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# The future of foods

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# The future of foods

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# Editorial: The future of foods

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Editorial on the Research Topic
The future of foods

Food production systems are currently facing a redefining moment filled with challenges and opportunities with the potential of rendering them more wholesome and sustainable. Thus, the future of foods can take many paths, from the product itself through the expanded use of new ingredient sources (e.g., grass, microalgae, acorns, insects, etc.) or by-products of the food industry (e.g., fruit pomace or brewer's spent grains), the development and optimization of novel processing technologies (e.g., additive manufacturing, precision fermentation, cell cultures and emerging processes) and the advancement of disruptive techniques for data management, integration, and food systems virtualization (e.g., machine learning, Generative AI (gen AI), large language models (LLMs) and digital twins).

Looking at the future of foods in a way that allows overcoming current challenges, and taking advantage of its opportunities, requires many considerations, such as factoring in customisation, security, extensive digitalization, and social, environmental, economic and health impacts of innovations. In particular, the development of new foods with sustainability in mind (e.g., alternative proteins) must consider health benefits as well as indulgence and sensory appeal, and these factors must be weighed against the cost of production processes and the ultimate cost to the consumer.

Contributing articles to this timely Research Topic explored the future of our food production systems, addressing key challenges ranging from food security affected by war and the political climate in grain-producing countries, or the impact of science and technology on food security in Ukraine, a hub for food and grain production. They also tackled concerns raising in other developing countries, specifically related to food safety practices in elderly facilities and the impact on foodborne disease outbreaks in South Africa (Tshegofatso and Ntsoaki) which can be generalised and extended worldwide as ageing populations are becoming a reality in many countries.

New unusual and disruptive food sources, such as cell-cultured or cultured meat, are considered from the point of view of increasing the sustainability of the process by using agricultural waste as low-cost scaffolds for cultured meat (Perreault et al.), to the detail of the molecular scale in a review by Azhar et al. and specificities, such as the impact of microplastics on fish muscle cell proliferation and differentiation (Sun et al.).

Other alternative soilless cultures and carbon fixers, such as spirulina and chlorella microalgae as food ingredients with high nutritional value, and proven sustainability, are covered in detail. In the study of Fratelli et al., the incorporation of spirulina biomass (SB) and spirulina residual biomass (RB) into wheat bread after extraction of C-phycocyanin was explored. They replaced 3% of wheat flour with either SB or RB to produce wheat bread with improved volume, high antioxidant capacity while also reducing the risk/presence of chemical contaminants (i.e., heavy metals). Baking with these novel ingredients also improved the digestibility of spirulina protein. Melcher et al. analysed and compared eight different commercial Chlorella products from distinct EU suppliers based on the identity of the production strain, macro- and micronutrient profiles, sensory properties, as well as microbial loads. The authors observed significant variations between white, yellow and green Chlorella products and concluded that advancing nutrient- and sensory comparative data sets could be helpful in the development of innovative foods.

From a microbiological perspective, the novelty of screening natural compounds for prophage induction in the control of pathogenic bacteria in food is extensively covered by the contribution of Tompkins et al. The authors emphasized the need to find natural approaches to combat pathogenic bacteria in food, rooted on the drive for clean-labels and the urge to maintain food safety. They proposed a high-throughput luminescent prophage induction assay as a valuable tool for the initial screening of natural bioactive compounds that have the potential to improve food safety and quality by inducing prophages. However, they recognised that further research is needed to understand the mechanism of bacterial cell death and to establish optimal concentrations for prophage induction in the context of food preservation.

The manuscript of Bazhal and Koutchma discussed the Impact of science and technology development in Ukraine on food security in the world. The solutions to enhance the stability of the grain and food supply are reviewed while aiding in reducing food and grain losses, improving food safety, and developing novel processing technologies. Another manuscript by Mottaleb and Govindan applied an ex-ante impact assessment procedure to examine the potential repercussions of the Russia-Ukraine conflict on the consumption and wheat trade and food security. In order to avoid hunger and supply shock from related disasters in the future, this study urges to search for alternative sources of grains, including wheat, particularly in import-dependent, resource-poor countries.

All these contributions to the Future of Foods Research Topic are novel and thought-provoking enough to attract your attention. Still, one manuscript, in the digital area remains, that will definitely convince you that this Research Topic gathers most of the key issues about the future of our food - Virtualisation of food: applications and perspectives towards optimising food systems. In this mini-review, Chen et al. discussed digital objects (models, ghosts and twins) as monitoring and improvement tools for food systems. By dissecting virtualisation requirements, including data collection and analysis using complementary techniques reaching from Machine Learning to physical models, the authors shed light on the applicability of digital objects at several levels, from product to distribution channels and their dynamic, economical and effective roles to inform the design and performance of food products, manufacturing processes and supply chain logistics to achieve healthy and sustainable food systems.

The editors are certain that this Research Topic of articles is a must-read for any aspiring or established food scientist/technologist/engineer.

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# Ukraine as a food and grain hub: Impact of science and technology development on food security in the world

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The challenges facing the world today caused by a growing population, reduced resources, global warming, climate shocks, and social and political crises are heavily affecting agri-food systems and supply chains. A global food crisis fueled by conflicts, global warming, climate shocks, and the COVID-19 pandemic is growing because of the bad effects of the war in Ukraine which is one of the world's major breadbaskets. Science and innovation are the key accelerators to achievingthe complex rapid change in food production, distribution, and consumption required to support the global food security. This article reviews the information on grains, crops, and food production in Ukraine and discusses how the development of food education, science, and technology in Ukraine may impact food security in the world. Ukrainian food science as a part of the global scientific community offers solutions to enhance the stability of the grain and food supply while aiding to reduce food and grain loss, improve food safety, develop novel processing technologies such as pulsed electric field technology (PEF), biotechnology, and extraction methods for biomass recovery or separation technologies, increase environmental safety, energy saving, management of food production and distribution, make advancement in the production of sugar and alcohol, and improvements of food attributes. In support of this conclusion, the main research and development achievements of Ukrainian food scientists are represented.

KEYWORDS

**food security, PEF, agriculture, grain, science and technology (S&T)**

## Introduction

According to the United States Department of Agriculture (USDA) definition, food security means ensuring that all people in the world have enough food to live a healthy and active lifestyle. Food sustainability is affected by social, economic, environmental, technological, and geopolitical conditions that may influence the food supply-chain in the long-run including agriculture, food processing, and distribution (Cole et al., 2018; Thomas et al., 2022). In 2020, the Food and Agriculture Organization of the United

Nations (FAO) that about 800 million people faced hunger (https://www.fao.org/state-of-food-security-nutrition/en/). FAO also noted that undernourishment climbed globally from 8.4% in 2019 to 9.9% in 2020. Meanwhile, according to the United Nations (UN), our planet's population is likely to increase up to more than nine billion people by 2050. Increase in food production needs by that time is estimated at 71% (Cole et al., 2018) to 100% (UN evaluation). This presents substantial challenges to achieving global food security goals (Moseley, 2022).

Conflicts and insecurity are the main drivers of food insecurity globally. A global food crisis fueled by conflicts, climate shocks, and the COVID-19 pandemic is growing because of the ripple effects of the war in Ukraine which is one of the world's major breadbaskets (Gross, 2022). Ukraine and Russia together supply about 30% of globally traded wheat (*Triticum aestivum*), 20% of maize (*Zea mays*) and 70% of sunflower (*Helianthus annuus*) supplies. A shortfall in export supplies is driving prices up, leaving import-dependent countries with higher food import bills or less food to eat (Hellegers, 2022).

In 2021, 36 out of 55 countries with food shortages depended on Ukraine and Russian exports for more than 10% of their total wheat imports, while some obtained almost the entire wheat imports from Ukraine and the Russian Federation. Director of the FAO Office of Emergencies and Resilience Mr. Rein Paulsen said: "Ukraine's farmers are feeding themselves, their communities, and millions more people around the world. Ensuring they can continue production, safely store and access alternative markets to sell their produce is vital to secure food availability, protect livelihoods, strengthen food security within Ukraine and ensure other import-dependent countries have a steady and sufficient supply of grain at a manageable cost."

To address the impacts of the war in Ukraine on the global agricultural sector, FAO has launched a new $17 million project to help Ukrainian farmers save the upcoming harvest in July and August of 2022 while ensuring the export of critical agricultural goods to international markets. The project aims to restore grain storage capacity and functionality of supply chains from harvest to export as well as maintain the productive capacity of Ukrainian farmers to enable the continuation of future productions.

Another important factor influencing the global food security is a food loss and waste (Saba and Patil, 2022). The USDA estimates that more than one-third of edible food in the United States is wasted. In developed countries, food waste mainly occurs at the retail and consumer ends of the supply chain (Spellman, 2021). Food loss and waste in agriculture are a pressing problem in developing countries, exacerbated by a lack of distribution infrastructure and problems in food storage such as refrigeration and processing (Cole et al., 2018).

One way to reduce the food waste is the continued development of preservation and stabilization post-harvest technologies (alone or in combination/synergy with other physical and chemical techniques) such as pulsed electric field (PEF) treatment (Bazhal et al., 2006; Barba et al., 2015; Zhang et al., 2020; Raso et al., 2022), high-pressure processing (Abera, 2019; Fam et al., 2021), sonication (Huang et al., 2020), drying (Mousakhani-Ganjeh et al., 2021; Hill et al., 2022), pressing, osmotic dehydration, freezing, cooking, or fermentation (Piergiovanni, 2012; Thatoi et al., 2022) which extend the shelf life of certain foods and can improve the quality of grains (Dar et al., 2022). Moreover, food processors develop new value-added extraction methods for biomass recovery or separation technologies to limit food loss (Feinbaum, 1999; Kumar et al., 2022). With the same purpose, selective recovery and extraction of inulin, starch, sugars, polysaccharides, proteins, polyphenols, flavor compounds, pigments, phytochemicals, and other high-value components present a special interest in the recycling of food wastes, sub-products, and non-food biomasses (Vorobiev and Lebovka, 2020).

Since the food security for people also depends on the nutritional status of livestock, the recycling of food waste into animal feed is also a promising way to rationalize using and reducing food waste (Noori et al., 2022; Sheppard and Rahimifard, 2019).

Actually, the challenges facing the world today are heavily affecting agri-food systems and supply chains, hence the need for complex rapid change in food production, distribution, and consumption (Rao, 2022). The Director-General of FAO, Mr. Qu Dongyu emphasized that the required transformation is critical, and science and innovation are key accelerators to achieve this change. Therefore, FAO initiates the first-ever Science and Innovation Strategy in achieving new levels of productivity, quality, storage, diversity, efficiency, and environmental sustainability.

However, science and innovation alone are not sufficient, enabling policies are also needed that respond to the needs of science results implementation for different level food producers including farmers. Moseley (2022) stated that a major rethink of the conventional food security paradigm has reigned for the past several decades and is needed. Beddington et al. (2012) highlighted that food science plays an integral role in global food security by informing concurrent, strategic investments to establish climate-resilient agricultural and food production systems, minimize greenhouse gas emissions, make efficient use of resources, develop low-waste supply chains, ensure adequate nutrition, encourage healthy eating choices and develop a global knowledge system for sustainability.

It is beyond the scope of this paper to consider all factors affecting food security. The objective of this paper is to review the information on grains, crops, and food production in Ukraine and discuss how the development of food education, science, and technology in Ukraine may impact food security in the world.

## Grain and food production in Ukraine

Historically, Ukraine was an agricultural country. According to FAO estimation, Ukraine has about 4,13,000 $km^2$ of agricultural area of the almost 6,04,000 of total area. Currently Ukraine is one of the five largest world grain exporters, annually supplying more than 45 million tons of grain to the world market. It is also the world's largest producer and exporter of vegetable oil sunflower, rapeseed (*Brassica napus*). Most of all, in 2019 Ukraine exported corn (32 million tons for $5.2 billions, EU—47%, China—13%, Egypt—12%, and others). Sunflower oil ranks second in terms of exports ($3.8 billion). Ukraine exported wheat for $3.65 billion, which makes it the third largest commodity of Ukrainian export (most sold to Turkey, Bangladesh, and Egypt). The eighth place in terms of Ukrainian exports is occupied by rapeseed ($1.26 billion), the ninth place is for soybeans ($1.16 billion), and sunflower seed cake is in the 10th position for exports ($0.975 billion). The Ministry of Agrarian Policy and Food of Ukraine reported that the export of agricultural products and food in 2021 increased by 22% compared to 2020 and reached about $31.3 billion, which accounted for 39.8% of the total exports from Ukraine.

At the same time, imports of agricultural products and food products in 2021 increased by 20% compared to 2020 and amounted to $6.9 billion, or 10.5% of total imports of goods to Ukraine.

The development of the food industry in Ukraine started mainly in the sugar from sugar beet (Beta vulgaris) production and flour milling. Further developments covered all essential branches of the food industry including flour-grinding, alcoholic beverages, brewing, baking, confectionery, meat, poultry and dairy, fishing, fat-and-oil, wine-making, canning, salt, vegetable, tobacco products, etc. (Gulyi, 2000; Mostenska, 2014; Petrushina, 2017). Over 22,000 large, medium and small size enterprises of various forms of ownership produced almost 20% of the total industrial output. Also, in Ukraine there are about 20 food enterprises with foreign capital: Cargill, Bunge (Suntrade), Glencore Agriculture, Nibulon, Delta Wilmar, Pfeifer andLangen, PepsiCo, Coca-Cola, Danone, Nestle, Mondelez (Craft Foods), Carlsberg, SUN InBev, Mareven Food Europe, Imperial Tobacco, JTI, B.A.T., Philip Morris and others.

The largest part of the food is beverages including meat and dairy products, tobacco products, bread and bakery products (such as bread, rolls, crackers, pies, donuts), oils and animal fats. In the total volume of food products sold in 2008, the products of the oil and fat industry accounted for almost 14%, dairy products and ice cream—13.6%, products of the meat processing industry—13.2%, bakery—5.5% (Sabluk, 2008).

In the frame of preparation for Ukraine's accession to the European Union, Ukraine developed and adopted some basic international laws that affected not only businesses, but also ordinary buyers. All food manufacturers have implemented a system of risk analysis, Hazards Analysis and Control of Critical Points (HACCP) aimed at improving food safety in the enterprises.

## Brief history of food science education

Food science and research in Ukraine have traditionally contributed to the development of the food industry and vice versa. In the 19th and early 20th centuries, there was no special education in food science, nutrition, processing, and engineering (Mostenska, 2014). Engineers and scientists that graduated from other universities (physicists, chemists) worked in the food industry. However, the growing sugar production determined a need for technical expertise that forced the opening of a program in a school (college) in the city of Smila (Cherkassy region), because it was the center of sugar production in Ukraine (Gulyi, 2000). This was the first specialized educational institution for the training of technologists, chemists, and technicians for the sugar industry. Later, this college became the Smila Institute of Sugar Industry. Also, the course on sugar technology began to be provided at Kyiv University and Kharkiv Technological Institute (Gulyi, 2000). In addition, the internships for some specialists in the laboratories of the Zurich (Switzerland) and Braunschweig Polytechnics, as well as at the Twulpstedf Plant (Germany) were offered (Gulyi, 2000). The number of such specialists was very limited, but this was the first successful experience of international cooperation in Food education.

In 1898, with the financial support of the owners of sugar factories, the Kyiv Polytechnic Institute (KPI, now the National Technical University of KPI) was founded. There was the first department of sugar technology (among mechanical, chemical, engineering, and agriculture) at the KPI, where engineers for the sugar industry have been educated. In 1930, based on the Smila Institute of Sugar Industry and the Department of Sugar Technology of the Kamyanetz-Podil'skyi Chemical Institute, and the Kyiv Polytechnic Institute, the Kyiv Institute of Sugar Industry (now called the National University of Food Technologies, NUFT) was established (Gulyi, 2000). All of the mentioned institutions are the educational and research branches of NUFT situated in different locations in Ukraine.

In total, more than 25,000 students study at the NUFT. Highly qualified specialists are educated in undergraduate, postgraduate, and doctoral programs. The University staff includes more than 750 professors with 21 academicians, 150 doctors of science, and 400 PhD degrees. The University has seven specialized academic councils for the defense of dissertations in 14 scientific specialties (Mostenska, 2014; Gulyi, 2000; https://nuft.edu.ua/).

## Food science, research, and technology development

Specialized research laboratories with a focus on fundamental and applied aspects of grain and food research have been functioning in the NUFT for 40 years (Gulyi, 2000; https://nuft.edu.ua/). Research activity is directed to the development of theoretical foundations of food technologies and processing intensification and optimization, development of advanced processing technologies and highly productive equipment, systems and means of mechanization of labor-intensive processes and operations, new power equipment and systems of rational power consumption as well as for the improvement of organizational activity and increase of effectiveness of food and processing enterprises (Mostenska, 2014; Gulyi, 2000; https://nuft.edu.ua/).

NUFT has a few dedicated research centers including (https://nuft.edu.ua/):

- Research institute of food technologies with six subsidiary research laboratories such as laboratory of construction and innovation projects, laboratory for studies on pectin properties, etc.
- Research engineering center of machines and technologies for packing of food production,
- Research engineering center for development and introduction of technologies and equipment for small food production,
- Research production center for quality evaluation of raw materials and finished products,
- Chair groups of research engineers working with economic problems in the food industry of Ukraine
- The institute for energy problems in the food industry

More than 3,000 patents of Ukraine and five license agreements on delivery and development of equipment, abrasive paste, and products have been received over the last 30 years (Gulyi, 2000; Mostenska, 2014). Thirteen (13) scientists of NUFT were awarded with the State Prize of Ukraine for achievements in the field of science and engineering. Four scientific journals are published at the NUFT: Ukrainian Journal of Food Science (in English, http://ukrfoodscience.ho.ua/), Ukrainian Food Journal (in English, http://ufj.nuft.edu.ua/indexen.html), Collection of scientific papers of NUFT (in Ukrainian), Food Industry (in Ukrainian).

In addition to NUFT with its above mentioned branches, there is Odessa Technological Institute of Food Industry (OTIFI) is a part of Odessa National Technological University (ONTU) in Ukriane (https://ontu.edu.ua/). The journal of Food Science and Technology, Economics of the Food Industry, and scientific papers are published by ONTU (in Ukrainian). Also, there is the International Center of Ukrainian-French Cooperation, the International Center of Ukrainian-Bulgarian Cooperation, and the International Center of Ukrainian-Turkish Cooperation at ONTU (https://ontu.edu.ua/#s5).

The main research and development achievements of Ukrainian food scientists are listed in Supplementary Material (Gulyi, 2000; Mostenska, 2014). This includes important contributions in the area of reducing food and grain loss, enhancing of food safety, developing of novel processing technologies such as pulsed electric field technology (PEF), biotechnology, and extraction methods for biomass recovery or separation technologies, improving environmental safety, energy saving, management of food production and distribution, and advancement in the production of sugar and alcohol, bakery and improvements of food attributes (Sabluk, 2008; Mostenska, 2014).

Despite the fact that Ukrainian food science has a huge research potential, it should be noted that industrial implementation of the scientific results and their impact is limited primarily by the lack of project management skills of Ukrainian scientists. Moreover, the challenging political and economic situation in Ukraine contributed to the deterioration of the situation in the food industry, and collaboration with research organizations. Most state-owned food enterprises went bankrupt over the last 30 years. There are only some of them such as bread production companies, for example, are supported through state subsidies. The basic needs of the population in food products are provided by national commercial enterprises and imported products.

In contrast to food production, the state of research in food science has deteriorated to a greater extent (Petrushina, 2017). Enterprises mainly used foreign technological lines, equipment and ready-to-use engineering and technological solutions and supply chains. At the moment, the food industry is experiencing a much greater need for production engineers and other specialists than scientists. However, in some situations of food businesses there is a need for special expertise and need in hiring scientific experts to solve some problems and provide assistance on a consultancy basis. There is also a trend when scientists develop their food technologies and start their business. This mainly takes place in beverage production, the confectionery industry, and meat products (e.g., First Private Brewery LLC, Kombucha LLC (production of beverages based on fermented kombucha), Nutrimed LLC (production of food additives), Ukrpectin LLC (processing of fruit and berry and vegetable products, production of pectin-containing products, and malt and polymalt extracts), MANZANA FOOD LLC (processing of fruit and jam production), etc.). Current government support of science in Ukraine is very limited. In 1991, funding for science was 2.4% of GDP, which gradually decreased to the current level of about 0.2% of GDP (Petrushina, 2017).

In Ukraine, starting in 2019, a new approach to research funding has been introduced on a competitive basis through the National Research Foundation (NRF) of Ukraine (https://nrfu.org.ua/en/). The Foundation determines and provides grant support for scientific research, and scientific and technical

(Experimental) developments of scientific organizations based on priorities. The largest number of grants have been received by scientific institutions of the National Academy of Sciences of Ukraine (108 grants), scientific institutions, and institutions of higher education of the Ministry of Education and Science of Ukraine (98 grants), which accounts for about 95% of the total grant support by the NRF (https://nrfu.org.ua/en/). Besides the fact that competitive funding of scientific activities through the NRF is the right step towards creating a competitive environment in the scientific field, this is not sufficient ($26 million USD in 2021) to effectively use and further develop the scientific potential of Ukraine (https://nrfu.org.ua/en/).

A few directions and next steps can be envisioned for successful Food Science and research development in Ukraine.

- changing the priority of government policy and supporting national science by placing government orders
- involving Ukraine in the FAO Science and Innovation Strategy including the following:

1) do better at monitoring the innovations, promoting adaptation to local needs, and scaling up successful implementations
2) introduction of better knowledge management and sharing as a key factor
3) improve research extension activities, using the best available technological solutions and expertise, including digital technologies, to effectively reach all food producers

- attracting foreign investment in the food industry and food research of Ukraine, to achieve a significant increase in companies with foreign capital
- developing targeted international collaborative programs and creating funds for cooperation with Ukrainian universities and research centers, allocation of grants, etc. that will address food security issues
- implementation of international internship programs for students and young Ukrainian scientists in the world's leading research universities and business centers
- allocation of international food research centers and laboratories in Ukraine

## Conclusion

Food science plays an integral role in global food security while we face challenges caused by a growing population, reduced resources, climate shocks, and social and political crisis such as the war. Ukrainian food scientific institutions and organizations as a part of the global scientific community can offer solutions, highly qualified experts, and substantial experience to contribute to and address these complex issues. This may include to enhance the stability of the food supply while aiding to reduce food and grain loss, enhance food safety, develop novel processing technologies such as pulsed electric field technology (PEF) and other preservation and transformation technologies, biotechnology, and extraction methods for biomass recovery or separation technologies, increase environmental safety, energy saving, management of food production and distribution, and make advancement in the production of sugar and alcohol, and improvements in food attributes. Ukraine has an enormous scientific potential, including food science and technology. Exploiting this potential requires extensive international support and wider involvement of Ukrainian researches in global scientific activity.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Supplementary material

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# How the ongoing armed conflict between Russia and Ukraine can affect the global wheat food security?

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**Introduction:** This study examines the potential impacts of Russia-Ukraine armed conflict on the consumption and trade of wheat and wheat food security. Russia and Ukraine jointly supply more than one-fourth of total wheat in the international market. Because of the ongoing armed conflict between Russia and Ukraine, wheat export from these two countries is heavily disrupted. Applying an ex-ante impact assessment procedure, this study examines the potential impacts of Russia-Ukraine armed conflict on the consumption and trade of wheat and wheat food security.

**Methods:** This study considered 115 countries and grouped them based on their geographic location. The sampled countries are grouped as: South Asia, Sub-Saharan Africa, Southeast Asia, Central Asia, North Africa, Middle East, and rest of the world. Simulation results are presented by the geographic location of the sampled countries. To assess the potential impacts of the ongoing Ukraine-Russia armed conflict on wheat food security in the sampled countries, this study mainly sourced data from FAOSTAT. This study first calculates the share of wheat consumption imported from Russia and Ukraine. Then, this study calculates the daily total calorie and protein intake exclusively from the imported wheat from Russia and Ukraine in the sampled countries by their groups. It is found that 1 kg of wheat provides roughly 2,839–2,965 kilocalories (kcal) of energy, and between 81 and 88 g (gm) of protein, in the sampled countries. Using the conversion factors, this study assesses the impacts of a reduction of wheat exports in the global market due to the ongoing armed conflict between Russia and Ukraine applying an ex-ante assessment process. Specifically, this study assumes a 100% and 50% reduction in wheat exports by Russia and Ukraine, and then estimates its impact on daily calorie and protein intake in the sampled countries.

**Results:** The ex-ante simulation shows that, under the assumption of a 100% reduction of wheat exports from Russia and Ukraine and assuming alternative wheat import sources are unavailable, yearly per capita wheat consumption would be reduced by 19% in South Asia, 57% in Sub-Saharan Africa, 26% in Southeast Asia, nearly 39% in Central Asia, the Middle East and North Africa, and 27% in other areas. Consequently, daily per capita calorie intake in South Asia would fall by more than 3%, in Sub-Saharan Africa by more than 6%, in Southeast Asia by 2.2%, in Central Asia, the Middle East, and North Africa by 14%, and in the other countries of our study by 6.2%. A 50% reduction of wheat exports by Russia and Ukraine without substitute supplies of wheat grain would also substantially reduce wheat

consumption as well as daily calorie and protein intakes from wheat, in the sampled countries.

**Discussions:** Malnutrition and hunger are widespread in many countries of Asia and Africa that depend on wheat and other imported cereals to meet their rising food demand. Rising wheat prices, particularly in countries that rely on imported wheat, can lead to violence and social unrest, as occurred during 2007-11. Based on the findings, to avoid hunger and supply shock related disaster in the future, this study urges to search alternative sources of wheat for the import-dependent, resource-poor countries. Eventually, as there are few alternatives to increase wheat supply other than enhancing yield gain, this study strongly suggests for steady public funding for adaptive and basic research to harness genetic gains for yield and climatic adaptation in wheat. Also, in the long run, it is necessary to explore the possibility of wheat area expansion in the suitable countries. For example, a recent study confirmed the potentiality of wheat area expansion in Argentina and Brazil. Also, there is also a possibility of expansion of wheat area in Sub-Saharan African countries. Exploring opportunities for the expansion and sustainable intensification of wheat production in suitable countries can be instrumental to ensuring self-sufficiency in wheat supplies in Sub-Saharan Africa.

KEYWORDS

**wheat, food security, consumption, import, nutrition**

## 1 Introduction

In 2019, 8.9% of the total 7.68 billion population of the world was undernourished (FAO et al., 2020). Alarmingly, the number of undernourished people has started to increase recently (FAO et al., 2020; United Nations, 2022a; von Grebmer et al., 2021). The major drivers of undernourishment, food insecurity and hunger are climate and economic shocks, crop diseases and pests epidemics, and war and conflicts (FAO et al., 2020). Among all factors, war and conflicts are the major drivers of hunger and food insecurity in the contemporary world. For example, in 2020, globally 155 million people were acutely food insecure, of which more than 64% of them (99.1 million) were residing in 23 countries where, war and conflicts were the principal causes of food insecurity (von Grebmer et al., 2021). Since February 2022, the world has witnessed a fresh armed conflict between the Russian Federation (hereafter Russia) and Ukraine. These two countries are the major producers and exporters of many essential commodities including sunflower oil, maize and wheat, and synthetic fertilizers (FAO, 2022). Because of the ongoing armed conflict, exports of commodities from these countries are severely interrupted, which has raised concerns about the possible effects on world food security (Table 7).

Applying an ex-ante impact assessment procedure, this study examines the potential impacts of Russia-Ukraine armed conflict on the consumption and trade of wheat and wheat food security. Wheat is the most widely grown crop in the world. In TE 2019 (triennium average, 2017–19 average), total land allocation to rice was less than 164 million ha, to maize less than 198 million ha, and to wheat 217 million ha (FAOSTAT, 2022a). Millions of resource-poor smallholder farmers in Asia and Africa rely on wheat cultivation for food, livelihoods, and income. Wheat also plays a crucial role in global food and nutritional security (Dixon, 2007; Dixon et al., 2009; Shiferaw et al., 2013), being consumed as food by inhabitants of at least 180 countries in 2019. The global triennium average ending 2019 (TE-average of 2017–19) *per capita* wheat consumption was nearly 67 kg and wheat-based food supplies more than 18% (543 kcal) of daily *per capita* dietary energy intake and nearly 20% (16.4 gm) of total daily protein intake per person, worldwide (FAOSTAT, 2022a). It is projected that by 2030 the yearly *per capita* wheat consumption in the world will be 70 kg, and in 2050 it will be 75 kg, will be propelled by increase in population and urbanization (Mottaleb et al., 2021a). It suggests that wheat will continue to play a significant role in global food security in the future.

Importantly, many countries in the world, particularly in Asia and Africa, rely on wheat imports to meet their demand. For example, Bangladesh, Sudan, Indonesia, Nigeria, Congo, Mali Madagascar, Chad, Ghana, El Salvador, Guinea, Lesotho, Djibouti, Rwanda and Cambodia heavily rely on wheat import for meeting demand. In fact, in 2020, at least 179 countries imported wheat to meet their domestic demand (FAOSTAT, 2022b). Food security situation in many of these wheat importing countries, is already precarious.

Because of the ongoing armed conflict between Russia and Ukraine, wheat export from these two countries is heavily disrupted. In 2021, Russia produced 75.5 million metric tons (MMT) of wheat (9.7% of global production) on 27.9 million ha of land (12% of world wheat area, TE 2021), making it the third largest wheat producing country. Ukraine ranked sixth in wheat production in 2021, accounting for 33 MMT (4.3% of world production) grown on 7.1 million ha (3.3% of world wheat area) (USDA, 2022). However, Russia is the world's number-one wheat exporting country, selling 32 MMT of wheat grain, or 16% of the world total 203 MMT, in 2021 (USDA, 2022). Ukraine was the fourth-largest exporter, accounting for 20 MMT of grain, or nearly 10% of the world total (USDA, 2022). Russia and Ukraine thus jointly supply more than a quarter of wheat in international markets. Many countries in the world heavily rely on Russia and Ukraine for wheat imports. Thus, the disruption in wheat exports from Russia and Ukraine due to the ongoing armed conflict will have devastating impacts on global food security.

**TABLE 1** Temporal changes in the wheat area (million ha) and production (million metric tons) during 1961–2020.

| Regions | 1963 | 1973 | 1983 | 1993 | 2003 | 2013 | 2020 |
|---|---|---|---|---|---|---|---|
| Area (million ha) | | | | | | | |
| Africa | 7.61 | 9.39 | 7.96 | 8.45 | 8.87 | 10.05 | 9.86 |
| Southern Africa | 1.50 | 2.11 | 1.91 | 1.11 | 0.92 | 0.56 | 0.53 |
| Americas | 37.7 | 37.3 | 53.5 | 46.9 | 40.1 | 36.8 | 35.4 |
| Asia | 60.9 | 71.8 | 80.3 | 97.1 | 95.4 | 101.3 | 99.0 |
| Eastern Asia | 25.7 | 26.7 | 29.2 | 31.4 | 24.0 | 24.8 | 24.4 |
| South Asia | 24.2 | 33.2 | 38.9 | 41.5 | 43.3 | 48.3 | 49.6 |
| Europe | 93.2 | 89.2 | 82.0 | 62.5 | 56.2 | 57.3 | 61.5 |
| Oceania | 6.51 | 8.00 | 12.19 | 7.99 | 11.66 | 13.51 | 10.44 |
| World | 206.0 | 215.7 | 236.0 | 222.9 | 212.3 | 219.0 | 216.3 |
| Production (million metric tons) | | | | | | | |
| Africa | 6.40 | 9.13 | 9.37 | 14.65 | 18.84 | 26.02 | 26.95 |
| Southern Africa | 0.88 | 1.80 | 2.21 | 1.83 | 2.21 | 1.96 | 1.85 |
| Americas | 56.4 | 71.2 | 117.7 | 111.0 | 98.3 | 114.5 | 116.3 |
| Asia | 49.7 | 89.5 | 152.5 | 228.9 | 248.7 | 310.8 | 336.9 |
| Eastern Asia | 18.3 | 35.4 | 71.2 | 102.6 | 91.3 | 121.5 | 134.5 |
| South Asia | 20.3 | 38.9 | 60.7 | 85.0 | 105.4 | 132.4 | 150.8 |
| Europe | 114.6 | 174.0 | 176.3 | 190.6 | 191.7 | 215.1 | 254.4 |
| Oceania | 8.2 | 9.4 | 16.1 | 14.1 | 19.2 | 27.2 | 18.1 |
| World | 235.3 | 353.3 | 472.0 | 559.2 | 576.8 | 693.6 | 752.7 |
| Yield (ton/ha) | | | | | | | |
| Africa | 0.84 | 0.97 | 1.18 | 1.73 | 2.12 | 2.59 | 2.73 |
| Southern Africa | 0.59 | 0.85 | 1.16 | 1.65 | 2.40 | 3.50 | 3.49 |
| Americas | 1.50 | 1.91 | 2.20 | 2.37 | 2.45 | 3.11 | 3.29 |
| Asia | 0.82 | 1.25 | 1.90 | 2.36 | 2.61 | 3.07 | 3.40 |
| Eastern Asia | 0.71 | 1.33 | 2.44 | 3.27 | 3.80 | 4.90 | 5.51 |
| South Asia | 0.84 | 1.17 | 1.56 | 2.05 | 2.43 | 2.74 | 3.04 |
| Europe | 1.23 | 1.95 | 2.15 | 3.05 | 3.41 | 3.75 | 4.14 |
| Oceania | 1.26 | 1.18 | 1.32 | 1.76 | 1.65 | 2.01 | 1.73 |
| World | 1.14 | 1.64 | 2.00 | 2.51 | 2.72 | 3.17 | 3.48 |

Note: all values are in triennium average format. For example, 1961–1963 average.
Source (FAOSTAT, 2022a).

There are a few opinion studies and blogs on the potential impacts of the ongoing armed conflict between Russia and Ukraine on the global food security (Abay et al., 2022; Bechdol et al., 2022; Bentley, 2022; Douglas, 2022; Sabaghi, 2022). The present study is a complete scientific study based on data and applies ex-ante impact assessment procedure to quantify the potential impacts of the disruption of wheat exports from Russia and Ukraine due the ongoing armed conflict on global wheat food security (Section 1). The rest of the study is organized as follows. Section 2 presents materials and methods; Section 3 includes temporal changes in wheat production and consumption by major regions; Section 4 presents major findings and Section 5 presents conclusions and policy implications.

# 2 Materials and methods

## 2.1 Data

This study relies on data from FAOSTAT (FAOSTAT, 2022a), a database administered by the Food and Agriculture Organization of the United Nations (FAO), as well as online data of the United States Department of Agriculture Foreign Agriculture Services (USDA-FAS) (USDA, 2022), and the United Nations "Comtrade" international trade statistics database (United Nations, 2022b).

To examine the potential impacts of a reduction in wheat exports by Russia-Ukraine on food and nutrition security of the wheat importing countries, this study considered the sampled 115 countries, their wheat imports from Russia and Ukraine in 2017–19, and wheat-related food consumption indicators such as domestic production, yield, total imports, yearly *per capita*, and aggregate total wheat consumption, and dietary energy intake (kcal and protein) from wheat. The name of the sampled countries can be seen in the Appendix. In this study sampled countries are grouped based on their geographic locations and the results are presented by groups.

## 2.2 Ex-ante impact assessment technique

To assess the potential impacts of the ongoing Ukraine-Russia armed conflict on wheat food security in the sampled countries, this study first calculates the share of wheat consumption imported from

**TABLE 2** Temporal changes in wheat consumption, and net exports (million metric tons) by major regions in the world.

| Regions | 1963 | 1973 | 1983 | 1993 | 2003 | 2013 | 2019 |
|---|---|---|---|---|---|---|---|
| Per capita consumption (Kg/*per capita*/year) | | | | | | | |
| Africa | 29.6 | 36.2 | 45.8 | 47.5 | 46.7 | 47.7 | 47.0 |
| Southern Africa | 41.6 | 55.2 | 62.2 | 57.7 | 50.3 | 57.8 | 54.8 |
| Americas | 57.0 | 57.2 | 61.1 | 62.5 | 63.2 | 61.4 | 60.6 |
| Asia | 30.2 | 43.2 | 59.6 | 67.7 | 63.7 | 62.9 | 65.6 |
| Eastern Asia | 26.9 | 37.9 | 66.1 | 74.3 | 65.4 | 60.8 | 63.2 |
| South Asia | 36.0 | 54.0 | 59.9 | 66.7 | 65.7 | 67.6 | 69.2 |
| Europe | 131.3 | 120.6 | 114.4 | 106.8 | 108.7 | 109.0 | 112.3 |
| Oceania | 96.9 | 81.5 | 77.3 | 70.6 | 71.6 | 70.8 | 67.1 |
| World | 55.4 | 59.0 | 67.5 | 69.9 | 66.8 | 65.4 | 66.7 |
| Total consumption | | | | | | | |
| Africa | 8.2 | 12.7 | 21.4 | 28.7 | 36.5 | 46.7 | 58.5 |
| Southern Africa | 0.90 | 1.44 | 2.05 | 2.41 | 2.83 | 3.46 | 3.60 |
| Americas | 25.1 | 29.5 | 38.7 | 46.4 | 53.8 | 58.6 | 60.7 |
| Asia | 50.3 | 89.9 | 151.3 | 220.2 | 246.1 | 262.7 | 298.9 |
| Eastern Asia | 20.0 | 37.8 | 76.4 | 104.0 | 101.6 | 97.9 | 105.2 |
| South Asia | 22.7 | 39.8 | 55.9 | 83.4 | 101.5 | 114.5 | 131.1 |
| Europe | 86.6 | 86.5 | 87.5 | 83.1 | 79.9 | 80.9 | 83.9 |
| Oceania | 1.35 | 1.51 | 1.56 | 1.61 | 1.83 | 2.15 | 2.75 |
| World | 171.4 | 220.2 | 300.4 | 380.0 | 418.1 | 451.1 | 504.7 |
| Net export (Export-import) | | | | | | | |
| Africa | −3.3 | −5.5 | −15.0 | −18.1 | −23.8 | −37.5 | −47.3 |
| Southern Africa | −0.15 | 0.04 | −0.20 | −0.94 | −0.54 | −1.76 | −2.08 |
| Americas | 26.6 | 34.9 | 56.7 | 48.0 | 29.3 | 37.2 | 35.6 |
| Asia | −16.5 | −22.2 | −36.1 | −40.5 | −30.1 | −48.2 | −64.4 |
| Eastern Asia | −8.5 | −12.9 | −21.7 | −21.5 | −10.6 | −16.6 | −15.5 |
| South Asia | −5.2 | −5.1 | −6.0 | −7.4 | −2.9 | −1.9 | −9.29 |
| Europe | −10.3 | −10.1 | −10.6 | 3.6 | 15.6 | 39.0 | 74.2 |
| Oceania | 5.1 | 7.8 | 9.8 | 9.6 | 12.9 | 19.0 | 13.5 |

Note: all values in triennium average.
Sources: FAOSTAT (2022c).

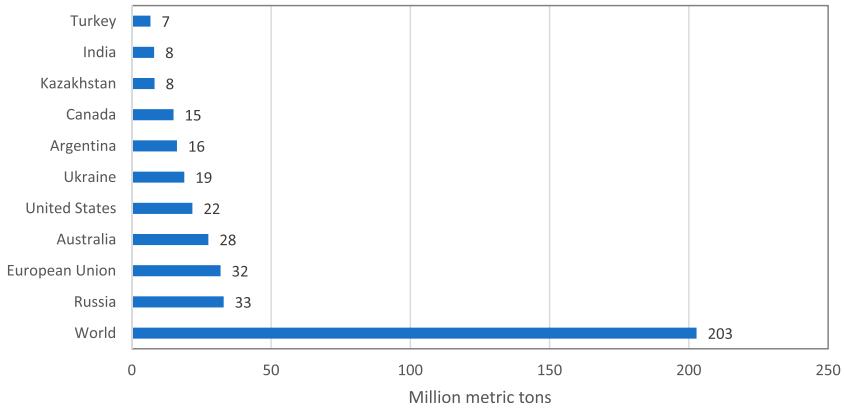


**FIGURE 1**
Top 10 wheat exporting countries in 2021/22. Source: Authors based on USDA (2022).

Russia and Ukraine. After that based on FAOSTAT data, this study calculates the daily total calorie and protein intake exclusively from the imported wheat from Russia and Ukraine in the sampled countries. It is found that 1 kg of wheat provides roughly 2,839–2,965 kilo calories (kcal) of energy, and between 81 and 88 gm of protein, in the sampled countries. Using the conversion factors, this study assesses the impacts of a reduction of wheat exports in the global market due to the ongoing armed

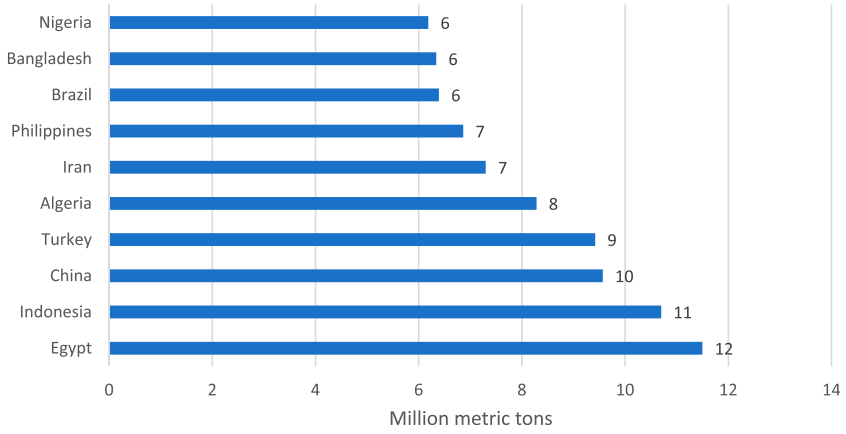


FIGURE 2
Top 10 wheat importing countries in 2021/22. Source: Authors based on USDA (2022).

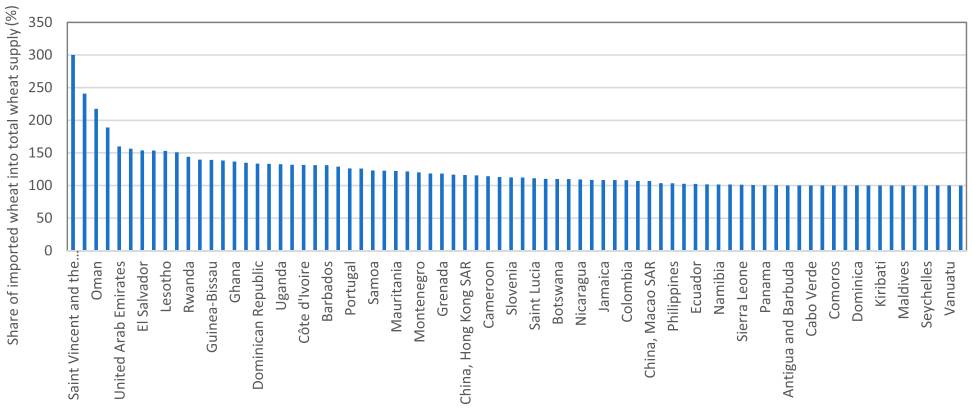


FIGURE 3
List of countries in 2019, where wheat consumption completely relies on imported wheat (imported wheat/Total domestic supply) (≥100%). Source: Authors based on FAOSTAT (2022d).

conflict between Russia and Ukraine. Specifically, this study assumes a 100% and 50% reduction in wheat exports by Russia and Ukraine, and then estimates its potential impacts on daily calorie and protein intake in the sampled regions.

## 3 Descriptive statistics: Temporal changes in wheat production, consumption, and trade

Global wheat production has increased and expanded over the last five decades, with area growing from 206 to 216 million ha during 1961–2020, due to mainly increases in wheat areas in Asia, Africa, and Oceania and area reductions in the Americas and Europe (Table 1). In TE 1963, wheat was cultivated on 206 million ha worldwide with an average yield of 1.14 t/ha, producing more than 253 MMT of wheat grain. In TE 1963 more than 45% of the world wheat area of 93.2 million ha was in Europe, producing nearly 49% of the global harvest for this crop (Table 1). Asia has since emerged as the dominant wheat-producing region, accounting by TE 2020 for 99 million ha (nearly 46%) of global area (216.3 million ha) and 45% of the annual average 753 MMT of grain produced (Table 1).

Notably, despite a modest global area increase of 5% during 1961–2020, global wheat production has increased by 220% (235 MMT in TE 1963 to nearly 753 MMT in TE 2020) driven by a more than 200% increase in average yield, from 1.14 t/ha in TE 1963 to 3.48 t/ha in TE 2020 (Table 1). This owes largely to the improved agronomic practices, irrigation facilities, application of fertilizers, the control of wheat diseases and pests and the development of high-yielding modern varieties.

Global average yearly wheat consumption *per capita* increased by 20% during 1961–2019, from slightly more than 55 kg to nearly 68 kg, due to mainly increased wheat consumption in the economically emerging countries of the Global South (Table 2), and particularly in Africa and South Asia, where the popularity of wheat-based food

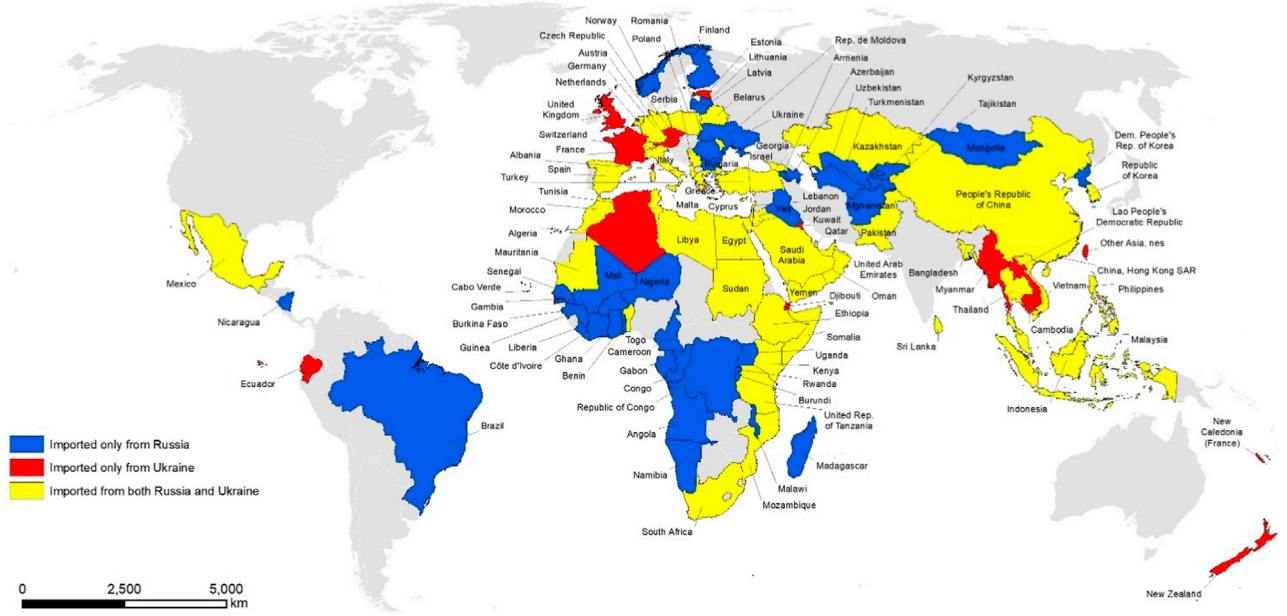


FIGURE 4
Countries that imported wheat from Russia and Ukraine in 2017–19. Source: Authors based on United Nations (2022b).

**TABLE 3 Estimated nutrition value of wheat per kg.**

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world | Sample average |
|---|---|---|---|---|---|---|
| Kilo calorie/kg | 2,944.6 | 2,916.8 | 2,928.6 | 2,956.9 | 2,839.9 | 2,900.5 |
| Protein (gm)/kg | 88.2 | 85.8 | 81.5 | 87.8 | 85.7 | 85.9 |

Source: Authors based on FAOSTAT (2022b).

