especially to safeguard antioxidant status. While it is true that the valorisation of co-products is a key way to achieve environmental sustainability, it is important to emphasise that they are characterised by high variability due to genetic variability, environmental conditions, but

above all to the different processing methods to which they are subjected. The solution, although very difficult to meet, could lie in the standardisation of manufacturing processes, which would reduce the variability of co-products and ensure homogeneity. This would optimise the nutritional and bioactive characteristics of these matrices, making them more reliable and suitable for use in animal diets. In addition, closer communication between co-product manufacturers and end-users would be crucial to ensure that information on the properties and benefits of co-products are correctly conveyed, thus facilitating their widespread adoption. The synergy between research, development and practical application could thus facilitate the integration of these co-products into food chains in a sustainable and efficient manner.

Microalgae represent an extremely interesting resource for the future of animal nutrition and the development of sustainable protein solutions. Their ability to grow rapidly in controlled environments makes them an environmentally friendly choice over traditional protein sources, which require more expensive and polluting agricultural land, water and other natural inputs. Microalgae not only contain high concentrations of protein and essential fatty acids, but are also rich in bioactive compounds that, as in the case of paramylon in *E. gracilis*, can provide additional benefits, such as immunomodulatory effects. This makes them a highly valuable source of nutrients for animal nutrition, with the potential to improve the health and welfare of farm animals. A crucial aspect that emerged from this thesis is the possibility of modulating the microalgae culture medium with the aim of improving its nutritional and functional profile, resulting in a final product with specific desired characteristics. In particular, *E. gracilis* culture media optimization allowed for the improvement of its antioxidant profile. Although microalgae have already been used in the feed industry for a long time, this ability to customise their composition to the specific needs of farm animals could make microalgae a key component of feeds, increasing their use while reducing the need for chemical supplements and improving nutritional efficiency. Modulation of the growing medium not only optimises biomass production, but also offers the potential to reduce the environmental impact of feed production, increasing the overall sustainability of the farming system.

When discussing microalgae, an important concern is their possible contamination by microplastics and nanoplastics, a phenomenon that can occur during the cultivation process in bioreactors, very often produced from plastic polymers. Plastic particles can transfer from

the walls of the bioreactors to the microalgae during the growth phase, accumulating inside the cells. This accumulation represents a significant problem, as nanoplastics can enter the food chain, posing a risk to food safety and human and animal health. In particular, the research conducted in this thesis has shown that plastic nanoparticles, despite their different behaviours, such as the agglomeration phenomenon, in different culture media, can persist in the digested fraction, remaining in the digestive system of animals consuming these feeds. Nanoplastics that are not eliminated during digestion can cause harmful effects, including inflammation, immune system alterations and cell damage. For this reason, it is essential to develop and adopt safer cultivation methods that avoid the use of plastics in bioreactors or significantly reduce the risk of contamination.

Cultured meat represents a promising biotechnological solution to meet the growing global demand for animal protein while reducing the environmental impact associated with conventional animal husbandry practices. This approach is based on the *in vitro* culture of muscle cells, making it possible to minimise the consumption of natural resources, such as water and soil, and to reduce GHG emissions from intensive livestock farming. Despite the first marketing application also at EU level, its large-scale implementation is still hampered by major scientific, technological and economic challenges. A central element of the production process concerns the cultured media composition, in particular the protein composition and the replacement of FBS, currently used as a supplement in cell culture media. The outcomes of this thesis have shown that co-products derived from the food industry, such as whey proteins, can effectively support the viability, proliferation and differentiation of C2C12 murine muscle cells, constituting a potential sustainable and ethical alternative. Although further studies are needed to validate the reproducibility of these results on other cell lines, optimise their efficiency and ensure compliance with food safety standards, with particular reference to the European regulatory framework, what has just been reported shows the importance of co-products in the sustainability of this process as well; realities that although independent of each other could find a strong synergy.

In cultured meat production, process scalability is another key challenge. Although the results obtained in the laboratory are promising, the transition to industrial production systems requires significant technological innovations, particularly in the area of bioreactor design, the formulation of economically sustainable culture media and the reduction of energy costs. Furthermore, consumer and institutional acceptance will depend on the

implementation of strategies aimed at communicating the transparency of the production process and improving the perception of the product's naturalness. In summary, cultured meat is an innovative and potentially sustainable solution, in line with the European strategic objectives of circular economy and reduction of GHG emissions. However, overcoming current limitations will require a multidisciplinary approach that integrates biotechnological advances, industry investment and the adoption of an appropriate regulatory framework. Only through this synergy will it be possible to translate promising research results into a concrete reality in the global food landscape.

Collectively, the findings of this thesis underscore the transformative potential of these alternative protein systems in addressing global food security and sustainability challenges. Hemp-based products and co-products represent a versatile and environmentally friendly solution, microalgae offer a scalable and nutrient-dense alternative, and cultured meat exemplifies the future of ethical and innovative protein production that could also be achieved through the valorisation of co-products of the food industry. Together, these approaches represent critical tools in mitigating the environmental impacts of traditional agriculture, alleviating feed-food competition, and ensuring resource efficiency. Future research must prioritize the standardization of analytical methodologies to ensure consistent evaluation and regulatory compliance of these novel protein sources.

In conclusion, this thesis has highlighted potential alternatives for the food and feed sectors, capable of addressing emerging global challenges. Through the application of biotechnologies, the adoption of sustainable agricultural practices, and the promotion of regulatory innovations, it is possible to accelerate the transition towards a circular and green economy. The insights gained through this work provide a robust foundation for future exploration, with the aspiration that hemp-based products, microalgae, and cultured meat will occupy central roles in shaping a sustainable, equitable, and resilient global food system.

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