**TABLE 4 Importance of wheat in the sampled regions.**

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|---|
| Average production per country (MMT) | 21.1 | 0.26 | 13.3 | 3.64 | 4.27 |
| Wheat consumed as food (MMT) | 18.9 | 0.88 | 11.7 | 3.74 | 2.12 |
| Yearly *per capita* consumption (kg/*per capita*) | 53.58 | 32.4 | 31.5 | 136.8 | 89.7 |
| Daily total calorie intake (kcal) | 2,625.3 | 2,478.1 | 2,885.1 | 3,063.8 | 3,164.2 |
| Daily total daily calorie intake from wheat (% share in total calorie intake) | 435.0 (17.2) | 261.5 (10.3) | 248.8 (8.32) | 1,104.0 (36.4) | 694.5 (21.7) |
| Daily total daily protein intake from wheat (% share in total protein intake) | 12.77 (20.1) | 7.72 (11.9) | 7.13 (8.6) | 32.9 (38.1) | 21.1 (21.9) |

Note: All values are presented in the triennium average ending 2019.
Source: Authors based on FAOSTAT (2022b).
[a]USDA (2022).

products has grown (Gandhi et al., 2004; Nagarajan, 2005; Mason et al., 2015; Mottaleb et al., 2018a). Annual *per capita* and national aggregate wheat consumption, as well as net wheat trade, have declined in Europe. Wheat consumption in Europe fell from more than 131 kg *per capita* yearly in TE 1963 to 112 kg in TE 2019 (Table 2). Yearly *per capita* wheat consumption in Oceania has

**TABLE 5** Share of wheat import, and wheat import from Russia and Ukraine into total wheat consumption (000 tons) in the sampled regions.

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|---|
| Wheat consumed as food (000 tons) | 18,932.4 | 875.7 | 11,655.9 | 3,470.4 | 2,120.4 |
| Total wheat imported (000 tons) | 2,403.9 | 783.2 | 3,784.3 | 2,453.7 | 2024.9 |
| Total wheat imported from Russia and Ukraine (000 tons)[a] | 1,421.9 | 301.6 | 866.0 | 1,187.5 | 142.6 |
| Value of imported wheat from Russia (million US$)[a] | 254.8 | 57.4 | 152.6 | 220.8 | 25.8 |
| Share of imported wheat from Russia and Ukraine into total import (%) | 59.2 | 38.5 | 22.9 | 48.4 | 7.04 |
| Share of imported wheat from Russia and Ukraine into total wheat consumed (%) | 7.5 | 34.4 | 7.4 | 34.2 | 6.7 |
| Wheat end stock, 000 MT (TE 2021) | 5,985.4 | 131.6 | 15,252.1 | 1,155.7 | 176.9 |
| No. of years stock can meet the demand (end stock/yearly consumption) | 0.39 | 0.12 | 0.41 | 0.34 | 0.12 |

Sources: Authors' based on FAOSTAT, (2022b).
[a]United Nations, (2022b).

declined from nearly 97 to 67 kg over the same period, but aggregate wheat consumption there rose from 1.35 to 2.75 MMT (Table 2).

South Asia and Sub-Saharan Africa have the world's highest share of undernourished population (FAO et al., 2020; von Grebmer et al., 2021); 22% or nearly 250 million of the population in Sub-Saharan Africa and 13.4% or 249 million in South Asia (FAO et al., 2020).

In Africa, annual wheat consumption was less than 30 kg *per capita* in TE 1963 (Table 2), supplying 233 kcal of daily dietary energy *per capita* or 11.5% of the average daily intake per person, and 7 gm of protein, or about 13% of daily intake (FAOSTAT, 2022c). In TE 2019, wheat consumption had increased to 47 kg *per capita* per year, supplying more than 374 kcal of dietary energy *per capita*, or nearly 15% of the average daily intake, as well as more than 11 gm of protein per person, or more than 17% of the daily intake (FAOSTAT, 2022d). In South Asia in TE 1963, yearly wheat consumption was 36 kg *per capita*, supplying 308 kcal of dietary energy daily or more than 15% of the average daily *per capita* intake, and nearly 9 gm of protein, around 17% of the daily *per capita* intake (FAOSTAT, 2022c). In TE 2019, yearly wheat consumption in the region had increased to more than 69.2 kg *per capita*, supplying over 585 kcal of dietary energy per day, or nearly 23% of the average daily intake, and 16.8 gm of protein, or more than 26% of the daily intake per person (FAOSTAT, 2022d).

An examination of the net trade (export-import) status for wheat producing and consuming countries revealed that, except for the Americas, Europe, and Oceania, all other regions are net wheat importers (Table 2). Importantly, although Asia has emerged as the world's largest wheat-producing region, it is also the top wheat importing region (Table 2). Despite significant wheat production increases since the early 1960s in Asia-South Asia in particular, and Africa, increases in *per capita* wheat consumption and population growth have widened the export-import gap of these regions (Table 2). China and India are the world's largest wheat-producing and consuming countries. In TE 2019, China and India jointly cultivated more than 54 million ha, 25% of world wheat area of the world, and produced 234 MMT of wheat, accounting for nearly 31% of the world total. However, with a joint total of 2.79 billion inhabitants, or 36% of the world population (7.76 billion), both countries consume their wheat harvest, with only sporadic wheat exports by India. Currently, less than 10% (14.2 MMT) of the wheat consumed (150.6 MMT) in South Asia is imported from international markets (USDA, 2022). Regarding major international exporters of wheat, Russia and Ukraine are the top exporters (Figure 1). Of 2021/22 global wheat export, totaling 203 MMT, Russia's share was more than 16% (33 MT) and Ukraine's share was more than 9% (19 MMT) (Figure 1). Russia and Ukraine altogether exported nearly 26% of the total wheat in the world in 2021/22 (Figure 1).

Among the major global importers, Egypt, Indonesia and China are the world's top three wheat importing countries (Figure 2). In 2021/22, Egypt imported 12 MMT, Indonesia 11 MMT and China 10 MMT (Figure 2).

However, many developing countries in Asia and Africa heavily rely on imported wheat for domestic consumption. Countries where the ratio of wheat import is more than or equal to 100% are presented in Figure 3. It shows that Oman, El Salvador, Lesotho, Rwanda, Guinea-Bissau, Uganda, Mauritania and many other countries completely rely on wheat imports for domestic consumption (Figure 3). A reduction of wheat supply in the international market due to the ongoing conflict between Russia and Ukraine can bring significant negative impacts on the food security situation of the countries, that rely completely on imported wheat to meet domestic demand.

Importantly, as Russia and Ukraine jointly are the source of nearly 26% of wheat in the international export market, many countries in the world rely on wheat imports from Russia and Ukraine for meeting domestic food demand (Figure 4). Using the Harmonized Commodity Description and Coding System (HS) code for wheat and meslin (HS code: 1001), a search of Comtrade revealed that, during 2017–19, 47 countries imported wheat from both Russia and Ukraine, 42 countries imported wheat from Russia alone, and

**TABLE 6 Importance of imported wheat from Russia and Ukraine in food and nutrition security of the sampled regions.**

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|---|
| Yearly wheat consumption (kg/*per capita*) | 53.58 | 32.4 | 31.5 | 136.8 | 89.7 |
| Import from Russia and Ukraine (yearly/*per capita*/kg)[a] | 10.2 | 18.5 | 8.2 | 52.9 | 24.5 |
| Share of imported wheat from Russia and Ukraine in wheat consumption (%) | 19.0 | 57.1 | 26.0 | 38.7 | 27.3 |
| Daily dietary energy intake from wheat (daily/*per capita*/kcal) | 435.0 | 261.5 | 248.8 | 1,104.0 | 694.5 |
| Daily dietary energy intake from wheat imported from Russia and Ukraine (daily/capita/kcal) | 83.4 | 150.0 | 62.5 | 431.9 | 195.2 |
| Share of kcal from imported wheat from Russia and Ukraine to total daily calorie intake from wheat (%) | 19.2 | 57.4 | 25.1 | 39.1 | 28.1 |
| Daily dietary protein intake from wheat (daily/*per capita*/gm) | 12.77 | 7.72 | 7.13 | 32.9 | 21.1 |
| Daily dietary protein intake from wheat imported from Russia and Ukraine (daily/*per capita*/gm) | 2.50 | 4.43 | 1.78 | 12.7 | 5.92 |
| Share of protein from imported wheat from Russia and Ukraine to total daily protein intake from wheat (%) | 19.6 | 57.4 | 25.0 | 38.6 | 28.1 |

Sources: Authors' based on FAOSTAT, (2022d).
[a]United Nations, (2022b).

**TABLE 7 Impacts of the complete ban (100% reduction of wheat export) of wheat exports from Russia and Ukraine on wheat consumption and nutrient intake in the sampled region.**

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|---|
| Yearly *per capita* total wheat consumption (kg/*per capita*) | 43.4 (−19.0) | 13.9 (−57.1) | 23.3 (−26.0) | 83.9 (−38.7) | 65.2 (−27.3) |
| Daily dietary energy intake from wheat (daily/*per capita*/kcal) | 351.6 (−19.1) | 111.5 (−54.4) | 186.3 (−25.1) | 672.1 (−39.1) | 499.3 (−28.1) |
| Daily dietary protein intake from wheat (daily/*per capita*/gm) | 10.27 (−16.6) | 3.29 (−57.3) | 5.35 (−25.0) | 20.2 (−38.6) | 15.18 (−28.1) |
| Daily total calorie intake (daily/*per capita*/kcal) | 2,542.0 (−3.2) | 2,328.1 (−6.1) | 2,822.6 (−2.2) | 2,631.9 (−14.1) | 2,969 (−6.2) |

Source: Authors' based on FAOSTAT (2022c).
Note: Values in parentheses are the percentage changes compared to the original values reported in Table 6.

**TABLE 8 Impacts of a 50% reduction in wheat exports from Russia and Ukraine on wheat consumption and nutrient intake in the sampled region.**

| Region | South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|---|
| Yearly *per capita* total wheat consumption (kg/*per capita*) | 48.5 (−9.5) | 23.2 (−28.5) | 27.4 (−13.0) | 110.4 (−19.3) | 77.5 (−13.7) |
| Daily dietary energy intake from wheat (daily/*per capita*/kcal) | 393.3 (−9.6) | 186.5 (−28.7) | 217.6 (−12.6) | 888.1 (−19.6) | 596.9 (−14.1) |
| Daily dietary protein intake from wheat (daily/*per capita*/gm) | 11.5 (−9.8) | 5.5 (−28.7) | 6.2 (−12.5) | 26.6 (−19.3) | 18.1 (−14.0) |
| Daily total calorie intake (daily/*per capita*/kcal) | 2,583.6 (−1.6) | 2,403.1 (−3.0) | 2,853.9 (−1.1) | 2,847.9 (−7.0) | 3,066.6 (−3.1) |

Source: Authors' based on FAOSTAT (2022c).
Note: Values in parentheses are the percentage changes compared to the original values reported in Table 6.

16 countries imported wheat from Ukraine alone (United Nations, 2022b).[1]

Applying an ex-ante impact assessment procedure this study specifically examines the potential impacts of a reduction of wheat exports from Russia and Ukraine, on wheat consumption and calorie and protein intake from wheat in the sampled countries.

## 4 Ex-ante impact assessments and discussions: major findings

The nutrition value of wheat is presented in Table 3, calculated based on FAOSTAT data. Our calculation shows that on average 1 kg of wheat provides, around 2900 kcal of dietary energy and nearly 86 gm of protein (Table 3). In assessing the impacts of the ongoing Russia-Ukraine armed conflict on wheat food security, we have used these conversion factors.

In Table 4, the wheat consumption pattern across regions is presented. Although countries in South Asia produce (21.1 MMT on average) and consume (18.9 MMT on average) more wheat than in any other region, the role of wheat as a staple food is more prominent in Central Asia, the Middle East, and North Africa, where yearly *per capita* wheat consumption exceeds 136 kg, supplying more than 36% of the daily total dietary energy and more than 38% of the daily total protein intake per person (Table 4). In Sub-Saharan Africa, annual average *per capita* wheat consumption just exceeds 32 kg, supplying over 10% of the daily dietary energy intake and 12% of protein intake, *per capita* (Table 4).

Many of the countries in our sample rely on wheat imports and particularly those from Russia and Ukraine. For example, a sampled South Asian country, such as Bangladesh, imported nearly 2,404 thousand MT (2.4 MMT) of wheat in TE 2019, of which nearly 1,422 thousand MT came from Russia and Ukraine and accounting for more than 59% of the country's total during TE 2019 (Table 5). Imports from Russia and Ukraine accounted for more than 38% of the wheat imported by countries in Sub-Saharan Africa, nearly 23% of national imports in Southeast and East Asia, more than 48% of imports by countries in Central Asia, North Africa, and the Middle East, and around 7% of imports by other countries in our sample (Table 5). In 2017, sampled countries imported nearly 51 MMT of wheat from Russia and Ukraine, which increased to 52 MMT in 2018, and 60 MMT in 2019. Importantly, the share of Russia and Ukraine wheat in the total consumed in South Asian countries is 7.5%, more than 34% of that consumed in Sub-Saharan African countries, 7.4% of that consumed in Southeast Asian countries, 34% of that consumed in Central Asian, North African, and Middle Eastern countries, and nearly 7% of the wheat consumed in other countries of our sample (Table 5). The ending stock of wheat in the sampled countries shows that, on average, none of them can meet their wheat demand for a year with their current stock of wheat (Table 5). It indicates the importance of Russian and Ukraine wheat exports for food security worldwide and, particularly, for South Asia and Sub-Saharan Africa.

On average, the yearly *per capita* consumption of imported wheat from Russia and Ukraine is more than 10 kg for the sampled South Asian countries, which is 19% of the yearly *per capita* wheat consumption of a sampled country in South Asia (Table 6). Yearly *per capita* consumption of Russian and Ukraine wheat in the Sub-Saharan Africa countries of our sample is more than 18 kg, or 57%, of the wheat they consume. Wheat imported from Russia and Ukraine constitutes 53 kg, or 39% of that consumed *per capita* each year in the Central Asian, Middle Eastern and North African countries in our sample. It is more than 24 kg, or 27% of yearly *per capita* wheat consumed in the other countries of our study (Table 6).

The average daily *per capita* dietary energy intake from wheat in a sampled South Asian country, such as Nepal is 435 kcal, 83.4 kcal (19.2%) of which is provided by wheat imported from Russia and Ukraine (Table 6). Of total daily dietary energy intakes in the countries of this study, the share provided by Russian and Ukraine wheat is more than 57% for Sub-Saharan African countries and more than 39% for Central Asian, Middle Eastern, and North African countries (Table 6). The imported wheat from Russia and Ukraine also contributes significantly to daily dietary protein intake in those countries. For example, in a sampled country in East and Southeast Asia, 1.78 gm, or 25% of the total 7.13 gm of average daily protein *per capita* from wheat, is provide by that imported from Russia and Ukraine (Table 6).

In Tables 7 and 8, two "what if" scenarios are presented, assuming a 100% reduction of wheat exports from Russia and Ukraine (Table 7) and a 50% reduction in wheat exports by Russia and Ukraine (Table 8). It shows that, under the assumption of a 100% reduction of wheat exports from Russia and Ukraine and assuming alternative sources are unavailable, yearly *per capita* wheat consumption would be reduced by 19% in South Asia, 57% in Sub-Saharan Africa, 26% in East and Southeast Asia, nearly 39% in Central Asia, the Middle East and North Africa, and 27% in other areas (Table 7). Consequently, daily *per capita* calorie intake in South Asia would fall by more than 3%, in Sub-Saharan Africa by more than 6%, in East and Southeast Asia by 2.2%, in Central Asia, the Middle East, and North Africa by 14%, and in the other countries of our study by 6.2% (Table 7). A 50% reduction of wheat exports by Russia and Ukraine without substitute supplies of wheat grain would also substantially reduce wheat consumption as well as daily calorie and protein intakes from wheat, in the sampled countries (Table 8).

Malnutrition and hunger are widespread in many countries of Asia and Africa that depend on wheat and other imported cereals to meet their rising food demand. In Afghanistan, Angola, Benin, Burundi, the Democratic Republic of Congo, Djibouti, Ethiopia, Guinea, India, Madagascar, Malawi, Mozambique, Nepal, Niger, Nigeria, Rwanda, Sudan, Tanzania, Yemen, more than one-third of children under 5 years of age are stunted (von Grebmer et al., 2021). Many of these countries rely on wheat imports from international markets, and particularly Russia and Ukraine. The degree to which wheat agri-food system disruptions will affect child nutritional status is subject to the national dependency on wheat for calories and protein. In Table 7, we observe the dependency on wheat in these poverty-stricken nations. Afghanistan, Yemen, Djibouti, and Sudan depend critically on wheat imports from Russia and Ukraine. The level

1 Annex A lists sampled countries.

of poverty in those countries also constrains their ability to mitigate the effects of food price crises.

At this point, a question arises as to whether import-dependent food insecure countries can switch to substitutes of wheat for the time being. Food consumption is embedded in cultural preferences. Changes in diets take place but at a slow pace. This is reflected in the negative but comparatively low own-price elasticities of demand for food items and the even lower cross-price elasticities of demand (Mottaleb et al., 2018b; Mottaleb et al., 2018c; Bairagi et al., 2020; Mottaleb et al., 2021b; Frija et al., 2021; Kruseman et al., 2021). This explains why the yearly *per capita* wheat consumption in Tunisia is nearly 199 kg, whereas rice consumption is 1.53 kg, in contrast rice consumption in Cambodia is 245.5 kg *per capita* per annum while wheat consumption is 2.9 kg (FAOSTAT, 2022d).

Overall, the findings of this study are consistent with the Russia-Ukraine armed conflict as resulting in disruptions in global wheat supplies that significantly threaten food and nutritional security in multiple countries. Rising wheat prices, particularly in countries that rely on imported wheat, can lead to violence and social unrest, as occurred during 2007–11 (Kliger, 2008; Clapp and Cohen, 2009; Zerbe, 2009; Kron, 2011; Sneyd et al., 2013). The findings of this study is supported by the findings of Araujo-Enciso et al. (2017), which asserted that a reduction in Russian wheat exports by 15%, Kazakh exports by 30% and Ukrainian wheat export by 38% below the projected baseline level, can lead to a reduction of wheat in the world market by 3% and it will result in an increase of wheat price by 7% in the world market.

## 5 Conclusion and policy implications

Currently, 864 million people- 8.9% of the world population, suffer from hunger (FAO et al., 2020) and 99.1 million are severely food insecure due to war and conflicts (von Grebmer et al., 2021). For the first time since 1990, the number of absolutely poor people in the world has started to increase and COVID-19 induced economic turmoil has exacerbated the situation. At least 47 countries are expected to fall short of the United Nations 2030 zero hunger goal (von Grebmer et al., 2021).

This study confirmed that during 2017–19 at least 115 countries have imported wheat from Russia and Ukraine. The findings of this study warn that a complete wheat export ban by Russia and Ukraine would significantly reduce yearly *per capita* wheat consumption and calorie and protein intakes from wheat. This will worsen the already precarious food and nutritional security of South Asia and Sub-Saharan Africa, two regions with the highest number of hungry and malnourished people in the world.

Based on the findings, to avoid hunger and supply shock related disasters in the future, this study urges to search alternative sources of wheat for the import-dependent, resource-poor countries. In the long run, as there are few alternatives to increase wheat supply other than enhancing yield gain, this study strongly suggests for steady public funding for adaptive and basic research to harness genetic gains for yield and climatic adaptation in wheat.

The average wheat yield in Africa in TE2020 was 2.73/ton, and in Asia it was 3.40 ton/ha, whereas the global average yield was 3.78 ton/ha. The presence of a big wheat yield gap in Africa points to a great opportunity to increase wheat production in the continent (Tittonell and Giller, 2013; Ittersum et al., 2016). This can be achieved by closing the yield gap through research and investment and intensification of wheat cultivation, which can be instrumental in achieving towards self-sufficiency in wheat and overall food security in Africa (Godfray et al., 2010; Otsuka and Larson, 2016).

In the long run, there is a possibility to explore pathways to wheat area expansion and sustainable intensification of existing production areas, particularly in Sub-Saharan African countries, East Asia, and in South America. In East Asia, around 100 million ha of cropland is potentially available, which is potentially suitable for wheat cultivation (Bruinsma, 2009). A recent study confirmed the potentiality of wheat area expansion in Argentina and Brazil (Colussi et al., 2022). Studies of Fischer and Shah (2010) and Deininger et al. (2011) identified more than 200 million ha with an additional 95 million ha of the rainfed area in Sub-Saharan African countries potential for crop cultivation with minimal investments on infrastructure (Fischer and Shah, 2010; Deininger et al., 2011). This underutilized area is 45% of the total global area potentially suitable for agricultural expansion in the world. The study of Negassa et al. (2013) specifically demonstrated that large regions in the DR Congo, Angola, Tanszania, Kenya, Madagascar and Nigeria are suitable for wheat cultivation. Policy that supports access to improved agronomy along with modern, high yielding varieties can markedly improve wheat production in these areas. Exploring opportunities for the expansion and sustainable intensification of wheat production in suitable countries can be instrumental to ensuring self-sufficiency in wheat supplies in Sub-Saharan Africa (Bentley, 2022). It is necessary to speed up the variety development process through modern breeding tools to accelerate rate of genetic gains through speed breeding and predictive modeling techniques using artificial intelligence models to identify the optimal plant variety for a particular place considering environmental, and climatic factors.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

KM: Conceptualization, data curation, model run, first draft and revision VG: Revision, supervision and fund raising. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frfst.2023.1072872/full#supplementary-material

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# Appendix

**TABLE A1 Name of the sampled countries.**

| South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|
| Afghanistan | Angola | Cambodia | Algeria | Albania |
| Bangladesh | Benin | China | Armenia | Austria |
| India | Burkina Faso | China, Hong Kong SAR | Azerbaijan | Belarus |
| Nepal | Burundi | Dem. People's Rep. of Korea | Egypt | Belgium |
| Sri Lanka | Cabo Verde | Indonesia | Iran | Belize |
| — | Cameroon | Lao People's Dem. Rep | Iraq | Brazil |
| — | Congo | Malaysia | Israel | Bulgaria |
| — | Côte d'Ivoire | Myanmar | Jordan | Cyprus |
| — | Dem. Rep. of the Congo | Philippines | Kazakhstan | Denmark |
| — | Djibouti | Rep. of Korea | Kuwait | Ecuador |
| — | Ethiopia | Thailand | Kyrgyzstan | Finland |
| — | Gabon | Viet Nam | Lebanon | France |
| — | The Gambia | — | Libya | Georgia |
| — | Ghana | — | Morocco | Germany |
| — | Guinea | — | Oman | Greece |
| — | Kenya | — | Saudi Arabia | Haiti |
| — | Liberia | — | Syria | Hungary |
| — | Madagascar | | Tajikistan | Iceland |
| — | Malawi | — | Tunisia | Ireland |
| — | Mali | — | Turkey | Italy |
| — | Mauritania | | Turkmenistan | Japan |
| — | Mozambique | | United Arab Emirates | Latvia |
| — | Namibia | — | Uzbekistan | Lithuania |
| — | Niger | — | Yemen | Malta |
| — | Nigeria | — | — | Mexico |
| — | Rwanda | — | — | Mongolia |
| — | Senegal | — | — | Netherlands |
| — | South Africa | | — | New Caledonia |
| — | Sudan | — | — | Nicaragua |
| — | Togo | — | — | Norway |
| — | Uganda | — | — | Peru |
| — | United Rep. of Tanzania | | | Poland |
| — | Zimbabwe | | — | Portugal |
| — | — | — | — | Rep. of Moldova |
| — | — | — | — | Romania |
| — | — | — | — | Serbia |
| — | — | — | — | Spain |
| — | — | — | — | Sweden |

(Continued on following page)

**TABLE A1 (*Continued*) Name of the sampled countries.**

| South Asia | Sub-Saharan Africa | Southeast Asia | Central Asia, North Africa, and the Middle East | Rest of the world |
|---|---|---|---|---|
| — | — | — | — | Switzerland |
| — | — | — | — | United Kingdom |
| — | — | — | — | Venezuela |

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# Cell-based meat: The molecular aspect

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Cellular agriculture is one of the evolving fields of translational biotechnology. The emerging science aims to improve the issues related to sustainable food products and food security, reduce greenhouse gas emissions and provide animal wellbeing by circumventing livestock farming through cell-based meat (CBM) production. CBM exploits cell culture techniques and biomanufacturing methods by manipulating mammalian, avian, and fish cell lines. The cell-based products ought to successfully meet the demand for nutritional protein products for human consumption and pet animals. However, substantial advancement and modification are required for manufacturing CBM and related products in terms of cost, palatability, consumer acceptance, and safety. In order to achieve high-quality CBM and its production with high yield, the molecular aspect needs a thorough inspection to achieve good laboratory practices for commercial production. The current review discusses various aspects of molecular biology involved in establishing cell lines, myogenesis, regulation, scaffold, and bioreactor-related approaches to achieve the target of CBM.

KEYWORDS

**cell-based meat, bioreactor, muscle cell, stem cell, cell lines**

## 1 Introduction

Proof-of-concept for large-scale production of cell-based meat (CBM) came into existence on live TV in 2013 when Prof Mark Post introduced the first-ever meat for consumption originated in the lab. The expansion of this technology, in combination with tissue engineering, has opened an avenue for the sustainable production of meat and meat products worldwide. In the coming decade, cellular agriculture is perceived as one of the important fields of biotechnology that may foster the world's growing population by exploiting stem cell and tissue engineering without sacrificing an animal (Post et al., 2020). Global meat consumption continues to increase owing to upward population growth, a rise in economic status, and urbanization. Recently, the Food and Agriculture

**Abbreviations:** AMR, Antimicrobial resistance; CDK, Cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, Epithelial growth factor; ECM, Extracellular matrix; FBS, Fetal bovine serum; FGF, Fibroblast growth factor; GHG, Greenhouse gas; HH stage, Hamburger Hamilton stage; IGF, Insulin-like growth factor; LIF, Leukemia inhibitory factor; MSC, Mesenchymal stem cell; MDC, Muscle derived cell; MDSC, Muscle-derived stem cell; MyHC, Myosin heavy chain; PDGF, Platelet-derived growth factor; SMSC, Skeletal muscle satellite cell; TERT, Telomerase reverse transcriptase.

Organization (FAO) of the United Nations anticipated the global demand for meat may extend to 455 million metric tons by 2050, while in 2005–06, it was 258 million tons (Alexandratos, 2012; Feeding the world in 2050 and beyond – Part 1: Productivity challenges, 2022).

Similarly, the consumption of fish is proposed to reach 140 million metric tons by 2050. Fish and seafood (crustaceans, mollusks, and other aquatic animals) support more than 20% of the global demand for the consumption of animal protein (Costello et al., 2020). Globally, more than 100 firms are developing their fish cell line or end product derived from fish line to manufacture CBM.

The majority of this increase is attributed to middle-income countries like India and China (FoodNavigator ASIA, 2022). The rising demand is challenging as the current livestock farming methods and aquaculture practices are linked to public health issues, environmental dilapidation, and animal welfare concerns. In the present review article, we mainly focus on molecular aspects of CBM which are currently being employed to establish cell lines and other molecular parameters such as transcription factors and muscle regulation.

## 2 Cell-based meat (CBM)

CBM has been recognized by many names, like cellular meat, cell culture meat, engineered meat, factory-grown meat, *in vitro* meat, fake meat, clean meat, neat meat, synthetic meat, lab-grown meat, and artificial meat. CBM is an emerging field of biotechnology that aspire to solve the greenhouse gas emissions (GHG), depletion of water bodies, cutting down grassland and forest land, and antibiotics misuse. A report from the FAO claims that the livestock segment is responsible for 14.5% of GHG emissions, blows out the earth's terrain (approximately 30%), and 8% of global freshwater (Gerber, 2013). From a source published by Our World in Data, India is the third largest country in GHG emissions. GHG emissions arise from electricity generation, infrastructure expansion, and animal agriculture. With the worldwide population probably double by 2050, the quest to reduce GHG emissions and augment a cheap source of protein in the form of livestock meat will be a daunting task and unable to support the growing demand. It is imperative to look for a sustainable system that emits minimum GHG, needs less water, requires minimum land space, and has minimum antibiotic use. In the United States, livestock animals mainly use 70%–80% of antibiotics (mainly used directly or indirectly by livestock animals to produce meat (CGDEV, 2022). Conventional livestock farming has led to regular misuse of antibiotics and, thereby, the selection of antimicrobial resistance (AMR) strains, posing significant health issues (Avesar et al., 2017; Tang et al., 2017). In 2015, colistin AMR originated in farms of pigs and was subsequently detected in chickens and other farm animals in South America (Nguyen et al., 2016; Monte et al., 2017; Reardon, 2017). Recent research predicted, AMR will be more accountable for deaths than cancer by the year 2050 (The Review on Antimicrobial Resistance, 2012).

Excessive livestock farming and growing animal welfare ethics have recently pushed traditional meat production into the back seat. Another primary concern of livestock farming is a foodborne disease from swine and avian influenza (Greger, 2007). Some common microorganisms found in the meat are *Escherichia coli*, *Salmonella*, and *Campylobacter* (Anomaly, 2015). CBM production in a sterile condition may check these troubles and improve food safety and security. Animal ethics and slaughtering animals are other aspects that drive toward CBM (van der Weele and Driessen, 2013; Sharma et al., 2015). The scientific community respects farm animals and their sentient beings with physical and psychological needs (Egg-Truth, 2022).

In the present review article, we aim to discuss various parameters of CBM, starting from the cell line establishment, feasibility, scaffolding, safety of CBM, regulatory framework, affordability, acceptability, and other aspects in detail. Figure 1 describes the general concept and methodology utilize to manufacture CBM of different species (chicken, fish, and mammals).

### 2.1 Stem cell

Stem cells are unspecialized cells possessing self-renewal capacity, and the discovery of stem cells paved the way for *in vitro* cell production and the concept of cultured meat. These cells have the potential to divide through mitosis to renew to other cell types throughout the life span of the multicellular organism. Subsequently, stem cell divides to create a cell population that can either stay as stem cells or separate lineage with more characterized capabilities such as blood, muscle, and neuronal cells. Usually, there are two types of stem cells, i) embryonic stem cells and ii) undifferentiated/substantial/grown-up stem cells. The embryonic stem cell is determined from the embryos, while undifferentiated cells reside in a tissue or organ at the side of other divided cells. The basic three properties of the stem are i) capable of dividing and renewing, ii) unspecialized, and iii) differentiate to other cell types. The cell typically passes through various stages during differentiation and specializes at each step. The signals that trigger the differentiation process inside and outside stem cells are still to be deciphered. The epigenetic regulation of genes typically controls the internal signal, while external signals such as physical contact with neighboring cells, growth factors or chemicals (specific to various receptors of stem cell) secreted by other cells, and the microenvironment are the main driving force for stem cell differentiation.

Pluripotent stem cells are difficult to handle and culture for CBM research as they require more time and resources to proliferate and differentiate into mature cell types compared to primary adult stem cells. Nevertheless, a pluripotent stem cell has the inherent capability to increase in large numbers and become immortal. Pluripotent stem cells derived from non-muscle sources can be isolated from diverse domesticated animals and expanded as a myogenic cell source for CBM. Recently, chemically and genetically modified porcine pluripotent stem cells have been transformed into myogenic cells possessing the ability to differentiate into embryonic muscle fibers (Genovese N. J. et al., 2017). Pluripotent muscle stem cells are attractive possible source cells; any CBM produced from these pluripotent cells must be appropriately screened for safety before consumption.

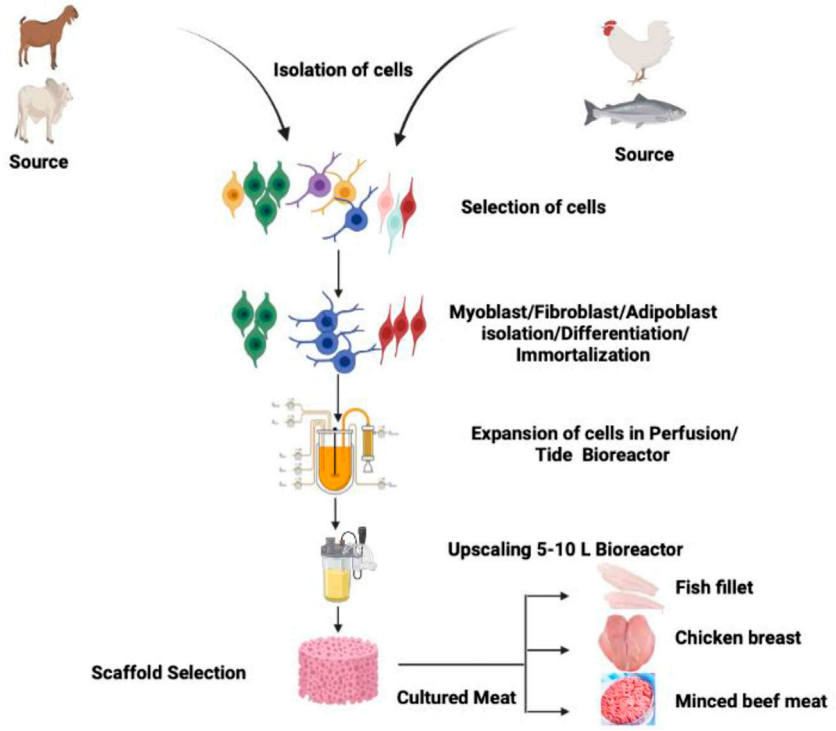


FIGURE 1
Schematic diagram showing the methodology of CBM production exploiting cells of chicken, fish, and mammalian species.

On the other hand, primary adult stem cells offer the benefit of being simply achieved from a biopsy of any animal species, such as sheep, buffalo, or cow, to acquire a cell population for any meat product; however, their proliferative potential is limited (Ding, 2019). Depending on the stem cell types, these cells can be stimulated to differentiate into muscle or fat cells. MSCs are considered reliable cells for skeletal muscle recovery *in vivo*, and their self-renewal capability maintains the population of stem cells and the generation of enormous numbers of myogenic cells. These myogenic cells proliferate, divide, fuse, and help produce new myogenic fibers (Brack and Rando, 2012; Yin et al., 2013). Mark post presented the meat hamburger prototype that amplifies the myoblast progeny of MSCs (Post, 2014). Multipotent progenitor cells deriving from porcine skeletal muscle exhibit higher doubling capacity than MSCs, presenting them as better source cells for CBM production. However, these cells require additional growth factors and are not able to differentiate into skeletal muscle fibers as proficiently as MSCs can perform (Wilschut et al., 2008).

## 2.2 Starting material for CBM

In 1961, Mauro first identified the *bona fide* satellite cells in frogs (Mauro, 1961). On appearance, satellite cells differ from muscle fiber in exhibiting chromatin-dense nuclei with minute cytoplasmic value. Satellite cells derive from the dermomyotome's cell population and are between the basal membrane and sarcolemma (Gros et al., 2005). Satellite cells add new nuclei in the growing muscle fibers by fusing with adjacent fibers during peri- and postnatal development (Relaix et al., 2005; Biressi et al., 2007). Afterward, satellite cells may acquire a quiescent stage. Upon muscle injury, they are activated for further muscle growth and development (Biressi et al., 2007; Fu et al., 2015; Almada and Wagers, 2016). A preplating procedure usually isolates bovine satellite cells (Li et al., 2011; Will et al., 2015). The purity of isolated satellite cells through the preplating method without further purification steps can lead to 31% of cells (depending upon the fusion index) and 95% by DESMIN staining (Coles et al., 2015; Will et al., 2015). The purification of satellite cells may be improved by FACS/MACS and in combination with positive selection (CD29 and CD56) and negative selection (CD31 and CD45) (Figure 2). However, the degree of high positivity may be deduced by their PAX7 positivity (Ding et al., 2018).

The mainstay material for producing CBM is myoblasts (satellite cells), which are challenging to grow *in vitro*. However, myoblasts can readily differentiate into myotubes (immature muscle cells) and myofibrils under specific environments. To expedite the replication of skeletal muscle satellite cells in a lab, cells are attached to an immobile substratum, for example, scaffold or microbeads, which may be coated with protein (collagen, chitosan, and laminin) to imitate the natural tissue. The scaffolds are usually biodegradable, edible, and re-usable during the culture method (Stephens et al., 2018).

Nutrient-rich medium is essential for growing satellite cells to provide a unique proliferation and differentiation phase, comprising antibiotics, antimicrobial agents, antifungal agents, and other

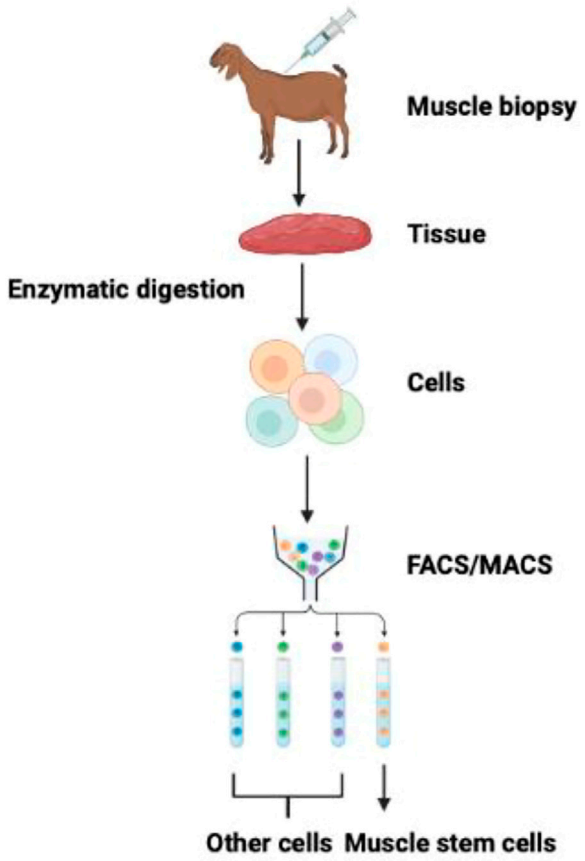


FIGURE 2
Isolation and purification of muscle stem cells. FACS and MACS methods can isolate muscle stem cells from the mixed population of cells exploiting cell-surface specific markers (Guan et al., 2022).

chemicals to prevent contamination. Culture media is typically optimized with varying amounts of fetal bovine serum (5%–10%) to optimize the growth and differentiation of satellite cells *in vitro*. Some laboratories designed serum-free media or chemically-defined media for culturing the satellite cells; however, these media components are expensive and possess proprietary issues (Edelman et al., 2005).

Muscle cell culture implicates significant challenges on an industrial scale in a large bioreactor. Stephens et al. reported that roughly eight trillion muscle cells are required to produce 1 kg of protein from a traditional bioreactor possessing a capacity of 5000 L (Stephens et al., 2018). Cultured muscle cells may reach a thickness of around 200 μm. In the thick muscle layer, oxygen and essential nutrients may not be able to penetrate the inner layer of cells, and due to an insufficient supply of oxygen and nutrients, cells may begin to die (Jones, 2010). Muscle strips are harvested and processed at this stage, and several supplementary chemical compounds are added to augment nutritional value, color, texture, and flavor. The production of a particular cut of meat, like chops, steaks, or roasts, needs additional technology to give the correct shape and structure to the muscle cells.

The paraxial mesodermal progenitor cells during fetal development give rise to muscle tissue. Upon sequential development process, paraxial mesoderm differentiates into myoblast, and the ensuing process is regulated by numerous growth factors (Chal and Pourquié, 2017). Through cell-to-cell fusion, myoblast generates muscle tissue, and part of them inhabits underneath the basal lamina of myofiber, which converts into quiescent satellite cells during the postnatal period. During muscle injury, the quiescent muscle cells are activated to differentiate into myoblast, leading to muscle regeneration. The quiescent satellite cells are characterized by the expression of Pax7, while Myf5 and MyoD are absent (Kuang et al., 2007). Upregulation of Myf5 and MyoD and downregulation of Pax7 occur by myogenic satellite muscle cells and make them proliferating myoblast during muscle injury. Myf5 plays a significant role in myoblast proliferation, while MyoD has a principal function in differentiation (Asakura et al., 2007; Gayraud-Morel et al., 2007). These intrinsic factors can be exploited as muscle stem cell markers to explore the cellular states of the cell. Muscle stem cells possess surface and cytoskeletal proteins, for example, vascular cell adhesion molecule, neural cell adhesion molecule (also known as CD56), integrin α7, β1 (CD29), CD34, desmin, and SM/C-2.6 (Wang et al., 2014). The synchronization of intrinsic and extrinsic factors plays a significant role in the fate of muscle stem cells. Hence, to maintain the functioning of muscle stem cells in the lab, the physiological conditions of muscle stem cells should be provided by mimicking the *in vivo* stem cell niche in the form of extracellular matrix (ECM) and paracrine factors.

## 2.3 Composition of muscle tissue

Skeletal muscle tissue comprises muscle fibers, connective tissues, and stem cell populations. Muscle stem cells usually reside on muscle fibers, and their isolation is a mainstay in upscaling in cellular agriculture, as described in the previous sections. Commonly used proteases for the purification of muscle stem cells are from muscle biopsy following physical dissociation, and meat mincing are trypsin, collagenase, pronase, and dispase. Numerous digestive enzymes with multiple combinations can be applied to digest muscle tissues. Collagenase and dispase have been extensively used as these enzymes specifically target ECM-containing collagen and fibronectin (Stenn et al., 1989).

### 2.3.1 Muscle fibers

The hallmark of any muscle fibers are due to their contractile properties (Klont et al., 1998; Lefaucheur, 2010). The contractility mainly depends on the amount of myosin heavy chain (MyHC) isoforms embedded within the thick filaments. Generally, mammalian skeletal striated muscles contain four types of MyHCs: I, IIa, IIx, and IIb. The speed and level of contraction of MyHCs depend on the ATPase activity, i.e., type I is slow while type IIa, IIx, and IIb are fast. Muscle fibers are dynamic in nature and can switch from one type to another and follow the pathway: I↔IIa↔IIx↔IIb (Meunier et al., 2010). Type I fibers show low-intensity contraction, are resistant to fatigue, and are found in respiratory and postural function muscles.

Strong expression of MyHC IIb was found in the skeletal muscle of pig breeds while absent in sheep horses (Lefaucheur et al., 1998; Picard and Cassar-Malek, 2009). Depending upon the species,

muscle fiber composition determines one of the critical factors of meat quality. The fiber composition varies from species to species; for example, pig Longissimus muscle possesses roughly 10% type I fibers, 10% type IIa, 25% IIx, and 55% IIb. Longissimus of bovine contains approximately 30% type I, 18% IIA, and 52% IIx. The factors determining muscle fibers' composition are breed, gender, age, physical activity, environmental condition (temperature), and feeding practices.

### 2.3.2 Connective tissue

The connective tissue primarily surrounds muscle fibers and fiber bundles and consists of cells and ECM comprising a composite network of collagen fibers enveloped in a matrix of proteoglycans (Abbott et al., 1977; Lawrie, 1989; Lefaucheur et al., 1998). Based on collagen type, the basic structural unit of collagen, tropocollagen, is a helical structure comprising three polypeptide chains coiled around one another to give a spiral structure. Interchain bonds stabilize the tropocollagen and form a fibril-like structure of 50 nm diameter. These fibrils are again stabilized by hydrogen and disulfide (intramolecular) or intermolecular bonds such as pyridinoline and deoxypyridinoline. These pyridinoline and deoxypyridinoline are known as crosslinkers. Various types of collagen found in skeletal muscles are fibrillar collagen I and III, abundant in mammals. While in fish, collagen I and IV predominate (Sato et al., 1991). Apart from collagen, the other components found in connective tissue are proteoglycans (PGs) (Nishimura, 2015). PGs are multifarious molecules consisting of core proteins in the range of 40–350 kDa. PGs are joined by covalent bonds to numerous dozen glycosaminoglycan chains, forming large complexes by attaching to other PGs and fibrous proteins. Glycosaminoglycans are negatively charged and bind with cations such as $Na^+$, $K^+$, $Ca^{2+}$, and water (Iozzo and Schaefer, 2015). The degree of intramuscular collagen crosslinking varies according to species, muscle types, genotypes, age, sex, and extent of physical exercise (Purslow, 2005). Collagen content differs from 1% to 15% of the muscle dry weight in adult cattle, 1.3 (*Posa major*) to 3.3% (*Latissimus dorsi*) of dry weight muscle in large while pigs for commercial slaughter stage. In the dry weight of poultry, only 0.75%–2% of the collagen was found (Liu et al., 1996), while variable content of collagen is reported in fish, depending upon the species (1%–10% between sardines and congers) (Sikorski et al., 1984; Sato et al., 1986).

### 2.3.3 Intramuscular fat

In fish, fat is present in subcutaneous positions and within the perimysium, myosepta. Myosepta contribute to the significant part of flesh and determine the quality of flesh. Intramuscular fat predominantly consists of structural lipids, phospholipids, and storage lipids (triglycerides). Approximately 80% of the triglycerides are stored in the muscle adipocytes between fibers and bundle fibers, and 5%–20% is stored as lipid droplets inside myofibers in the cytoplasm (Essén-Gustavsson and Fjelkner-Modig, 1985). The content of phospholipid is relatively constant at 0.5%–1% of the fresh muscle of pigs; however, muscle triglyceride content is highly variable depending upon the species (Wood et al., 2008; Shingfield et al., 2013). The size and number of intramuscular adipocytes determine the intramuscular fat content. The interindividual disparity in intramuscular fat content of a particular muscle between animals of comparable genetic makeup has been connected with variation in the intramuscular adipocyte in pigs and cattle. On the other hand, variation in the intramuscular fat content of a particular muscle of the same genetic origin in animals raised in different dietary components has shown differences in the size of adipocytes (Gondret and Lebret, 2002). In fish, the upsurge in myosepta width is possibly associated with an increase in the size and number of adipocytes (Weil et al., 2013). The intramuscular fat content also fluctuates depending upon the muscle origin, genotype, age, breed, diet, and the rearing conditions of the livestock (Mourot and Hermier, 2001; Lebret, 2008; Bonnet et al., 2010; Hocquette et al., 2010; Shingfield et al., 2013). Chinese pigs (Meishan), American pigs (Duroc), and European local pig breeds (Iberian and Basque) possess higher contents of intramuscular fat as compared to European conventional genotypes, for example, Large White, Pietrain, and Laandrace (Bonneau and Lebret, 2010). Fresh *Longissimus* muscle of conventional genotypes of pigs slaughtered at commercial slaughterhouses have intramuscular fat in the range of 1%–6% and sometimes up to 10% in certain breeds (Lebret, 2008). *Longissimus* muscle of cattle, the intramuscular fat content varies from 0.6% in Belgian Blue to 23.3% in Black Japanese at 24 months of age (Gotoh et al., 2009). It has been noticed in French cattle breeds that selection on muscle mass is strongly connected with a decrease in collagen and intramuscular fat content. Popular breeds such as Charolaise and Blonde d'Aquaintaine possess less intramuscular fat content compared with a hardy breeds such as Aubrac and Salers (Schreurs et al., 2008). The intramuscular fat content also varies in fish between species, such as 'lean' species carrying 3% (cod), while fatty species contain more than 10% (Atlantic salmon) (Hocquette et al., 2010).

## 2.4 Myogenesis and regulation

Myogenesis is a highly ordered and complex process of MSCs. The process is regulated by the co-expression of paired box transcription factors (Pax3/Pax7) and myogenic regulatory factors (such as Myf5, Mrf4, MyoD, and myogenin) (Zammit and Beauchamp, 2001; Relaix et al., 2005; Baig et al., 2019). Myogenesis is illustrated by various factors such as cell cycle arrest, increased nuclear sizes, cell alignment, myogenic activation, multiple cell fusion, and peripheral localization (Chargé and Rudnicki, 2004). However, skeletal muscle regeneration depends on interactions between MSCs and their microenvironment composed of basal lamina and sarcolemma (Kuang et al., 2008).

Animal meat is composed of skeletal muscle tissues, so tissue engineering of skeletal muscle tissues has been exploited to produce CBM. Due to the non-proliferating capability of adult skeletal muscle cells, MSCs are utilized as a precursor for replication. MSCs exhibit high responsiveness and migratory abilities; MSCs are precarious for preserving skeletal muscle's functional and structural integrities and are also accountable for muscle regeneration through a coordinated myogenic program (Lee et al., 2018). The discoveries of MSCs led to the production of cells *in vitro* and the development of CBM. Therefore, MSCs provide a viable source of cells for skeletal muscle recovery (*in vivo*). The ability of MSCs to self-renew and self-sustain the stem cell

population and the production of an enormous number of myogenic cells, which again proliferate, multiply, and fuse to form new myofibers (Shaikh et al., 2021). MSCs are typically located between the basal lamina and sarcolemma and are active in regulating myofiber growth and development under the influence of myogenic regulatory factors (Ahmad et al., 2020; Shaikh et al., 2021). The first CBM production model was established on bovine MSC, and the principle is still applied in bioreactor-based cultured meat production (Verbruggen et al., 2018). In the following subsection, we discussed the molecular parameters involved in the regulation of muscle and development.

### 2.4.1 Pax3

Paraxial mesoderm gives rise to skeletal muscle in the trunk and limbs and subsequently segments into repetitive epithelial structures termed somites. Pax3 has already been transcribed in the presomitic mesoderm stage adjoining the first somite and afterward newly formed somites (Schubert et al., 2001). As time progresses, somite matures, and the ventral domain endures an epithelial to mesenchymal transition, subsequently down-regulating Pax3 and activating pax1/9 to form the sclerotome. The sclerotome forms the cartilage and bone of the vertebral column and ribs, while the neighboring subdomain forms the tendon. The dorsal domain of somite maintained its epithelial structure and termed it a dermomyotome. Pax3 expression is now limited to dermomyotome and remains present in myogenic progenitor cells, which delaminate and travel from the somite to other distant parts, such as the limb, during the myogenesis (Goulding et al., 1991; Buckingham and Relaix, 2007). Myotome, the first differentiated skeletal muscle, forms within the central domain of the somite (under the dermomyotome) and functions as a scaffold for successive waves of cells of myogenic origin. Subsequently, myogenic cells activate the myogenic determination genes, such as *Myf5*, *MRF4,* and *MyoD*, and delaminate from the edges of the dermomyotome. At the same time, there is a downregulation of Pax3. The epaxial part of the myotome forms a deep back muscle, and the hypaxial myotome gives rise to the muscle of the body wall and trunk. The level of Pax3 expression is high in the hypaxial domain of the dermomyotome (Bober et al., 1994; Relaix et al., 2004).

The *Pax3* gene codes for the Pax3 protein and is characterized by a highly conserved paired box motif. The *Pax3* gene is also known as WS1, WS3, CDHS (Craniofacial-deafness-hand syndrome), and HUP2). The PAX family of transcription factors is characterized by a highly conserved pair of DNA binding domains, and it was first identified in *Drosophila* segmentation genes (Tremblay and Gruss, 1994). Based on the similar functional organization and degree of sequence homology, humans and murine possess nine Pax members (Pax1-Pax9) and comprise a subfamily called group III (Stuart et al., 1994).

Transcriptome study ascertains the related developmental gene expression pattern between cattle and mice. Just after the commencement of gastrulation (day 14 of the embryonic stage), Pax3 mRNA is identified in the bovine conceptus, indicating the initial stages of mesoderm formation (Pfeffer et al., 2017). In this stage, only Pax3 is visible and detected, while Myf5 (myogenic factor 5), MyoD (myogenic differentiation factor D), MRF4 (myogenic regulatory factor 4), and Pax7 are not detected. Somites are visible by day 21 of the embryonic stage and evident with 5 and 14 somite pairs (Maddox-Hyttel et al., 2003; Richard et al., 2015). On day 23 of gestation, at least 24 pairs of somite pairs are visible, comprising presumptive forelimb bud, otic and optic placodes as well as five visible branchial arches (Gonzalez et al., 2020).

### 2.4.2 Pax7

Pax7 (paired box 7) is one of the satellite cell's mainstay homeobox-containing transcription factors and lineage markers (Seale et al., 2000). Proliferating mouse satellite cells exhibit the expression of Pax7 and are typically absent in myotubes. Pax7 is also expressed in the dermomyotome and presumptive myoblast with a partially overlapping expression pattern of Pax3 in the mouse embryo (Relaix et al., 2004; Horst et al., 2006). The subpopulation of satellite cells also expresses Pax3; however, Pax3 is unable to substitute for Pax7 either in adult muscle precursor cells or embryonic stage (Conboy and Rando, 2002; Relaix et al., 2004; Kuang et al., 2006). Transcriptome studies reveal that similar gene expression patterns exist between mice and cattle. On an embryonic day 14, *Pax3* mRNA is identified in the bovine conceptus indicating the initial stages of mesoderm formation, while Myf5, MyoD, MRF4, myogenin, and Pax7 are not expressed (Pfeffer et al., 2017). By embryonic day 21, somites are apparent (5–14 pairs) (Maddox-Hyttel et al., 2003; Richard et al., 2015) and near gestation day 23, at least 24 pairs of somites are visible, including a presumptive forelimb bud, five visible branchial arches, otic and optic placodes. These developmental and morphological characteristics are equivalent to a Hamburger Hamilton (HH) stage 21 chick embryo and embryonic 9.5 days in the mouse (Hamburger and Hamilton, 1951). The presence of MyHC myotome suggests MRF expression within the dermomyotome at the time of the developmental window covering days 14–23 of the gestation period. Demonstration of cryosection and their analysis indicates the presence of Pax7 immunopositive cells within the dermomyotome. As the gestation time increases, the number of Pax7 continues to decline (Gonzalez et al., 2013; Sun et al., 2015). Satellite cells isolated from the semitendinosus muscle of neonatal calves express the combination of Pax7 and Myf5 (Li et al., 2011). Satellite cells isolated from longissimus 4–6 week-old pigs possess a large amount of Pax3 immunopositive cells (Sebastian et al., 2015). It is still not known why satellite cells have such a diverse population of cells. Scientists are pondering and tempted to speculate that the total number of muscle fiber increase during the development process (Bérard et al., 2011).

### 2.4.3 Presence of Pax3 and Pax7 in adult skeletal muscles

Pax3 and Pax7 are two closely related transcription factors in the maintenance of progenitors of skeletal muscle lineage (Chi and Epstein, 2002; Robson et al., 2006). In the late fetal stage, myogenic progenitor cells comprising Pax3/7-positive cells start taking a position on the muscle fibers beneath a basal lamina (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). This is the hallmark niche of myogenic progenitor cells (so-called satellite cells) of adult muscle which determine muscle regeneration (Montarras et al., 2013). Adult satellite cells are quiescent, while fetal and postnatal myogenic progenitor cells divide actively. The quiescent

satellite cells undergo quick activation on account of injury or in tissue culture experiments. Under these circumstances, there is marked upregulation of the myogenic determination factor (MyoD), downregulation of Pax7, activation of myogenin, and muscle fiber differentiation.

Previously, satellite cells have been isolated from single fiber and exploiting flow cytometry of Pax3-positive satellite cells from the trunk muscle of $Pax3^{GFP/+}$ mice (Collins et al., 2005; Montarras et al., 2005). The capacity of these cells was found to be efficient in self-renewal *in vivo*. Satellite cells marked by Pax7 expression are necessary for the regeneration of muscle (Tedesco et al., 2010).

Pax3 and Pax7 have also been investigated for promoting cell survival, proliferation, and regulating the skeletal muscle program in various time intervals, such as during embryogenesis, postnatal development, and adulthood (Buckingham and Relaix, 2015). During embryogenesis, Pax3 functions as an antiapoptotic, preferably in the hypaxial somite (Borycki et al., 1999). Double mutant of Pax3/Pax7 exhibited the importance of these transcription factors, and most of the myogenic cells in the somite lost, and at the same time, muscle failed to form (Relaix et al., 2005). After parturition, satellite cell experience apoptosis in the Pax7, even in the diaphragm muscles where Pax3 is expressed (Relaix et al., 2006).

### 2.4.4 MyoD

The transcription factors of MRFs have a role to play in the progression of muscle in vertebrates and are expressed temporally in muscle tissue in a regulated way. This is due to the presence of the E box, which is a DNA consensus sequence "CANNTG" (Dechesne et al., 1994). The MRFs such as MyoD, MRF4, Myf5, and Myogenin work in an interdependent regulatory and cascading manner (Liu et al., 2010).

The very first MRF to be discovered was myoblast determination protein 1 (MyoD). This discovery used in the differentiation of myocytes as MyoD during the differentiation process showed sequence regulatory gene expression (Hernández-Hernández et al., 2017). MyoD and myogenin are widely used as a marker of differentiation in myogenic development. MyoD is the well-known and verified transcription factor in the development of the myogenic cell-lineage specification. In small vertebrates, the phylogenetic study on amino acids showed more than 50% similarity between MyoD and Myf5 before differentiation (Megeney and Rudnicki, 1995). Among these regulators, MRF4 was highly expressed in mature myofibers (Zammit, 2017). MyoD is a sequence-specific DNA-binding protein that plays a significant role in skeletal muscle development.

The activation, differentiation, and proliferation mechanisms are regulated in an orchestral manner. This activation depends on the consensus sequence E-box and E proteins such as E47. A higher binding affinity of MyoD-E47 to the dsDNA bHLH domain was observed during the mammalian muscle cell activation (Zhong et al., 2022). As discussed, the Pax7 shows positive expression during the cell cycle's G0 phase (quiescent stage) in the myogenic stem cells or satellite cells (SCs). At this stage, along with Pax7, 90% of these cells express Myf5 and are therefore selected to form myogenic cells. After the activation of these SCs with various growth factors and signaling pathways, such as Ras-Erk, TGF-β, Notch, JAK-STAT, and HGF, the expression of MyoD can be detected. In the proliferation stage, the MRFs, myogenin, and MRF4 showed their expression (Asfour et al., 2018). Although the muscle tissue-specific genes reside in different chromosomal loci, they work in a much-regulated manner both during embryogenesis and in culture cells. Therefore in this regulated mechanism, the chromatin remodeling enzymes, a subunit of SW1/SNF, activated Brg1 and MyoD play an essential role in making inter-chromosomal interactions (Harada et al., 2015). As mentioned before, these MRFs are regulated through various factors. One such study by Latimer and coworkers revealed that the lack of methionine downregulated the MyoD and myogenin expression and obstructed the differentiation of fish muscle cells (Latimer et al., 2017). Another transfection study revealed that the MRFs, after introducing into the cells, fibroblast expressed muscle-specific genes compared with hepatocytes (Schäfer et al., 1990). In a mouse model, scientists have proved that MyoD also functions as a genome organizer in muscle cell development (Wang et al., 2022). In bovine skeletal muscle development, the transcriptional regulation is different, which includes additional genes such as Myoz2, confirmed by siRNA interference techniques (Wei et al., 2022).

### 2.4.5 Myogenin

*Myog* is also a crucial gene during the terminal differentiation of muscle development. The regulation of differentiation in pluripotent P19 cell lines was observed with the association of the MEF2C (myocyte enhancer factor 2C) gene. This gene triggers the increment of expression of Myog more than 20 folds and takes part in the positive regulation of these cells (Ridgeway et al., 2000). Along with muscle development, Myog also regulates neurogenic atrophy with the help of other associated TFs such as histone deacetylases (HDACs) 4 and 5 (Moresi et al., 2010). The epigenetic regulation of Myog has also been observed during muscle development at the larval stage in Atlantic salmon (Burgerhout et al., 2017). In general, the histone modification enzymes, chromatin remodelers, cofactors, and other specific TFs have a role to play in the regulation of muscle proliferation to differentiation from the quiescent stage. In the mouse model, the development of the sternum and rib was also visible other than muscle development, and the deletion of the *Myog* gene exhibits muscle scarcity and lethality (Vivian et al., 1999; Meadows et al., 2008). This observation supports a study that revealed the critical function of Myog in skeletal muscle development. The mutation in Myog in mice showed muscle scarcity and death instantly after birth; however, the mutation in other MRFs does not show such results (Hasty et al., 1993). Figure 3 depicts the comparative roles of various myogenic factors during myogenesis.

### 2.4.6 Myogenic factor 5

Myf5 is considered the early expressed gene with MyoD, and its crucial function in the commitment and proliferation of the cells that direct the myogenic process (Giordani et al., 2007). Myf5 is vital for the satellite cells in the initialization of the myogenesis process. The double mutant of the *MYF5* study confirms its importance in the formation of muscle dystrophy, although the mice were not lethal (Ustanina et al., 2007). Myf5 has a role to play in chromatic remodeling, which provides access to other associated TF factors to be activated (Gerber et al., 1997). At least six distinct sequences control myf5 expression in the somite, and even within a presumably uniform structure like the myotome, more than one

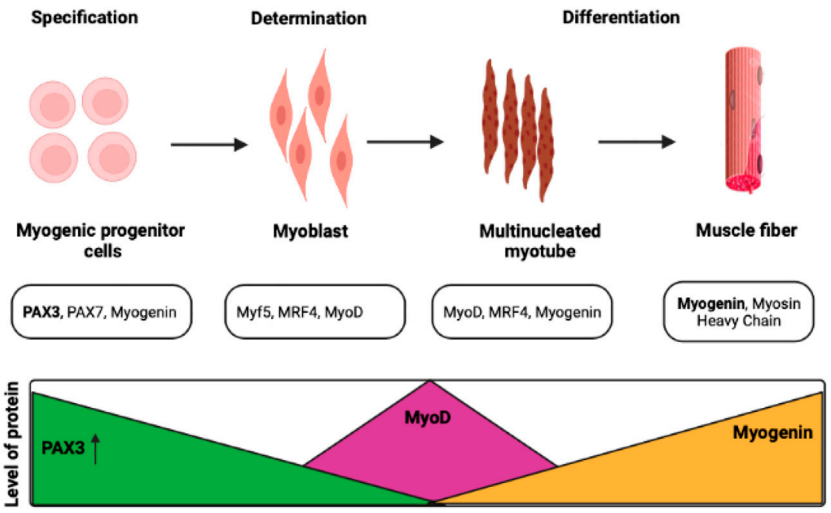


FIGURE 3
Transcription factors control myogenesis at various stages. Satellite cells proliferate, differentiate, and renew the population of progenitor cells to maintain muscle function (Darabi and Perlingeiro, 2008; Olguín and Pisconti, 2012).

regulatory module is necessary (Hadchouel et al., 2003). From a gene homology point of view, the *Myf5* gene is well conserved between fish and mammals (Ustanina et al., 2007). In recent research on rat skeletal muscle cells, compressive stress has a time-dependent effect on how Myf5 expression is regulated. They found that on prolonged stress stimulation, the expression of *Myf5* and *MyoD* genes was downregulated (Lu et al., 2020). The course of feeding pattern (under-feeding, long-term under-feeding, and re-feeding) in sheep provided a differential expression pattern of myokines, MRFs, and TFs, where Myf5 transcript showed an overexpression (Jeanplong et al., 2003). A study concluded that the factors governing adult Myf5 expression can be genetically distinguished from those governing Myf5 during development and may even be different (Zammit et al., 2004).

## 2.5 Functions of myokines in skeletal muscles

Myokine is a cytokine comprising a molecular weight in the range of 5–20 kDa. During muscle contraction, skeletal muscle cell produce and release myokines. Table 1 briefly describes the function of myokines and cytokines implicated in myogenesis.

## 3.1 *In vitro* culture of muscle cells

*In vitro* stem cells need a culture environment like growth media, cell substrates, antibiotics, antimycotic agents, and incubators. The culture environment provides optimum *in vitro* niche conditions to grow cells, mimicking *in vivo* (ECM, hormones, and cytokines). Recapitulations of media components are performed by exploiting synthetic chemicals and artificial devices. In the next section, we will discuss i) media, ii) cell substrates, iii) serum and their replacements, iv) antibiotics, and v) additional nutrients and supplements.

## 3.2 Extracellular matrix (ECM)

ECM is a multilayered environment that provides structural support, signals responses to injuries, helps cellular communication, and presents architectural preservation of skeletal muscle cells. During myogenesis, ECM interacts with, adheres to, safeguards muscle cell, assist in biochemical signaling, and offers structural support (Lee et al., 2018). Some of the ECM proteins assist in cell-matrix interactions and matrix assembly regulation (Gillies and Lieber, 2011). Besides its biological function, ECM comprises nutrients such as proteins (collagen) and glycosaminoglycans that impact the texture of tissue and overall meat quality (Table 2) (The Good Food Institute, 2022).

In myotubes' developmental regulation during myogenic differentiation's early stages, ECM is essential in regulating MSC's phenotypic expression (Zhang et al., 2021). The basal lamina comprises a three-dimensional ECM network and is directly linked to MSC (Kuang et al., 2008). The majority of ECM comprises collagen fibers and proteoglycan matrix; however, ECM also contains elastin, fibronectin, and laminins (Thorsteinsdóttir et al., 2011). Three-dimensional scaffolds are critical to stabilize cells and impersonate the ECM during tissue formation. To augment the quality, taste, and tenderness of CBM, it may be advisable to co-culture preadipocytes with myoblast, owing to their effective increase in intramuscular fat content of cultures meat.

Collagen forms an intramuscular connective tissue network, and it is the most abundant fibrous protein in skeletal muscle (10% by weight) (Gillies and Lieber, 2011). Collagen provides elasticity, tensile strength, strengthens bones, regulates cell attachment, and role in differentiation (Ahmad et al., 2020). Collagens are necessary for the self-renewal of MSC and differentiation *in vivo* in mice. For example, the knockout of collagen VI impaired regenerating capacity of MSC following muscle injury (Urciuolo et al., 2013).

**TABLE 1 Functions of cytokines and myokines in myogenesis.**

| Cytokines and myokines | Functional role | References |
|---|---|---|
| IL-6 | A multifunctional cytokine that regulates myogenesis of the proliferative capacity of muscle stem cells via IL-6 signaling; Myoblast differentiation in C2C12 cells. Differentiating C2C12 cells, STAT3 or mRNA knockdown of IL-6 exhibit a reduced level of expression of MyHC IIb and myogenin leading to disruption of fusion of myotube | Baeza-Raja and Muñoz-Cánoves (2004), Muñoz-Cánoves et al. (2013) |
| IL-15 | IL-15 is expressed at higher levels (mRNA and protein) and interconnected between adipose tissue and muscle tissues. mRNA levels of IL-15 are 10-fold upregulated in C2C12 cells in differentiated cells compared with undifferentiated cells. IL-15 stimulates protein synthesis and inhibits protein degradation in cultured skeletal myotubes | Quinn et al., 1997 (2002) |
| MyoG | Overexpression of MyoG and low DNA methylation under thermal regulation in fish. The mature myofibers showed a low level of MyoG | Burgerhout et al. (2017), Zammit (2017) |
| LIF (Leukemia Inhibitory Factor) | One of the members of the IL-6 cytokine family. LIF affects myoblast proliferation, regeneration, and differentiation. LIF was recognized as an upstream constituent stimulating myoblast differentiation by activating the JAK2/ STAT3 signaling pathway. LIF is essential for the survival of embryonic muscle cells and myoblast proliferation in mice and rats | Spangenburg and Booth (2002), Sun et al. (2007), Yang et al. (2009), Broholm et al. (2011) |
| IL-4 | Function as a myoblast recruitment factor during muscle growth. Regulated cell fuse by acting on myoblast through IL-4R. IL-4Rα is also expressed by both myoblast and myotube, essential for muscle development. Myoblasts devoid of IL-4Rα are not recruited by IL-4-secreting nascent myotubes and develop normally. However, these myotubes are smaller in size with lower myonuclear numbers | Horsley et al. (2003) |
| IL-1β | Injury in the skeletal muscle causes infiltration of immune cells in the extracellular space leading to a rise in cytokine levels. IL-1β may increase up to 20-fold, and elevated cytokine levels may stimulate muscle satellite cell proliferation *via* the NF-kB signaling mechanism | Otis et al. (2014) |
| Myostatin | Belongs to TGF-β family and negatively regulates skeletal mass. It inhibits differentiation and muscle growth by thwarting the progenitor cells | Rodgers and Ward (2022) |
| Irisin | It is a polypeptide hormone that gets activated during physical work out in response to exercise this myokine is produced in skeletal muscle abundantly. Its precursor is also a cleaved product of FNDC5 (fibronectin type III domain containing 5) overexpressed in skeletal muscle *via* autocrine signaling of irisin. Mainly functions in regulating muscle homeostasis and bone turnover | Colaianni et al. (2017) |
| Myonectin | FAM132b, or myostatin, is a skeletal muscle expressing secretory myokine, also a precursor of irisin; endorses the transfer of fat from adipocytes to the liver organ. A study found higher levels of myonectin in rats after exercise | Seldin and Wong (2012), Peterson et al. (2014) |
| Decorin | It is a negative regulator of myostatin in the regulation of muscle growth. During fetal bovine development (2.5 months), decorin was observed to be expressed in skeletal muscle. It has the role of a regulatory secreted proteoglycan expressed in myotubes during exercise-related activities | Nishimura et al. (2002), Kanzleiter et al. (2014) |

Collagens are found in numerous forms, and several of them have been revealed in skeletal muscles, such as fibrillar collagens I, III, V, IX, and XI. Collagen I and III account for more than 75% of total skeletal muscle collagen (McKee et al., 2019). Collagen and gelatins are widely applied in the pharmaceutical and food industries owing to their biodegradability, biocompatibility, and low antigenicity (Liu et al., 2015).

## 4 Immortalization

Efforts are being made to develop cell lines for CBM to cultivate and facilitate the research and development of novel food products. Recently, the Good Food Institute, in collaboration with Kerafast, has standardized and developed terrestrial as well as aquatic cell lines suitable for CBM (The Good Food Institute, 2019). The main

**TABLE 2 Stem cell differentiation into muscle cells and cultured meat production in livestock.**

| Animal origin | Cell source/type | Summary in brief | References |
|---|---|---|---|
| Sheep | Adult muscle tissue/Satellite cell | A successful system has been established to isolate, purify, and identify sheep skeletal muscle satellite cells using two steps enzymatic digestion (Type 1 collagenase and Trypsin) and differential adhesion methods with media containing 20% FBS+10% horse serum. These cells can also differentiate into osteogenic and adipogenic lineage with respective induction media | Wu et al. (2012) |
| | Adult muscle tissue/Satellite cell | Enrichment of ovine miRNA database and the sheep miRNA transcriptome outline during skeletal muscle development. They have shown that satellite cell proliferation and myogenic differentiation are affected by miR-192 *via* the downregulation of retinoblastoma 1 in total of 2396 miRNAs present | Zhao et al. (2016) |
| | Adult muscle tissue/Satellite cell | Zhang and coworkers investigated the isolation method for muscle-derived stem cells (MDSCs) using XI collagenase and trypsin enzymatic digestion from fetal sheep skeletal muscle and a differential attachment method to purify the cells. The MDSCs were able to differentiate into adipocytes, osteoblasts, chondrocytes, and neuron-like cells when cultured in the respective optimized induction medium. They have shown that MDSCs were multipotential and are significant players in muscle repair, and they can be used in tissue engineering research and clinical applications | Zhang et al. (2019) |
| | Fetal muscle tissue/Adult muscle tissue/Satellite cell | Naturally grazing Wuzhumuqin sheep, the Skeletal Muscle Satellite Cells (SMSCs) were isolated and investigated the levels of Muscle Regulatory Factors (MRFs) at different stages of fetal stage, from semitendinosus muscle of 0 month old and 6 month old sheep. The levels of Myf5 and MyoD decreased in proliferating SMSCs as the generations increased. The Myf6 and Myog levels were more at month 0 in sheep muscle, and at 6 months, the levels were low | Rihan et al. (2020) |
| | Embryonic muscle tissue | Study elucidates protein function and its role in sheep embryonic skeletal muscle growth and development. Total 5520 proteins were identified and in that 1316 were in differential abundance by tandem mass tag analysis in longissimus dorsi at embryonic ages Day 85, Day 105, and Day 135 of Chinese merino sheep | Xinyue Wang et al. (2020) |
| | Adult muscle tissue/Satellite cell | Skeletal muscle cells were isolated using enzymatic digestion of the hind limb muscle of a sheep fetus. Results have shown that a culture medium with 5% FBS allows satellite cells to grow without differentiation, but with 10% FBS, the cells get differentiated | Rashidian et al. (2020) |
| Goat | Adult muscle tissue/Satellite cell | Successfully established goat skeletal muscle cell culture and their differentiation into myogenic and adipogenic lineages when provided the required conditions. Basic fibroblast growth factor will suppress terminal myogenic differentiation of goat satellite cells | Yamanouchi et al. (2007) |
| | Adult muscle tissue/Satellite cell | Enzymatic digestion of intercostal muscle of goat was performed to isolate skeletal muscle stem cells that reside between the muscle fibers' basal lamina and plasma membrane. It has shown that adipogenic differentiation of satellite cells was induced when hyper- contraction in the isolated fine fibers | Yamanouchi et al. (2009) |
| | Adult muscle tissue/Satellite cell | Study showed reciprocal interactions between muscle-derived cells (MDC) and bone-marrow derived mesenchymal stem cells (MSCs) in autologous conditions. MSC contributes to the formation of myotubes when co-cultured directly with MDC, but the myogenic nature is not acquired in MSC when only soluble factors of MDC are used | Kulesza et al. (2016) |
| | Adult muscle tissue/Satellite cell | miR-27b promotes differentiation and inhibits the proliferation in skeletal muscle stem cells of the Anhuai goat. In a reverse way, the pax-3 inhibits the differentiation and enhances the proliferation. Goat satellite cells | Ling et al. (2018) |

(Continued on following page)

**TABLE 2 (*Continued*) Stem cell differentiation into muscle cells and cultured meat production in livestock.**

| Animal origin | Cell source/type | Summary in brief | References |
|---|---|---|---|
| | | myogenic proliferation and differentiation are regulated through miR-27b by targeting Pax3 | |
| | Adult muscle tissue/Satellite cell | They used pronase enzyme digestion method and stratified liquids between 40% and 90% of percoll to isolate the SMSCs. Isolation, purification, and identification of goat skeletal muscle satellite cells were successfully established and demonstrated the potential of these cells to be induced into myoblasts and adipocytes | Wang et al. (2020) |
| Chicken | Egg embryo muscle tissue/primary muscle cells | Delta-like protein-1 (gDLK1) expression is more in broilers' muscles than layers, indicating that this gene is a marker for high muscle growth in chickens. There is ample gDLK1 in muscle tissue at embryonic stage but decreased in both layers and broilers after hatching period. The induction of gDLK1 gene was confirmed using histological studies after injury to the muscle | Shin et al. (2009) |
| | Adult (7-day old) muscle tissue/satellite cells | Here they examined the muscle LIM protein role in skeletal muscle proliferation and differentiation, and by focusing on TGF-β signaling determined its mechanism of action. By regulating Smad3 phosphorylation in the TGF-β signaling pathway knockdown of cysteine and glycine-rich protein 3 suppressed chicken satellite cell differentiation | Han et al. (2019) |
| | Adult muscle tissue/satellite cells | Nucleic acid, amino acid composition, and taste characteristics of the cultured muscle tissue and traditional meat were investigated in this study. The glutamic acid and Inosine-5′- monophosphate concentrations were significantly lower in cultured meat tissue than traditional meat. Cultured meat tissue from chicken and cattle has significantly lower taste characteristics like umami, bitterness, and sourness when compared with traditional meat which were assessed using an electronic tongue system | Joo et al. (2022) |
| Bovine | Adult muscle tissue/satellite cells | Culturing the bovine muscle satellite cells for improved proliferation and early differentiation on Glycosaminoglycan and fibrous protein-coated surface mimics natural ECM. They observed there is re organization of Golgi complex in differentiated cells | Rønning et al. (2013) |
| | Adult muscle tissue/satellite cells | In bovine skeletal development they investigated the profile of miR-1 and miR-206 and their biological function. MiR-1 and miR-206 positively regulate bovine SMSCs myogenic differentiation via Pax7 and histone deacetylase 4 downregulation | Dai et al. (2016a) |
| | Adult muscle tissue/satellite cells | In this study, they investigated the miR-128 biological functions in skeletal muscle development. In bovine SMSCs, miR-128 negatively regulates myogenic differentiation by inhibiting Sp1, an activator of MyoD | Dai et al. (2016b) |
| | Fetal bone marrow | Bovine fetal mesenchymal stem cells (bfMSCs) derived from bone marrow was evaluated for *invitro* myogenic differentiation using 3 different protocols. Levels of MRFs or more when bfMSC cultured using 100um of 5-Aza. When bfMSC cultured using Gal-1 and SkGM-2 found that there is upregulation of intermediate and late MRFs and downregulation of early MRFs | Okamura et al. (2018) |
| | Adult muscle tissue/satellite cells | They used fluorescence-activated cell sorting method to enrich bovine SMSCs in isolation of the cells. They found that p38 mitogen-activated protein kinase signaling and Pax7 expression are reciprocal as the culture age increases. They examined the proliferation of the cells using p38 inhibitor in the culture for a long time. The cells proliferating more and longer generations in the presence of p38i. SMSCs culture for large-scale cultured meat production relies on cell purity and inhibition of p38 mitogen-activated protein kinase signaling | Ding et al. (2018) |

(Continued on following page)

**TABLE 2 (*Continued*) Stem cell differentiation into muscle cells and cultured meat production in livestock.**

| Animal origin | Cell source/type | Summary in brief | References |
|---|---|---|---|
| | Adult muscle tissue/satellite cells | The role of Podocon an ECM protein role in MDSCs differentiation examined through b-Catenin inhibition and activation conditions along with transfected cells with its overexpression and inhibition using different analytical techniques. Podocan, regulates the Wnt4/b-catenin signaling pathway and promotes bovine MDSCs differentiation | Shuang Li et al. (2019) |
| Buffalo | Inner cell mass/Embryonic stem cells | In buffalo ESC-like cells embryoid bodies when cultured on gelatin-coated plates in the presence of retinoic acid ($10^{-7}$ or $10^{-8}$M) or DMSO (1or 2%) for 25 days will induce skeletal myogenesis which confirmed by marker expression using RT-PCR. | Singh et al. (2013) |
| Porcine | Adult muscle tissue/muscle progenitor cells | The porcine muscle progenitor cells were cultured for >120 population doublings in the presence of 5 ng/ml basic fibroblast growth factor while maintaining a normal karyotype. Co-culture of porcine Murine progenitor cells with murine C2C12 myoblasts induced myogenic differentiation to form myotubes. The progenitor cells ability to differentiation into adipogenic and osteogenic lineages also confirmed using qRT-PCR and respective staining of the cells | Wilschut et al. (2008) |
| | Adult muscle tissue/satellite cells | In porcine muscle, there are 2 cell types those that express alpha 6 integrin and those not expressing it. The alpha 6 integrin expressing cells are able to form more myotubes this is confirmed using qRT-PCR. They also showed that inhibition of alpha 6 integrin reduces the myogenic stem cell differentiation | Wilschut et al. (2011) |
| | iPSCs | Early skeletal muscle transcription program in porcine iPSCs is activated by CHIR99021, a glycogen synthase kinase-3b inhibitor, in amalgamation with a DNA methylation inhibitor 5-aza-cytidine. Terminal differentiation to form myotubes was induced by ectopic expressed MyoD1 activation | Nicholas J. Genovese et al. (2017) |
| | Adult muscle tissue/satellite cells | Long non-coding ribonucleic acid MSTRG.59589 which is highly expressed in skeletal muscle cells role was investigated using knock down model. The knock down model given the differential gene expression patterns, which further analyzed revealed that they are mainly elevated in muscle contraction, actin cytoskeleton and other pathways of the muscle development. In porcine satellite cells; myogenic differentiation is promoted by the palladin gene, which is regulated by MSTRG.59589 | Long Li et al. (2019) |
| | Adult muscle tissue/satellite cells | One Gram of neonatal pig muscle tissue they isolated $5.3 \times 10^4$ porcine muscle stem cells. Porcine muscle satellite cells stem cell ness is maintained using Ascorbic acid −2 phosphate in the medium and cultured cells on polydimethylsiloxane molds to form 3D tissue networks to mimic pork meat structurally, and this process is scalable for industry-level | Zhu et al. (2022) |
| | Adult muscle tissue/satellite cells and 3T3 L1 adipocyte cells | Liu and coworkers investigated the edible 3D porous gelatin microcarrier (PoGelat-MC) as a scaffolding system for culturing and expanding the porcine skeletal muscle cells and murine myoblast cells. The cells cultured in spinner flasks with PoGelat-MC have shown spontaneous myogenesis in spite of absence of the myogenic reagents. The centimeter scale meat balls which exhibited similar mechanical and higher protein content when compared with conventional ground-pork meat produced by assembly of microtissues on 3D printed mold using cross-linker transglutaminase. The proliferation and differentiation of 3T3L1 pre-adipocytes into mature adipocytes were also done on PoGelat-MCs. The fat micro tissues are produced as a modular assembly unit to produce fat-containing engineered meat | Liu et al. (2022) |

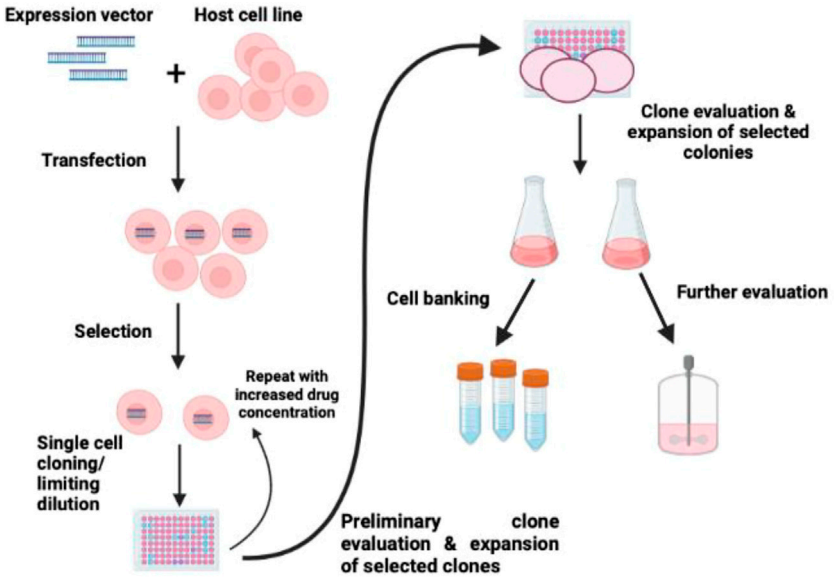


FIGURE 4
Schematic diagram of immortalization of cells. The strategy of immortalization can be achieved by the induction of telomerase expression and inactivating p53/p16/Rb (Maqsood et al., 2013).

objective of any firm is to develop a novel cell line that is immortal, loses its cell cycle checkpoint pathways, and bypasses the senescence process. There are at least three approaches in establishing cell lines, i) expression of the catalytic subunit of telomerase, ii) introduction of viral genes that inactivate p53/p14/Rb, and iii) serendipitous discovery of immortalized cell lines. Every approach exploits either the expression of telomerase, the circumventing/inactivation of cell cycle checkpoint, or a combination of both (Maqsood et al., 2013).

The insertion of telomerase in immortalizing cell lines has been utilized since 1999 (Ouellette, 2000). The telomere extension helps cells escape cell death triggered by telomere shortening. This can be achieved by ectopic telomerase expression encouraging immortalization of human esophageal keratinocytes (normal) without deactivating the p53 pathway (Harada et al., 2003). Immortalized fibroblast cell lines are also generated from human embryonic stem cells under undifferentiated cell growth conditions, thus creating a system for the culture of hESCs (Xu et al., 2004).

DNA damage and other stress activate transcription factor p53 causing cell cycle arrest until the cell establishes that DNA can be repaired. If the DNA damage is irreversible, p53 plays a role in activating and triggering apoptosis and cell cycle arrest (Chen, 2016). Consequently, activation of p16 and Rb halts other proteins from initiating DNA replication, resulting in apoptosis (Takahashi et al., 2007). Mutating the p16 or Rb gene may allow cells to continue DNA replication leading to immortalization (Maqsood et al., 2013).

Previously, the immortalized cell line was established through inactivation or bypassing the p53/p16/Rb stress response by transforming viral genes (Figure 4). Here in this method, simian virus 40 (SV40) large T-antigen were planned to bind and inactivate p53/Rb and other tumor suppressor factors in a variety of species and organ types (Jin et al., 2006; Yamada et al., 2019; Zhang et al., 2020). Customarily, T-antigen reactivates the host cell to stimulate the replicate SV40 virion (Ahuja et al., 2005). However, in most mammalian systems, the T-antigen can transform the host cell without viral assembly and cell death leading to stable transformation and immortalization in the host cell (Chou, 1989). Apart from SV40 T-antigen, a few other viruses and viral proteins, such as E1A and E1b protein of adenovirus, E6 and E7 ORFs of human papillomavirus, and Epstein-Barr virus, have been employed to produce immortal cell lines through inactivation of cell cycle checkpoints (Shay et al., 1991; Counter et al., 1992; Klingelhutz et al., 1994; Oh et al., 2003).

Recently, Upside Foods, a United States-based cultured meat firm, submitted a patent employing telomerase reverse transcriptase (TERT) overexpression utilizing CRISPR to knock out the expression of p16 and p16 in chicken skeletal muscle cells (Thorley et al., 2016). The proliferative capacity of cells increases owing to the knockout of p15 and p16 alone; however, adding the ectopic TERT gene has augmented the overexpression of TERT indefinitely. Some approaches to immortalizing the myogenic cell lines may evade telomere shortening and the p16 stress pathway by ectopic expression of TERT and the Rb inhibitors cyclin D1 and cyclin-dependent kinase 4 (Stadler et al., 2011; Genovese N. et al., 2017).

## 4.1 Challenges in establishing a unique cell line

CBM needs unique cell lines prepared from agriculturally effective systems to scale up on an industrial scale. One of the first daunting tasks in CBM is establishing its cell line, which can be used in each cycle to produce meat. Few firms (Kerafast, United States and ESCO ASTER, Singapore) manufacture cell lines of various species for commercial purposes. MACK1 (myoblast) adherent cell line derived from the mature muscle of Atlantic mackerel (*Scomber scombrus*) for useful cellular agriculture (Saad et al., 2022). ICAR-National Bureau of Fish Genetic Resources, India, has a repository of

about a cell line of 50 fishes, especially *Catla*. Freshwater fish species of Bluegill fry cell line (adherent fibroblast), Rainbow trout, and embryonic cell line of Nile tilapia have already been established (NRFC, 2023). Researchers are manipulating the stem cell expression markers in a suitable culture medium to develop unique methods for myogenesis induction in the cell lines (Thorley et al., 2016). Presently, few labs are working on establishing particular cell lines for various organisms like bovine, seafood, and aquatic species (Better ways to start cultivating meat | Research (2020-2022) | GFI, 2021). One of the main obstacles in developing a cell line is the limited knowledge of surface genetic markers and the non-availability of species-specific antibodies to assist in identifying a suitable cell line. However, few firms have developed the cell lines of various species like chicken, fish and turkey, and are commercially available (Kerafast) (Kerafast, 2023). Due to limited growth of the primary existing cells (not growing beyond 30–40 passages) in the defined media, is the main reason for developing the cell lines. In November 2022, UPSIDE Foods reported the establishment of myoblast and fibroblast cell lines with demonstrated differentiation capacity in the suspension culture (Ding et al., 2018). Immortalization has been carried out through the introduction of a cis gene expressing chTERT. Based on their presented data, US FDA gave the green light to conduct further research to develop CBM.

Developing a cell-based fish cell line to meet the growing demand for alternative proteins has several advantages over mammalian and avian CBM approaches. Firstly, fish cells may undergo less senescence and have more doubling with regard to mammalian and avian species (excluding embryonic stem cells) (Klapper et al., 1998; Strecker et al., 2010; Graf et al., 2013; Kim et al., 2018). Secondly, fish cell lines are known for maintaining karyotypic stability with respect to mammalian and avian species (Barman et al., 2014; Fan et al., 2017). Thirdly, a fish cell may easily grow under atmospheric air and possess high intracellular buffering capacity (Bols and Lee, 1991; Rubio et al., 2019). In cell culture bioreactor avian and mammalian cells require carbon dioxide and bicarbonate to control the pH in addition to air, oxygen, and nitrogen (Warner et al., 2015). Managing only three gases (air, oxygen, and nitrogen) is an advantage of fish cells over mammalian cells as it simplifies scale-up challenges and mitigates the issue of $CO_2$ stripping (Sieblist et al., 2016).

## 5 Culture media components

Culture media is one of the crucial parameters of the final cultured meat product that maintains cells in *ex vivo* (Post et al., 2020). Depending upon media components, the taste and texture of the cultured meat are decided. The following section discusses various media types commonly used in CBM. Basal media formulations are sufficient to keep the cell alive for a limited period; however, various media are used to proliferate for extended periods. Minimal essential medium (MEM) is frequently used to maintain cells in tissue culture comprising amino acids, vitamins, glucose, and salts (Eagle, 1959). Minute variations in MEM have created a new media commonly used for mammalian cell cultures, Dulbecco's modified Eagle's medium (DMEM) (van der Valk et al., 2010)

In cell culture, 70% of the glucose is converted into lactate by highly proliferating cells; however, 20%–30% of the remaining glucose is available for tricarboxylic acid (Ryan et al., 1987). The lack of nutritional components like vitamins D, E, and selenium may cause degenerative changes in muscle (Braga et al., 2017).

The proliferating cells require a special type of media compared to differentiating cells. Energy requirement changes from general nutrient usage to highly specialized protein production depending on cell types. Cell culture media poses a challenge to sustainable cellular agriculture.

Fetal bovine serum (FBS) is an animal-derived component commonly used as media; the possibility of contamination violates ethics and is unsustainable for CBM. FBS is perceived as a universal supplement comprising 200–400 kinds of proteins and numerous small molecules with undefined concentrations.

Chemically defined media components such as proteins, sugars, growth factors, and fatty acids can replace FBS with previously established procedures (van der Valk et al., 2010).

Growth factors regulate cellular activities like proliferation, differentiation, and stimulation as they activate signaling pathways. The commonly employed growth factors for stem cell research are fibroblast growth factor (FGF), epithelial growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor, bone morphogenic proteins, and platelet-derived growth factor (PDGF). For proper muscle development, hepatocyte growth factors, FGF, IGF, and PDGF are also pertinent (Goonoo and Bhaw-Luximon, 2019).

Some commercially available growth factors for bioactive compound production or therapeutic application are mainly produced with research-grade or cGMP benchmarks. To meet the quality of the food industry especially concerned with cell culture expression, the growth factors require cost-effective management on an industrial scale.

Glucose and amino acids are major components in high concentrations and strongly affect the environmental footprint. Glucose as a substrate gives rise to amino acids through fermentation (Ikeda and Nakagawa, 2003). The production of glucose on an industrial scale has been well-established since centuries ago, with modest waste production and a high level of integration (An and Katrien, 2015). The approach is based on the hydrolysis of starch which is naturally produced by photosynthesis. Scientists are utilizing an alternative source of peptides, amino acids that are usually obtained from the bacterial, fungal, and algal biomass that is enriched with fats, amino acids, vitamins, and minerals (Xu et al., 2006; Ramos Tercero et al., 2014; Matassa et al., 2016). Recycling culture media is one of the vital aspects of CBM with a promising results concerning cost-effective and extended batch duration (Yang et al., 2018; Zhu et al., 2018). In perfusion systems, this method may appreciably curtail the use of sterile water; however, media recycling in mammalian systems is still in its infancy.

The investigations done by Kolkmann et al. on serum-amended DMEM to culture bovine myoblasts have shown the potential of FBM (Fibroblast Basal Medium), FBM/DMEM, and Essential 8™ Medium to become alternative (Kolkmann et al., 2020). This group recently developed chemically defined media which supports 97% of the growth of the primary bovine myoblast cells compared with golden standard culture medium. The composition of the media is DMEM/F12 as a basal medium, supplemented with L-ascorbic acid 2-

phosphate, fibronectin, hydrocortisone, GlutaMAX™, albumin, ITSX, hIL-6, α-linolenic acid, and growth factors such as FGF-2, vascular endothelial growth factor, IGF-1, HGF, and PDGF-BB (Kolkmann et al., 2022). Insulin or IGF1, FGF2, and TGF-β1 are the three key signaling components in nutritionally rich E8 serum-free media (Amit et al., 2004; Kuo et al., 2020). Novel media formula (B8) was introduced by Chen Y et al. to support a high growth rate under low seeding density conditions and to grow iPSCs for more than 100 passages (Chen et al., 2021). Research studies are developing small molecule cocktail (chroman 1, emricasan, polyamines, and trans-ISRIB—CEPT) patents and Rho Kinase inhibitors—ROCKi to increase the cell yield cellular survival during differentiation (Watanabe et al., 2007).

# 6 Scaffolding

It is an agent that mimics the *in vivo* system (biomechanical and biophysical) and enables the final product's potential vascularization and spatial heterogeneity. Scaffolding provides structural and mechanical support to the cell types, ensuring their proper growth and adherence to the flasks. Most current scaffolds are based on mammalian-derived biomaterials; other than that, non-mammalian sources, namely, salmon gelatin, alginate, and additives, including gelling agents and plasticizers, are also being used. The mechanical strength arises from the network structure rather than the properties of individual collagen fibers. To achieve the texture of conventional meat, either by using mechanically similar scaffolding materials or by inducing cells to secrete their own ECM is necessary (Bomkamp et al., 2022). For CBM production, biomaterials, such as biopolymers, growth factors, enzymes, and various additives, are considerably used on a commercial scale. These biomaterials should be inexpensive, environment friendly, and cost-effective. The porous biomaterial allows the exchange of oxygen, nutrient inflow, and waste product removal to continue the cell's metabolic function and avoid necrotic formation during the process. A complete balance of morphology, structure, and chemistry is needed. Customarily, scaffolding was established for medical purposes in tissue engineering and regenerative medicine (Owen and Shoichet, 2010; Garg and Goyal, 2014; Aamodt and Grainger, 2016). Here CBM requires different standards such as degradable, safe for consumption, palatable, texture, taste, and nutritional values. Essentially, the scaffold should be safe, readily available, and cost-effective for industrial production.

Manipulating the biologically sourced material such as collagen and ECM should be kept at a minimum as they are non-replicative and need livestock for their generation. Few promising materials such as cellulose, starch (amylose and amylopectin), chitin, chitosan, alginates, and hyaluronic acid are commonly used (Cunha and Gandini, 2010; Ben-Arye et al., 2020). Protein-based systems, for example, fibrin, collagen, keratin, gelatin, or silk, are also preferred. Other types of material, for instance, the derivatives of polyester, polyhydroxyalkanoates, and proteins expressed in the bacterial system, are currently being utilized (Bugnicourt et al., 2014). Plant-based proteins (lignin), decellularized leaves, and fungal mycelia are also actively pursued (Modulevsky et al., 2014). Apart from biopolymers, various synthetic polymers, including a range of polyesters, are favored owing to their tailored degradation through chemical hydrolysis in the human body (Woodard and Grunlan, 2018).

Biopolymers extracted from a non-mammalian source such as algae (alginate or agar) and fish species (gelatin) have been commercially used in tissue engineering (Nagai et al., 2008; Yamada et al., 2014). Alginate and agar allow the cultures of mammalian cells due to the non-availability of cell recognition sites the-Arg-Gly-Asp (RGD), which stimulate cell adhesion and migration (Bedian et al., 2017; Schuster et al., 2017). However, gelatin possesses RGD sequences, and a promising approach is to blend algae-derived polymers having fish-derived gelatin. One prominent and attractive ingredient to produce edible and biodegradable scaffolds is salmon gelatin (Enrione et al., 2012). The physical properties of salmon gelatin allow blending with other biopolymers to form copolymers and stable polyelectrolyte complexes owing to lower melting temperatures than other mammalian gelatin (Acevedo et al., 2015).

## 6.1 Microcarriers

Microcarriers are beads comprising various materials, porosities, and topographies that provide a surface for anchorage to the cells to hold (McKee and Chaudhry, 2017). Microcarriers offer a large surface-to-volume ratio and are perceived as critical for upscaling in CBM. Microcarriers suspended in a medium provide a 3D culture environment.

Since the inception of the microcarrier concept for the culture of adherent cells in 1967, numerous microcarriers have been established and commercialized (Van Wezel, 1967). Generally, microcarriers have been used for the expansion of cells fabricating molecules of interest, such as monoclonal antibodies, vaccines, and proteins (Phillips et al., 2008). In a recent development in the field of cell and gene therapy, emphasis has been given to developing microcarriers for the culture of human stem cells for therapeutic purposes (Cui et al., 2009; Chen et al., 2013; Gümüşderelioğlu et al., 2013; Li et al., 2016).

Microcarriers exploited for CBM production should fulfill the food regulation of the country while proposing optimal topography and surface chemical properties for target cell types. Preferably, microcarriers should be animal-free components to prevent the use of animal products throughout the production of CBM.

Microcarriers may also work as nutrient carriers, such as essential growth factors, amino acids, *etc.*, to meet the satellite cell's nutrient demand. This can help minimize the number of medium exchange steps and reduce the risk of contamination and cell loss. Successful loading of sol-gel-derived bioactive glass microcarriers in combination with basic FGF-2 and cytochrome c were sustainably released spanning several weeks (Perez et al., 2014). Sustained release and microencapsulation of bioactive molecules are currently given the utmost importance in the food industry (O'Neill et al., 2014; Shishir et al., 2018). The principle may also apply to microcarriers-based cell culture in meat production. Physical parameters like temperature and pH can be tuned to control *in vitro* release kinetics from loaded microcarriers (Zhou et al., 2018; Matsumoto et al., 2019).

Satellite cells are anchorage-dependent; thereby, these cells require microcarriers surface for the attachment. The attachment of cells is a critical parameter that affects the entire process of yield (Bock et al., 2009). Cell attachment encompasses the association between cell adhesion molecules and substrates on the surface of the microcarriers (Goldmann, 2012). The integrin protein regulates cell adherence (Derakhti et al., 2019). These are heterodimeric glycoproteins comprising a and b subunits, each with various

isoforms depending on the expression of isoforms (Rowley et al., 1999). Integrins bind to a diverse class of proteins with different combinations like $\alpha_1\beta_1$ has a specific affinity to collagen, $\alpha_5\beta_1$ to fibronectin, and $\alpha_v\beta_3$ to vitronectin (Barczyk et al., 2010).

Recently, Norris et al. developed edible microcarriers possessing tunable mechanics as well as a surface topology for CBM (Norris et al., 2022). They are made-up microcarriers employing gelatin and food-grade crosslinking enzyme (transglutaminase). The inexpensive method does not require the application of any synthetic polymeric materials, instead needs small crosslinking agents or modified chemical groups. The scalable process to produce edible microcarriers by exploiting water-in-oil emulsions enables readily fabricating hydrogel microparticles with a spherical shape and smooth surface.

## 6.2 Bioreactors (scale-up)

The crucial aspects of the scale-up are bioprocess modeling and optimization, for which spinner flasks act as small-scale systems, especially in defining mass and energy transfer models. Because of this, attempts were made to mimic the 3D environment in the spinner flasks for the animal cell culture by internal mixing (Verbruggen et al., 2018). There are different lab-scale reactors for use in tissue engineering. Biopharmaceuticals are stirred-tank, hollow fiber, roller bottles, rocking bed, fluidized-bed and fixed (packed) bed systems (Odeleye et al., 2020). Vijay Singh first described the rocking bed bioreactor or wave reactor intended for the cultivation of animal cells (Singh, 1999). Wave bioreactor contains a flexible polymeric bag with special ports for allowing us to introduce air, oxygen, or medium or to withdraw samples under sterile conditions. Fluidized bed reactors (FBRs) are rarely applied in tissue culture, but homogenous bed expansion behavior, related to good mass transfer characteristics, as well as lower shear stress, compared to stirred tank reactors and simpler scale-up procedures, makes them advantageous to others in the production of the cultured meat (Ellis et al., 2005). In hollow fiber bioreactors, hollow fibers act as a semi-permeable membrane by allowing the water and nutrients for cell growth while removing metabolic products and serve as a cell immobilization base by not allowing cells to pass through it (Baba and Sankai, 2017).

The large-scale production of cultured meat requires bioreactors. The ultimate bioreactor will have properties like mass transfer, oxygen level, shear stress, and medium flow at optimum levels to produce high output and support the cells with the scaffold. Bioreactors with specific functions are available. Studies have been carried out on bioreactors, like rotating wall vessel bioreactors, direct perfusion bioreactors, and microcarrier-based bioreactors for producing cultured meat. Porous scaffolds that allow media flow through it are used in direct perfusion reactors, and gas exchange will happen in a fluid loop located externally. These reactors maintain the scaffolds-based culture with high mass transfer and great shear stress (Carrier et al., 2002). For the differentiation phase, animal cell immobilization on an edible scaffold or MCs with perfusion mode operation method is a promising approach for the production of CM (Allan et al., 2019; Bellani et al., 2020; Letti et al., 2021). The limitations of these bioreactors are membrane fouling which leads to heterogeneous cell growth, mass transport limitations, and non-uniform nutrient and inhibitor gradients (Yang et al., 2006; Detzel et al., 2010).

*In vivo*-like conditions are maintained in rotating wall vessel bioreactors by regulating the rotating speed in a row, balances the centrifugal force, drag force, and gravitational force, and finally allowing the 3D culture to be submerged in the medium (van der Weele and Tramper, 2014). This type of reactor bears high mass transfer with reduced shear stress. A well-mixed environment that controls bioprocess conditions precisely, such as pH value, dissolved oxygen concentration, and concentration of nutrients in the cell culture broth, will be provided in Stirred tank reactors (Badenes et al., 2016; Bellani et al., 2020; Hanga et al., 2021). Bovine adipose-derived stem cells are used as precursor cells for both adipose and muscle cells in a single-use, disposable STR vessel (Mobius CellReady 3 L, MilliporeSigma, Burlington, MA, United States) to expand the cells in 100 mL-spinner flask to 3 L-STR resulted in a notable fold increase of 114.19 ± 1.07 for bovine adipose-derived stem cell number (Hanga et al., 2021).

Microcarrier-based reactors support the cells in 3D environments. Microcarriers are used in two ways one is suspension, and the other is packed bed reactors. In the case of packed bed reactors, the medium flow should be continuous and oxygenated before entering the reactor; the limitation of this type of reactor is it is only useful for up to 30 L of volume. In the case of suspension-based microcarriers, the cells should grow on them, and the characteristics of the cell will vary according to the seeding density. The formation of aggregates and shear stress due to agitation are the major problems with these reactors (Moritz et al., 2015).

# 7 Conclusion

CBM is an emerging field of cellular agriculture. The biotechnological advancement utilized skeletal muscle tissue engineering technology to bypass livestock farming. CBM came into existence after growing concerns of animal welfare, ethical approval, and sustainable livestock management. Technological advancements like bioprocessing engineering and tissue engineering lead to the isolation and propagation of stem cells, identification and modification of suitable biomaterials, and designing culture systems with different cell types like muscle and fat cells. Specialized industrial bioreactors equipped with state-of-the-art technology utilizing serum-free media components are prerequisites for commercial CBM production. Shortly cultured meat or the food industry will be one of the essential parameters in food security in developing countries like India and Southeast Asia. The research and development in cellular agriculture are in the right direction. Still, we need robust scientific, industrial, and commercial connecting links to create a full spectrum of CBM industries to flourish.

# Author contributions

AA initiated the concept, wrote checked the draft and finalized the manuscript. SB helped in writing. SK prepared the tables and wrote few sub-section. SS edited the manuscript. GP edited, checked and finalized the draft version. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

Authors AA, SB and SS were employed by company Neat Meatt Biotech Pvt. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Repurposing agricultural waste as low-cost cultured meat scaffolds

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Growing meat *in vitro* using tissue engineering and bioproduction techniques (cellular agriculture) has become an increasingly promising solution to the global food security challenge. Our lab has established methods to cultivate bovine muscle tissue on decellularized plants, representing a viable low-cost, sustainable method to grow meat on edible scaffolds. Most work in this area has focused on the use of edible plant materials (i.e., spinach leaves, apple, broccoli) with inherent economic value. Harvest waste such as corn husk or jackfruit represent abundant sources of cellulose for scaffold production and may be a viable alternative. The present study aims to investigate production of cultured meat through tissue engineering and bioproduction on decellularized, edible samples of corn husk and jackfruit rind. Corn husks and jackfruit rinds were exposed to immersion decellularization. DNA quantification and histological analysis demonstrated sufficient decellularization (0.17 ± 0.06 and 0.07 ± 0.00 ug DNA/g tissue for corn husk and jackfruit rinds, respectively). Following decellularization, corn husk scaffold stiffnesses decreased from 56.67±16.71 MPa to 12.95±2.43 MPa in fiber-aligned direction, while jackfruit decreased from 7.54 ±2.42 MPa to 2.47±1.47 MPa. Seeded scaffolds with bovine satellite cells (BSCs) (11.45±2.24 ug/ul lysate/Gram) and avian (QM7s) (12.90±1.99 ug/ul lysate/Gram) demonstrated increased protein yields on jackfruit scaffolds. QM7 cultured on corn husk scaffolds yielded increased protein but PBSCs seeded on corn husks did not yield protein content higher than controls (QM7 on corn husk: 16.28±3.55, PBSCs on corn husks: 9.57±1.56 ug/ul lysate/Gram, control: 6.35±1.43 ug/ul lysate/Gram). Additionally, cell transfer from scaffold to scaffold (bead-to-bead transfer) was observed on corn husk scaffolds in a dynamic environment. These results suggest that decellularized harvest waste scaffolds may aid in realization of cultured meat products that will contribute to a more robust and environmentally sustainable food supply.

KEYWORDS

**cellular-agriculture, edible scaffolds, decellularization, agriculture waste, cultured meat, corn-husk, jackfruit, bead-to-bead-transfer**

## 1 Introduction

The rising world population, expected to exceed 9 billion people by 2050, is inducing unsustainable strain on current food production systems, which are already hampered by increasingly limited arable land, fresh water, and environmental concerns exacerbated by climate change (Goodland, 1997; Tuomisto, 2011). Further, animal-based protein consumption worldwide has surged in the last 50 years, rising from 61 g per person per

day in 1961 to 80 g as of 2011, placing additional pressure on our agricultural systems (Sans & Combris, 2015; Newman et al., 2023). A major paradigm shift in the production of food is critical for retaining food stability.

Cellular agriculture has been proposed as a more environmentally sustainable, less resource-intensive method for expanding food production, which could help reduce greenhouse gas emission, water use, and significant global deforestation and biodiversity loss caused by conventional animal agriculture (Holmes et al., 2022; Joo et al., 2022; Knežić et al., 2022). Through these processes, meat would be generated *in vitro* by expanding cells acquired from skeletal muscle biopsies or other methods and expanded in bioreactors to produce meat. Despite the high capital influx and lofty predictions of marketable cultured meat products becoming available in the near future, many technological and economic barriers continue to hinder mass production (Stephens et al., 2018; Holmes et al., 2022). Radically low-cost technologies must be developed and leveraged to reduce the production cost of cultured meat.

Cellular agriculture requires a cell source and, for anchorage dependent cells, scaffold materials to offer a stiff substrate upon which cell expansion and differentiation can occur (Bhat et al., 2014; Bar-Shai et al., 2021; Andreassen et al., 2022). Bioprocesses for preparation of industrial quantities of anchorage-dependent cells employ microcarriers as scaffolds to facilitate high-volume cultivation within suspension bioreactors. Cell expansion using microcarriers is well-established for pharmaceutical applications; however, traditional carriers were not designed in consideration of the unique demands of the cellular agriculture use-case (Wang & Ouyang, 1999; Verbruggen et al., 2018; Bodiou et al., 2020; Andreassen et al., 2022). Such considerations are well reviewed and highlight unique emphases: scaffolds must be palatable and safe for consumption, nutritious, low cost, and readily-available, while still facilitating cell adhesion and growth. Additionally, exhibition of cell migration from populated carriers to unpopulated carriers within bioreactors, a phenomenon referred to as bead-to-bead transfer, may further justify use of the carrier in scaling the bioprocess.

In previous studies, our laboratory has established that decellularized plants can be leveraged as biomaterials to support mammalian cell growth (Gershlak et al., 2017). We have established that bovine satellite cells can grow and differentiate readily on decellularized spinach-derived scaffolds, and further demonstrated the use of broccoli florets as a microcarrier for suspension culture within a bioreactor (Jones et al., 2021; Thyden et al., 2022). Such plant-derived scaffolds are primarily cellulose and therefore edible. Additionally, decellularization process time and cost is minimal, requiring only readily-available detergents that are already utilized in pharmaceuticals and food production (Harris et al., 2021; Thyden et al., 2022). However, these previous studies, in addition to similar efforts by other researchers, largely employ traditional foods as starting materials for scaffolds (Modulevsky et al., 2014; 2016; Hickey & Pelling, 2019; Jones et al., 2021; Thyden et al., 2022). These foodstuffs have an implicit market value on their own, and therefore may not be ideal for cellular agriculture application.

Agricultural harvest waste materials, (plant parts such as corn husks) and food waste like fruit rinds may be alternative sources for edible cellulose scaffolds. Globally, plant waste generated by the food industry represents a major underutilized source of lignocellulosic material, with an estimated 3.8 billion metric tons of crop residue generated per year (Galiwango et al., 2019; Xu et al., 2019; Santolini et al., 2021; Araya-Chavarría et al., 2022). Repurposing these wastes as a component of a cultured meat product represents a significant opportunity to both add value back to conventional agricultural crops within the framework of a cellular agriculture economy, while simultaneously reducing pollution created from either burning or depositing the agricultural waste into landfills (Santolini et al., 2021).

In this work, we evaluate two common harvest wastes - corn husks and jackfruit rind—as scaffolds for use in cellular agriculture applications. Corn husk is a monocot leaf with parallel striations that visually resemble the anisotropy of skeletal muscle, which may be advantageous for *in vitro* muscle tissue growth (Pengelly et al., 2011). Jackfruit is well-established as a fibrous, nutritious meat alternative on its own (Norris et al., 2021), and the inner rind (known as jackfruit rags) is similarly fibrous and may provide a scaffold with texture that more closely mimics structured meat. We investigated decellularization efficacy, scaffold stiffness, and cell seeding and with intermittent agitation to simulate a bioreactor environment. Generally, we concluded that decellularization successfully removes cellular material and DNA, and reduces scaffold stiffness.

Bovine satellite cells (BSCs) and a quail myoblast cell line (QM7) (Antin & Ordahl, 1991) are able to adhere to both scaffolds, display bead-to-bead transfer when seeded in an environment with intermittent agitation, and contribute additional protein content to the edible scaffold. While both scaffolds present viable materials for cultured meat production, we found jackfruit to be the more promising option with respect to mechanical similarity to native bovine muscle and post-culture protein content. These results suggest that the incorporation of decellularized agricultural waste products into novel bioprocesses may successfully yield large quantities of a cell-scaffold hybrid meat product. Thus, we propose a novel closed bioreactor system for cellular agriculture products.

# 2 Materials and methods

## 2.1 Cell culture

All primary bovine satellite cells used in each study were isolated from skeletal muscle of adult cows, sourced from a local slaughterhouse, as previously described (Jones et al., 2021). During expansion, BSCs were maintained in growth media (GM) consisting of DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA, United States) that has been supplemented with 10% FBS (Thermo Fisher Scientific),1% Penicillin/Streptomycin (Thermo Fisher Scientific), and the following growth factors (Thermo Fisher Scientific): 4 ng/mL recombinant human fibroblast growth factor—2 (FGF2), 2.5 ng/mL recombinant human hepatocyte growth factor (HGF), 10 ng/mL recombinant human epidermal growth factor (EGF), and 5 ng/mL recombinant human insulin-like growth factor—1 (IGF)). QM7 satellite cells, an immortalized cell line derived from quail muscle, were purchased (ATCC, Manassas, VA, United States) and maintained under the same

conditions as the bovine satellite cells. Differentiation media consisted of DMEM/F12, 2% heat-inactivated fetal bovine serum, and 1% P/S and the growth factors consistent with GM.

## 2.2 Decellularization

Fresh whole jackfruit was acquired from a local supermarket and, using a pair of iris scissors, rind fibers, exclusive of the edible fruit surrounding the large seeds, were manually separated from the fruit's pith and rinsed with $DIH_20$. Rind fibers were submerged in 10% SDS (Sigma-Aldrich, 124 St. Louis, MO, United States), 3% Polysorbate-20 (Sigma-Aldrich), and 10% bleach (The Clorox Co., 125 Oakland, CA, United States) in a 50 mL conical tube and placed on a laboratory roller for 48 h. Samples were then rinsed in DIH20 for 24 h and stored at 4C in DI H20. Similarly, decellularized corn husk samples were segmented using iris scissors and submerged and continuously agitated in 1% SDS in DI water for 5 days, followed by 2 days in 1% Polysorbate-20, 3% bleach, and 1 day in DI H2O. Samples were stored at 4°C in DI water until used. Solutions were exchanged daily.

## 2.3 DNA content

The total DNA content of the fresh and decellularized samples of jackfruit and corn husk was analyzed using a CYQUANT® DNA assay kit (Thermo Fisher Scientific, Waltham, MA, United States), per the manufacturer's instructions.

Prior to the test, fresh and decellularized samples were sequentially flash-frozen using liquid nitrogen, minced using iris scissors to ensure homogenization of the samples, and stored in 1x PBS at −80°C for up to 1 month, until analysis was performed. DNA analysis was performed using a BioTek Cytation 1 Imaging Plate Reader (Winooski, VT, United States) at 480 nm excitation and 520 nm absorption.

## 2.4 Histology

Fresh and decellularized samples of jackfruit and corn husk were submerged in 4% paraformaldehyde for approximately 20 min and rinsed with phosphate buffer saline. Samples were processed, embedded in paraffin, sectioned via microtome, stained using Safranin-O and fast green, and imaged via inverted microscope as previously described (Thyden et al., 2022). Additionally, paraffin sections were deparaffinized using xylene, rehydrated, and stained in 0.02% toluidine blue for 5 min and imaged brightfield.

## 2.5 Mechanical testing

To evaluate both the impact of decellularization on scaffold stiffness, and to compare scaffold elasticity to published values of native bovine muscle tissue, tensile stiffness of scaffolds was evaluated. To do so, fresh and decellularized jackfruit and corn husk samples were pulled to failure using an Instron 68TM-30 universal testing system (Instron, Norwood, MA) equipped with a 100 N load cell. Jackfruit samples were axially affixed into the machine and pulled at a constant 10 mm/min until failure. Young's modulus was calculated via slope of the linear region of the resultant stress/strain graphs for both fresh and decellularized samples ($n \geq 4$).

Corn husk, which is classified as a monocot, possesses longitudinally striated leaf venation (Pengelly et al., 2011), causing the material to be structurally anisotropic. To best characterize its mechanics, samples were pulled to failure both parallel to leaf venation and orthogonal to it. To do so, both fresh and decellularized corn husk samples were cut into dog-bone shapes with ~1.5 cm width in the center, and tested under the same conditions as jackfruit ($n \geq 4$).

## 2.6 Cell seeding and visualization of seeded scaffolds

Decellularized cornhusk and jackfruit scaffolds were sterilized via submersion in 70% ethanol for a minimum of 10 min. Scaffolds were then submerged in sterile 1X PBS for 5 min three times to eliminate trace quantities of ethanol. The scaffolds were then conditioned in growth media and equilibrated for a minimum of 30 min at 37C and 5% $CO_2$. In sterile, vented, 15 mL conical tubes BSCs or QM7s, jackfruit fibers or corn husk, and GM was inoculated at a ratio of $10^6$ cells/100 mg scaffolds/1 mL GM, based on the weight of the scaffold. Each reactor was manually agitated every 15 min for at least 1 h. After 24 h, 5 additional mL of growth media was added to each reactor and each reactor was manually agitated every subsequent 24 h until the completion of each study. Scaffolds were then visualized using Phalloidin and Hoechst 33342 and fluorescent microscopy as previously described (Thyden et al., 2022).

## 2.7 Protein analysis of recellularized scaffolds

For cell and scaffold visualization and protein analysis, samples were maintained in GM for 72 h, and were then maintained for 1 week in differentiation media. Media was changed every 2–3 days. Upon completion of the culture, samples were either frozen at −80C for protein analysis, or immediately fixed in 4% paraformaldehyde for staining. Additional samples were fixed for imaging after only 3 days in GM.

A BCA analysis was performed on fresh, decellularized, and recellularized samples of jackfruit and corn husk. Additional samples of decellularized jackfruit and cornhusk that had been conditioned in growth media for 1 hour and once frozen were assessed as well. Jackfruit fiber masses were recorded, and then placed into 100uL of NP-40 cell lysis buffer (Thermo Fisher Scientific) with 1ul of protease inhibitor cocktail (Thermo Fisher Scientific), for 30 min. Samples were vortexed and lysates were analyzed for total protein via Pierce BCA Assay (Thermo Fisher Scientific) against a bovine serum albumin standard curve. Absorbance at 562 nm was quantified using a BioTek Cytation 1 Imaging Plate Reader 1 plate reader. All samples were normalized to their total (final) mass in mg (3 samples were tested from 3 bioreactors for each condition).

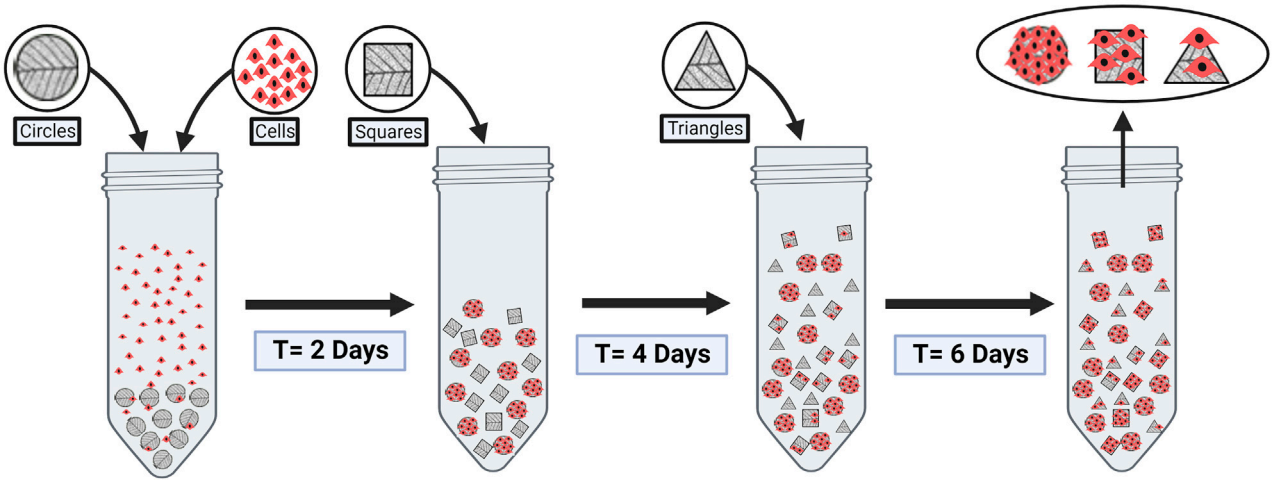


FIGURE 1
Bead-to-bead transfer schematic showing introduction of new scaffolds at different time points, differentiated by shape. Figure made in BioRender.

## 2.8 Bead-to-bead transfer

To study the ability for cells to migrate from one scaffold to another, decellularized corn husk was shaped into circles, squares, and triangles. 100 mg of circle-shaped decellularized corn husk was sterilized and conditioned as described above, and simultaneously inoculated into 15 mL conical tubes with growth media and QM7 cells at a ratio of 2 × $10^6$ cells/mL in 1 mL of growth media. The suspension was incubated for 24 h, and the initial cell media was replaced with fresh growth media. 100 mg of square and triangle shaped decellularized corn husk scaffolds were inoculated into the culture after 48, and 96 h, respectively (Figure 1). 48 h following the inoculation of triangle shaped scaffolds, all scaffolds were fixed with 4% paraformaldehyde for 15 min. Samples were stained for using Phalloidin and Hoechst 33342 as described above, and imaged using a BioTek Cytation 1 Imaging Plate Reader.

An automated tiling and stitching strategy was used to image the entirety of each scaffold at 10x to image the entirety of the scaffolds. Additionally, for each tile, z-stacks of ten images spanning the thickness of the sample were captured. The maximum intensity of each pixel from the z-stack was projected into a new image for nuclear counting. To distinguish nuclei from background autofluorescence, the Trainable Weka Segmentation plugin of FIJI was used to generate binary masks of cell nuclei, which were then counted. Nine shapes from a total of 3 bioreactors were quantified.

## 2.9 Statistics

Statistical analysis was performed using GraphPad Prism 9. Analysis of variance (ANOVA) with multiple comparisons was used in all tests, using two-sided t-tests for *post hoc* analyses. Significance was considered for *p*-values under 0.05.

# 3 Results

## 3.1 Sample decellularization

Jackfruit fibers and cornhusk segments were prepared, and decellularized as described in the protocol above. Each scaffold progressively lost its color and attained a translucent white color as the protocol was completed (Figure 2A).

### 3.1.1 DNA quantification of decellularized plant scaffolds

A nearly complete loss of DNA was observed when Jackfruit fibers and cornhusk segments were submitted to the decellularization process when compared to fresh samples (Figure 2B). Fresh corn husk segments and jackfruit fibers contained an average of 39.41 ± 4.46 and 4.00+/1.64 ug DNA/g tissue respectively, and decellularized corn husk segments and jackfruit fibers contained an average of 0.17 ± 0.06 and 0.07 ± 0.00 ug DNA/g tissue respectively.

### 3.1.2 Histological analysis

Safranin-O and Fast green staining was performed on fresh and decellularized jackfruit fibers and cornhusk segments (Figure 2C). The absence of intracellular green color and dark purple nuclei in decellularized samples complement the quantitative results, thus validating the decellularization protocols for each scaffold.

### 3.1.3 Scaffold mechanics

Fresh and decellularized corn husk and jackfruit rind (Figure 3A) exhibited stiffnesses above native bovine muscle (~8 kPa), in the MPa range. Corn husk stiffness was reduced post-decellularization when pulled parallel to the aligned venation (56.67±16.71 MPa vs. 12.95±2.43 MPa before/after decellularization) but not in the perpendicular direction (10.78±6.42 MPa vs. 6.18±2.42 MPa before/after

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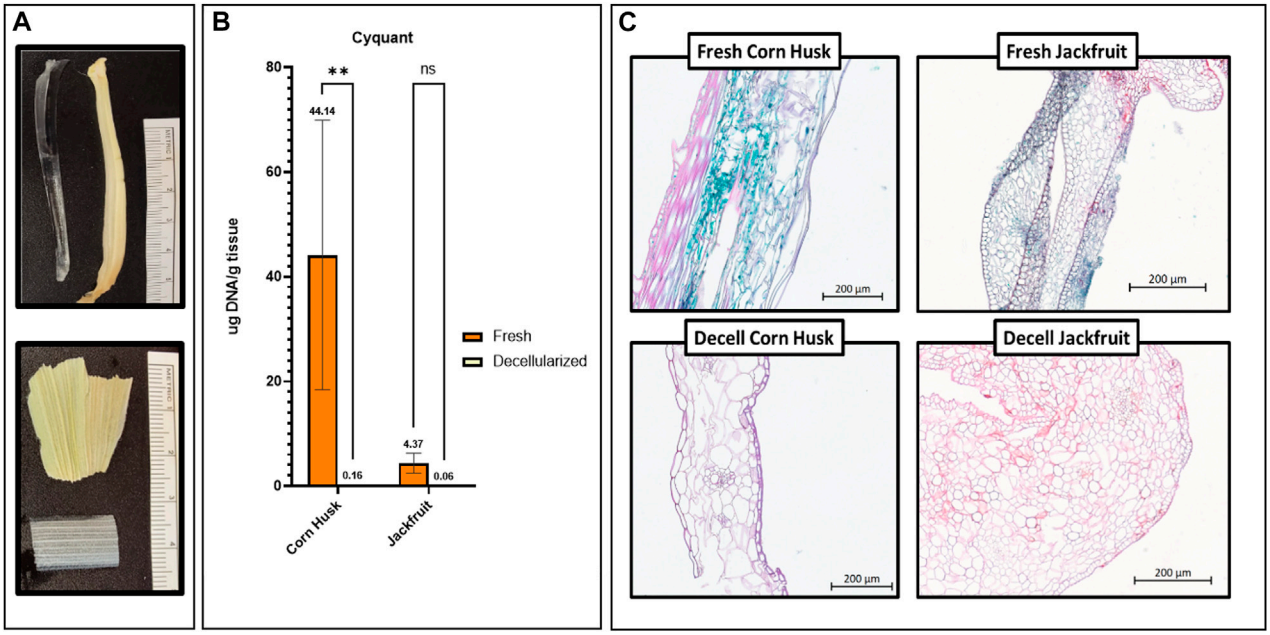


FIGURE 2
**(A)** Images of Decellularized and Fresh jackfruit (top) and corn husk (bottom). **(B)** Cyquant DNA assay results Fresh and Decellularized corn husk and jackfruit. **** = $p < 0.0001$, NS = no significance. $n = 3$, all groups. **(C)** Safranin-O and Fast Green stained corn husk and jackfruit, both Fresh and Decellularized. Absence of cellular material (green/blue) but retention of matrix (pink) is evident in decellularized sample images. Scale bars indicated on images.

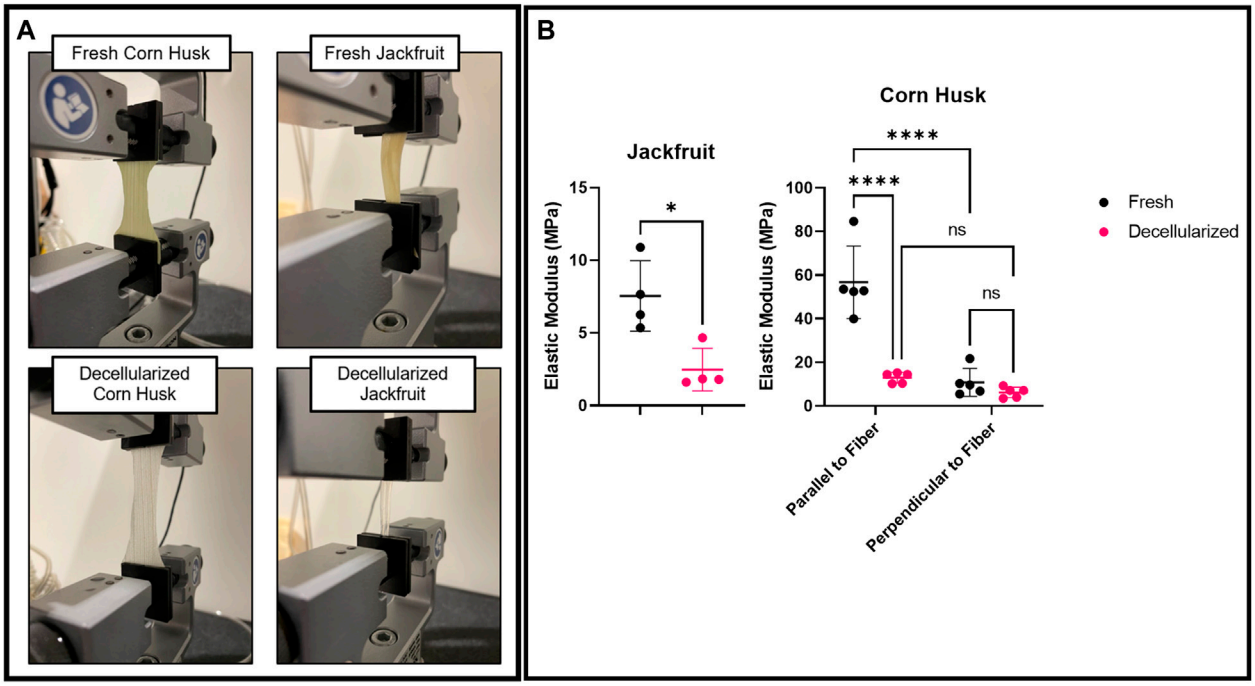


FIGURE 3
Uniaxial tissue mechanics on **(A)** Fresh corn husk and jackfruit (top) and Decellularized corn husk and jackfruit (bottom). **(B)** Young's modulus calculations for corn husk in fiber parallel and fiber perpendicular directions, and for jackfruit. Fresh and Decellularized samples are compared in each group. $n = 4$ for all samples, * indicates $p < 0.05$, and **** indicates $p < 0.0001$, both in a two-sided Tukey *post hoc* *t*-test, NS indicates no significance.

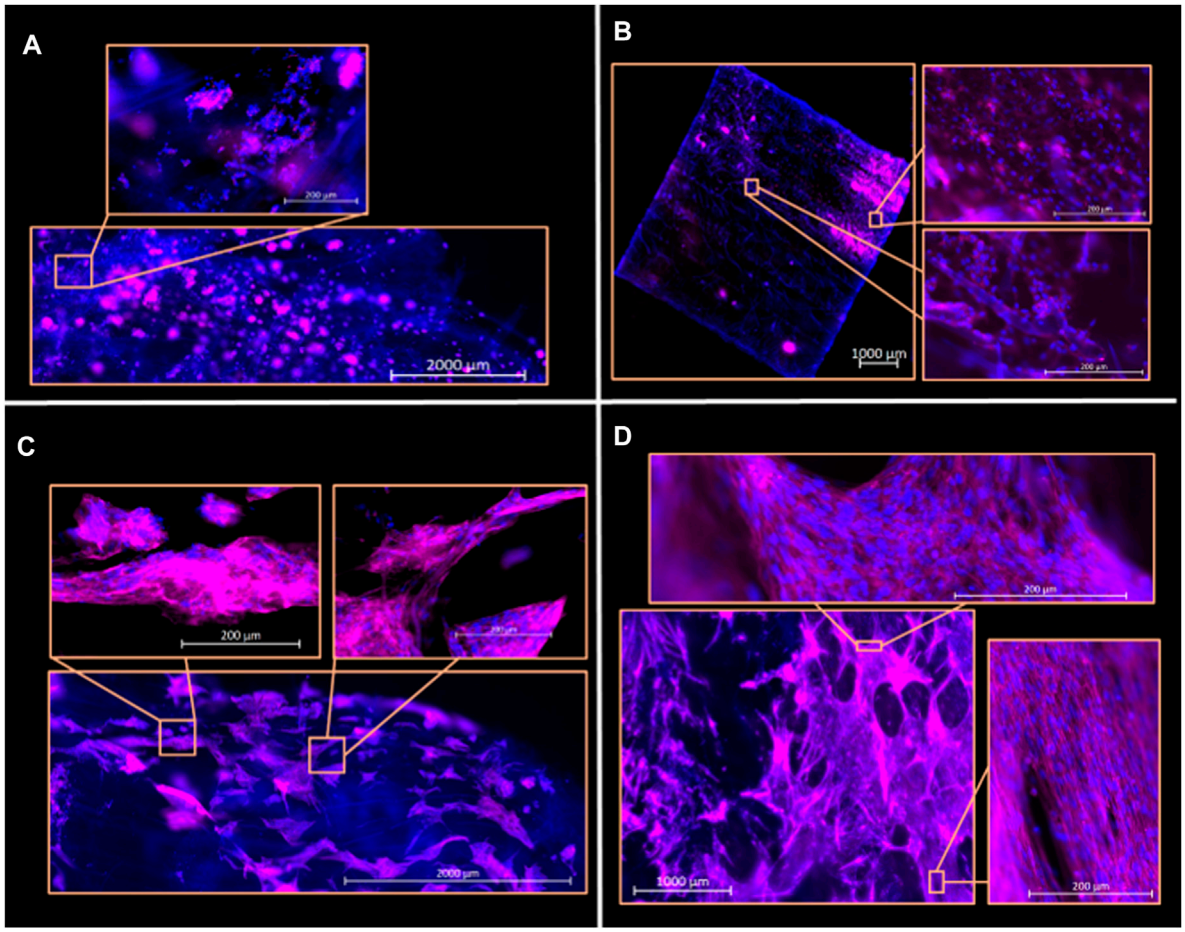


FIGURE 4
Hoechst 33342 (blue) and phalloidin (pink) fluorescent imaging of PBSCs and QM7s on decellularized corn husk and jackfruit scaffolds at Day 3 of culture. **(A)** QM7s on jackfruit scaffold, presenting as aggregated clumps across the length of the sample. **(B)** Seeded deposits of QM7s on corn husk scaffold. **(C)** PBSCs on jackfruit scaffolds, with inserts showing cells with elongated F-actin striations. **(D)** PBSCs on corn husk scaffold qualitatively showing extensive scaffold colonization and cell alignment. Scale bars indicated on images.

decellularization). Jackfruit stiffness was also significantly reduced post-decellularization (7.54±2.42 MPa vs. 2.47±1.47 MPa, fresh vs. decellularized) (Figure 3B).

### 3.1.4 Cell seeding

BSCs and QM7 cells were seeded onto decellularized jackfruit fibers and decellularized corn husk segments and observed for cell nuclei and cytoskeletal filaments after having been cultured for 3 days in proliferation media. Both QM7s and BSCs were observed to have adhered over extensive scaffold regions (up to 2.5 cm$^2$) of the decellularized jackfruit and decellularized corn husk fibers after 3 days in proliferation media (Figures 4A–D).

Subsequent culture was conducted for 7 days in low-serum differentiation media and cells remain adhered after the additional 7 days (Figures 5A–D). Qualitatively, it was observed that BSCs demonstrate localized regions of cytoskeletal alignment on both scaffolds, a common metric for characterizing skeletal muscle organization, whereas it was observed that QM7 cells adhered with less organization. Additionally, when seeded onto jackfruit fiber samples, QM7 cells appeared as multicellular agglomerations.

## 3.2 Protein content analysis

Protein content of cell seeded scaffolds was compared against untreated decellularized scaffolds and media-conditioned scaffolds primed for 1 h in media with 10% FBS. Untreated jackfruit scaffold protein content was negligible. Culture media-conditioned jackfruit samples had an average of 0.61 ± 0.35 ug/ul lysate protein per Gram of tissue mass while PBSC-seeded and QM7-seeded samples had 11.45±2.24 and 12.90±1.99 ug/ul lysate/Gram respectively (Figures 6A,B).

There was no significant difference between the protein detected in jackfruit samples seeded with QM7 cells and PBSCs. PBSC-seeded corn husk protein content was not significantly different to corn husk conditioned in culture media. However, corn husk seeded with QM7s had a measurable increase in protein content (16.28±3.55 ug/

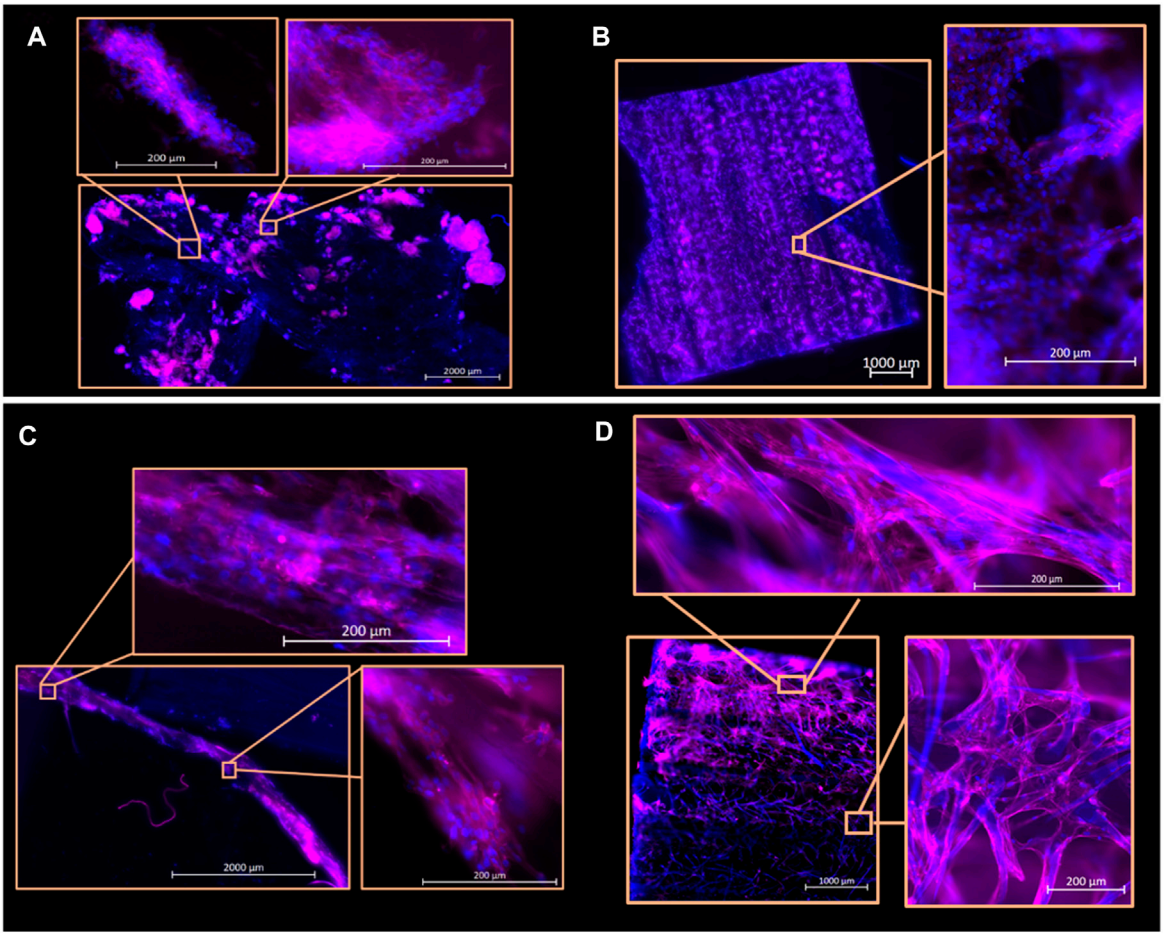


FIGURE 5
Hoechst 33342 (blue) and phalloidin (pink) fluorescent imaging of PBSCs and QM7s on decellularized corn husk and jackfruit scaffolds at Day 7 of culture. **(A, B)** indicating QM7 scaffold colonization on jackfruit and corn husk scaffolds, respectively. **(C)** PBSCs on jackfruit scaffolds, with inserts presenting cell deposition across the fiber length of the scaffold. **(D)** PBSCs on corn husk scaffold indicating localized cell alignment. Scale bars indicated on images.

ul lysate/Gram) to both samples seeded with PBSCs, and media-conditioned samples (9.57±1.56 and 6.35±1.43 ug/ul lysate/Gram, respectively) (Figures 6A,B). There was no significant difference between the protein detected in jackfruit samples seeded with QM7 cells and PBSCs; however, there was a significant difference between mammalian cell seeded jackfruit and jackfruit that was conditioned in growth media.

## 3.3 Bead-to-bead transfer on corn husk scaffolds

Upon completion of the study described in Figure 1, circular corn husk scaffolds maintained in media for the full culture time contained 811±618.1 nuclei/mm$^2$, square scaffolds seeded at Day 2 acquired 117±108 nuclei/mm$^2$, and triangle scaffolds introduced at Day 4 acquired 36±35 nuclei/mm$^2$. There was a significant difference in the nuclei density between circle and both square and triangular shapes and there was no significant difference between the nuclei density on square and triangle shapes (Figures 7A,B).

# 4 Discussion

The realization of cellular agriculture may aid in satisfying protein demand while sustainably reducing dependence on unsustainable meat production strategies. Consumer transition from traditional meat products to products of cellular agriculture will depend on a number of factors, notably, the nutritional characteristics and cost parity. Several techno-economic analyses suggest that radical reductions in the bioproduction costs of mammalian cells, at scale, are necessary for cultured meat products to reach an economically competitive price for consumers (Bhat et al., 2014; Raman, 2021; Garrison et al., 2022). For reference, the USDA reports that the average cost of lean beef in 2021 was approximately $6.17/kg, *versus* approximately $63/kg for cultured meat (*Daily Beef Reports | Agricultural Marketing Service*,

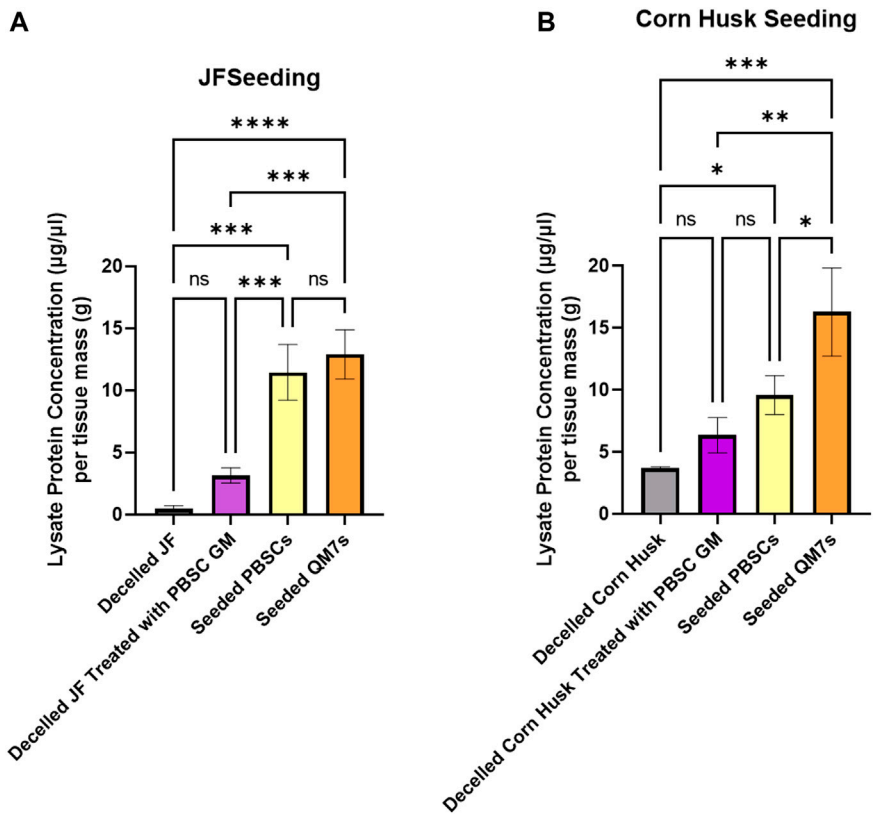


FIGURE 6
BCA protein analysis measured as protein lysate concentration in µg/µL, normalized by total tissue (cells and scaffold) mass in g, on jackfruit rind fibers **(A)** and corn husk **(B)**. Groups for both scaffold types include untreated decellularized scaffolds, decellularized samples treated for 1 h in growth media (GM), scaffolds with seeded PBSCs cultured for 10 days, and scaffolds with QM7s cultured for 10 days * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, *** = $p < 0.0001$, NS = no significance between groups. $n = 3$ for all groups, statistics are derived from ANOVA with *post hoc* Tukey *t*-test.

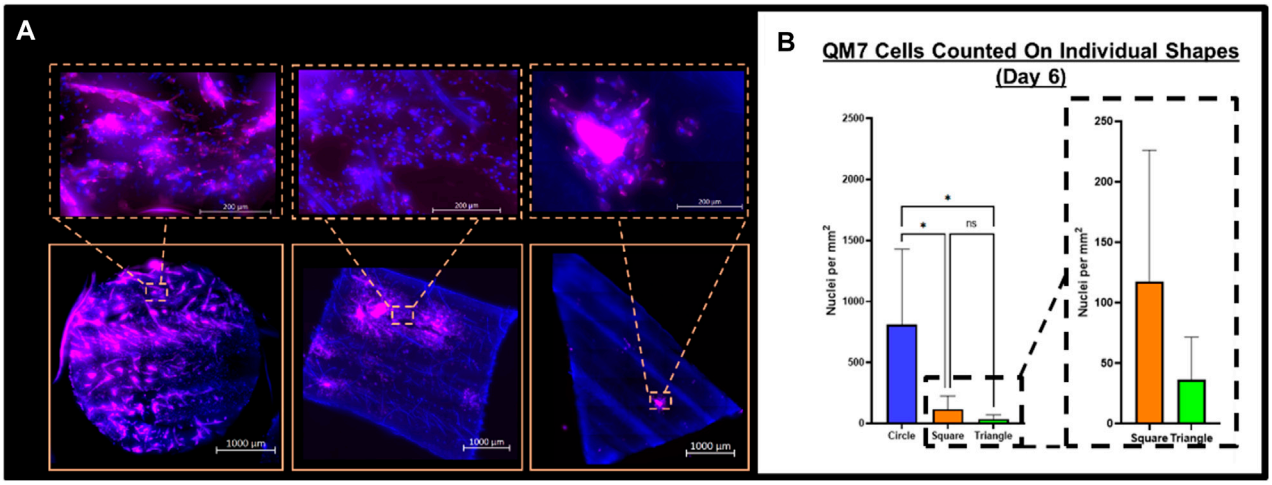


FIGURE 7
**(A)** Bead-to-bead transfer images showing transfer of QM7 cells at each time point. **(B)** QM7 cell count on scaffolds at each time point. $n = 9$, * indicates $p < 0.05$ with Tukey two-sided *t*-test, NS indicates no significance.

n.d., 2022). For this reason, scaffolds, among other reagents, and production methods for cultivation of cell-based meat must reach an exceptionally low cost. In this study, we demonstrated that plant-based scaffolds derived from agricultural waste can be used as affordable, edible scaffolds for cultured meat, and are usable as edible cell carriers for scale-up. Researchers' efforts to yield cell masses for the many tissue constituents of meat—lean skeletal muscle (Ben-Arye et al., 2020; Stout et al., 2023), adipose tissue (Huang et al., 2023; Yuen Jr et al., 2023), or connective tissue (Pasitka et al., 2023; P; Wood et al., 2023)—consistently depend on these types of carrier scaffolds to satisfy the anchorage dependence of such cell types. While some efforts to acclimate traditionally anchorage dependent cells to suspension culture have been proposed for myoblast proliferation, adherence to stiff binding surfaces are still employed during differentiation phases. Given this current need for cell-binding scaffolds, an opportunity for the development of "hybrid-meat products", which retain the scaffold upon which cells are expanded and differentiated is valuable and may offer an environmentally sustainable protein production method that limits the economic constraints of more complex bioproduction systems.

It is important to note that cellular agriculture presents a widely different use-case for traditional bioproduction strategies than those typically used for manufacturing pharmaceutical products. For example, single use bioreactors, which are widely employed for monoclonal antibody production, are advantageous for their low start-up investment, flexibility of use, and prevalidation. Such bioreactors are disadvantageous for cellular agriculture due to their inability to sustainably and repeatedly support frequent high-throughput cell expansion (Stephenson & Grayson, 2018; Allan et al., 2019). Similarly, a cell carrier that is to be used for supporting anchorage dependent cells for cellular agriculture applications may be justified by its advantageous qualities unique for cultured meat production. The characteristics of traditional cell carriers, such as supporting cell adhesion and cell viability over time are necessary for yielding the cells and cell products desired (Nienow et al., 2014); however, for cultured meat applications, economic qualities such as the affordability and availability of the cell carrier must be considered. Pharmaceutical bioprocesses seek to yield cell products, whereas a cultured meat bioprocess seeds to yield the cells themselves. Additionally, for cultured meat products that incorporate its scaffold as hybrid-meat products, the edibility and nutritional characteristics of the scaffold are important.

We have identified corn husk and jackfruit rind as two potential sources for affordable and readily available cell carriers for hybrid-meat products. The process outlined in this study yielded decellularized samples that support satellite cells from both bovine and avian sources (Figure 2). This study exemplifies the option for farmers to further commoditize corn husks or jackfruit rinds from what may have been treated as waste, to a vital input for cellular agriculture. Decellularization is a simple process that can be completed with common affordable reagents in many regions and improves the accessibility of lab grown meat production. Thus, future studies on decellularized plant scaffolds derived from additional, site-specific, agricultural waste products are justified.

Lab grown meat must offer substantial quantities of protein to meet expectations of an equitable and environmentally sustainable approach to food production. Thus, continuous evaluation of each lab grown meat product's total protein content is vital for judging the adequacy of the product and the efficiency of the process by which it was prepared. We demonstrated via BCA assay that cellular adhesion and culture on decellularized plant tissue increases the total protein content of the product (Figure 6). However, continuous improvements designed to optimize the amino acid profile and maximize the total protein content per unit of product is justified. There are many additional factors that may contribute to the total protein content including seeding efficiency, growth density, cell-to-scaffold mass ratio, myocyte fusion index as a measurement for differentiation efficiency, media evaluation, and electrostimulation and cell hypertrophy.

To date, many bioengineering publications describing plant decellularization have focused on fruits and vegetables like apple, asparagus, spinach, parsley, or broccoli. These are selected based upon their morphological and compositional characteristics, to meet the requirements for the biomaterial scaffold for which they are used. For example, spinach leaves, possessing an extensive vasculature with a central vessel that is easily cannulated, can be employed for studies that require scaffold perfusion (Gershlak et al., 2017). Conversely, broccoli florets are lightweight and roughly spherical, and can be used as effective microcarriers in bioreactor culture (Thyden et al., 2022). Corn husk and jackfruit similarly present unique structural characteristics we believed would be beneficial for cultured meat production. Corn husk presents uniaxial striations following its vasculature that were observed, albeit not homogenously, to encourage myocyte alignment, following during culture (Figures 4C,D, 5C,D). Microscopically, corn husk trichomes can be observed, small fibrous hairs that extend from the plant surface. We observed cell binding to trichomes, which may increase available surface area for cell proliferation, rendering the material a strong candidate for cell expansion. Jackfruit is composed of multiple loose fibers. This texture makes it a staple in vegan alternatives to meat dishes like pulled chicken or pork. The loose, fibrous structure of jackfruit may have promoted increased cell integration with the scaffold, and contributed to the higher protein content we observed compared to cell-seeded corn husk (Figure 6A). In additional support of jackfruit, the plant has previously been identified, by AI, as the plant most likely to resemble the marbling characteristics of many cuts of meat (Ong et al., 2021).

In this study, we particularly highlight the stiffness of corn husk and jackfruit scaffolds, a critical factor for both cell growth and behavior on the scaffold, and for consumer considerations like texture and mouthfeel of the final meat product. We found that decellularized jackfruit had a considerably lower modulus of elasticity *versus* corn husk (Figure 3), supporting its candidacy as a viable scaffold for development of structured muscle tissue. Both scaffolds were an order of magnitude higher modulus of elasticity than native bovine muscle tissue (<20 kPa) (Lapin et al., 2013; L. K; Wood et al., 2014), which may be due to properties of cellulose in these tissues, as a dense fibrous polysaccharide (Galiwango et al., 2019; Hickey & Pelling, 2019). While this study did not investigate scaffold mechanics post-cell seeding, increasing cell content on these scaffolds, in addition to inclusion of fat tissue and connective tissue into engineered tissues will likely impact scaffold mechanics (Boots et al., 2021, p. 23; Fraeye et al., 2020; K; Handral et al., 2022; Schätzlein & Blaeser, 2022). It is also likely that long-term culture of

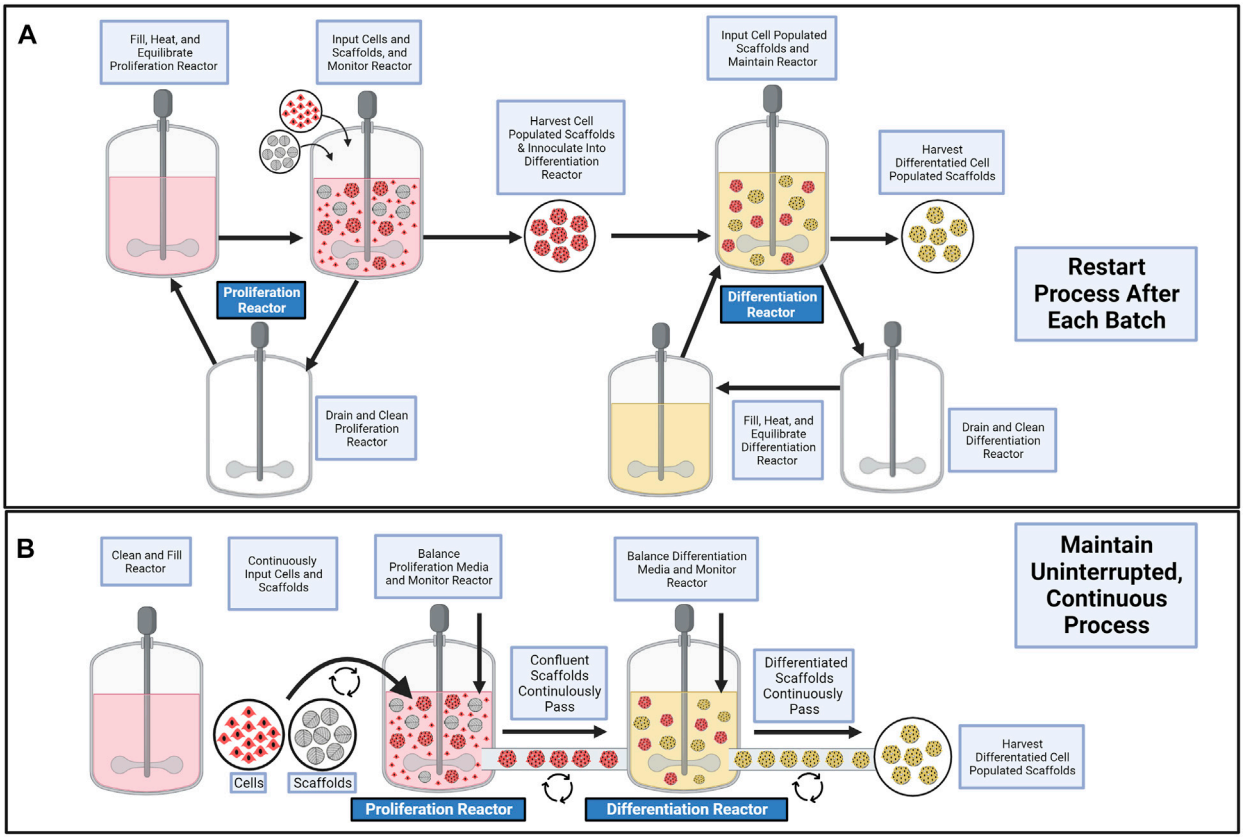


FIGURE 8
Schematics of batch processes for cultured meat scale up. **(A)** Conventional process requiring multiple systems for proliferation and differentiation steps. **(B)** Proposed continuous process using bead-to-bead transfer to reduce labor steps in cultured meat cultivation. Figure made in BioRender.

cellulose scaffolds may induce some mechanical degradation (Märtson et al., 1999; Helenius et al., 2006; Modulevsky et al., 2014). Future work should target methods to reduce scaffold stiffness, and encourage cell incorporation of matrix proteins like collagen and elastin to promote improved meat texture and increase scaffold elasticity. Similarly, it is imperative to evaluate the texture and mechanics of engineered tissues as cell seeding is optimized and tissue density increases, to determine how closely these tissues recapitulate the feel of conventional meat.

Having demonstrated that agricultural waste can serve as an edible cell carrier for satellite cells, that cells exhibit bead-to-bead transfer upon them (Figure 7), and understanding that for theoretical hybrid products, the scaffolds are included in the final product, the context for unique bioprocess design can be established. For example, a traditional, non-continuous bioprocess (Figure 8A) could be improved upon by developing a closed, two bioreactor, continuous bioprocess which consists of a proliferation bioreactor for cell expansion on decellularized plant scaffolds, a sensor for detecting confluent scaffolds, a differentiation reactor to which confluent scaffolds are continuously passed, and a final sensor for detecting fully differentiated units of the final hybrid product (Figure 8B).

## 5 Conclusion

Bioprocesses for manufacturing cultured meat products must be designed for affordability. Decellularized plant scaffolds maintain many unique characteristics that are advantageous to cellular agriculture applications, such as their availability, simple preparation, and edibility. We have demonstrated the preparation of decellularized plant scaffolds derived from waste products, thus exemplifying affordable acquisition of a critical bioprocess component. We have demonstrated the variability in decellularized plants in regard to their stiffness, a key characteristic of traditional tissue engineering scaffolds. Additionally, we have noted the ability of cells to both contribute protein to decellularized plant scaffolds, as well as migrate between them. These results justify further investigation into methods for improving the affordability of the decellularization process, preparation of scaffolds from other agricultural waste products, establishment of methods for improving the cellular contribution of total protein, and designing novel bioreactors in consideration of access to decellularized plant-based scaffolds derived from agricultural waste. Ultimately, such efforts may improve the affordability of cultured meat bioprocesses and enable the realization of cellular agriculture.

## Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## Author contributions

Conceptualization GG, TD, LP, RT, and JJ; planning GG, TD, LP, RT, and JJ; data curation LP and RT; formal analysis LP and RT; funding acquisition GG, TD, and RT; project supervision GG, TD, LP, and RT; imaging LP and RT; histology RT; DNA quantification RT, LP, AP, and DR, decellularization and scaffold preparation LP, RT, AP, DR, JN, and JK; protein quantification LP and RT; writing LP and RT; editing LP, RT, AP, DR, JK, JJ, GG, and TD; Review LP, RT, AP, DR, JN, JK, JJ, TD, and GG. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Virtualization of foods: applications and perspectives toward optimizing food systems

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Food production cannot be decoupled from human and planetary wellbeing. Meeting safety, nutritional, sensorial, and even price requirements entails applying an integral view of food products and their manufacturing and distribution processes. Virtualization of food commodities and products, i.e., their digital representation, offers opportunities to study, simulate, and predict the contributions of internal (e.g., composition and structure) and external factors (e.g., processing conditions) to food quality, safety, stability, and sustainability. Building virtual versions of foods requires a holistic supporting framework composed of instrumental and computational techniques. The development of virtual foods has been bolstered by advanced tools for collecting data, informing and validating modelling, e.g., micro-computed tomography, to accurately assess native food structures, multi-omics approaches, to acquire vast information on composition and biochemical processes, and nondestructive and real-time sensing, to facilitate mapping and tracking changes in food quality and safety in real-world situations. Comprehensive modeling techniques (including heat and mass transfer, thermodynamics, kinetics) built upon physic laws provide the base for realistic simulations and predictions of food processes that a virtual food might undergo. Despite the potential gaps in knowledge, increasing the adoption of food virtualization (data-based, physics-based or hybrid) in manufacturing and food systems evaluation can facilitate the optimal use of resources, the rational design of functional characteristics, and even inform the customization of composition and structural components for better product development. This mini-review focuses on critical steps for developing and applying virtual foods, their future trends, and needs.

KEYWORDS

**virtualization, multiscale modeling, optimization, food quality, food processing, shelf-life, food supply chain**

## Introduction

Efficient use and transformation of resources along the food supply chain are critical to achieving sustainability goals such as food security and waste reduction. To this end, food systems must be integrally conceptualized to acknowledge their dynamic nature, complex interactions, and transformations, and the effects of external factors on their safety, quality, stability, and even consumer acceptance (Erdogdu et al., 2017; Vitrac et al., 2021; Marra, 2022). Adopting advanced modeling, high-resolution, and real-time data acquisition techniques plays an important role in virtualizing, simulating, and predicting phenomena in foods so that such milestones can be achieved (Saguy, 2016; Datta et al., 2022).

Although the role of virtualization of the food supply chain as an improvement tool has been introduced for over 10 years, its applications are still limited (Marra, 2016). 'Virtual foods' or '*in silico* foods' can be defined as digital representations with the same characteristics (e.g., physical properties, chemical composition) as their real-life counterparts (Verdouw et al., 2016). They are digital twins constrained to the food product itself (Verboven et al., 2020). To be effective and useful, these digital twins of specific commodities or products should be built so that all physical principles, transport phenomena, and food reactions are applicable to them. As such, virtual foods should respond to simulated stimuli in 'virtual environments' in ways that accurately mimic the behavior of their real-world versions. The 'virtual environments' can be tuned to reflect relevant scenarios throughout the food supply chain to i) evaluate external effects on food structure, quality, and safety, ii) develop and optimize processes to attain high efficiency and sustainability (Marra, 2023), iii) predict the occurrence and impact of failure events during distribution and storage (Peleg et al., 2011); and iv) reduce the time-to-market of new products (Saguy, 2016; Mengucci et al., 2022). Building virtual foods is intricate since it relies on the availability of extensive databases of physical, structural, chemical, and biological attributes and a holistic supporting framework of advanced characterization, modeling, data processing, and management approaches (Datta et al., 2022). This mini-review aims to identify the critical components, summarize the available approaches, and discuss the challenges, needs, and future trends in developing and applying food product virtualization.

## Building a virtual food

Food undergoes transformations from harvest to consumption (Singh and Corradini, 2023); some are intentional and rapid such as those imposed by processing, and others progressive and cumulative, such as the ones that ensue from a food's inherent deterioration processes. Regardless of their triggers, these transformations highly depend on a food's intrinsic properties, such as physical attributes, chemical composition, hierarchical organization of its structural components, and natural microbiota. External factors, such as temperature, humidity, atmosphere composition, and airflow (Duan et al., 2020), also influence the rate and extent of the transformations a food undergoes along the supply chain. Linking these factors coherently provides the foundation to build a virtual version of an actual product. Even if a physics-based digital twin is being built, it requires knowledge of actual food properties to assist in its development and validation (Datta, 2016; Erdogdu et al., 2017; Erdogdu, 2023). Thus, accurate and careful spatial and temporal mapping of food properties, components, structures, and surrounding factors is required to create a reliable avatar of a product. This thorough assessment will allow accounting for the effects of intrinsic and extrinsic factors and their dynamic nature on transport phenomena and reactions kinetics modeling. The following sections will briefly present techniques valuable to build upon information on intrinsic factors and modeling approaches, as seen in Figure 1, and to incorporate this information into data-based, physics-based, or hybrid food models.

## Physical properties and structural features

Knowing the physical properties of food materials (e.g., density, size, shape, thermal properties) is one of the cornerstones of effectively simulating natural and manufacturing processes. Measuring techniques for these properties have been extensively documented (e.g., Figura and Teixeira, 2007). Although important progress has been achieved on every front, advances in size, shape, and structural determinations have probably benefited the most due to the advent of high-resolution image acquisition, analysis, and reconstruction. Hence, they will be primarily discussed in the following section.

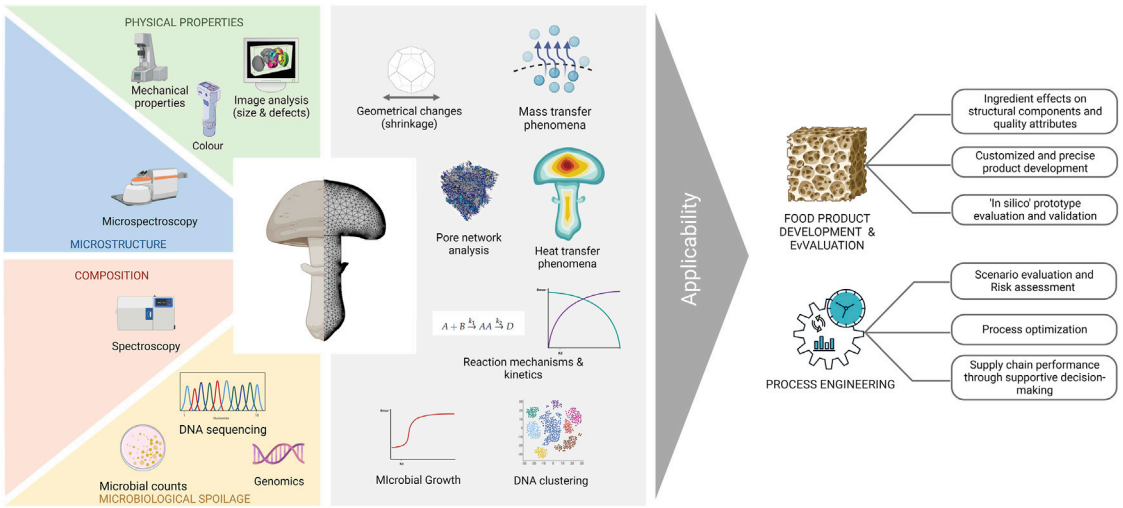


FIGURE 1
Contributions to the development of a virtual food and potential applications.

## Data acquisition

Food (micro)structure is complex and heterogeneous. The irregular arrangement of different structural units, e.g., lignocellulosic backbones and protein networks, and component compartmentalization result in materials with irregular shapes and composition (Piovesan et al., 2021). Regarding size and shape, 3D laser scanning has been identified as an improved tool for collecting information to develop geometric models of foods with irregular shapes at high morphological resolution (Zhang et al., 2020). Similarly, over the past 20 years, high-resolution techniques have improved the scouting of food microstructure (Gruyters et al., 2020). Microstructural features percolate into macroscopic properties, transport phenomena, and biochemical processes at different stages of a product's life (Mahiuddin et al., 2018). Therefore, accurate visualization and realistic representation of microstructural constituents are useful in developing or validating an *in silico* food. Several techniques traditionally used for medical applications, such as magnetic resonance imaging (MRI) and X-ray micro-computed tomography (X-ray μCT), have been consistently gaining popularity for the nondestructive and noninvasive assessment of food structure (Kirtil et al., 2017; Van Dael et al., 2019).

Based on the differences in components' molecular mobility within a food matrix, MRI has been employed to characterize food structure and track moisture distribution and loss during processing (Schork et al., 2020). X-ray μCT has been applied to capture the 3D microstructure of various foods and track the deformation of, for example, porous networks' specific surface area, size, and fragmentation during processing, including drying, cold storage, and ripening (Cantre et al., 2014; Gruber et al., 2021). Despite the efficacy of these techniques in characterizing microstructural features in solid foods, their application to liquid/semi-solid, soft materials (e.g., multiphase products such as weak gels, emulsions, froth, or frozen foams) still encounters several hurdles due to these materials' optical opacity, low shear strength, and susceptibility to beam effects due to high energy synchrotronic radiation that can induce melting, deformation, and damages (Metilli et al., 2021). These limitations can be overcome by clever sample preparation (e.g., inclusion within sheltering structures), pre-tempering samples at freezing conditions (e.g., −15°C), or rapid data acquisition (Guo et al., 2017). However, alternative techniques should be applied to soft materials for proper characterization. It should also be noted that *in-situ* and real-time characterization of food structures during processing is still rare.

## Data use

Food properties and quality attributes have been linked to features revealed from X-ray μCT images by complementing them with other measuring techniques and multivariate analysis. For example, internal defects, disorders, and injuries were detected in vegetables and fruits using this approach. Even the position- and porosity-dependent effective $O_2$ diffusivity have been accurately determined in this kind of product based on high-resolution images (Chigwaya et al., 2021; Nugraha et al., 2021).

Extensive research has been conducted on reconstructing food structures and modeling deformation using empirical and mechanistic-based approaches from high-resolution data. X-ray CT images have been used to rebuild food geometry at different levels of granularity, from a single corn kernel microstructure to bulk packing of pears in a box (Suresh and Neethirajan, 2015; Verdouw et al., 2016; Gruyters et al., 2020). The reconstructed geometric models can be implemented into computational fluid dynamics (CFD) and transport models to simulate, for example, the performance of cooling systems on a food's temperature during storage (Yin et al., 2022). Machine learning approaches (e.g., artificial–ANN- and convolutional neural networks - CNN) have also been applied to rebuild structures from images (Wu et al., 2019; Röding et al., 2020).

Regarding modeling structural deformation during processing, Mahiuddin et al. (2018) provided a comparative analysis of mechanistic-based approaches to account for these changes. Proper material characterization allows the informed selection of a model for different foods (e.g., elastoplastic, linear-elastic, viscoelastic, or hyper-elastic), resulting in more realistic simulations. Novel hybrid models, as proposed by Pacheco-Aguirre et al. (2015), who introduced a shrinkage-deformation algorithm to simulate size reduction, shape change, and microstructure deformation more realistically during drying and evaluate the effect of these changes on moisture diffusivity, will increasingly play a more important role in reproducing food structure more realistically and accurately simulating deformations in virtual foods.

# Food composition and microbiota

Foods have complex compositions, with specific compounds contributing to their nutritional, organoleptic, and quality attributes. Not only does chemical composition varies among food items, but it also changes throughout a food's production and shelf-life. A series of chemical and biochemical reactions, whose rates are dependent on external factors, progressively modify food composition (van Boekel, 2008). Additionally, foods are natural hosts of various microorganisms and are susceptible to spoilage by bacteria, yeasts, and fungi. The comprehensive study of food composition, reaction mechanisms, microbial-host interactions, and kinetics, thus, is essential for accurately simulating food quality changes and predicting the remaining shelf life in digital renderings of a food.

## Data acquisition

Similar to physical properties, there is no lack of methods to report on food composition accurately (Nielsen, 2017). However, the comprehensive assessment of major and minor food components requires innovative and multidisciplinary approaches to address the interconnected reactions occurring alongside food-related phenomena.

Nondestructive and real-time sensing based on spectroscopic techniques, e.g., machine vision and hyperspectral imaging (HSI) in several modes, allows the simultaneous and continuous acquisition of composition and spatial information. These spatially resolved spectra are fast and easy to acquire noninvasively and in-line, being capable of monitoring from compositional changes to the effectiveness of processing operations (Colaruotolo et al., 2021).

Foodomics refers to applying high-throughput omics techniques (e.g., genomics, proteomics, lipidomics, interactomics) to monitor biomolecules in foods (Cifuentes, 2012; Pollo et al., 2021). Currently, it is impossible to think about food safety without genomics. However, its applications have expanded beyond surveillance to allow characterizing the complex microbial communities in foods involved in potential spoilage and safety risks (Beck et al., 2021; Palevich et al., 2021). A foodomics-based approach has also been used to gain insights into underlying mechanisms involved in food quality losses (Hu et al., 2022) and bioactivity (Ding et al., 2023), as well as mapping changes in composition and microbial loads during processing (Sinanoglou et al., 2014; Lerma-García et al., 2016; Topcam et al., 2023). It has also been proposed as a potential replacement for costly and subjective methods, such as sensory analysis. By implementing a foodomics approach, arrays from electronic noses and tongues coupled with chemometrics can result in accurate sensory prediction models (Tan and Xu, 2020). These models could be integrated with transport and reaction kinetics models to predict the 'virtual smell and taste' of the virtual foods. Hence, foodomics will progressively play a more prominent role in holistically mapping the relationships between composition and food phenomena, contributing to developing representative and accurate virtual foods.

## Data use

Chemical formation and degradation kinetics, as well as microbial growth, and inactivation models are critical to developing representative virtual foods. Although novel analytical techniques progressively allow better elucidation and monitoring of these processes, this progress has not permeated to data analysis or characterization. Microbial inactivation and chemical reactions (e.g., Maillard reaction, lipid oxidation, vitamin degradation) kinetics in foods have been historically described using fixed-order kinetic models, particularly first-order (van Boekel, 2008; Corradini, 2018). Microbial growth is routinely modeled using several empirical models, e.g., Verhulst's model variants (Peleg et al., 2011). The parameters of these primary models have been primarily estimated on model systems or liquid foods, and their dependence on state variables has been primordially established using models with limited physical meaning, for example, the Arrhenius equation (Peleg et al., 2012; Saguy, 2016). Hence, to adequately mimic changes in composition and microbial load in a virtual food, some advances need to be implemented. Since most kinetic models are valid only under the assumption that the involved reactions are elementary, which is not always the case, a comprehensive study of complex reaction networks and mechanisms is needed. Also, coupling food reaction kinetics with multidimensional and multiscale transport models should become the norm rather than the exception (Ranjbaran et al., 2021). Such efforts would help provide a more realistic view of chemical and microbiological changes in the entirety of a food matrix (Peleg, 2023). Finally, recent advances in deep learning techniques have enabled the extraction of kinetic parameters from limited data, which could potentially accelerate the accurate modeling of these reactions in real and virtual foods.

## Transport models for the utilization of virtual foods

Food exposure to different and fluctuating environmental factors, e.g., temperature and relative humidity, throughout the supply chain lead to thermal and water content gradients within food matrices, significantly influencing reaction rates and non-homogenous changes in quality and safety attributes (Sun et al., 2021). Therefore, transport models are important building blocks to ensure the development and utilization of a virtual food.

Food materials are multiscalar matrices with unique shapes, porosity, and cellular microstructures (Janssen et al., 2020). To realistically and accurately represent a food matrix, transport models should be multiscalar too. They should be composed of a hierarchy of interconnected sub-models, each of them defining the food's behavior and properties at a specific scale (Rahman et al., 2018). At a macroscopic scale, transport phenomena are governed by heat and mass balance principles, grounded in classic theory such as Fourier's law of thermal conduction, Fick's law of diffusion, and Darcy's law of pressure-driven flows (Turner and Mujumdar, 1996; Erdogdu et al., 2017). Applying these principles allows for characterizing and simulating the spatial-temporal distribution of state variables (e.g., temperature, pressure). The integrated application of these principles within a multiphase transport approach allows for modeling water evaporation/condensation within solid porous media as driven by local dynamically and under nonequilibrium gradients of water vapor pressure, for example,. Hence, heat and moisture exchange between a food and its environment can be estimated more accurately and realistically than ever before (Chen and Pan, 2021).

By using finite element (FE) or finite volume (FV) methods, modeling transport phenomena in bodies with irregular geometries, material discontinuity, and heterogeneous composition, such as foods, can be effectively achieved (Curcio et al., 2016; Chen et al., 2022). Mesh-free methods (MFM), including pore network, dissipative particle dynamics, and smooth particle hydrodynamics, can be applied to overcome some of the limitations imposed by FE or FV methods due to the rigidity of the mesh itself. MFM allows diving into an assumed 'homogenized region' and studying microscopic deformations and transport phenomena at the microscale (Karunasena et al., 2015). This results in accurate descriptions of pore geometry, dynamic tracking of the gas-liquid interfaces and gas distribution within porous media, and determining moisture diffusivities at a cellular level (Metzger, 2019; Welsh et al., 2021; Panda et al., 2022).

Building interconnected multiscale transport models could provide valuable insights into composition-property-structure-environment relationships in foods and food processes. However, applications of such models are still few, and several challenges remain to be surmounted to expand their use. They will require i) increased accessibility to rapid, high resolution, nondestructive data acquisition of structural attributes and components in real time, ii) obtaining accurate food properties and state variables data at multiple scales for model validation; ii) expanding the modeling of metabolic gases (e.g., $O_2$, $CO_2$) transport in porous food matrices, and iv) increased use of deep learning approaches to determine governing equations and critical parameters for processes such as diffusion in a heterogeneous media (Im et al., 2023). It is envisaged

that these advances and hybrid approaches will contribute significantly to the development of virtual foods (Sun and Shi, 2022).

## Applications of a virtual food

The value of developing virtual foods is profound for scientific research and practical applications. Conceived as comprehensive *in silico* models of real foods, they will allow us to better study and understand composition-structure-property-performance relationships. From a practical perspective, and as a crucial component of more extensive digital twins comprising a process, sets of thereof or even the whole supply chain, these avatars can be paraded through different virtualized environments to acquire realistic responses of a commodity or product to different scenarios as discussed below.

### Novel product design and development

Accurate *in silico* foods could facilitate the development of novel food products, including packaging (Marra, 2022). Desirable geometries, microstructural features, and nutrient stability can be pinpointed, designed, and tested through modeling and simulation to meet sensorial attributes, nutritional requirements (e.g., personalized needs), and sustainability goals (Nikitina and Chernukha, 2020; Marra, 2023). Additive manufacturing could be applied to build actual prototypes for validation. Packaging allows preserving quality and freshness, *in silico* food models can be used to scout and predict the performance of packaging materials virtually.

### Process development, improvement, and optimization

Food processing is critical for producing safe, nutritious, and sustainable products. However, thermal and nonthermal processing result in lower food quality, functionality, or nutrition. Additionally, several conventional treatments, such as hot air drying, pose sustainability challenges, e.g., excessive energy consumption. Thus, as a food avatar embedded within a process digital twin can help develop efficient food processes and optimize operating conditions (Verboven et al., 2020; Erdogdu, 2023; Topcam et al., 2023). A virtual food could serve as a new 'pre-check' approach to test whether a novel processing approach works. Using a multiphysics approach, optimal operating conditions could be matched to specific commodity varieties or products with different composition. This will allow the simultaneous optimization of food and process.

### Supply chain mapping and smart logistics

Food virtualization provides a feasible, rapid, and efficient way to assess potential sources of concern throughout the supply chain that might shorten a product's shelf-life or present a higher risk of exposure to potential contamination (Tagliavini et al., 2019; Defraeye et al., 2021). By simulating these situations and retrieving valuable information from the food avatar, a smarter distribution/storage system with integrated measures to minimize risks can be enacted and customized for different types of foods to prevent food waste and outbreaks, as recently reported by Shrivastava et al. (2022).

## Conclusions and recommendations

Several elements that contribute to the virtualization of a food product, from databases to modeling approaches, are already available. Advances in computational power, characterization techniques, and data integration strategies are completing the jigsaw puzzle. Despite their potential value, the implementation this powerful tool has been demonstrated on limited occasions. Numerous challenges remain for their full implementation as a routine tools to optimize the performance of the food supply chain.

Full virtualization of a food product requires interdisciplinary contributions. Expanding collaborations beyond strictly food disciplines at the academic and industrial level can facilitate this process. The data needed to inform each component of a virtual food are substantial and require contributions that reflect spatial and temporal variability. Thus, cloud-based storage with the capability to integrate categorized information should be established and shared among stakeholders.

Additionally, development and operation of virtual food models will require specific knowledge and skills, which may limit their applicability. Translation and dissemination of the outcomes (Kansou et al., 2022), user-friendly graphical user interfaces (GUI), and proper training in relevant skills are critical to expanding their future use and building more sustainable and secure global food systems.

## Author contributions

CC, AH-J, and MC contributed to the conceptualization of the manuscript. CC and MC compiled the literature review, and all authors organized the sources. AH-J developed the illustration. CC wrote the first draft of the manuscript; subsequent versions reflect the contributions of all authors. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author MC declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

## Publisher's note



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# *Spirulina* and its residual biomass as alternative sustainable ingredients: impact on the rheological and nutritional features of wheat bread manufacture

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**Introduction:** Following consumers' demand for healthier alternatives, *Spirulina* biomass has been included as a functional ingredient in different types of food as a sustainable alternative to improve physicochemical characteristics and nutritional aspects in the diet. This study aimed to explore the use of *Spirulina* biomass (SB) and residual biomass (RB) obtained after C-phycocyanin (C-PC) extraction in the production of wheat bread (WB).

**Methods:** The dough rheology, texture, color, nutritional aspects, and bioaccessibility of the different manufactured bread samples were accessed to achieve this goal.

**Results and Discussion:** This is the first research to exploit RB. It was possible to replace 3% of wheat flour for SB or RB in bread-making; this substitution did not impact the rheological characteristics of the dough, even though the pH increased with the addition of SB or RB (5.63 and 5.70, respectively). SB and RB addition improved bread volume and enhanced the nutritional profile, increasing the antioxidant capacity (DPPH and FRAP) compared to WB. Heavy metals (Ni, Cd, and Pb) were not found in any of the samples (<0.2 mg/100 g). The *in vitro* protein digestibility in bread was better than in SB and RB raw materials, which indicates that bread manufacturing may contribute to improving protein digestibility. In addition, SB needs greater appreciation for sustainable food practices worldwide and in Brazilian exploration, requiring management strategies with industry and society working together. Further studies are necessary, focusing on acceptability to understand the viability of these ingredients to target consumers' preferences.

KEYWORDS

**wheat bread development, *Arthrospira platensis*, functional ingredients, rheological properties, *in vitro* digestibility, sustainability**

# 1 Introduction

Since 6000 B.C., bread-making has been stated as a symbolic tradition of different cultures around the world (Belderok, 2000; Nawar, 2020). Bread has been recognized as a secular daily food consumed over the years, contributing not only to the macro and micronutrient requirement but also because of the physical and, consequently, sensorial aspects (Dewettinck et al., 2008).

Although bread has a high nutritional value, mainly comprising carbohydrate and protein content and micronutrients such as lipids, vitamins, minerals, and bioactive compounds (Shewry and Hey, 2015), nowadays, the quality of wheat flour for bread manufacture has undergone several changes, such as genetic statistics, cytogenetic research, hybrid and mutation breeding, phenotyping advances, and genomic selection (Venske et al., 2019).

Thus, to achieve food requirements and positive repercussions on health (Amoah et al., 2022), the addition of plant-based ingredients in wheat bread production—besides the use of flour, water, salt, sugar, and yeast ingredients—such as fibers, proteins, enzymes, bioactive compounds, and algae, has been demonstrated as a reality (Onwezen et al., 2021; Zain et al., 2022; Ferreira et al., 2023).

Moreover, healthy products and habits have been increasing in consumer demand (Asioli et al., 2017; Feil et al., 2020; Burt et al., 2021), not only individually but alongside the environmental preservation of fauna and flora, constituting the One Health concept (Banwo et al., 2021). The whole use of biomass produced globally, such as *Spirulina (Arthrospira platensis),* stimulates the circular economy in a more sustainable manufacturing path, mainly using renewable sources such as cyanobacterium and algae (Amarante and Braga, 2021). To change this scenario, it is necessary to adopt changes in the food and bioprocessing industries (Godfray et al., 2010; Braga et al., 2020).

In this way, *Spirulina* biomass has been included as a functional ingredient in crostini (toasted bread) (Niccolai et al., 2019) and other bread (Saharan and Jood, 2021), breadsticks (Uribe-Wandurraga et al., 2019), cookies (Batista et al., 2017), wheat crackers (Batista et al., 2019), pasta (Raczyk et al., 2022), and dairy products (Hernández et al., 2022) to increase the food industry's repertoire, generally providing an intense color (generally green) and flavor, and also to improve nutritional and technological aspects (Lafarga et al., 2020).

It is essential to highlight that this is one of the few studies demonstrating the potential of *Spirulina's* residual biomass (RB) for food production. Two previous works from our research group (Fratelli et al., 2022; Braga et al., 2023) mentioned its potential, and the first report on use of residual biomass as an ingredient was recently published.

*Spirulina* has already been regarded as a sustainable protein source since it contains up to 70% protein in its dry mass, grows faster than terrestrial plants, and has higher protein productivity per area than other crops such as soybean, legumes, or wheat. Given the developments in the circular economy based on Spirulina biomass (SB) and RB after extracting the C-Phycocyanin (C-PC) for better diets and food industry innovation, the current work is an important step in this approach (Braga et al., 2023).

Furthermore, there has been a greater emphasis on optimizing *Spirulina* production in order to promote economic feasibility and environmental sustainability. Even when C-PC is extracted from *Spirulina* biomass, the residual biomass (RB) retains significant levels of antioxidants, vitamins, and minerals, making it a viable raw material in high-value goods (Fratelli et al., 2021; Fratelli et al., 2022).

The use of *Spirulina* in the formulation of wheat bread with the addition of 10% of SB was demonstrated in the literature by Burcu et al. (2016). However, the sensory analysis tests showed that the higher the percentage of SB, the lower the acceptability, as confirmed by Morsy et al. (2014), indicating that the highest overall acceptability of *Spirulina* snack food was found with a *Spirulina* biomass addition rate of 2.5% and corn flour addition rate of 97.5%.

In a more recent paper by Saharan and Jood (2021), it was shown that wheat flour bread could be formulated with a 'liked moderately' overall acceptability, fortified with *Spirulina platensis* powder in 2, 4, and 6% levels up to 2 days of storage. The values of acceptability of the control bread (without *Spirulina* powder supplementation) and bread with the addition of 2% of *Spirulina* powder were very similar, which may indicate that consumers prefer lower values of *Spirulina* fortification. The similarities and differences between bread groups could be assured with statistical analysis, which the authors did not present. In addition, several authors have reported that even low-percentage additions of SB and C-PC present a significant improvement of the manufactured products in terms of bioactivity, particularly considering antioxidant activity (Capelli and Cysewski, 2010; El Baky et al., 2015; Palanisamy et al., 2019; Amarante et al., 2020; Niccolai et al., 2021; Braga et al., 2023). Since consumer acceptance is limited regarding the flavors related with microalgae as ingredients (Sanjari et al., 2018), for now, the addition of low percentages is a strategy to include *Spirulina* and other alternative sources of nutrients in the daily diet of people.

Therefore, this study aimed to apply two innovative ingredients, SB and RB, obtained after C-PC extraction, in the production of wheat bread (WB) to study and compare the dough rheology, texture, color, nutritional aspects, and bioaccessibility between the different manufactured bread samples.

# 2 Material and methods

## 2.1 Raw materials

The ingredients used in the bread-making process were obtained from the local Portuguese market, which included commercial wheat flour of type 65 without yeast (Pérola®, Porto, Portugal), distilled water, white cane sugar in sticks (Modelo Continente Hipermercados, S.A., Senhora da Hora, Portugal), refined salt (Modelo Continente Hipermercados, S.A., Senhora da Hora, Portugal), and dehydrated yeast in powder (Fermipan®, Lallemand Iberia, Portugal). The organic and biodynamic *Spirulina* biomass was supplied by Fazenda Tamanduá® (Paraíba, Brazil).

## 2.2 Obtention of residual biomass (RB) from *Spirulina*

According to Fratelli et al. (2022), after the solid–liquid C-PC extraction and centrifugation (for 120 min, with breaks every

30 min, at 10,000 × g (NT 816, Nova Analítica®, Brazil), the RB was obtained for application in bread for the full utilization of SB. Thereby, the supernatant, C-PC, was separated from the remaining biomass, named residual biomass in this work, and stored in an ultra-freezer at −38°C. After RB collection, the RB samples were freeze-dried for 72 h in a freeze dryer (Liobras® K105, São Paulo, Brazil).

## 2.3 Wheat dough samples: pH and rheological characteristics

The pH measurements were performed in triplicate in the unleavened dough of WB samples using a pH-Meter Basic 20 potentiometer (Crison Instruments®, Spain) until stabilization.

The wheat flour moisture quantification (moisture analyzer PMB 202—Adam Equipment Co. Ltd., United Kingdom) with or without 3% of SB or RB was determined to evaluate the rheological analysis of the unleavened dough. Mixing and pasting characteristics were analyzed in the Micro-doughLAB 2800 equipment (Perten Corp., Sweden) on a small scale (4 g ± 0.01, standardized at 14% of moisture). The viscoelastic behavior was assessed in a HAAKE MARS 60 rheometer (Thermo Scientific®, Karlsruhe, Germany), coupled with a UTC—Peltier system (for temperature control at 18°C), using a serrated parallel-plate sensor system with 20 mm diameter (PP20). All tests were performed at least in triplicate.

The mixing curves were based on the AACC International Method 54–70.01 (AACC, 2014) with laboratory adaptations (at a speed of 120 rpm, 30°C, for 10 min). The water absorption (WA) was adjusted to obtain the maximum torque value of 130 mN.m ± 4% for each dough (target peak). The following parameters were evaluated: I) water absorption (WA); II) degree of softening (DS); III) dough development time (DDT); IV) stability time (ST).

The pasting characteristics were determined according to the AACC International Method 54–60.01 (AACC, 2010), with adaptations by Dang and Bason (2015) and Martins et al. (2020). Thus, the method is based on a constant mixing rate at 63 rpm for 43 min to understand each dough's behavior after adding water, previously predetermined by water absorption (%) in the Micro-doughLAB 2800 equipment (Perten Corp., Sweden), through a series of temperature cycles: I) 30°C for 6 min; II) temperature increment to 90°C for 15 min; III) constant temperature of 90°C for 7 min; IV) decrease to 50°C for 10 min; and V) constant temperature of 50°C for another 5 min, until the end of the test.

The dough viscoelastic behavior parameters were evaluated by small-amplitude oscillatory shear measurements (SAOS). First, a stress sweep test was performed at 6.28 rad/s (1 Hz) to determine the linear viscoelastic region (LVR). In all the samples, a constant shear stress of 10 Pa was applied to proceed to the frequency sweep test conducted inside the LVR from 0.063 to 396 rad/s. Dough samples were coated with paraffin to prevent dryness during the measurements.

## 2.4 Production and image analysis of wheat bread samples

The WB formulation was performed in six steps. I) The thermoprocessor (position 3 for 30 s) was used to activate the dehydrated yeast with sugar and warm water at 37°C. II) The addition of wheat flour and refined salt with or without 3% of SB or RB was carried out in WB, WB-SB, and WB-RB doughs. The mixture was processed in the thermoprocessor (position 6 at room temperature). III) The WB doughs were kneaded in the thermoprocessor (cob position for 2 min). IV) WB samples were molded (220 g each) and placed into greased trays (9.1 × 18 cm of width and length dimensions). V) WB samples were fermented for 60 min at 37°C in a fermentation chamber. VI) WB samples were baked for 60 min at 160°C in an electric convection oven. The samples were cooled down for 1 hour and stored in plastic food bags.

**TABLE 1 Proportion of ingredients in % of flour basis (fb) in each WB formulation.**

| Ingredient | WB | WB-SB | WB-RB |
|---|---|---|---|
| Wheat flour (T 65) (%) | 100.00 | 97.00 | 97.00 |
| *Spirulina* biomass (%) | 0.00 | 3.00 | 0.00 |
| Residual biomass after C-PC extraction (%) | 0.00 | 0.00 | 3.00 |
| Distilled water (%)[a] | 54.00 | 54.20 | 54.70 |
| Dried yeast (%) | 3.50 | 3.50 | 3.50 |
| Refined salt (%) | 1.68 | 1.68 | 1.68 |
| White cane sugar (%) | 1.00 | 1.00 | 1.00 |

[a]Determined by Micro-doughLAB, 2800 equipment (Perten Corp., Sweden).

The equipment used in the bread-making process were Bimby® TM31 thermoprocessor (Wuppertal, Germany), Arianna XLT133 Unox® fermentation chamber (Cadoneghe, Italy), and Arianna XFT133 Unox® electric convection oven (Cadoneghe, Italy). The ingredients' proportion can be seen in Table 1, according to preliminary tests based on the study of Graça et al. (2018).

The WB image samples were captured using a mobile camera (SAMSUNG® S 22 Plus). The binary images were set in the central point of each image with a resolution of 72 dpi, both horizontal and vertical. The images were analyzed using ImageJ (Available at: http://rsb.info.nih.gov/ij/) as described by Rahimi et al. (2020).

## 2.5 Wheat bread physical property evaluation during storage time

The specific volume was determined as a ratio between the volume and weight according to the AACC Method 10–05.01 (AACC, 2010). The crumb moisture content was measured in an air oven at 100°C, and the baking loss was calculated after 48 h, according to the AACC Method 44–15.02 (AACC, 2009). All the physical analyses for estimating bake loss and crumb moisture were performed in triplicate and at six replicates for specific volumes. The $a_w$ at 20°C of bread samples were measured in triplicate using a HygroPalm HP23 portable water activity analyzer (Rotronic®, São Paulo).

The texture of WB was determined by texture profile analysis (TPA) using a TA-XTplus texturometer (Stable MicroSystems®, Surrey, United Kingdom), with a load cell of 5 kg. TPA consists of double compression of a piece of food that mimics the action of

the jaw, as described by Graça et al. (2018). Each WB sample was cut into a 20-mm-thick slice, and the analysis was performed with an acrylic cylindrical probe of 10 mm diameter piercing 5 mm of the sample at 1 mm/s of crosshead speed, with a resting time of 5 s between cycles. The texture profile of WB, WB-SB, and WB-RB samples was compared regarding hardness (N) and cohesiveness (dimensionless), considered the most representative parameters in these samples, calculated by the TA.XTplus software. The measurements were repeated six times for each sample at 0 h, 24 h, and 48 h after bread production.

The WB crumb and crust samples were evaluated in a portable Chroma-Meter CR400 colorimeter (Konica Minolta®, Japan) according to the CIE $L^*a^*b^*$ system (International Commission on Illumination) using the color parameters $L^*$, $a^*$, and $b^*$, where $L^*$ indicates a brightness of 0 (blackness) or 100 (whiteness), $a^*$ indicates the degree of redness (+60) or greenness (−60), and b* indicates the degree of yellowness (+60) or blueness (−60). The measurements were replicated 9 times. Data were demonstrated by the total color difference ($\Delta E$) that was measured as in Eq. 1. $L_o^*$, $a_o^*$, and $b_o^*$ were the values obtained from WB:

$$\Delta E = \left[ \left(L_o^* - L^*\right)^2 + \left(a_o^* - a^*\right)^2 + \left(b_o^* - b^*\right)^2 \right]^{1/2}. \quad (1)$$

(Jusoh et al., 2009).

The color differences between the samples were classified as $\Delta E \leq 1$—imperceptible by human eyes; $1 < \Delta E \leq 2$—cognizable by close observation; $2 < \Delta E \leq 10$—cognizable at a glance; $10 < \Delta E \leq 50$—a clear difference noticeable in colors; $\Delta E \geq 50$—exactly two different colors (Li et al., 2017).

## 2.6 Proximate composition, phenolic compounds, and antioxidant activity of wheat bread samples

The proximate composition of WB samples was determined through the American Association of Cereal Chemists with adaptations, formerly the AACC International Method (AACC, 2023). The ash, fat, protein, and mineral contents were analyzed in dried WB samples in triplicate. The ash content was determined by muffle furnace combustion at 550°C overnight. Soxhlet extraction with petroleum ether as the solvent at a medium temperature for 6 h determined the total fat content. The protein content was determined using the Dumas method (Thermo Quest® NA 2100 Nitrogen and Protein Analyzer, Interscience, Breda, Netherlands), considering the protein-to-nitrogen conversion factor of 6.25. The mineral and heavy metal profiles were evaluated by inductively coupled plasma optical emission spectrometry—ICP-AES (Thermo Fisher® Scientific iCAP PRO-7000 Series, Massachusetts, United States) (Khemiri et al., 2021). The total content of carbohydrates was calculated by difference as in Eq. 2:

$$\text{Total carbohydrates} = \left[100 - \left(\text{moisture} + \text{ash} + \text{protein} + \text{fat}\right)\right]. \quad (2)$$

The phenolic compound and antioxidant activity analysis was performed in triplicate. Methanolic (80%) extracts of WB, WB-SB, and WB-RB dried samples were resuspended in 20% DMSO. The final extracts were concentrated at 20 mg/mL. The total phenolic compound content was determined by the Folin–Ciocalteu methodology, as described by Mohankumar et al. (2018). The antioxidant activity was performed against two free radicals: DPPH by Brand-Williams et al. (1995) and FRAP by Benzie and Strain (1996).

## 2.7 Bioaccessibility of wheat bread: *in vitro* digestion by the INFOGEST protocol

The *in vitro* digestibility of WB, WB-SB, and WB-RB samples was evaluated following the INFOGEST protocol described by Minekus et al. (2014) and Brodkorb et al. (2019). The INFOGEST—*in vitro* tool was used to calculate the solution amount in each stage (available at: http://www.proteomics.ch/IVD/). Thus, 2.5 g of each sample and blanks were measured in triplicate, and the phases were run to mimic digestion: *a) oral phase* (2 min at pH 7)—with simulated salivary fluid (SSF) containing amylase. The samples required adding more water to allow their homogenization to form a paste-like consistency. The water quantities were noted; *b) gastric phase* (2 h at pH 3)—the bolus was diluted with simulated gastric fluid (SGF) containing the gastric enzyme pepsin; *c) intestinal phase* (2 h at pH 7)—the simulated intestinal fluid (SIF) containing pancreatin and bile salts was added into the mixture. The entire protocol was performed at 37°C in a continuous and gentle mixing process in a rotary agitator.

Then, the intestinal phase was stopped using protease inhibitor 4-(2 aminoethyl) benzensulfonylfluoride (Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland). Then, the samples were finally centrifuged (8.000 rpm at 10°C for 10 min), and the undigested pellets were collected and dried in Falcon tubes at 80°C for 3 h and then at 45°C for 1 h until constant weight. The digested part (supernatant) was filtered using a Buchner funnel with Whatman® qualitative filter paper (125 mm). The filter paper was weighed before and after filtering the contents of each Falcon tube. The crude protein *in vitro* digestibility (IVD %) of wheat bread (WB, WB-SB, and WB-RB) and raw material (SB and RB) was calculated from the difference between the initial biomass and the undigested biomass, divided by the initial biomass and multiplied by 100, as described by Khemiri et al. (2021).

## 2.8 Statistical analysis

Analyses of variance between samples were performed by one-way ANOVA with Tukey's post-hoc test at a significance level of 95% ($p < 0.05$) using the STATISTICA 8.0 software (StatSoft, Inc., Tulsa, United States). The percentage variation (PV) was calculated for firmness using Eq. 3:

$$\text{PV} = [\,(\text{value of bread firmness at 48 h} - \text{value of bread firmness at 0 h}) / \text{value of bread firmness at 0 h} \times 100]. \quad (3)$$

The Windows version of ImageJ was used for measurement of WB binary images of alveolar cell structure ($n = 150$), performed independently, and expressed as the mean and ± standard deviation.

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**TABLE 2 Rheological parameters and pasting curves (C1–C5 with standardized time and temperature) of developed dough in Micro-doughLAB values are shown in mean ± standard deviation.**

| Dough rheological parameters | | | | | C1[a] (torque mN.m) | C2 (torque mN.m) | C3 (torque mN.m) | C4 (torque mN.m) | C5 (torque mN.m) |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Water absorption % | Degree of softening (mN.m) | Development time (min) | Stability (min) | 30.0°C | 62.2°C | 90.0°C | 82.5°C | 50.0°C |
| | | | | | 4 min | 14 min | 22 min | 30 min | 43 min |
| WB | 54.0$^{b}$ ± 0.2 | 17.0$^{a}$ ± 3.0 | 2.6$^{a}$ ± 0.6 | 4.1$^{a}$ ± 0.1 | - | 62.7$^{a}$ ± 3.2 | 237.8$^{c}$ ± 1.5 | 184.2$^{c}$ ± 2.1 | 428.2$^{c}$ ± 33.1 |
| WB-SB | 54.2$^{b}$ ± 0.2 | 9.1$^{a}$ ± 6.0 | 1.9$^{a}$ ± 1.2 | 6.0$^{b}$ ± 0.7 | - | 62.8$^{a}$ ± 1.9 | 287.8$^{a}$ ± 4.9 | 275.8$^{a}$ ± 2.8 | 540.0$^{a}$ ± 5.6 |
| WB-RB | 54.9$^{a}$ ± 0.3 | 12.3$^{a}$ ± 3.1 | 2.9$^{a}$ ± 1.5 | 5.3$^{ab}$ ± 0.6 | - | 60.8$^{a}$ ± 4.1 | 268.2$^{b}$ ± 3.5 | 247.0$^{b}$ ± 3.0 | 521.7$^{b}$ ± 4.6 |

[a]Values of the torque peak (C1) have been adjusted for 130 mN.m ± 4% for all samples using the Micro-doughLAB 2800 equipment (Perten Corp., Sweden), just as it has been used for water absorption.

Values followed by different superscript letters in each column are significantly different ($p \leq 0.05$), determined by one-way ANOVA and Tukey's post-hoc test (STATISTICA 8.0), as demonstrated for water absorption.

# 3 Results and discussion

## 3.1 Impact of *Spirulina* fractions (SB and RB) in wheat flour dough samples

The rheological characteristics of the doughs with or without *Spirulina* counterparts, described in Table 2, showed that the water absorption (%) required in WB-RB was higher than in WB and WB-SB, which showed the same significant value to reach the predetermined standard torque value of 130 mN.m, which is the ideal torque for wheat bread dough. Amoriello et al. (2021) showed that functional bread with *Spirulina* requires the same values for water absorption as described in our study, between 50.9% and 53.9%.

The differences between the water absorption capacity of the samples (WB, WB-SB, and WB-RB) could be explained by the addition of other protein sources, such as SB and RB in wheat flour, which compete for water with other components in the dough system, resulting in doughs with increased farinograph water absorption, as described by Graça et al. (2018) with the addition of *Chlorella vulgaris* in different concentrations (1.0, 2.0, 3.0, 4.0, and 5.0 g/100 g of wheat flour). With values higher than 3 g, the amount of water required was high. In addition, water quantity is an important factor to be adjusted for the distribution of the dough ingredients, their hydration, and the gluten protein network development, as well as to increase the production of yield (Graça et al., 2018).

The results for degrees of softening (the difference in torque between the maximum torque and the medium line of the curve 8 min after the development time) and the dough development time (time to reach the torque value of 130 mN.m) were similar for all samples; thus, the addition of SB or RB did not impact these dough characteristics. The dough stability time was higher in WB–SB than in WB, which suggests that SB affected the tolerance of the flour to withstand torque, which resulted in a positive effect on the physical properties of bread, such as higher specific volume and lower bake loss for WB-SB and WB-RB, which is in line with the results of Amoriello et al. (2021), due to the presence of hydrocolloids, mainly alginate, agar, and carrageenan. However, it is different from what was shown by Graça et al. (2018) with *Chlorella vulgaris* in higher concentrations (≥3%), whereby the protein content of wheat flour was somehow disrupted, which resulted in lower dough strength and loaf volume, which had a negative effect on the quality characteristics of bread.

It is important to know that the course of wheat dough behavior was described by five basic torque points C1–C5, expressed in mN.m (AACC, 2010). The results showed that until C2, the dough behavior was equal among the samples. However, in C3, C4, and C5, the SB and RB impacted with higher values, and WB showed the lowest values. These points (C3 to C5) are related to bread characteristics (Table 2).

It is known that *Spirulina* interferes in the starch gelatinization process because the components in this food matrix are mainly polysaccharides, considering its origin and quantity (Amoriello et al., 2021). Other factors depend on the particle properties, such as size and distribution, morphology, and hardness (Martínez-Sanza et al., 2020). The particle size of the added raw materials (SB ≤ 106 μm and RB > 106 μm) was not standardized, which could impact pasting curve parameters as the nutritional profile of the dough with SB and RB addition.

The mechanical spectra presented in (Figure 1A) suggest that adding 3% of SB or RB did not interfere with the rheological parameters of unleavened dough of WB, WB-SB, and WB-RB. All samples showed a higher elastic behavior (G′) than a viscous behavior (G″), which can be evidenced by Figure 1B. This demonstrates that the tan <1 corresponds to a predominant elastic behavior for all samples. It is essential to highlight that this viscoelastic behavior depends on the frequency, usually associated with poorly structured systems, as seen in pasta with *Spirulina* (Fradinho et al., 2019). The values of G′ at 6.283 rad/s (1 Hz) and 62.83 rad/s (10 Hz) were similar ($p > 0.05$ ANOVA and Tukey's post-hoc test) for all samples, suggesting that the *Spirulina* counterpart's addition did not impact the elastic behavior of doughs.

Nunes et al. (2020a) demonstrated that the incorporation of *Chlorella vulgaris* at 1% in wheat bread led to higher G′ values, suggesting that the microalgae caused a strengthening of the dough structure, and the dough with the incorporation of *Tetraselmis chuii* showed lower G′ values than the control (Nunes et al., 2020b), suggesting a destabilization of the structure by the addition of microalgae, contrary to what was observed in this study that WB

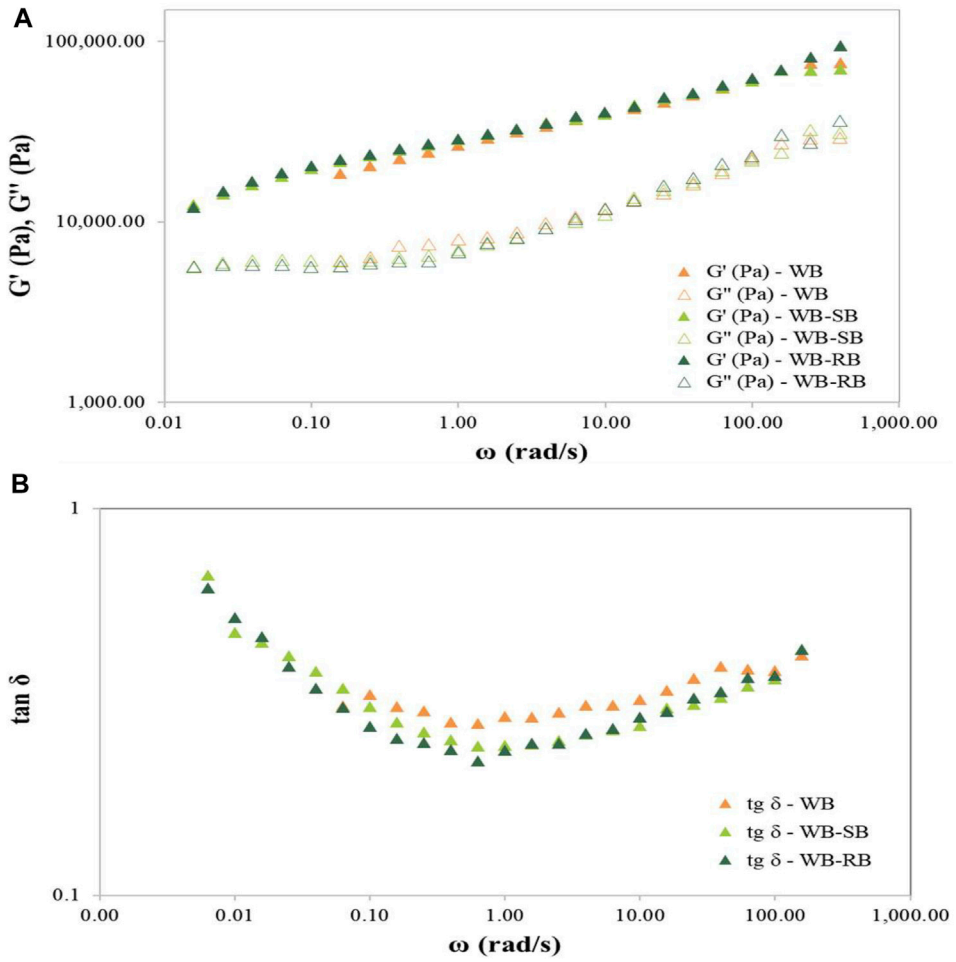


FIGURE 1
Mechanical spectra **(A)** and tan δ vs. angular frequency **(B)** of wheat bread dough with 3% of *Spirulina* biomass (WB-SB) or residual biomass (WB-RB) compared to the control wheat bread (WB). G′ corresponds to the elastic modulus, and G″ corresponds to the viscous modulus. The test was performed in triplicate, and the most representative curve for each sample is presented.

with SB or RB, or without *Spirulina,* showed the same performance. Thus, this behavior is in accordance with *Spirulina* suspensions (more elastic) in comparison to other algae *Microchloropsis gaditana and Scenedesmus almeriensis,* with more viscous (liquid) rheological characteristics (Martínez-Sanza et al., 2020).

Dough pH demonstrated an increase with the addition of 3% of *Spirulina* fractions SB ($5.63^{a} \pm 0.05$) or RB ($5.70^{a} \pm 0.05$) when compared to WB ($5.42^{b} \pm 0.05$). Differently from the study conducted by Garzon et al. (2021), it was observed that the wheat flour doughs, conventionally prepared using compressed baker's yeast, demonstrated superior pH when compared to the dough samples with the addition of 1, 2, or 3% of *C. vulgaris* powder. However, it is essential to note that the algae type influences the pH behavior and the type of yeast, which are different between the studies. Morsy et al. (2014) highlighted that *Spirulina* presents a pH of 6.93, which can enhance food pH levels. As we could see in this study, this can be stated because *Spirulina* is naturally found in tropical regions with a high concentration of NaCl and bicarbonates, mainly found in inhabiting alkaline lakes (pH 11) (Volkmann et al., 2008).

## 3.2 Physicochemical properties of wheat bread samples with or without *Spirulina* fractions (SB and RB)

The inclusion of SB or RB in WB development did not affect the $a_w$ and crumb moisture of the produced bread, indicating that the addition of *Spirulina* and its counterpart does not imply these two physicochemical properties of bread. The same results were found by García-Segovia et al. (2017) with the addition of different types of algae in wheat bread formulation (*Isochrysis galbana, Tetraselmis suecica, Scenedesmus almeriensis,* and *Microchloropsis gaditana*). In rice flour gluten-free bread with 1% or 4% of *Spirulina* and additives in different concentrations (1.5 or 2.2% of methylcellulose and 0.2 or 0.6% of transglutaminase), the average crumb moisture was 45%, as shown by Belkina et al. (2022a) with the addition of 0.5% of *Spirulina* in wheat bread formulations, a little bit higher than what was found in this paper.

The specific volume, considered a bread expansion measurement, was positively affected by SB and RB addition, as described by Amoriello et al. (2021), adding 1, 2.5, or 4% of *Spirulina*

**TABLE 3** Means ± standard deviation of the physical properties of wheat bread (WB) formulations with 3% of *Spirulina* counterparts (WB-SB and WB-RB) or without (WB).

| Parameter | | WB | WB-SB | WB-RB |
|---|---|---|---|---|
| Bread properties | Crumb moisture (%) | $39.72^{a} \pm 0.52$ | $40.57^{a} \pm 0.35$ | $39.68^{a} \pm 0.60$ |
| | Bake loss (%) | $30.57^{a} \pm 0.00$ | $28.57^{b} \pm 0.00$ | $29.52^{b} \pm 0.02$ |
| | Specific volume ($cm^3/g$) | $2.58^{b} \pm 0.12$ | $3.14^{a} \pm 0.24$ | $3.14^{a} \pm 0.21$ |
| | Water activity ($a_w$) | $0.86^{a} \pm 0.02$ | $0.86^{a} \pm 0.01$ | $0.86^{a} \pm 0.02$ |
| Firmness (N) | 0 h | $1.97^{Bb} \pm 0.26$ | $2.12^{Bb} \pm 0.43$ | $3.00^{Ba} \pm 0.28$ |
| | 24 h | $4.21^{Aa} \pm 0.81$ | $3.71^{Aa} \pm 0.54$ | $4.17^{ABa} \pm 1.69$ |
| | 48 h | $4.39^{Aa} \pm 0.71$ | $4.17^{Aa} \pm 0.47$ | $5.15^{Aa} \pm 1.52$ |
| Cohesiveness | 0 h | $0.77^{Aa} \pm 0.04$ | $0.75^{Aa} \pm 0.02$ | $0.69^{Ab} \pm 0.03$ |
| | 24 h | $0.47^{Ba} \pm 0.06$ | $0.48^{Ba} \pm 0.05$ | $0.47^{Ba} \pm 0.06$ |
| | 48 h | $0.41^{Ba} \pm 0.06$ | $0.39^{Ca} \pm 0.08$ | $0.34^{Ca} \pm 0.03$ |
| Color parameters of the crust | $L^*$ | $66.55^{a}$ (±0.54) | $37.16^{b} \pm 1.81$ | $36.72^{b} \pm 0.73$ |
| | $a^*$ | $8.11^{a}$ (±0.66) | $-2.71^{b} \pm 0.90$ | $-0.58^{c} \pm 0.19$ |
| | $b^*$ | $34.41^{a}$ (±1.10) | $14.31^{c} \pm 1.12$ | $15.58^{b} \pm 0.22$ |
| | $\Delta E$ | - | 34.90 | 33.86 |
| Color parameters of the crumb | $L^*$ | $63.26^{a}$ (±1.72) | $36.04^{b} \pm 0.73$ | $35.58^{b} \pm 0.62$ |
| | $a^*$ | $-0.62^{a}$ (±0.02) | $-2.24^{a} \pm 0.27$ | $-1.18^{a} \pm 0.08$ |
| | $b^*$ | $17.60^{a}$ (±0.57) | $19.26^{a} \pm 0.64$ | $22.39^{a} \pm 0.29$ |
| | $\Delta E$ | - | 27.32 | 28.11 |

Values followed by different superscripts and lowercase letters in each row are significantly different between samples ($p < 0.05$). Values followed by different superscript capital letters in each column are significantly different between storage time ($p \leq 0.05$), determined by one-way ANOVA with Tukey's post-hoc test (STATISTICA 8.0).

in the soft wheat bread development. These differences in outputs with SB and RB addition could be due to the extensibility enhancement and gluten network improvement related in the literature. In addition, the presence of algae could positively affect specific volumes, except for alginate, mainly because of polysaccharides and fiber content (Rosell et al., 2001; Belkina et al., 2022b).

One reason for the cessation of dough expansion during baking is the resistance of the dough to extension, which depends on rheological properties like elasticity and viscosity. Starch gelatinization in the cell membranes occurs above 65°C, increases dough viscosity, and impairs the extensibility of the dough, which results in increased pressure in closed gas cells, leading to rupture of the cell membranes. As a result, the gas molecules will exchange between adjacent cells and ultimately be transported to the outside of the dough, resulting in a loss of gas and presumably limited capacity for expansion (Mondal and Datta, 2008), as seen in WB. The bake loss of WB was higher than that in bread with the SB and RB addition, as shown in Table 3.

These results disagree with the results of Montevecchi et al. (2022), which indicated that the % weight loss during baking was similar in wheat bread with or without *Spirulina* addition, demonstrating a baking loss reduction of around 13.76%, whereas in our study, the baking loss (%) was higher (around 30%).

As Kotoki and Deka (2010) highlighted, when a high percentage of moisture is lost, bread will present a dry crust with less weight, which we can see in the WB, WB-SB, and WB-RB samples. The inclusion of SB and RB helped in the reduction of baking loss, which is still high, which confirms that the addition of baking agents and dough improvers may overcome these problems, as well as can help with the freshness maintenance of bread and other baked goods, which age earlier and become stale because of a high moisture loss (Kohajdová and Karovičová, 2009; Moayedallaie et al., 2010).

Hardness (bread firmness) represents the maximum resistance to probe penetration, and cohesiveness is recognized as the force that can deform a material before it breaks; in other words, it is the strength of the internal bonds that make up the body of the product (Rosenthal and Thompson, 2021), which is obtained using the area of the first bite divided by the area of the second bite.

The results of WB and WB-SB firmness (N), demonstrated in Table 3, at 0 h of storage were lower when compared to that of WB-RB, indicating that adding 3% of RB negatively affected bread firmness, making the bread crumb harder. Graça et al. (2018) found similar results at 0 h time with *C. vulgaris* addition, instead of *Spirulina*, showing that the added algae concentration did not influence bread firmness. However, for RB, the conclusions have been controversial, and it is a matrix that is poorly elucidated in the literature.

Bread firmness at 24 h and 48 h showed similarity after 24 h storage time in all samples. However, it is crucial to highlight that the percentage variation of WB showed the highest increase in bread firmness during 48 h (55.12%), the lowest for WB-RB (41.71%), and

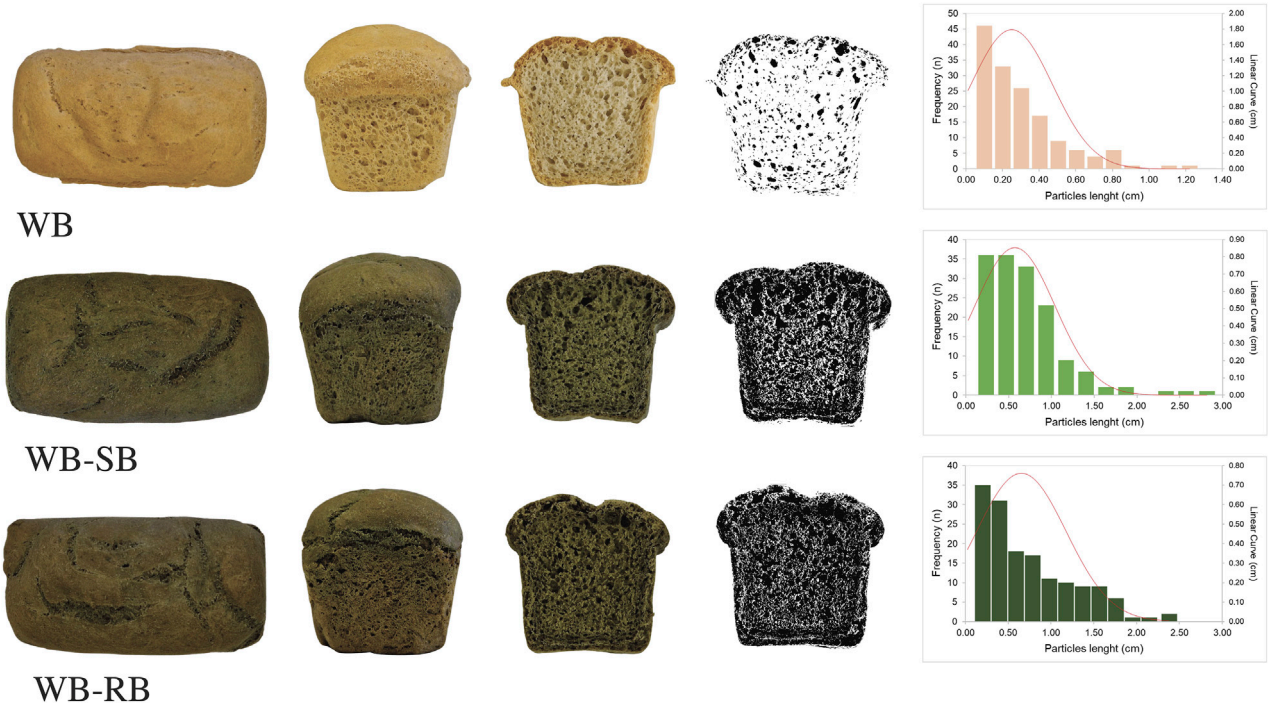


FIGURE 2
Representative images. Binary images in black and white of the region of interest (ROI) to represent the whole surface of slices of each bread loaf (72 × 72 DPI) and frequency (n) of particle length (cm) of formulated WB, WB-SB, and WB-RB samples.

moderate for WB-SB (49.17%), which can also justify the use of *Spirulina* and its counterparts to help in bread firmness during 48 h of the storage period, which was differently observed by Graça et al. (2018) during 72 h, that showed a competition for water absorption by the *C. vulgaris* in the wheat bread that should cause acceleration in the aging kinetics of bread, expressed in terms of firmness increase within short time.

Bread cohesiveness showed higher and similar results for WB and WB-SB, the same as described by Sanjari et al. (2018) with *Spirulina* powder in baguette bread; comparable results of bread cohesiveness were also found in sourdough wheat bread made by García-Segovia et al. (2017), and lower results of bread cohesiveness were found for WB-RB. After 48 h of storage, all values become similar for all samples, with a percentage of variation (PV) between 47% for WB and 50% for WB-RB, which indicates that after 48 h, bread with *Spirulina* and its counterparts needed less force to deform before it breaks when compared to WB. These changes may be due to several factors related to aging in bread hardness and cohesiveness, such as starch retrogradation, protein denaturation, decrease in moisture content, and formation of the bond between starch and protein (Sanjari et al., 2018).

In addition to the use of *Spirulina* as an ingredient in the development of functional foods, these cyanobacteria are also used as a marketing strategy or as a coloring agent due to the presence of colored compounds such as chlorophylls, carotenoids, and phycobiliproteins, which is among the top trends in the food industry these days (Lafarga et al., 2020).

As shown in Table 3, WB bread has a whiter crust and crumb ($L^*$); WB-SB and WB-RB have greener crust and crumb, although WB also showed negative values for $a^*$ WB, with SB and RB being higher; and the crust of WB is yellower than the other samples ($b^*$), but for the crumb, all samples showed yellowness with positive values for $b^*$. ΔE of WB-SB and WB-RB, when compared to WB, demonstrated that $10 < \Delta E \leq 50$; this can guarantee that the color difference is clearly perceived by human eyes, which can be seen in Figure 2. Other studies show these color differences in algae products (Batista et al., 2019; Khemiri et al., 2020; Lafarga et al., 2020).

## 3.3 Nutrition profile, phenolic compounds, and antioxidant activity of wheat bread

From the results shown in Table 4, it could be observed that the carbohydrate and ash content in WB, without the *Spirulina* counterparts, showed higher values. This may indicate that substituting 3% of wheat flour—in flour basis—for SB or RB influenced the amount of these nutrients. Ash is one of the most critical indicators of wheat flour's quality and use and consists of mineral compounds such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), and copper (Cu) (Czaja et al., 2020). Thus, the ash values are high because its main constituents, Fe and K, were around 1% and 45% in ash analysis, respectively, which were also higher in WB. Boron (B) was higher in WB than in WB-SB and WB-RB.

The total proteins of SB and RB raw materials, in dry weight, used in this study are 57.54 (±0.24) and 66.58 (±0.39) g/100 of dried samples, respectively. The result for SB agrees with that found in the

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**TABLE 4** Macronutrients (g/100 g of dried sample), micronutrients, and heavy metals (mg/100 g of dried sample) of control wheat bread (WB) formulated bread with 3% of *Spirulina* biomass (WB-SB) and 3% of residual biomass (WB-RB). The results are demonstrated as average ± standard deviation.

| Components | | WB | WB-S | WB-RB |
|---|---|---|---|---|
| Macronutrients | Carbohydrates (g) | $57.30^{a} \pm 0.29$ | $55.06^{b} \pm 0.17$ | $54.60^{b} \pm 0.21$ |
| | Proteins (g) | $13.73^{b} \pm 0.36$ | $14.96^{a} \pm 0.16$ | $15.50^{a} \pm 0.04$ |
| | Ash (g) | $3.11^{a} \pm 0.10$ | $2.74^{b} \pm 0.06$ | $2.66^{b} \pm 0.14$ |
| | Lipids (g) | $0.27^{b} \pm 0.04$ | $0.52^{a} \pm 0.09$ | $0.15^{b} \pm 0.12$ |
| Micronutrients and heavy metals | Na (mg) | $28.57^{b} \pm 3.41$ | $28.06^{b} \pm 1.99$ | $100.43^{a} \pm 6.30$ |
| | K (mg) | $178.85^{a} \pm 8.92$ | $139.59^{b} \pm 12.08$ | $136.73^{b} \pm 6.59$ |
| | Ca (mg) | $6.04^{a} \pm 0.58$ | $7.98^{a} \pm 1.55$ | $8.72^{a} \pm 2.82$ |
| | Mg (mg) | $5.75^{a} \pm 0.51$ | $4.82^{a} \pm 0.45$ | $4.87^{a} \pm 0.32$ |
| | P (mg) | $40.84^{b} \pm 3.95$ | $36.08^{b} \pm 2.53$ | $88.76^{a} \pm 5.95$ |
| | S (mg) | $31.34^{a} \pm 1.59$ | $31.27^{a} \pm 1.65$ | $33.27^{a} \pm 1.65$ |
| | Fe (mg) | $2.20^{a} \pm 0.12$ | $1.75^{b} \pm 0.16$ | $1.81^{b} \pm 0.05$ |
| | Cu (mg) | $0.43^{a} \pm 0.03$ | $0.38^{b} \pm 0.01$ | $0.39^{ab} \pm 0.01$ |
| | Zn (mg) | $0.43^{a} \pm 0.03$ | $0.33^{b} \pm 0.03$ | $0.46^{a} \pm 0.03$ |
| | Mn (mg) | $0.13^{a} \pm 0.01$ | $0.11^{b} \pm 0.01$ | $0.12^{ab} \pm 0.00$ |
| | B (mg) | $0.23^{a} \pm 0.02$ | $0.14^{b} \pm 0.02$ | $0.10^{c} \pm 0.02$ |
| | Cr (mg) | $0.32^{a} \pm 0.04$ | $0.27^{a} \pm 0.02$ | $0.28^{a} \pm 0.01$ |
| | Ni (mg) | $0.18^{a} \pm 0.01$ | $0.15^{a} \pm 0.02$ | $0.16^{a} \pm 0.01$ |
| | Cd (mg) | $0.01^{a} \pm 0.00$ | $0.02^{a} \pm 0.00$ | $0.01^{a} \pm 0.00$ |
| | Pb (mg) | $0.23^{a} \pm 0.01$ | $0.22^{a} \pm 0.01$ | $0.22^{a} \pm 0.01$ |

Values followed by different superscript letters in each row are significantly different ($p < 0.05$), determined by one-way ANOVA and Tukey's post-hoc test (STATISTICA 8.0).

literature (ranging from 17% to 73%, more commonly up to 60%, but with high nutritional variability, requiring further optimization of cultivation and post-processing conditions) (Muys et al., 2019). The proximate composition of RB was not found in the literature, but the increase of protein in this raw material could be explained by the dry matter concentration, even without C-PC phycobiliproteins after extraction. For this reason, WB-SB and WB-RB showed higher values of proteins than WB. The lipid content of SB ranges between 9% and 17% (Muys et al., 2019), and in wheat flour, it ranges between 0.1% and 2.1% (Prabhasankar and Rao, 1999); hence, WB-SB showed higher lipid values than WB and WB-RB. The C-PC extraction possibly influenced the lipid content in RB, which could explain the lower lipid values in bread.

Na and P contents are higher in the WB-RB sample, which could not be explained by Na and P values of raw materials that showed 1333.38 mg and 1120.39 mg of Na in SB and RB, respectively, and 963.18 mg and 664.51 mg of P in SB and RB respectively. Cu, Zn, and Mn were higher in WB and WB-RB samples. One of the hypotheses for WB mineral higher levels is the concentration of minerals, due to greater water loss in WB. None of the bread samples showed relevant heavy metals such as Ni, Cd, and Pb values.

FRAP analysis (Table 5) showed higher results in WB-SB and WB-RB. DPPH showed higher values in WB-SB. However, the analysis of total phenolic compounds showed the highest values in WB-RB. The lowest values for antioxidant analysis through FRAP and DPPH radicals and phenolic compounds were found in WB; therefore, 3% of SB and RB in substituting wheat flour for wheat bread development can improve the antioxidant capacity and phenolic compounds. This can be explained because the antioxidant properties of *Spirulina* are attributed to molecules such as phycocyanin, β-carotene, tocopherol, γ-linolenic acid, and phenolic compounds (Kumar et al., 2022), that showed inhibitory activity against lipid peroxidation radical scavenging and metal chelating potential (Bermejo et al., 2008).

*Spirulina* is a photosynthetic organism and, like all photosynthetic organisms, has developed defense mechanisms to protect the chloroplast from reactive oxygen species. Despite *Spirulina* having low concentrations of phenolic compounds, there are still some in its cellular matrix; few studies have recently investigated the role of phenolic compounds in algae and microalgae (Kepekçi and Saygideger, 2012). Even in low concentrations, the phenolic compounds were higher in WB-SB and WB-RB than in WB, which could also enhance antioxidant capacity.

## 3.4 Image analysis of wheat bread with or without *Spirulina* counterparts

It was possible to develop WB by adding 3% of SB or its counterpart, RB, as seen in the WB images in Figure 2, where we can also see the binary images of the WB samples with

**TABLE 5 Total phenolic content and antioxidant activity of three formulated wheat breads with (WB-SB and WB-RB) or without (WB) *Spirulina* counterparts. The results are shown as average (± standard deviation) made with 1 g of dry material bread samples.**

| Analysis | | Formulated bread | | |
|---|---|---|---|---|
| | | WB | WB-SB | WB-RB |
| Total phenolic content (mg GAE) | | $27.70^{c}$ ± 0.00 | $51.70^{b}$ ± 0.00 | $66.30^{a}$ ± 0.00 |
| Antioxidant activity methods (μM TE/g) | DPPH | $3.28^{c}$ ± 0.01 | $36.32^{a}$ ± 0.04 | $34.91^{b}$ ± 0.09 |
| | FRAP | $2.06^{b}$ ± 0.02 | $7.49^{a}$ ± 0.10 | $7.38^{a}$ ± 0.19 |

Values followed by different letters in each row are significantly different ($p < 0.05$), determined using one-way ANOVA and Tukey's post-hoc test (STATISTICA 8.0).

measurements of the alveolar cell structure ($n = 150$) of each loaf. It was possible to establish that the particle length of WB-RB, in general, and the average size was larger (0.65 cm ± 0.52), showing a maximum and minimum length of 2.38 cm and 0.05 cm, respectively. WB-SB showed similar measurements to WB-RS, with an average size of 0.57 cm ± 0.47, a maximum length of 2.82 cm, and a minimum length of 0.02 cm. WB had the smallest measurements, with an average of 0.57 cm ± 0.47, a maximum size of 1.22 cm, and a minimum size of 0.01.

In addition, as described by Rahimi et al. (2020), dough rheological characteristics and gas cell stability are the two factors that are responsible for impacting the baking process and bread quality. Gluten protein in wheat flour helps generate a strong dough network that retains the incorporated air, prevents the product from collapsing, and maintains a nice porous structure. The pore size homogeneity delivers a product with an appealing texture, appearance, and other quality characteristics. The main ingredients incorporated in developing bakery products are flour, water, and chemical or biological leavening ingredients, and the gas cells are occluded as small nuclei in the dough (Rahimi et al., 2020). SB and RB helped maintain the bread structure.

## 3.5 *In vitro* digestibility of bread and raw material for bread production

*In vitro* digestibility, also known as bioaccessibility, as described by Demarco et al. (2022), is related to the digestive transformations in food through the use of enzymes to understand the maximum fraction from food matrix release into the digestive tract to measure the intestinal absorption and pre-systematic metabolism of food. The method used in this paper to analyze the protein bioaccessibility in WB samples, INFOGEST static *in vitro* simulation, is the most reliable and standardized protocol for bioaccessibility analysis in food samples (Minekus et al., 2014). Therefore, more studies are needed to comprehend microalgae digestibility in food products (Demarco et al., 2022).

In Figure 3, it can be seen that protein *in vitro* digestibility (IVD) in dry matter was similar in all WB samples (an average of 94%), but it was lower in raw materials SB and RB (an average of 57%). After digestion, the values of IVD (%) were similar in all WB samples (WB, WB-SB, and WB-RB), with an average of 81% lower than in dry matter. This also happened in raw materials (SB and RB), representing an average IVD (%) of 56% with no differences

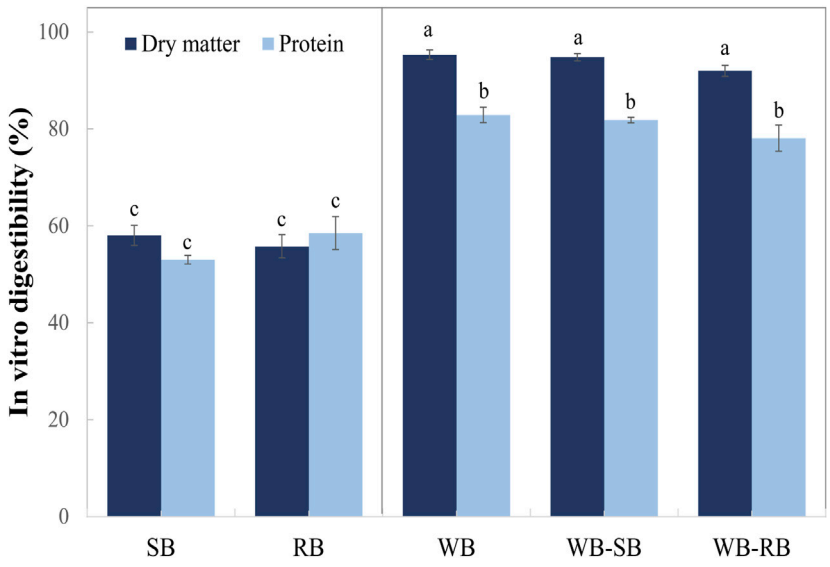


FIGURE 3
*In vitro* digestibility (%) of *Spirulina* microalgae fractions (*Spirulina* biomass—SB and residual biomass—RB) and wheat bread with (WB-SB and WB-RB) or without (WB) Spirulina counterparts. The colored bars are the average, and the error bars are the standard deviation. Different letters in each sample indicate significant differences between samples by ANOVA and Tukey's post-hoc test ($p < 0.05$).

between SB and RB. Some studies have demonstrated the digestibility of proteins in *Spirulina* food products, as cited in the review by Demarco et al. (2022), such as chocolate biscuits (86%—with 1% of *Spirulina* biomass), snacks (89%—with 2.6% of *Spirulina* biomass), and bread wheat pasta (showed an increase in protein digestibility; the authors did not show the percentage).

*Spirulina* biomass showed a protein digestibility of 82% in other studies, higher than that observed in our study (Demarco et al., 2022). Moreover, none of the studies showed similar results to wheat bread with *Spirulina* counterparts, but as shown in this study with WB samples, no differences were found between microalgae cookies and the control (IVD 87%–95%) (Batista et al., 2017). The nature of the formulated product can explain these results. The pre-treatment in *Spirulina* biomass may facilitate cell disruption, and the cooking techniques could help the protein digestibility of microalgae (Demarco et al., 2022).

It should be emphasized that introducing *Spirulina* and its counterparts in recipes requiring algae addition could be easier in human diet acceptance. The replacement of wheat flour by *Spirulina* fractions (SB and RB) does not decrease the protein digestibility, which is an interesting finding in this study, so even the RB (residual biomass of *Spirulina*) is digestible, but not yet appreciated.

## 4 Conclusion

SB and RB can be applied as innovative ingredients to replace 3% of wheat flour in wheat bread development. It can be noted that both raw materials from microalgae affected the technological aspects of WB in a positive way, mainly in the rheological parameters of the dough and quality properties of WB. SB and RB enhanced the nutritional profile, with the highest values of proteins, some minerals, and phenolic compounds in WB-BS and WB-RS compared to the control WB, improving the antioxidant capacity. Heavy metals (Ni, Cd, and Pb) were not found in any of the samples (values <0.2 mg/100 g of dried samples).

Moreover, the *in vitro* protein digestibility was better in WB than in the raw materials (SB and RB), which indicates that the bread-making process may help with protein digestibility. However, no difference was found in the digestibility of bread with or without the addition of 3% of SB or RB, which indicates that RB is digestible, but has not yet been valued. Therefore, the authors expected this work to draw researchers' attention to RB, an unexplored ingredient. In addition, SB needs greater appreciation for sustainable food practices worldwide and in Brazilian exploration, requiring management strategies involving the industry and society working together. It is essential to highlight that further studies focusing on acceptability are needed to understand the viability of these ingredients in targeting consumers' preferences.

## Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## Author contributions

CF: Data curation, Methodology, Writing–original draft, Investigation, and Writing–review and editing. MN: Data curation, Writing–original draft, Writing–review and editing, and Investigation. VD: Conceptualization, Methodology, Visualization, and Writing–review and editing. AR: Data curation, Supervision, Validation, Writing–original draft, and Writing–review and editing. AB: Conceptualization, Investigation, Methodology, Supervision, Visualization, and Writing–review and editing.

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## Conflict of interest

The authors CN, AR and ARCB declared that they were editorial board members of Frontiers at the time of submission. This had no impact on the peer review process and the final decision.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# High-throughput screening of natural compounds for prophage induction in controlling pathogenic bacteria in food

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**Introduction:** The clean label trend emphasizes the need for natural approaches to combat pathogenic bacteria in food. This study explores the potential of inducing prophages within bacterial genomes as a novel strategy to control pathogenic and spoilage bacterial growth.

**Methods:** A luminescence-based high-throughput assay was developed to identify natural compounds capable of inducing prophages. Bioactive compounds from four chemical libraries were screened at a final concentration of 10 μM. The assay measured luminescence production in *Escherichia coli* BR513, a genetically modified strain producing β-galactosidase upon prophage λ induction. Luminescence values were normalized to cell concentration (OD600) and the interquartile mean of each 384-well plate. A cut-off for normalized luminescence values, set at 2.25 standard deviations above the mean, defined positive prophage induction.

**Results:** Four naturally-derived compounds (osthol, roccellic acid, galanginee, and sclareol) exhibited positive prophage induction, along with previously identified inducers, rosemary, and gallic acid. Dose-response experiments were conducted to determine optimal concentrations for prophage induction. However, the results could not distinguish between prophage-induced cell death and other mechanisms, making it challenging to identify ideal concentrations.

**Discussion:** The high-throughput luminescent prophage induction assay serves as a valuable tool for the initial screening of natural bioactive compounds that have the potential to enhance food safety and quality by inducing prophages. Further research is required to understand the mechanism of bacterial cell death and to establish optimal concentrations for prophage induction in a food preservation context.

KEYWORDS

**clean label trend, sustainable approaches to food safety, natural compounds, prophage inducers, food safety**

# 1 Introduction

In developed countries, consumers are increasingly demanding that foods only contain natural and easily recognizable ingredients (Asioli et al., 2017). This consumer demand, known as the clean label trend, is largely driven by an increasing awareness of the potential toxicity of certain synthetic food additives (Carocho et al., 2014). As such, consumers tend to choose additive-free foods or foods containing natural additives over products that contain synthetic additives (Carocho et al., 2014). The food industry is thus shifting toward the use of naturally-sourced antimicrobials as replacements for commonly-used synthetic compounds, such as benzoates, sorbates, propionates, nitrites, and parabens (Carocho et al., 2015) that have traditionally been used to control the growth of foodborne bacterial pathogens (Emerton and Choi, 2008; Garcia et al., 2008).

The food industry's transition to the strict use of naturally-sourced additives should not compromise food safety. Natural compounds from plants, animals, and microorganisms are known to have antimicrobial properties (Carocho et al., 2015). Varying mechanisms for the antimicrobial action of natural compounds have been proposed, which may involve interactions with proteins, enzymes, or membrane function (Fan et al., 2018). Bacteriophages (phages) have also emerged as effective natural antimicrobials (Sillankorva et al., 2012; Quinto et al., 2019) that can be added to foods to eliminate foodborne bacterial pathogens. Virulent phages introduce their DNA into cells of the target bacterial pathogens to produce progeny phage that ultimately lyse the host cells, in what is termed the lytic cycle (Salmond and Fineran, 2015; Moye et al., 2018). A number of commercialized phage products are employed to reduce the presence of bacterial pathogens in foods (Moye et al., 2018).

One limitation of the current phage control approach is the fact that due to the limits of phage host range, only a single bacterial species can be targeted at a single time. This limits applications of phage control in the food industry. For example, the use of phages to control bacterial spoilage is impractical because many different bacterial species are typically involved in the spoilage of a food (Odeyemi et al., 2020). Induction of temperate phages is an alternative phage-based antimicrobial mechanism of action (MOA) that was recently proposed for controlling bacterial pathogens on foods (Cadieux et al., 2018). Temperate phages are viruses of bacteria that infect their hosts by integrating their DNA into the bacterial host chromosome and immediately enter a lysogenic life cycle, in which no harm is inflicted on the host bacterial cell (Feiner et al., 2015). Various environmental stresses trigger the chromosomally-embedded phage (prophage) to enter into the lytic cycle, in which the phage DNA excises from the bacterial DNA and forms progeny that eventually lyse the host cell (Salmond and Fineran, 2015). Activation of the lytic cycle, known as prophage induction, can be initiated by the addition of bioactive compounds, which could be used as an approach for eliminating bacterial pathogens in food (Cadieux et al., 2018), since the majority of bacteria possess prophages (Kang et al., 2017).

It is well known that certain antibiotics induce prophages of foodborne bacterial pathogens (Cone et al., 1976; Raya and H'Bert E, 2009; McDonald et al., 2010); however, fewer natural compounds have been shown to induce prophages. We previously demonstrated that various teas, coffee, gallic acid, rosemary and cranberry juice are prophage inducers of prophage λ (Tompkins et al., 2018). These natural compounds have been described in the literature as having antimicrobial activity against bacterial pathogens (Côté et al., 2011; Nieto et al., 2018), and our results suggest that the MOA of the compounds is through the induction of prophages in the target bacteria.

In this work, a high-throughput luminescent prophage induction assay was modified in order to render it amenable to automated systems so that thousands of known bioactive compounds from chemical libraries could be screened. The high-throughput luminescent prophage induction assay serves as a preliminary screen to identify natural compounds that could be used to induce prophages from foodborne pathogenic and spoilage bacteria, thereby improving the safety and quality of foods using sustainable approaches.

# 2 Materials and methods

## 2.1 Bacterial strain

The assay used in this work is based on the use of the genetically engineered *E. coli* BR513 (ATCC 33312). *E. coli* BR513 carries a *lacZ*-prophage λ gene fusion, and cleavage of the lambdoid phage repressor, CI, results in the synthesis of β-galactosidase (Elespuru and Yarmolinsky, 1979). The cleavage of the CI repressor, and thus prophage induction, may be caused by treatments that cause DNA damage. β-galactosidase produced by *E. coli* BR513 can be measured using luminescent substrates and taken as an indication of prophage λ induction. *E. coli* BR513 was stored at −80°C in 20% glycerol and cells were revived by streaking a loopful of frozen stock onto tryptic soy agar (TSA). Plates were incubated at 37°C for 18–24 h.

## 2.2 Compounds tested in the assay

Compounds from the Centre for Microbial Chemical Biology (CMCB, McMaster University, Hamilton, Ontario, Canada) bioactives collection were screened for their ability to induce prophage λ using the luminescent high-throughput prophage induction assay. The CMCB bioactives collection (Table 1) contains 3,747 compounds from four vendor libraries, including the Prestwick Chemical Library, the BIOMOL2865 Natural Products Library, the Lopac1280 (International Version) Library, and the Spectrum Collection, and comprises Food and Drug Administration (FDA)-approved drugs, off-patent drugs, natural products, and other compounds with demonstrated biological activity (Miller et al., 2009; Davenport et al., 2014; Torres et al., 2016).

## 2.3 High-throughput luminescent prophage induction assay

The high-throughput luminescent prophage induction assay was adapted for use with an automated robotic system at the CMCB. To begin the assay, duplicate cultures of *E. coli*

**TABLE 1** The Centre for microbial chemical biology bioactives collection consisting of four vendor libraries was used in the high-throughput luminescent prophage induction assay. *The total number does not include duplicate compounds.

| Library | Source | Number of compounds | Average molecular weight (g/mol) |
|---|---|---|---|
| Prestwick Chemical Library | Prestwick Chemical, Illkirch-Graffenstaden, France | 1,120 | 372 |
| BIOMOL2865 Natural Products Library | Enzo Life Sciences Inc., Farmingdale, New York, United States | 502 | 401 |
| Lopac1280 (International Version) Library | Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada | 1,280 | 337 |
| Spectrum Collection | MicroSource Discovery Systems Inc., Gaylordsville, Connecticut, United States | 2,000 | 354 |
| | Total* | 3,747 | |

BR513 were grown overnight in trypticase soy broth (TSB) supplemented with 0.2 M glucose (TSB 0.2 M glucose) at 37°C with shaking. The next morning, cells were diluted in fresh TSB 0.2 M glucose to an optical density ($OD_{600}$) of 0.1. The cultures were grown at 37°C with shaking at 250 RPM until the $OD_{600}$ reached 0.5. A Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to add 50 µL of culture to the wells of 384-well plates. Next, 0.5 µL of each test compound from the CMCB bioactives library (Table 1), having been dissolved in dimethyl sulfoxide (DMSO), were added to the cultures using a Biomek $FX^P$ liquid handler (Beckman Coulter, Indianapolis, Indiana, United States) to achieve a final concentration of 10 µM. Plates were incubated statically at 37°C for 24 h. Positive and negative controls were included in each 384-well plate. Streptonigrin (final concentration of 2 µg/mL) was added to appropriate wells as a positive control and DMSO was used as the negative control.

The $OD_{600}$ was measured following the 24-h incubation period, followed by measurement of luminescence. For this, 50 µL of the Gal-Screen β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific) were added to each well. This assay system includes a lysis buffer and the β-galactosidase substrate, Galacton-Star®, which are used in a single-step reaction that emits light proportional to the amount of the lactose-degrading enzyme. Luminescence readings were measured 5 min after the addition of the Gal-Screen β-Galactosidase Reporter Gene Assay System using an Envision plate reader (Perkin Elmer, Waltham, Massachusetts, United States).

## 2.4 Data analysis

Relative light units (RLUs) generated by the *E. coli* BR513 cultures incubated with the respective compounds were normalized to the $OD_{600}$ to account for any effects of cell concentration on β-galactosidase production. Additionally, the $OD_{600}$-normalized RLU results were further normalized to the interquartile mean to account for variation between plates. For this, the $OD_{600}$-normalized RLU from each well were divided by the interquartile mean of the corresponding 384-well plate (excluding controls). The interquartile mean is defined as the mean of the middle 50% of the rank-ordered data (Mangat et al., 2014). All bioactive compounds for which both replicates had a value greater than 2.25 standard deviations from the mean of treated *E. coli* BR513 samples were considered positive for prophage induction, as described by the following equation:

$$\frac{RLU/OD}{\mu_{iq}} > 2.25\ standard\ deviations_{mean}$$

Where; *RLU/OD* is the $OD_{600}$-normalized RLU, $\mu_{iq}$ is the interquartile mean.

## 2.5 Dose response experiments

Dose response experiments were performed to investigate the effect of compound concentration on prophage induction capacity and to verify whether naturally-derived compounds with inconclusive results (i.e., compounds for which only one replicate generated a normalized RLU value above the cut-off) were prophage-inducing agents. These compounds included osthol, sclareol, galangine, and roccellic acid. In addition to generating RLU vales above the cutoff in one of the two replicates, these compounds were also included in the dose response experiments because they all have antimicrobial activity (Choudhary et al., 2006; Cushnie and Lam, 2006; Sweidan et al., 2017; Tan et al., 2017), and we hypothesized that the mode of action of these compounds at the appropriate dose could be explained by prophage induction and subsequent cell death. Rosemary and gallic acid were also included in the dose response experiments because they were previously identified as being prophage inducers. Since they are also natural food-grade compounds, they were included in the dose response screen so they could be directly compared to the compounds selected from the high-throughput luminescent prophage induction assay.

The response to varying doses of selected compounds (Table 2) was evaluated by incubating duplicate cultures of *E. coli* BR513 with 11 concentrations (half-log dilutions of the preceding concentration) of each compound following the methods for the high-throughput luminescent prophage induction as described in Section 2.3. The highest tested concentration of each compound varied between 2 and 100 µg/mL. Streptonigrin, ciprofloxacin, chloramphenicol, and ampicillin (dissolved in DMSO) were also included in the dose response experiments as positive controls since they have been described in the literature as being prophage-inducing antibiotics (Levine and Borthwick, 1963; Goerke et al., 2006; Maiques et al., 2006).

**TABLE 2** Prophage λ inducers, based on results from the high-throughput luminescent prophage induction assay. *Naturally-derived compounds.

| Antibiotics | Antifungals | Antiseptics |
|---|---|---|
| Chlortetracycline hydrochloride | Bithionate sodium | Alexidine hydrochloride |
| Demeclocycline hydrochloride | Dichlorophene | Hexachlorophene |
| Doxycycline hyclate | Econazole nitrate | |
| Doxycycline hydrochloride | Miconazole nitrate | **Miscellaneous compounds** |
| Meclocycline sulfosalicylate | Niclosamide | *α-mangostin |
| Minocycline hydrochloride | Pentachlorophenol | Biochanin A |
| Minocycline hydrochloride | Phenylmercuric acetate | Bromoacetyl alprenolol menthane |
| Oxytetracycline | Salinomycin sodium | Calmidazolium chloride |
| Tetracycline hydrochloride | Sulconazole nitrate | CGP-7930 |
| Chloramphenicol | | CP-55940 |
| Florfenicol | **Antiparasitics** | Enoxolone |
| Thiamphenicol | Bithionate sodium | Fiduxosin hydrochloride |
| Colistimethate sodium | Dichlorophene | GBR-12909 dihydrochloride |
| Colistin sulfate | Niclosamide | L-162,313 |
| Polymyxin B sulfate | Ronnel | NNC 55-0396 |
| Fusidic acid sodium | Triclabendazole | Ononetin |
| Lasalocid sodium | | PQ-401 |
| Lasalocid sodium salt | **Antipsychotics** | *Tschimganidin |
| Monensin | Fluspirilene | Tyramine |
| Mupirocin | Pimozide | Tyrphostin A9 |
| Nigericin | Penfluridol | Tyrphostin Ag 879 |
| Tyrothricin | | 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1h-pyrazole |
| | **Antibacterials** | |
| | Chloroxine | |
| | Triclosan | |

# 3 Results

## 3.1 The high-throughput luminescent prophage induction assay identified 61 prophage-inducing agents

The entire CMCB bioactives collection, consisting of 3,747 compounds was screened using the high-throughput luminescent prophage induction assay to identify natural compounds that could be used in the induction of prophages from bacterial pathogens and spoilage bacteria that contaminate foods. Compounds were considered positive hits for prophage induction when both replicate signals were greater than the respective cut-offs. The hit cut-offs, calculated as 2.25 standard deviations from the mean, were 2.7 and 2.6 for replicates 1 and 2, respectively. Several structurally-related compounds were identified (Table 2; Figure 1), indicating that certain chemical classes contribute to prophage induction activity. Sixty-one bioactive compounds were identified as potential prophage-inducing agents, including 22 antibiotics, 9 antifungals, 5 antiparasitics, 3 antipsychotics, 2 antibacterials, 2 antiseptics, and 18 miscellaneous compounds, two of which were of natural origin (α-mangostin and tschimganidin). In addition to the positive hits, there were 22 compounds that had inconclusive results (Table 3), which included 5 antibiotics, 2 antifungals, 1 antiparasitic, 3 antibacterials, 1 antiseptic, and 10 miscellaneous compounds. Inconclusive, or non-replicating hits, were those for which only one replicate signal surpassed its cut-off. Six of the non-replicating hits were natural compounds that are derived from plants and lichens, including osthol, roccellic acid, galanginee, sclareol, leoidin, and xanthone (data not shown). The results suggest that similarities in chemical structure amongst the identified compounds may nevertheless indicate which chemical moieties may be responsible for prophage λ induction.

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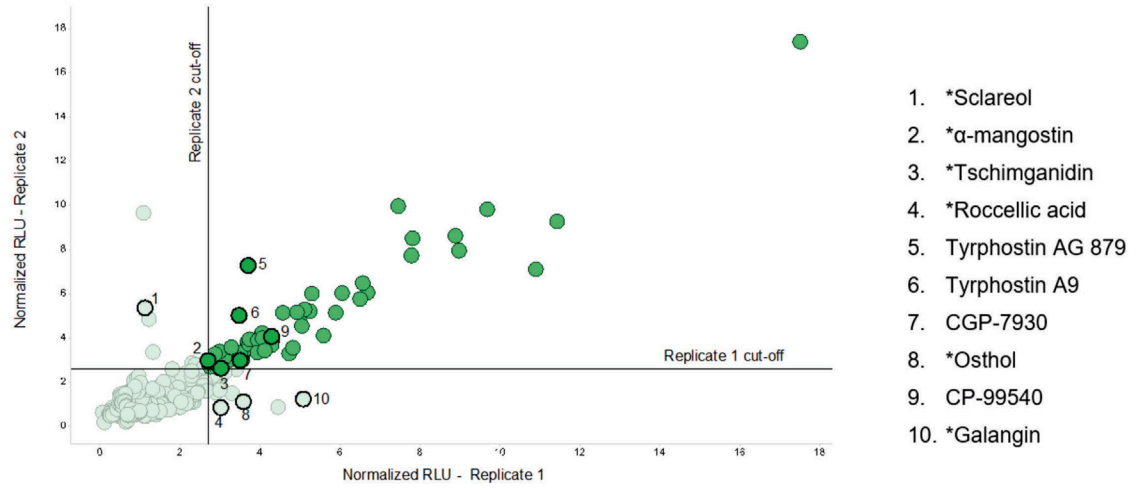


FIGURE 1
Replicate plot showing the normalized RLU signal in the prophage indicator strain *Escherichia coli* BR513 resulting from the addition of each compound tested in the high-throughput luminescent prophage induction assay. For each compound, two replicates were conducted. The *x*-axis represents the signal from replicate 1 and the *y*-axis represents that of replicate 2. RLU values were normalized to $OD_{600}$ and the interquartile mean, and those in solid green represent positive hits, or compounds that had normalized RLU values greater than 2.25 standard deviations of the mean of each replicate. The numbered points outlined in solid black highlight positive and inconclusive hits that were further tested in the dose response experiments. *Naturally-derived compounds.

**TABLE 3 Compounds identified as inconclusive hits in the high-throughput luminescent prophage induction assay. *Naturally-derived compounds.**

| Antibiotics | Antiseptic |
|---|---|
| Calcymicin | Methylbenzethonium chloride |
| Chloramphenicol hemisuccinate | |
| Monensin sodium | **Miscellaneous compounds** |
| Nonactin | *Galanginee |
| Diphenyleneiodonium chloride | *Leoidin |
| | Lynestrenol |
| **Antifungals** | Perhexiline maleate |
| Butoconazole nitrate | *Roccellic acid |
| Miconazole | *Sclareol |
| | Tamoxifen citrate |
| **Antiparasitic** | Thapsgargin |
| Ivermectin | *Xanthone |
| | 7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-day]pyrimidin-4-ylamine |
| **Antibacterials** | |
| Narasin | |
| *Osthol | |
| Salinomycin | |

## 3.2 Dose response

A subset of positive hits from the high-throughput assay was selected for evaluation in dose response experiments. The selection included compounds with structural similarities, as well as compounds that were natural in origin, since they could have potential for use in clean label food applications. For example, two tyrphostins and CGP-7930 all contain a di-tert-butyl phenol group and were thus evaluated and compared. Four of the inconclusive hits that were compounds of natural origin (osthol,

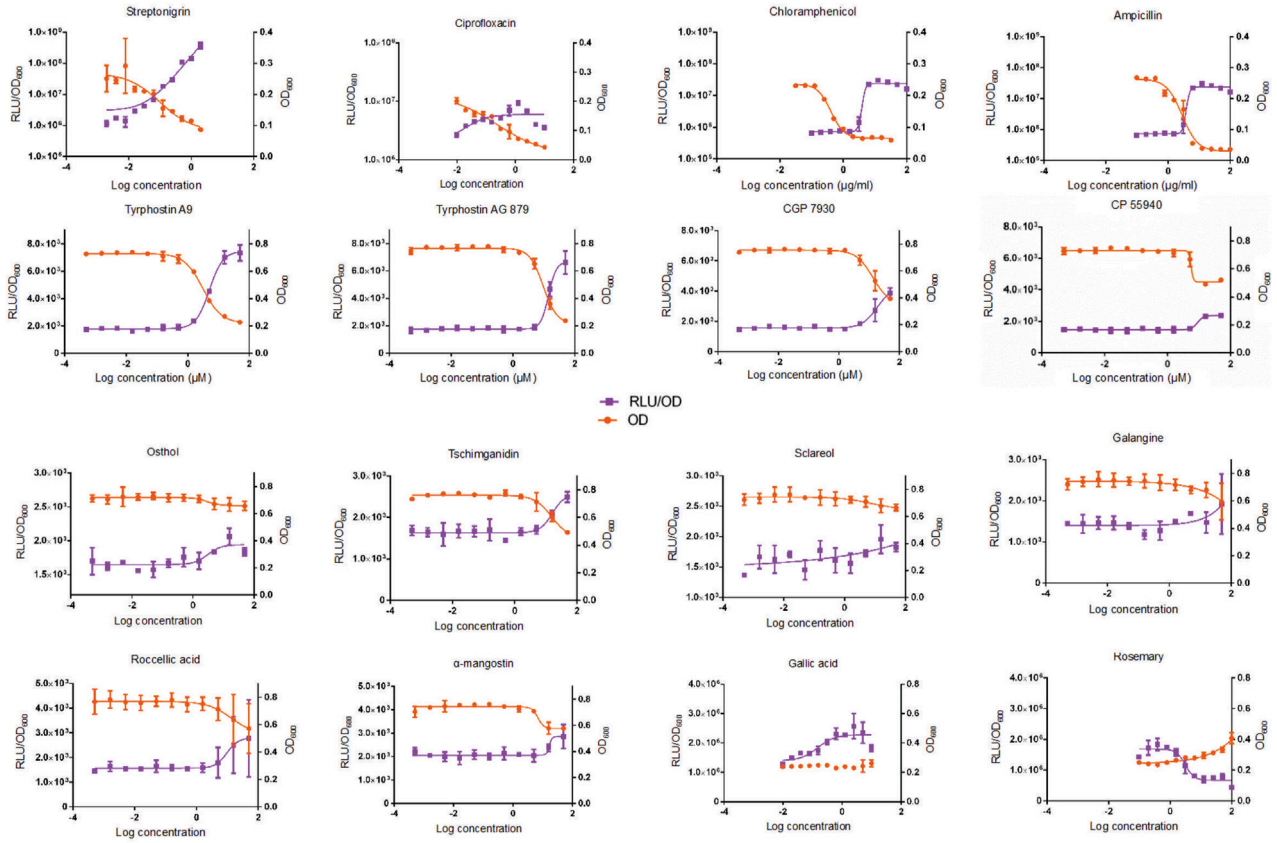


FIGURE 2
The average response in RLU/$OD_{600}$ and in $OD_{600}$ in *Escherichia coli* BR513 after 24-h exposure to varying concentrations of bioactive compounds from the Centre for Microbial Chemical Biology (CMCB, McMaster University, Hamilton, Ontario, Canada) bioactives collection, four antibiotics that were used as positive controls (top row) and two compounds (gallic acid and rosemary) that were previously shown to be prophage inducers. The purple squares represent values that correspond to RLU/$OD_{600}$ values, while the orange squares represent values that correspond to $OD_{600}$. Error bars represent standard deviations of the duplicate cultures that were incubated in the dose response experiments. Compounds in the bottom two rows are naturally-derived.

roccellic acid, galanginee, and sclareol) were also included to confirm whether they were indeed capable of inducing prophage λ. Tschimganidin and α-mangostin were selected from the positive hits since they are naturally-derived compounds, whereas streptonigrin, ciprofloxacin, chloramphenicol and ampicillin were used as positive controls since they have all been described as agents that can induce prophages (Levine and Borthwick, 1963; Goerke et al., 2006; Maiques et al., 2006).

The dose response curves (Figure 2) confirmed the four natural compound inconclusive hits as being prophage λ-inducing agents, since the 10 μM concentration, as used in the initial screen, generated a normalized RLU response greater than the 2.25 standard deviation cut-off for both replicates. For most of the compounds included in the dose response experiments, the $OD_{600}$-normalized RLU increased and the $OD_{600}$ decreased as the concentration of bioactive compounds increased. This was the case for streptonigrin, indicating that a higher concentration resulted in higher production of β-Galactosidase due to the induction of prophage λ. The only compound that did not generate a dose response in RLU/$OD_{600}$ was rosemary, which was included in the dose response experiments because it is a known antimicrobial compound and was previously shown to induce prophage λ. Gallic acid, an organic acid naturally found in teas, was the only other compound that did not cause a significant response in $OD_{600}$, suggesting that a higher concentration may be required to cause cell death.

## 4 Discussion

In this study, a high-throughput luminescent prophage induction assay was developed to identify compounds capable of inducing prophages as a way to inhibit the growth and survival of pathogenic and spoilage bacteria that contaminate foods. The use of automated equipment improved the efficiency of the assay, and allowed for rapid screening of thousands of bioactive compounds. The compounds that resulted in the highest normalized luminescent signals were considered positive hits for prophage induction. Data analysis involved normalization to the interquartile mean to reduce variation between plates, as systematic error is inherent in high-throughput screening (HTS) data (Mangat et al., 2014). Luminescence was measured using the Gal-Screen β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific) because this system was specifically designed for HTS

applications. The dose response experiments were conducted to confirm the prophage inducing capacity of inconclusive hits, as well as two natural compounds (gallic acid and rosemary) that were selected based on previous results.

Most of the compounds identified by the assay are not suitable for use in foods, however some natural compounds were positive hits. The most abundant class of compounds identified by the assay were antibiotics, but antiseptics, antifungals, antiparasitics, antipsychotics, antibacterials, antiseptics and miscellaneous drugs were also identified as prophage inducers. Although not all the bioactive compounds identified in the screen may be used in foods, the results are still useful because they identify which types of compounds and/or which chemical moieties may possess prophage inducing characteristics. The notion that chemical structure may be correlated with the ability to induce prophages is supported by the fact that three compounds identified as positive hits in the current screen, tyrphostin A9, tyrphostin AG 879, and CGP-7930, contain a common chemical moiety: a di-tert-butyl phenol group. In total, twenty tyrphostin analogues were screened in the assay, but the only two tyrphostins (A9 and AG879) identified as positive hits contain di-tert-butyl phenol groups. Although these positive hits are not natural compounds, the fact that they were positive hits suggest that naturally-derived compounds with similar chemical structure may be useful as suitable food additives for the control of the growth of bacterial pathogens by means of prophage induction. For example, 2,4-di-tert-butylphenol (DTBP) is an antioxidant that may be extracted from seeds, fruits and sweet potato and can be produced as a fermentation product of certain lactic acid bacteria (Choi, et al., 2013; Varsha et al., 2015). It has been shown to exhibit antioxidant and antifungal activity, thus demonstrating its potential as a natural food additive. (Varsha et al., 2015). Contrasting these results, other compounds that contain a di-tert-butyl phenol group were not observed to induce prophages. The widely-used synthetic phenolic antioxidant, butylated hydroxytoluene (BHT), also contains a di-tert-butyl phenol group. BHT is used as a preservative in foods and in food packaging and acts as an antioxidant as well as an antimicrobial (Ayaz et al., 1980), but its antimicrobial activity has not yet been linked to prophage induction. In this study, we did not observe prophage induction during incubation with BHT. As an antioxidant BHT likely binds free radicals, thereby reducing DNA damage, which is a major cause of prophage induction (Nanda et al., 2014).

The synthetic cannabinoid, CP-55940, was also identified as a positive hit in the high-throughput luminescent prophage induction assay. CP-55940 was developed by Pfizer, Inc. in the 1970s but was never marketed. CP-55940 shares similar structural features and mimics the euphoric effects of the naturally occurring cannabinoid, tetrahydrocannabinol (THC) (Debruyne and Le Boisselier, 2015). Since the two compounds exhibit similar toxicological effects and are structurally similar, the naturally-occurring cannabinoids, THC and cannabidiol (CBD), could be screened for their ability to induce prophages. Cannabis became legal in Canada with the assent of the Cannabis Act in June 2018 (Cannabis Act, 2018). Cannabis edibles became legal in 2019, and a number of foods and drinks that have been infused with cannabis may be legally sold. Therefore, naturally-occurring cannabinoids that are structurally similar to CP-55940 may serve as antimicrobials to reduce the presence of bacterial foodborne pathogens in cannabis food products.

Two natural compounds that originate from plants within the *Umbelliferae* or *Apiaceae* family were identified as positive hits. A terpenoid, tschimganidin was identified in the assay, and a coumarin derivative, osthol, was an inconclusive hit that was subsequently confirmed as a prophage-inducing agent in the dose response experiment. Tschimganidin and osthol may be extracted from the roots of *Ferula tschimganica* and *Ferula campestris*, respectively (Kadyrov et al., 1972; Basile et al., 2009). Both compounds are also found in other umbellifers, but have a common source: root resin of *Ferula persica* (Kerminov, 1992). The plant may therefore represent an important source for the extraction of effective natural prophage-inducing compounds.

Tan and others (2017) elucidated the structures of osthol and nine other prenylated coumarins by spectroscopic methods and direct comparison with reference compounds, and showed that the root extract and its prenylated coumarins exhibited antimicrobial activity against clinically relevant bacteria including the foodborne pathogens *Bacillus subtilis* and *Klebsiella pneumoniae* at concentrations between 5 and 125 μg/mL. Interestingly, green teas contain coumarins (Yang et al., 2009), and our group identified various green tea as potent inducers of prophage λ (Tompkins et al., 2018). We previously identified gallic acid as a strong natural prophage inducer. However, in this study, gallic acid was not identified as a positive hit. The reason for the discrepancy is likely due to the fact that different concentrations of gallic acid were used in the different studies. For example, the 10 μM concentration used in the current study is equivalent to 1.7 μg/mL, while the concentration used in our earlier work was 10 μg/mL. These results suggest that the prophage induction activity of gallic acid may be dose-dependent, and gallic acid was thus included in the dose response experiment, in which an increase in RLU/OD was observed with increasing concentration. Increased concentration of gallic acid did not however, significantly affect the $OD_{600}$. Higher concentrations should be tested to confirm whether gallic acid is able to cause cell death. Gallic acid is a component of green tea, meaning that green teas may represent an important group of food-grade ingredients that should be the focus of future prophage induction studies.

The fact that gallic acid is a known antimicrobial (Borges et al., 2013) with DNA-damaging properties (Hossain et al., 2013) elucidates, at least partially, its effectiveness as a prophage inducer, as DNA damage is the first step in the predominant mechanism of prophage induction. DNA damage, whether caused by exposure to UV irradiation or treatment with a specific compound, triggers the SOS response in bacterial cells, thus initiating the production of the RecA protein, in turn causing autoproteolytic cleavage of prophage repressors that are responsible for the lysogenic state (Nanda et al., 2014). The cleavage of phage repressors thus allows the prophage to excise from the bacterial host chromosome and enter the lytic cycle (Sassanfar and Roberts, 1990; Campoy et al., 2006).

Mitomycin C, streptonigrin and norfloxacin are antibiotics that are known to activate the recA-dependent pathway and prophage induction (Levine and Borthwick, 1963; Raya and H'Bert E, 2009; McDonald et al., 2010). These compounds were also included in the current screen as part of the BIOMOL 2865 Natural Products Library and the Prestwick Chemical Library. However, in this work they were not identified as positive hits as the normalized

RLUs for streptonigrin, mitomycin C and norfloxacin did not surpass the required cut-offs to be considered a positive result. Earlier, we confirmed each antibiotic as being a prophage-inducing compound (Tompkins et al., 2018). The concentration used in the earlier work was 2 μg/mL for each antibiotic, while in the current screen, the concentrations used were 5.1 μg/mL, 3.6 μg/mL, and 4.4 μg/mL for streptonigrin, mitomycin C and norfloxacin, respectively. Since the concentrations of the three antibiotics used in the current screen were higher than the previous work, it was expected that all three compounds would be identified as prophage inducing compounds. False negative results may be attributed to the age of the chemical stocks used in the bioactives library, as freezing and thawing, as well as hydration of compounds stored in DMSO over time may diminish compound concentration, solubility, and potency (Kozikowski et al., 2003). This is supported by the fact that the streptonigrin used as a positive control and in the dose response experiment was prepared as a fresh stock and resulted in a positive hit for prophage induction.

Dose-response experiments are used to determine the response of an organism, to a stimulus or stressor, following exposure for a set exposure time (Peleg, 2021). In this study, "dose" is defined as the quantity of a compound that results in prophage induction. Determining the dose response, and developing dose–response models, is typically used to assess the concentrations of drugs required to inhibit or kill microorganisms. Dose–response data can be visualized by dose–response curves, which relate the magnitude of a dose (stimulus or stressor) to the response of a biological system. The applied dose is usually plotted on the *X*-axis and the biological response is plotted on the *Y*-axis (Hamilton et al., 1977; Altshuler, 1981).

In this study, the dose response experiment demonstrated that prophage inducing compounds inhibited growth of the indicator strain, *E. coli* BR513, as previously reported (Elespuru and Yarmolinsky 1979), but could not distinguish between the causes of cell death. Since RLU values were normalized to $OD_{600}$, the responses in normalized RLU could have been caused by cell death as a result of other direct mechanisms. Although certain compounds at sublethal concentrations induce prophages and lyse the host cell from the inside, additional mechanisms could also cause cell death when a given compound is used at higher concentrations. For example, chloramphenicol, which was identified as a positive hit, is known to generally inhibit protein synthesis (Shinagawa et al., 1977), suggesting it could inhibit the production of repressor proteins that keep prophages in their lysogenic state, thus causing induction of the lytic cycle. This may be the case when used at sublethal levels, but at higher concentrations, chloramphenicol kills bacterial cells by inhibition of protein synthesis required for cell wall formation, which is an additional pathway to cell death that does not involve phages (Schwarz et al., 2016.

In order to determine whether differing concentrations of a respective compound kill bacterial cells through prophage induction or another mechanism, the dose response experiment will need to be modified by including a negative control strain that does not carry prophages. In this scenario, if the compound kills bacterial cells through prophage induction, we would expect that the OD of the prophage negative control strain would not decrease, as there would be no prophages to induce and subsequently lyse the cell. Future experiments aimed at further studying the prophage inducing activities of the natural compounds we identified will include a prophage negative *E. coli* strain.

Overall, the naturally-derived compounds that were included in the dose response experiments did not exhibit as marked of a response as the synthetic compounds, which parallels the results from the initial screen. Sclareol, roccellic acid, osthol, and galangine were all inconclusive hits in the high-throughput luminescent prophage induction assay, thus their bacterial killing capacity was expected to be less potent than the synthetically-derived compounds included in the screen. While these compounds were confirmed to be positive for bacterial inhibition in the dose response experiments, they should be considered as hits of lower intensity, since their normalized RLU values were closer to the positive cut-off values (data not shown). Since these plant-derived and lichen-derived (roccellic acid) bioactive compounds generally demonstrated the expected profile and are known to exhibit antimicrobial properties (Cushnie et al., 2003; Hayet et al., 2007; Sweidan et al., 2017; Tan et al., 2017), they should be considered for use in future prophage induction screens.

The dose response curves for osthol, α-mangostin, and CP-55940 reached a plateau in both $RLU/OD_{600}$ and $OD_{600}$ signals, suggesting that these compounds elicited a maximum response, or that higher concentrations would have no significant effect on inhibition of cell growth or ability to induce prophages. In contrast, the dose response for rosemary demonstrated the opposite effect: as the normalized $RLU/OD_{600}$ values decreased the $OD_{600}$ values increased. This is due to the fact that the signals were likely skewed due to the colour of the rosemary extract powder used for its preparation. The $OD_{600}$ and the $RLU/OD_{600}$ results in the dose response experiments for rosemary should thus not be considered accurate, as an increased concentration of the pigmented solution could have caused an increase in $OD_{600}$ as well as a decrease in the luminescent signal.

In conclusion, several libraries of bioactive compounds were screened for capacity to induce prophage λ, as indicated by the production of β-galactosidase in the indicator organism *E. coli* BR513. This work identified several compounds that could be used to induce prophages in foodborne bacterial pathogens and spoilage bacteria. The naturally-derived prophage-inducing compounds identified in this work, as well as other naturally-derived compounds with chemical structures similar to that of the synthetically-derived positive hits should be further tested for their ability to induce prophages, as a sustainable approach to improvement of food safety and quality.

## Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## Author contributions

ET conducted experiments, analyzed data, and developed the initial manuscript draft. BC provided advice on experimental design, and analyzed data. LG provided conceptualization of the study,

experimental resources, funding acquisition, project administration, parts of initial manuscript draft, significant review and editing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Enhancing food safety and cultivated meat production: exploring the impact of microplastics on fish muscle cell proliferation and differentiation

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Cultivated meat, a sustainable alternative to traditional livestock farming, has gained attention for its potential environmental and health benefits. However, concerns about microplastic contamination pose challenges, especially when sourcing cells from marine organisms prone to microplastic bioaccumulation. Additionally, the pervasive presence of microplastics in laboratory settings, ingredients, and during the production, increases the risk of unintentional contamination. This study focused on Atlantic mackerel (*Scomber scombrus*) skeletal muscle cell lines to examine the effects of microplastic exposure, represented by fluorescent polyethylene microspheres (10–45 µm) on cell performance including cell proliferation, cell viability, gene expression, and differentiation processes critical for cultivated meat production. The results revealed significant impacts on cell attachment and proliferation at microplastic concentrations of 1 µg/mL, 10 µg/mL, and 50 µg/mL. Notably, the 10 µg/mL concentration exerted the most pronounced effects on cell viability during both attachment and proliferation phases. While the results indicated that both microplastic concentration and size influence cell viability, cell differentiation remained unaffected, and additional contributing factors require further investigation. These findings underscore the necessity of thoroughly exploring microplastic-cell interactions to ensure food safety and safeguard health within the burgeoning cultivated meat industry.

KEYWORDS

**cultivated meat, cell-based seafood, microplastics, cell growth, gene expression**

## 1 Introduction

Cultivated meat, derived from the cultivation of animal cells, presents an innovative shift in food production with potential environmental and health benefits (Rischer et al., 2020; Eibl et al., 2021; Dupuis et al., 2023; Jahir et al., 2023). Produced within controlled environments, this approach not only minimizes the risks associated with conventional farming contaminants but also promises a more resource-efficient methodology (Stephens et al., 2018). Recent studies indicate that, with renewable energy integration, cultivated meat could achieve up reductions of global warming by 92%, air pollution by 93%, land use by 95%, and water consumption by 78% compared to traditional beef farming (Vergeer et al.,

2021; Kim et al., 2022; Sinke et al., 2023). As the industry advances, cultivated meat is projected to command a substantial portion of the $1.7 trillion conventional meat and seafood market, addressing pressing challenges like deforestation, biodiversity loss, and antibiotic resistance (Vergeer et al., 2021; Sinke et al., 2023).

In controlled laboratory environments, cultivated meat is produced from cells, such as those from animals. These cells undergo proliferation in specialized growth mediums to form muscle tissue, representing a potentially safer, more ethical, and environmentally sustainable alternative to conventional meat production (Chriki and Hocquette, 2020; Ong et al., 2021). However, a potential safety concern in this innovation is the contamination of microplastics. One avenue of potential contamination arises from the source animals. Marine ecosystems, for instance, are known reservoirs of microplastics (Andrady, 2011; Cole et al., 2011; Ivar do Sul and Costa, 2014). This results in bioaccumulation within marine life, such as fish and oysters (Galloway et al., 2017; Sharma and Chatterjee, 2017; Bhuyan, 2022; Courtene-Jones et al., 2022). When such marine organisms serve as the source animals for cell extraction, undetected microplastics could be inadvertently introduced into the cultivation process. Existing analytical methodologies often fail to detect smaller microplastic particles, leading to potential underestimations of their abundance in source organisms (Huppertsberg and Knepper, 2018; Lv et al., 2021; Vivekanand et al., 2021; Adhikari et al., 2022). Another significant source of contamination is the laboratory environment itself. Studies have underscored the pervasive nature of microplastics in laboratory settings, emanating from the degradation of ubiquitous plastic equipment, containers, and consumables (Löder et al., 2017; Schymanski et al., 2018; Koelmans et al., 2019). The production process of cultivated meat necessitates the use of various plastic-based apparatus, including bioreactors, pipettes, cell culture flasks, and other equipment that come in direct contact with the medium and growing cells (Allan et al., 2019; Lee et al., 2022).

Microplastics, tiny fragments of plastic less than 5 mm in size, have garnered significant attention due to their ubiquity in the environment and the potential risks they pose to human health (Lim, 2021; Diamantidou et al., 2022; Leslie et al., 2022; Lwanga et al., 2022; Osman et al., 2023; Tsochatzis et al., 2023). Upon ingested, these particles can traverse the gastrointestinal tract, and some evidence suggests that smaller micro- and nanoplastic particles may even penetrate tissues, entering the circulatory and lymphatic systems (Campanale et al., 2020; Hirt and Body-Malapel, 2020; Jiang et al., 2020; Kannan & Vimalkumar, 2021; Yee et al., 2021; Diamantidou et al., 2022; Leslie et al., 2022; Fournier et al., 2023; Li et al., 2023; Ramsperger et al., 2023; Tsochatzis et al., 2023). These fragments can act as carriers for various toxicants, including heavy metals, polycyclic aromatic hydrocarbons, and endocrine-disrupting chemicals (Campanale et al., 2020; Abbasi et al., 2021; Amelia et al., 2021; Yee et al., 2021; Karla Lizzeth et al., 2023), thereby introducing these harmful agents into the human body. From a cellular perspective, the risks of microplastics become more intricate. The direct interaction between cells and microplastics can lead to physical disruptions, such as membrane damage (Fleury and Baulin, 2021; Dai et al., 2022; Wang et al., 2022), and chemicals inherent to or leached from these plastics are known to induce oxidative stress, inflammatory responses, and genotoxic effects (Hirt and Body-Malapel, 2020; Goodman et al., 2021; Alqahtani et al., 2023; Cao et al., 2023; Herrala et al., 2023; Jeyavani et al., 2023; Mattioda et al., 2023). Potential risks of such interactions encompass DNA lesions, organ dysfunctions, metabolic irregularities, immunological aberrations, neurotoxicity, and perturbations in reproductive and developmental processes (Galluzzi et al., 2018). Furthermore, previous research has indicated a potential link between microplastic exposure and the development or exacerbation of certain chronic diseases such as cardiovascular diseases including thrombosis, atherosclerosis, cancer, and diabetes (Lee et al., 2023; Wu et al., 2023). Given the documented adverse effects of microplastics upon ingestion, understanding and mitigating these risks is paramount for the cultivated meat industry (EFSA Panel on Contaminants in the Food Chain CONTAM, 2016; Rubio-Armendáriz et al., 2022; Mamun et al., 2023; Ziani et al., 2023).

While the presence and potential hazards of microplastics are increasingly acknowledged, understanding the exact mechanisms by which they influence cellular functions remains a critical research frontier (O'Neill and Lawler, 2021; Thornton Hampton et al., 2022). To understand the cellular impacts of microplastic exposure more comprehensively, we utilized Atlantic mackerel (*Scomber scombrus*) skeletal muscle cell lines, previously established and characterized by Saad et al. (2023), given their relevance to cultivated meat processing. This study employed fluorescent polyethylene microspheres (10–45 μm) as representative microplastics, a size range previously documented in fish (Thiele et al., 2021; Makhdoumi et al., 2023). We aimed to elucidate the effects of microplastics on cellular performance, emphasizing cell viability during the attachment and growth phases, as well as cell differentiation, which are pivotal processes in cultivated meat production (O'Neill et al., 2021; Reiss et al., 2021). The study employed microplastic concentrations of 1 μg/mL, 10 μg/mL, and 50 μg/mL. Preliminary results indicated that all treatments significantly affected cell attachment (on day 2) and proliferation (on day 4), with no discernible effects on cell differentiation after 2 weeks. Variables such as microplastic size and concentration potentially influenced these outcomes. These findings, albeit initial, provide foundational insights for subsequent research, emphasizing the importance of understanding microplastic-cell interactions for ensuring food safety, protecting human health, and mitigating environmental impacts.

## 2 Materials and methods

### 2.1 Cell lines preparation and maintenance

The mackerel cell lines (MACK2) used in this study were obtained from Dr. David L. Kaplan Lab at Tufts University. The cell preparation followed the protocol described in Saad et al. (2023). Briefly, frozen cells at passage 81 were thawed using 9 mL of complete growth medium, which comprised Leivovitz's L-15 medium (Gibco™, Billings, MT, United States) and supplemented with 20% fetal bovine serum (FBS, Gibco™, Billings, MT, United States), 1 ng/mL FGF2-G3 (human) growth factor (Defined Bioscience, San Diego, CA, United States), 20 mM

HEPES (Gibco™, Billings, MT, United States), and 1% Antibiotic–Antimycotic (Gibco™, Billings, MT, United States). The cell suspension was then centrifuged at 500 RCF for 6 min, and the resulting pellet was resuspended in 10 mL of growth medium. The cells were incubated in a 75 $cm^2$ culture flask (Thermo Fisher Scientific, Waltham, MA, United States) at 27°C in a $CO_2$ free incubator. Maintenance of the cells involved regular passaging at approximately 70% confluency and seeding at a density of approximately 5000 cells/$cm^2$. Alternatively, cells were stored by freezing in growth medium supplemented with 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, US).

## 2.2 Microplastic preparation and exposure

Fluorescent green polyethylene microspheres obtained from *Cospheric LLC* (Goleta, CA, United States) were used in this study. The microspheres exhibited a size range of 10–45 µm. Prior to experiments, a sterilization process was undertaken using 91% isopropyl alcohol (IPA), allowing excess fluid to drain as the spheres gradually underwent evaporation. Subsequently, the sterilized microspheres were integrated into the completed growth medium supplemented with 0.01% Tween 20. Microspheres were introduced into the experimental setup at concentrations of 1, 10, and 50 µg/mL based on previous studies (Schirinzi et al., 2017; Hwang et al., 2020; Palaniappan et al., 2022). An experimental control consisting of the complete growth medium with 0.01% Tween 20 but devoid of microspheres was included in the study. To achieve a consistent distribution of the microspheres within the growth medium, a pre-experimental step involved subjecting the microsphere medium to sonication prior to each experiment.

## 2.3 Effects of microplastic on cell attachment and viability

Mackerel cells were seeded in triplicate in the 6-well plates at a seeding density of approximately 5000 cells/$cm^2$. The cells were cultured at a constant temperature of 27°C and devoid of $CO_2$. To investigate the influence of microplastics on different stages of cell growth, two distinct experimental conditions were employed. In the first scenario, microplastics were incorporated into the cell medium prior to seeding to assess their influence on cell attachment, a crucial initial step in cell proliferation. After 48 h of incubation, cells reached the logarithmic growth phase and were detached from the plate surface using 0.25% trypsin–EDTA (Thermo Fisher Scientific, Waltham, MA, United States). The cell viability was evaluated using the Trypan Blue Assay and the Countess 3 FL Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, United States). This assay exploits differential cellular uptake of Trypan Blue dye to discern and enumerate viable *versus* non-viable cells. Instrument parameters, including counting thresholds and dye dilution ratios, were set in accordance with the manufacturer's specifications to ensure accuracy and reproducibility of the viability metrics, In the second scenario, microplastics were introduced to the cell medium after the logarithmic growth phase was attained, with the old medium replaced by either microplastic-containing or control fresh medium. Subsequent to the introduction of microplastics, the cells remained undisturbed for a period of 4 days, allowing for the exploration of potential interactions between microplastics and the cells. After this interaction period, the cells were detached, and the viable cell count was determined, offering insights into the effect of microplastics on cell growth after the initial proliferation stages.

TABLE 1 Oligonucleotides used for RT-qPCR of mackerel cells.

| Primer | Sequence (5′–3′) |
|---|---|
| HPRT fwd | GTCTACGTTGACAGGCAAGAATGT |
| HPRT rev | GTCTGGTCGGTAGCCAACACT |
| MYOD1 fwd | TTGGAGCACTACAGCGGGGA |
| MYOD1 rev | GCTGGTGTCGGTACTGATCCG |
| MYOG fwd | GGAGCACCCTGATGAACCCC |
| MYOG rev | CGCTTGACGACGACACTCTGG |
| TNNT3A fwd | TCAGCGCGGTAAGTTTGCAG |
| TNNT3A rev | CTCCTCTTCTACGGCCTCGACA |

## 2.4 Effects of microplastic on cell differentiation

### 2.4.1 RT-qPCR gene expression analysis

Mackerel cells at passage 82 were detached from 6-well plates using trypsin for 3–4 min and pelleted by a 7.5 min centrifugation at 500 RCF. RNA was extracted from samples using the NucleoSpin RNA kit (Mackerey-Nagel, Dueren, Germany) and quantified with a Qubit 4 Fluorometer using the Qubit RNA High Sensitivity (HS) assay kit (Thermo Fisher Scientific, Waltham, MA, United States). cDNA libraries for each sample were constructed from 100 ng of RNA using the PrimeScript RT master mix (Takara Bio, Kutatsu, Japan) under manufacturer specifications. A minus reverse transcriptase control (-RT) was made from the sample exhibiting the higher RNA yield by not adding the master mix to the sample. The PrimeScript RT master mix contains both random hexamers and oligo dT primers. RT-qPCR was performed in a CFX Opus 96 thermocycler (Bio-Rad, Hercules, CA, United States) using the TB Green Advantage qPCR premix (Takara Bio, Kutatsu, Japan) and the oligonucleotides specified in Table 1. Primers were designed using a reference genome for southern bluefin tuna (Thunnus maccoyii; NBCI RefSeq GCF_910596095.1) by Saad et al. (2023). Three technical replicates were performed per sample and gene, as well as for the—RT and no template controls (NTC). Amplification conditions were as follows: initial step of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C, and a final dissociation analysis of 15 s at 65°C and 0.5°C/s increases until reaching 95°C. Absolute gene expression values were calculated as $2^{(-\Delta Ct)}$ using the hypoxanthine guanine phosphoribosyltransferase (HPRT) gene as the housekeeping gene. Relative gene expression values were calculated as $2^{(-\Delta\Delta Ct)}$ over the 0 ng/mL microplastics control.

### 2.4.2 Immunostaining

To observe the influence of microplastics on cell differentiation, immunostaining for myosin heavy chain was conducted following the protocol outlined in Saad et al. (2023). Mackerel cells were cultured at 100% confluency in growth medium and exposed to microplastics at varying concentrations, with subsequent observation over a 14-day differentiation period. Following culture, cells were fixed with 4% paraformaldehyde at room temperature for 30 min (Thermo Fisher Scientific, Waltham, MA, United States). Subsequently, the cells were rinsed with Phosphate Buffered Saline (PBS, Sigma Aldrich, Burlington, MA, United States) and permeabilized for 10 min using 0.1% Triton-X (Sigma Aldrich, Burlington, MA, United States). Subsequent to permeabilization, the cells were blocked for 30 min using 1 × blocking buffer (Abcam, Cambridge, United Kingdom), followed by an additional PBS wash. The primary antibody solution, MF-20 (4 µg/mL), was applied to the cells and allowed to incubate overnight at 4°C. After a subsequent PBS wash, the cells underwent an additional 30-min blocking step using 1 × blocking buffer and were then incubated for 1 h with secondary antibodies—Goat Anti-Mouse IgG H&L (Alexa Fluor® 594, Abcam, Cambridge, United Kingdom), and Phalloidin-iFluor 488 Reagent (Abcam, Cambridge, United Kingdom)—each diluted at 1:1000 in 1 × blocking buffer. After a final wash with PBS, the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/mL, Thermo Fisher Scientific, Waltham, MA, United States) in PBS for 15 min at room temperature. Imaging was conducted using a fluorescence microscope (DP27, Olympus Life Science, Tokyo, Japan) equipped with an LED Illumination system (CoolLED, Andover, United Kingdom). Multiple objective lenses (4x, 10x, 20x) were utilized to evaluate the cell morphology and structure at various levels of detail. Images taken with 4x and 10x lenses were used for general orientation and lower-resolution overviews, while the 20x lens was employed for detailed visualization necessary for the conclusions.

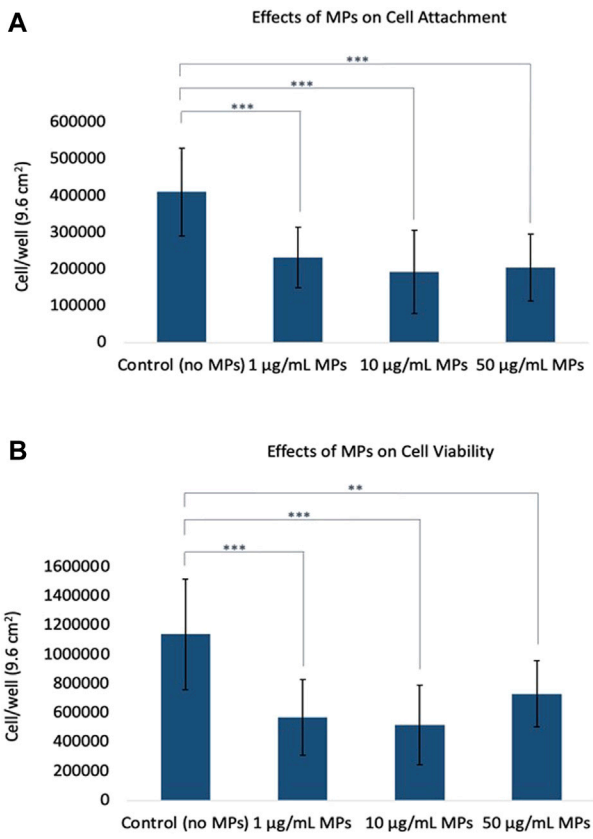


FIGURE 1
Effects of microplastics on cell viability. **(A)** Influence of microplastics on initial cell attachment when introduced to the cell medium prior to seeding. **(B)** Impact of microplastics on cell proliferation after cells reached the logarithmic growth phase. Error bars represent standard deviation. The *t*-test was used to evaluate the differences between each MPs treatment with experimental control; asterisks indicate a significant difference at $p < 0.01$ (**) and $p < 0.001$ (***).

## 2.5 Statistical analysis

Statistical analysis was conducted using One-way ANOVA, with a *t*-test utilized for comparisons between two parameters using Minitab Statistical Software.

# 3 Results and discussion

## 3.1 Cell viability

The viability of cells during both the attachment and growth phases is paramount to the cultivation of meat. In the attachment phase, cells must successfully anchor to a scaffold or matrix to prevent their loss during media changes, establishing a robust foundation for subsequent stages. Following successful attachment, it is imperative for these cells to proliferate efficiently, ensuring an adequate cellular population for muscle tissue formation. Any significant cell mortality or diminished proliferation during these phases could undermine the entire production yield and efficiency (Allan et al., 2019; Bodiou et al., 2020). In order to investigate the effects of microplastics on mackerel cell viability at attachment and growth phases, microplastics were introduced to cells at different times in this study. Figure 1A highlighted the outcomes of microplastics on initial cell attachment when introduced into the cell medium before seeding, and statistical analysis revealed a highly significant difference in cell counts between the groups (ANOVA, $p < 0.001$). Conversely, Figure 1B depicted the impact of microplastics on cell proliferation when added after the cells had reached the logarithmic growth phase, also demonstrated statistically significant alterations in proliferation rates (ANOVA, $p < 0.001$). The initial number of cells for seeding was ~5000 cells/cm$^2$, and the surface area of the 6-well is 9.6 cm$^2$. Data collection occurred on Day 2 for Figure 1A and Day 4 for Figure 1B, corresponding with a marked increase in cell death and detachment. In Figure 1A, the experimental control group (no microplastic) showed a cell count of 410,000, whereas microplastic-treated cells evidenced a decline to approximately half this number. Similarly, Figure 1B displayed a control cell count of 1,100,000, with those treated with microplastics showing a range from 510,000 to 730,000. Notably, the presence of microplastics led to a decrease in cell counts, with the 10 µg/mL

concentration consistently showing the lowest viability. The underlying causes and potential mechanisms responsible for this trend are explored in detail in the subsequent sections.

### 3.1.1 Concentration effects on cell viability

This study elucidates the intricate dynamics of microplastic (MP) exposure and its impact on the viability of mackerel cells, revealing a potentially non-linear dose-response relationship. Methodologically, the cells underwent exposure to MPs at concentrations of 1, 10, and 50 μg/mL. Remarkably, the most pronounced decrease in cell viability was observed at the intermediate concentration (10 μg/mL), challenging the conventional dose-response paradigm that posits increased toxicity with higher concentrations. This was evidenced by the cell viability trends where, for Figure 1A, the order was control >1 μg/mL > 50 μg/mL > 10 μg/mL, and for Figure 1B, control >50 μg/mL > 1 μg/mL > 10 μg/mL. These findings contrast with several prior studies where a direct dose-dependent toxicity of MPs was reported. For instance, Palaniappan et al. (2022) performed an investigation involving L929 murine fibroblasts and MDCK epithelial cell lines and noted a dose-dependent decrease in cell viability when exposed to 1, 10, or 20 μg/mL of PE or PS microspheres. Furthermore, their study highlighted amplified oxidative stress at higher MP doses, as evidenced by increased SOD3 gene expression. In another study (Lee et al., 2021), human umbilical vein endothelial cells (HUVECs) were exposed to polystyrene microplastics (PS-MPs, 0–100 μg/mL), revealing that higher doses markedly reduced cell viability and disrupted angiogenic tube formation in the short term, while inducing autophagic and necrotic cell death after prolonged exposure. Besides, in a distinct investigation focusing on the human intestinal milieu (Herrala et al., 2023), researchers assessed the toxicological ramifications of ultra-high molecular-weight polyethylene particles (250–1,000 μg/mL) on human colorectal adenocarcinoma Caco-2 and HT-29 cells. A 48-h exposure to these polyethylene particles precipitated a dose-dependent decline in cell viability and a concomitant upsurge in oxidative stress. The oxidative damage was particularly pronounced in the mitochondria, illuminating the broader health concerns.

The intricate interplay between MP concentration and its potential cytotoxic effects is evident. While a substantial proportion of literature supports a dose-dependent decrease in cell viability, certain exceptions persist. In a recent *in vitro* study examining the impact of microplastics (PVC and PE) on gilthead seabream and European sea bass head-kidney leucocytes (HKLs) (Espinosa et al., 2018), it was observed that exposure to varying concentrations of microplastics for 1 and 24 h did not significantly affect HKL cell viability. Additionally, high doses of microplastics resulted in minimal changes to key cellular innate immune functions, including a decrease in phagocytosis and an elevation in respiratory burst activity. These divergent findings underline the significance of further investigations, accounting for microplastic type, size, and the specific cellular environment, to draw conclusive inferences on the broader impacts of MPs on cellular health. Our findings contribute to this evolving narrative, suggesting that the interaction between MPs and biological systems may be more complex than previously understood.

### 3.1.2 Influence of microplastic size and aggregation

In addition to concentration, we observed that the size and aggregation state of MPs played a pivotal role in mediating their interaction with mackerel cells. Specifically, at a concentration of 10 μg/mL, MPs were prone to adhere to the cell surface, either individually or as small aggregates, leading to the most significant reduction in cell viability. Conversely, the 50 μg/mL concentration resulted in larger MP aggregates that remained suspended in the culture medium, limiting their contact with cells, as evidenced in Figure 2. This lack of cellular interaction, particularly in scenarios where MPs were introduced post-cell attachment, corresponds with the minimal impact on cell viability observed at this higher concentration (Figure 1B cell count trend: control >50 μg/mL >1 μg/mL >10 μg/mL). Therefore, the pronounced effects at 10 μg/mL highlight the potential for specific MP sizes that maintain close cellular contact to disrupt cell viability. Reflecting on the role of microplastic size and aggregation in cell viability, our results align with the emerging research that examines the complex effects of microplastics on cellular health. A systematic review assessed dose–response relationships regarding microplastics and cell viability by evaluating studies up to March 2021 (Danopoulos et al., 2022). Of the 17 studies reviewed, 8 were included in a meta-regression analysis. The review identified four MP-associated effects: cytotoxicity, immune response, oxidative stress, and barrier attributes, with genotoxicity showing no effect. Key predictors of cell death were irregular MP shape, exposure duration, and MP concentration. Notably, Caco-2 cells displayed heightened susceptibility to MPs. Concentrations as low as 10 μg/mL (5–200 μm) affected cell viability, while 20 μg/mL (0.4 μm) influenced cytokine release. These findings are in line with our observations, specifically the significant cell viability reduction at 10 μg/mL, suggesting that not only the concentration but also the physical form of MPs modulates their cytotoxicity.

In a detailed investigation by Zhang et al. (2022), the impacts of polystyrene microspheres (PS-MPs) and nanospheres (PS-NPs) were explored across four distinct sizes: 0.1, 0.5, 1, and 5 μm. This research identified a marked preference for cellular uptake among the smaller nanoparticles compared to their larger counterparts. Notably, the PS-MPs presented minimal effects on cell viability and apoptosis. However, subtle indications of oxidative stress were discernible in high-concentration groups. A striking differentiation was evident in membrane damage, with PS-MPs inducing substantially greater damage than PS-NPs, emphasizing the size-dependent cellular responses to MPs. Building on the effects of microplastic size on cells, an investigation into polystyrene (PS) particle toxicity further elucidated these intricate relationships (Hwang et al., 2020). Researchers found that PS particles act as potential immune stimulants, triggering cytokine and chemokine production in a manner determined by size and concentration. Larger PS particles (10–100 μm in diameter) exhibited negligible cytotoxicity. In contrast, smaller particles, specifically those at 460 nm and 1 μm, detrimentally affected red blood cells. Their increased surface area, which facilitates stronger interactions like van der Waals forces, was pinpointed as the cause for hemolysis. Furthermore, exposure to these smaller PS particles resulted in elevated IL-6 secretion, signifying potential early-stage inflammation. However, the study also highlighted no

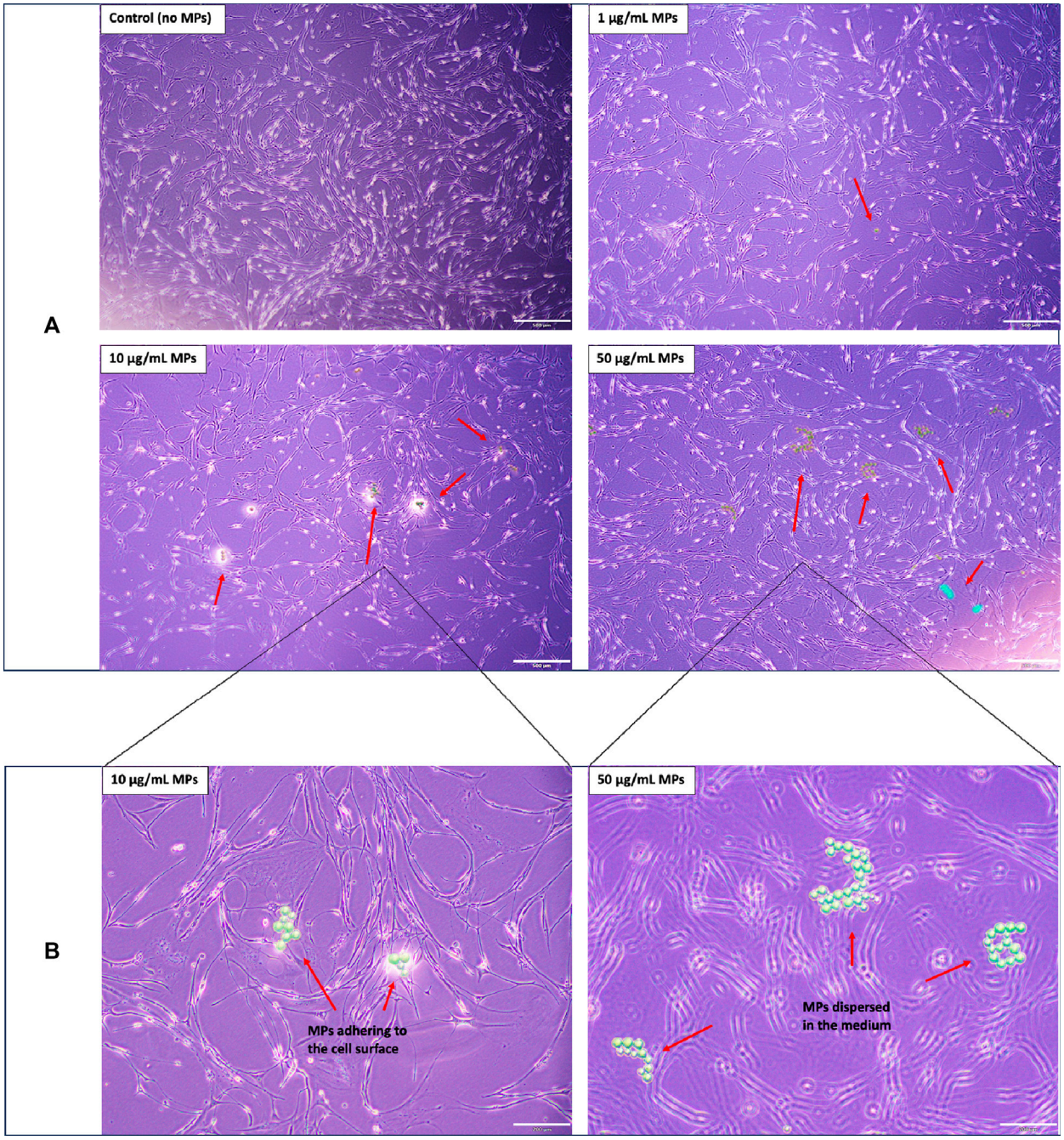


FIGURE 2
Morphological analysis of mackerel cells with and without MP treatments: **(A)** Low-magnification (4X objective) images showcasing the morphology of mackerel cells after 4 days of incubation with varying concentrations of MPs. Red arrows highlight observable MP or MP aggregates. Scale bar represents 500 µm. **(B)** High-magnification (10X objective) images providing detailed views of cell morphology for the 10 µg/mL and 50 µg/mL MP treatments, corresponding to the samples depicted in Figure 2A. Scale bar represents 200 µm.

substantial rise in histamine secretion, mitigating concerns of histamine-driven inflammation or allergic reactions. Particle uptake predominantly occurred through endocytosis and phagocytosis by phagocytic cells, leading to localized inflammation via pro-inflammatory cytokine release, rather than inducing direct cytotoxicity (Hwang et al., 2020). In addition to the examples described, other studies have consistently indicated that the size of microplastic particles significantly influences cellular interactions. Notably, smaller particles are linked to heightened cellular uptake, more pronounced inflammatory responses, increased apoptosis rates, and enhanced cellular stress responses (Wright and Kelly, 2017; Revel et al., 2018; Yong et al., 2020). These findings highlight the potential health risks associated with finer microplastic particles.

### 3.1.3 Potential interactions and other involved factors

The interaction between cells and microplastics is a multifaceted process, influenced by a confluence of factors, such as the physicochemical properties of microplastics, cellular characteristics, toxicity of the monomers, additives and oligomers in particles and prevailing environmental conditions (Revel et al., 2018; Smith et al., 2018; Campanale et al., 2020; Leslie et al., 2022). This dynamic interplay gains significance when contemplating the potential ramifications of microplastics on cellular health (Lee et al., 2023; Li et al., 2023). While this study predominantly illustrated the influence of microplastic size and dose on cell viability, it is vital to embed these insights within the expansive framework of factors modulating microplastic-cell interactions. The polymer composition (e.g., polymer type, presence of additives, and potential for the microplastics to absorb other environmental contaminants) of microplastics, for instance, is often associated with discrete cytotoxic effects (Duis and Coors, 2016; Revel et al., 2018; Hwang et al., 2020). The shape of microplastics further refines this interaction spectrum. Fibrous microplastics have been reported to potentially introduce physical harm, further affect tissues or induce blockages (Wright et al., 2013; Watts et al., 2015; Diamantidou et al., 2022; Leslie et al., 2022; Tsochatzis et al., 2023), whereas spherical microbeads, prevalent in personal care products, could facilitate a smoother cellular internalization (Wright et al., 2013). Additive blooming in polymer materials such as plasticizers, lubricants, stabilizers, antoioxidants, which are added by the manufacturer to improve the properties of the polymers, may immigrate to the surface of the polymers due to the phase separation (Nouman et al., 2017). Generally, these additives are unsubstituted amides derived from long-chain fatty acids. Reports have indicated that the cytotoxicity associated with these additives can arise from the leaching of bloomed materials in a soluble form or from direct contact between the bloomed substances and the cells (Kim et al., 2003).

Nanoscale microplastics can lead to the creation of reactive oxygen species (ROS), suggesting that they may cause stress to cells by promoting oxidative reactions (Campanale et al., 2020; Paul et al., 2020; Yee et al., 2021). Concurrent inflammatory responses may destabilize cellular homeostasis, possibly marking the onset of apoptosis (Elmore, 2007; Wright et al., 2013; Lamichhane et al., 2023). There could also be direct physical effects, such as potential damage or blockages in tissues, especially in organisms with multiple cell types (Bhagat et al., 2021; Yee et al., 2021). Certain cellular stress signals, specifically p-JNK and p-p38, emphasize that microplastics can be regarded as stress-causing agents (Jeong et al., 2016; Jeong et al., 2017; Scopetani et al., 2020). It's also worth noting that different organisms and cell types can react differently to these plastics (Jeong and Choi, 2019; Bhagat et al., 2021). Comprehending these multifaceted interactions is crucial for elucidating the effects of microplastics on cellular viability and for devising strategies to mitigate their potential adverse impacts.

## 3.2 Cell differentiation

Cell differentiation is paramount in the cultivated meat production process, serving as an indispensable determinant of the final product's organoleptic and nutritional characteristics. This involves guiding pluripotent or multipotent cells, predominantly stem cells, through specific differentiation pathways to yield the requisite specialized cell types constituting meat, such as myocytes, adipocytes, and fibroblasts (Allan et al., 2019; Zakrzewski et al., 2019; Reiss et al., 2021). The meticulous orchestration of myocyte differentiation is pivotal for the formation of myofibrils, conferring the unique texture and mouthfeel characteristic of meat (Listrat et al., 2016; Lee et al., 2021). In parallel, the directed differentiation of adipocytes is crucial for the deposition of intramuscular fat, a critical determinant of flavor profile and marbling (Li et al., 2020). Additionally, fibroblast differentiation and subsequent connective tissue formation offer essential structural integrity and have implications for meat tenderness (Purslow, 2020). Therefore, an in-depth understanding and precise control over these differentiation processes are indispensable for the optimization and scalability of cultivated meat, ensuring both its commercial viability and alignment with consumer expectations (Reiss et al., 2021; Bomkamp et al., 2023).

### 3.2.1 Differential gene expression

In this study, we assessed the impact of microplastics on muscle cell differentiation by measuring the expression levels of three key myogenic markers: MYOD1, MYOG, and TNNT3A. MYOD1 is characteristic of myogenic progenitors (myoblasts) during the early phases of muscle cell differentiation, MYOG is expressed at the myocyte stage, and TNNT3A acts as a late marker associated with skeletal muscle function. Figure 3A illustrates the absolute gene expression values for MYOD1, MYOG, and TNNT3A of mackerel cells cultivated in varying concentrations of microplastics (0, 1, 10, and 50 μg/mL). Remarkably, a heightened expression of MYOG compared to MYOD1 was observed, denoting that the cells were in a well-differentiated myocyte state. However, the absence of TNNT3A expression implies that full maturation into functioning muscle fibers had not occurred. Figures 3B,C depict the fold-change expression of MYOD1 and MYOG genes, represented as $2^{(-\Delta\Delta Ct)}$, over the control treatment with no microplastics. HPRT served as the housekeeping gene for expression normalization. Notably, a general lack of statistical difference was observed between the treatments in gene expression under varied microplastic concentrations, as calculated by one-way ANOVA. The exception was a single noteworthy difference in MYOD1 expression between 10 μg/mL and 50 μg/mL concentrations; however, this observation was tenuously supported with a $p$-value bordering on 0.05, indicating a weak statistical significance.

The observed increase in MYOG expression, coupled with the non-expression of TNNT3A, indicated a state of well-differentiated cells that had not yet reached the later stages of muscle development. This observation could be attributed to the potential onset of cellular senescence or reduced cell viability, especially considering that the mackerel cell line utilized was at passage #82, a stage at which cells often exhibit altered differentiation patterns due to accumulated genetic and epigenetic changes (Di Micco et al., 2021). Despite the variations in microplastic concentrations, the overall gene

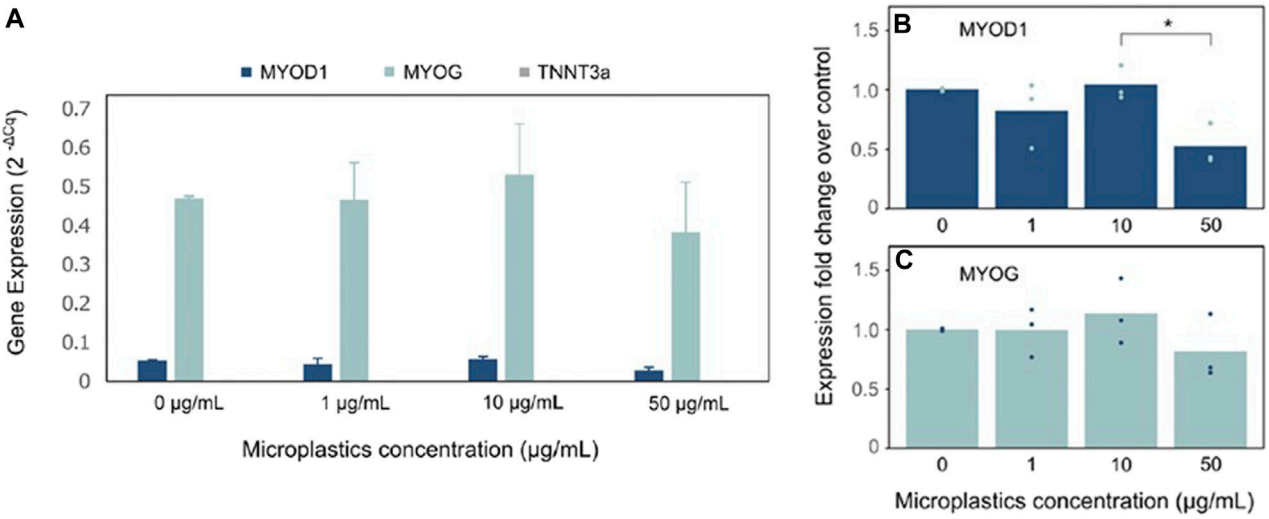


FIGURE 3
Expression of muscle differentiation gene markers in mackerel cell growing with/without MPs: **(A)** Absolute gene expression values for MYOD1, MYOG and TNNT3A of MACK cells. Gene expression is represented as 2(-ΔCt). **(B and C)** Fold-change expression of MYOD1 and MYOG genes over the control treatment. Points indicate single data points for each biological replicate and bars indicate their mean value ($n$ = 3 experimental, $n$ = 3 technical). HPRT was used as the housekeeping gene for expression normalization. Statistical significance calculated by one-way ANOVA is indicated by asterisks, in which $p < 0.05$ (*).

expression exhibited minimal statistically significant differences, emphasizing the need for further exploration into the interactions between microplastics and cell differentiation processes.

### 3.2.2 Immunocytochemical analysis

Complementary to our qPCR findings, immunocytochemical assays provided a visual confirmation of cell differentiation processes. Cells subjected to various microplastic concentrations (0, 1, 10, 50 µg/mL) over 14 days and were subsequently probed with MF20, DAPI, and Phalloidin, targeting myosin, cellular nuclei, and actin filaments, respectively. As depicted in Figure 4, each experimental condition displayed characteristic staining patterns across the trio of molecular markers. Notably, elongated structures positive for myosin, specifically stained by MF20, were evident in all conditions. This consistent staining pattern underscores mackerel cell differentiation into muscle cells. Importantly, the similarities observed between MP-exposed groups and the control suggest that the tested MP concentrations had a negligible impact on mackerel cell differentiation.

The observed minimal variation in gene expression across different MP concentrations suggests that muscle cell differentiation may possess an inherent resilience to microplastic-induced stress. Such resilience has been documented in various cellular systems that maintain homeostasis and continue differentiation despite environmental perturbations (Gugliuzza and Crist, 2022). Alternatively, the consistent gene expression might imply a threshold effect, where only microplastic concentrations or particle sizes above/below a specific level are disruptive enough to perturb cellular processes (Campanale et al., 2020). For instance, literature suggests that cellular stress responses can be size-dependent when it comes to nanoparticle interaction, which will be elaborated upon in the subsequent section.

### 3.2.3 Effects of microplastics on cell differentiation

While our findings demonstrated cell differentiation, the effects of microplastics on this process remained indiscernible; all treatment groups and controls yielded analogous outcomes. In contrast, earlier research has highlighted distinct impacts of microplastics on cell differentiation. For instance, Najahi et al. (2022) examined the influence of polyethylene terephthalate microplastics (MPs-PET, <1 µm and <2.6 µm) on human mesenchymal stromal cells, revealing a 30% reduction in cell proliferation and alterations in the differentiation potential of adipose and bone marrow cells. Concurrently, Han et al. (2020) found that polyvinyl chloride (PVC) and acrylonitrile butadiene styrene (ABS) microplastics influenced non-adhesive peripheral blood mononuclear cells (PBMCs) to differentiate into dendritic cells, implying that such plastic exposures might instigate human immune responses. In another study, Hua et al. (2022) highlighted that PS microplastics could disrupt cortical layer differentiation in cerebral spheroids, emphasizing the potential neurotoxic implications. Similarly, Im et al. (2022) reported that polystyrene nanoparticles, especially those with decreased crosslinking density, influenced reactive oxygen species activity and notably promoted adipogenic differentiation in mesenchymal stem cells. Moreover, it was reported that the stage of cell differentiation could influence the cell's interaction with microplastics, especially regarding the uptake of these particles (Peng et al., 2023). In that study, researchers demonstrated that 2-µm polystyrene (PS) microplastics affected human cell lines differently based on their differentiation state, with undifferentiated Caco-2 cells showing significant PS uptake, whereas differentiated cells presented a reduced capacity for PS internalization. Collectively, these findings underscore the multifaceted impacts of micro/nanoplastics on cell differentiation across different cell types, emphasizing the

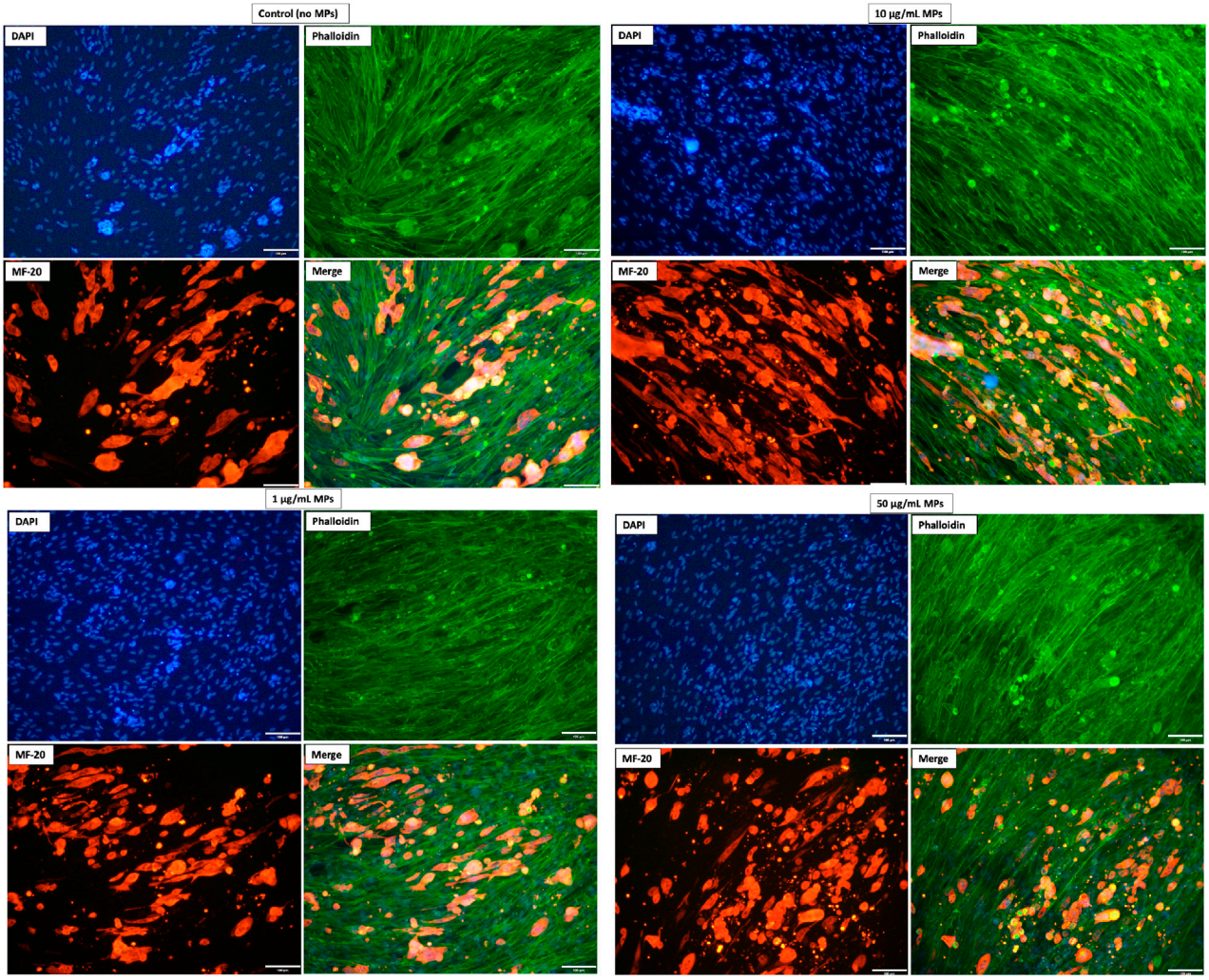


FIGURE 4
Representative immunostaining images of mackerel cells with and without MP exposure. DAPI (blue) labels nuclei, Phalloidin (green) highlights actin filaments, and MF20 (red) detects muscle fiber differentiation. Captured using a 20X lens; scale bar = 100 µm.

need for comprehensive understanding and vigilant monitoring. Additionally, variables including the specific plastic type, exposure duration, and plastic concentration remain crucial determinants that could further modulate these effects (Smith et al., 2018).

To unravel the complex dynamics between microplastics and muscle cell differentiation, it is crucial for future research to investigate the molecular interactions that support this process. Investigations should extend beyond traditional myogenic markers to include an array of molecular targets potentially influenced by microplastic exposure. A comprehensive approach that encompasses the study of cellular senescence, epigenetic modifications, and a wide spectrum of differentiation-related biomarkers will be instrumental in advancing our understanding of the implications of microplastic exposure. It is imperative that future studies incorporate these aspects to elucidate the cellular and molecular mechanisms affected by microplastics, which will ultimately refine our understanding of their impact on cell fate decisions and tissue development.

## 4 Conclusion

In conclusion, this study evaluated the impact of microplastics on Atlantic mackerel (*S. scombrus*) skeletal muscle cell lines, using fluorescent polyethylene microspheres (10–45 µm) as model microplastics. The focus was primarily on understanding the effects of microplastic exposure on essential cellular processes, namely, cell viability during attachment and growth phases and cell differentiation, which hold paramount significance in cultivated meat production. Utilizing microplastic concentrations of 1 µg/mL, 10 µg/mL, and 50 µg/mL, alongside a control devoid of microplastics, the study adopted the Trypan Blue Assay for cell viability assessment. The findings highlighted a marked difference in cell viability among microplastic-exposed treatments compared to the control. In parallel, cell differentiation was investigated using RT-qPCR for gene expression analysis and immunostaining methodologies. Notwithstanding the observable cell differentiation, the study discerned no pronounced influence of microplastics on cell differentiation.

The findings of this study elucidate the interplay between microplastics and cellular mechanisms, highlighting the potential

ramifications for cellular processes. As preliminary data, this investigation lays a foundation for subsequent, more detailed studies. Given the pervasive presence of microplastics in contemporary environments, it is imperative to explore their broader effects on cellular systems. Recognizing and understanding these implications is not only vital for advancing biotechnological applications but also for discerning potential long-term impacts on broader ecological and human health contexts.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials, further inquiries can be directed to the corresponding author.

## Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

## Author contributions

TS: Data curation, Investigation, Methodology, Project administration, Writing–original draft. AT: Investigation, Methodology, Writing–original draft, Formal Analysis, Software. AB: Investigation, Methodology, Writing–original draft. RO: Investigation, Methodology, Conceptualization, Data curation, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Assessing food safety and hygiene practices in old age homes in Mangaung and Lejweleputswa regions, free state

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**Introduction:** Poor food handling, improper cooking, and inadequate storage practices contribute to the spread of harmful pathogens, particularly in vulnerable environments such as old age homes. Ensuring compliance with food safety regulations is essential to protect elderly residents from foodborne illnesses.

**Methods:** This study assessed food safety practices in 14 old age homes (N = 14) in the Free State, South Africa. Data were collected using questionnaires (N = 80) to evaluate food handlers' knowledge, attitudes, and behaviors. Additionally, a food safety checklist was used to assess compliance with hygiene and safety standards.

**Results:** The study identified gaps in food safety practices and regulatory compliance. Non-compliance was observed in 5 facilities (35%), with key issues including inadequate handwashing facilities, limited access to hot water, and insufficient personal protective equipment. While 9 facilities (65%) adhered to food safety regulations, stricter enforcement of measures is necessary to ensure consistent adherence. Although participants demonstrated a good understanding of personal hygiene, proper food handling, sanitation, and microbial contamination prevention, a gap remained between knowledge and practical application.

**Discussion:** The findings highlight the need for improved food safety measures in old age homes. Enhancing hygiene infrastructure, conducting frequent inspections, providing regular food safety training, and enforcing standard operating procedures (SOPs) are critical for mitigating risks. Strengthening these aspects will contribute to safeguarding the health and well-being of elderly residents.

KEYWORDS

**food handlers, food handling practices, food safety knowledge, quality assurance, hygiene compliance, legislative compliance, foodborne illnesses, elderly health**

## Introduction

Food safety in institutional settings, particularly in old age homes, is a critical public health issue. Elderly individuals are particularly vulnerable to foodborne illnesses due to age-related changes in the immune system, chronic conditions such as diabetes or hypertension, and the potential for multiple medications that weaken the body's

defence mechanisms (Elbehiry et al., 2023; Mphaga et al., 2024). According to Manafe et al. (2023) and Mphaga et al. (2024), the elderly population in care facilities faces a disproportionate risk of foodborne illnesses, which can lead to prolonged recovery periods, complications, and, in some cases, death. The World Health Organization (WHO) identifies foodborne diseases as one of the major contributors to global morbidity and mortality, with the elderly representing one of the most susceptible groups in food safety risk assessments (World Health Organization, 2024).

In South Africa, the Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972), along with the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018), provides the regulatory framework for food safety in institutional settings. These regulations are designed to establish standards for hygiene, food handling, storage, and preparation, aiming to protect consumers from foodborne illnesses. However, despite the presence of these guidelines, compliance in old age homes remains inconsistent, posing a serious public health risk. Research by Pakdel et al. (2023) has highlighted that non-compliance with food safety standards in elderly care facilities can lead to food contamination, which further exacerbates the vulnerability of elderly residents to foodborne illnesses.

Previous studies have indicated that food safety compliance in institutional settings often varies due to factors such as staff training, resource availability, and management practices (Mphasha et al., 2024; Moghnia et al., 2021; Teffo and Tabit, 2020). According to Aljasir (2023), even when food safety protocols are in place, they are sometimes poorly implemented or inconsistently monitored, leading to gaps in food safety practices. In old age homes, the role of food handlers is crucial, as their knowledge and training directly impact the implementation of safe food handling practices. Studies by Insfran-Rivarola et al. (2020) and Manafe et al. (2023) indicate that many staff members are inadequately trained, which hinders their ability to effectively adhere to food safety guidelines. This deficiency in training is particularly concerning in environments where residents are highly susceptible to contamination, highlighting the importance of providing staff with adequate training and ensuring strict compliance with hygiene protocols.

Moreover, environmental factors within old age homes, such as kitchen cleanliness, temperature control for food storage, and pest management, have been identified as key determinants of food safety (Afriyie et al., 2022). Studies by Gürsu, (2024) and Kirchner et al. (2021) show that even when food is prepared with care, improper storage and cross-contamination from unsanitary surfaces or equipment can lead to foodborne outbreaks. Refrigeration, in particular, plays a crucial role in preventing the growth of harmful bacteria such as *Salmonella* and *Escherichia coli*, which can thrive if food is not stored at the correct temperature. However, cleanliness of refrigerators and freezers often falls short in many facilities, as reported by Ehuwa et al. (2021), contributing to an increased risk of contamination.

Food handlers' personal hygiene, particularly hand hygiene, plays a role in preventing foodborne illness outbreaks. Studies by Bhagwat (2019) and Pakdel et al. (2023) highlight the importance of proper handwashing, glove use, and compliance to hygienic clothing standards. However, compliance with these practices is often insufficient, as many food handlers fail to follow recommended hygiene practices due to either a lack of awareness or insufficient enforcement of protocols.

In terms of pest and waste management, effective control measures are essential in maintaining a hygienic environment in old age homes. According to the National Environmental Health Norms and Standards for Premises and Acceptable Monitoring Standards for Environmental Health Practitioners promulgated under the National Health Act (Guidelines for Environmental Infection Control in Health-Care Facilities, 2003), pest control systems must be in place to prevent contamination from rodents, insects, and other pests. According to Bingham and Hagstrum (2023) and Kattiyapornpong et al. (2023), proper waste management systems, including safe disposal of food scraps and waste oil, are critical in controlling pests and maintaining hygiene. However, research indicates that many facilities still struggle with pest control and waste management, putting food safety at risk (Raphela et al., 2024; Viljoen et al., 2021).

In line with these findings, this study aims to explore how food safety practices are implemented in old age homes in the Mangaung and Lejweleputswa regions in the Free State Province, with a specific focus on assessing food safety knowledge, hygiene practices, and compliance with national food safety regulations. The study will contribute to the growing body of research by identifying the gaps in food safety practices that need to be addressed to better protect elderly residents from foodborne illnesses. By improving food safety practices and ensuring better compliance with established regulations, these facilities can significantly enhance the quality of life and safety of their residents.

# Research design and methods

## Study design

A cross-sectional quantitative study was conducted to assess food safety practices in old age homes in the Mangaung and Lejweleputswa regions of the Free State.

## Study population and sampling strategy

The study focused on old age homes in the Mangaung and Lejweleputswa areas of the Free State. These areas were selected due to the proximity to the researchers' institution, making it easy to visit the facilities and collect data. As shown in Figure 1, all the selected old age homes had Certificates of Acceptability (CoA), which confirm compliance with South Africa's hygiene standards for food premises, as outlined in the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018). Although a list of old age homes was obtained from the Department of Social Development, only 14 were included in the study, as they were the only facilities with a valid CoA at the time. Some other homes were excluded because they were temporarily closed. Food handlers at the selected homes were given questionnaires to complete, with the researcher guiding them to ensure understanding without influencing their responses. The food handlers filled out the questionnaires independently to

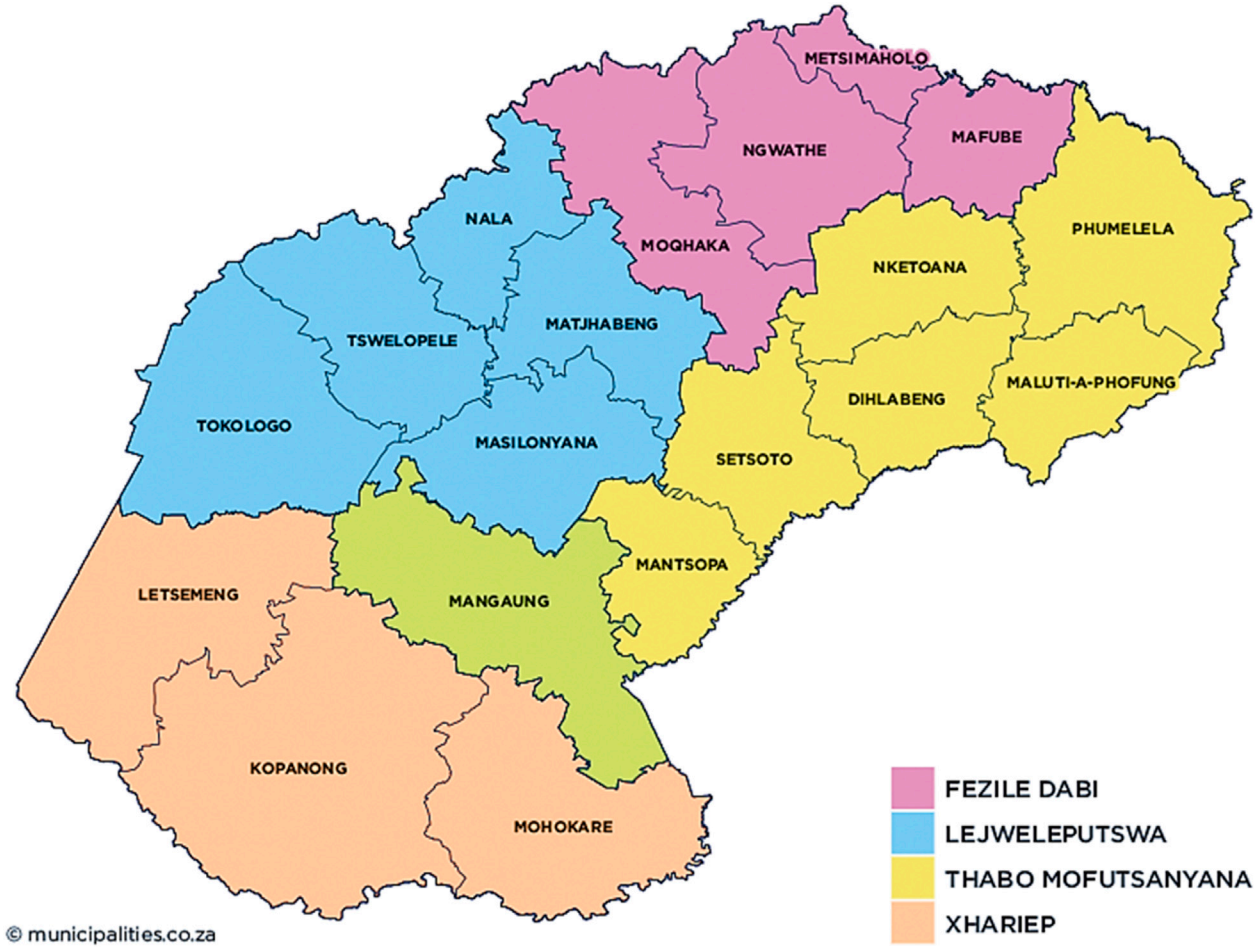


FIGURE 1
Study region.

maintain the authenticity of their answers. Additionally, a food safety checklist was used, which the researcher filled in through direct observation of food handling practices at the facilities, providing further validation of the data collected.

## Data collection

A structured questionnaire was used to collect data, and it was divided into four sections: Personal Hygiene, Food Safety Management, Cleaning Practices, and Pest Control. The questions were carefully designed to be clear and consistent, ensuring they aligned with food safety regulations to assess whether the required standards were being met. This format allowed food handlers to answer the questions independently, ensuring their responses were not influenced by the researcher. In addition to the questionnaire responses, food safety management documentation in these facilities was also consulted, particularly when using the food safety checklist, to provide a comprehensive assessment of compliance with food safety standards. Throughout the data collection process, the researcher worked closely with a registered EHP to ensure that all food handlers were interviewed. After the interviews, the researcher then reviewed the completed questionnaires to confirm that all responses were accurate and complete.

## Reliability

The questionnaire was reviewed by an EHP who specializes in food premises and food handling inspections. The EHP ensured that the questions were clear, relevant, aligned with the study's goals, accurately captured key aspects of food safety practices, and complied with food safety regulations. To further ensure the data's consistency and reliability, the researcher took steps to guarantee that the responses provided by food handlers were uniform across all participants. While food handlers were encouraged to answer independently, the researcher remained present during the data collection process to provide clarification if needed and to make sure the responses were unbiased.

Additionally, the researcher used a food safety checklist during direct observations of food handling practices and the overall condition of the facilities, which allowed for an objective assessment to determine whether the practices observed were

consistent with the responses provided in the questionnaire. This two-step approach, combining the questionnaire and the observational checklist, ensured that the information collected was consistent and aligned with the study's objectives.

## Validity

To assess knowledge that supports compliance with legislation, the tool was shared with a practicing EHP who regularly uses similar tools and is familiar with the requirements of the Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972), with particular emphasis on the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018) stipulated under it. These regulations provide detailed guidelines for food handling, hygiene practices, sanitation protocols, and waste control measures to ensure public health safety. The tool was designed to align closely with these regulatory standards to ensure that the questions it contained were both relevant and in compliance with established food safety practices.

## Data analysis

The questionnaire data were coded and entered into an Excel spreadsheet, then imported into the Statistical Package for the Social Sciences (SPSS) version 29. Responses were coded as "1.00" for "Yes" and "2.00" for "No" to make analysis easier. Descriptive statistics were used to summarize the data and assess hygiene practice compliance. This coding helped organize the data, allowing comparisons and identifying common trends related to hygiene practices, food safety management, and infrastructure in old age homes. SPSS outputs also showed summarized results for key areas, like handwashing facilities, temperature checks, and cleaning protocols, highlighting the percentage of respondents following each practice.

# Results

## Data analysis methodology

### Questionnaire

The data for this study were analyzed using SPSS software, focusing on means and frequencies to assess food safety and hygiene practices in old age homes. The results were grouped into themes that emerged from the data, including hygiene and sanitation practices, food safety practices, cleaning and maintenance, pest and waste management, and notable gaps in practices. These themes helped identify key areas of compliance and non-compliance across the study participants.

Since no formal statistical tests for significance (such as chi-square or t-tests) were performed, the results presented here are based on descriptive statistics, providing an overview of the compliance rates for different food safety practices. Frequencies and percentages are used to describe the extent to which food safety standards are being followed.

## Food safety checklist

In addition to the questionnaire, a detailed food safety checklist was used to assess how well the 14 old age homes followed the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018). The checklist looked at important areas like personal hygiene, food storage, food preparation, equipment maintenance, and waste management. The results from the checklist gave additional insights into the food safety practices in the homes, working alongside the data from the questionnaire and observations.

Compliance was scored from 1% to 100%, with higher scores indicating better adherence to the prescribed standards. A score below 50% indicated areas requiring improvement. The checklist was supplemented with observational data and interview findings, providing a reliable analysis of actual food safety practices in the homes. This also helped address potential discrepancies between self-reported practices and observed behaviors.

## Compliance overview

Table 1 summarizes the compliance rates for various food safety and hygiene practices across different themes in old age homes from the study participants. These rates are specifically compared to the standards set under the Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972), particularly the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018). These standards outline the requirements for maintaining hygiene in food handling, storage, and preparation, which are critical to preventing foodborne illnesses.

The results presented in Tables 1, 2 highlight both areas where facilities are performing well and areas that require improvement. The following sections will provide a detailed discussion of these findings.

## Non-compliance overview

Table 2 summarizes the areas of non-compliance observed across the study participants, grouped by key themes. It highlights the percentage of facilities that did not fully meet important food safety and hygiene practices, emphasizes areas that require improvement to ensure the safety and well-being of residents in old age homes.

TABLE 1 Summary of food safety and hygiene practices compliance[1].

| Theme | Compliance overview | Compliance rate (%) |
|---|---|---|
| Hygiene and Sanitation | - Handwash basins with essentials (water, soap, drying) | 83.8 |
| | - Hygienic drying method | 83.8 |
| | - Changing and storage facilities | 83.8 |
| | - Designated restroom with wash basin | 83.8 |
| Food Safety Practices | - Correct stock rotation | 100 |
| | - Separation of raw and cooked foods | 100 |
| | - Defrosting, preparation, and cooking on-site | 100 |
| | - Cooling methods and reheating on-site | 100 |
| Temperature and Monitoring | - Temperature checks on chillers/freezers | 60.0 |
| | - Monitoring temperature of reheated food | 71.3 |
| | - Temperature checks on food delivery | 60.0 |
| Cleaning and Maintenance | - Easy-to-clean surfaces | 75.0 |
| | - Cleaning schedules | 78.8 |
| | - Maintenance staff available | 75.0 |
| Pest and Waste Management | - Pest control systems | 78.8 |
| | - Waste and refuse disposal facilities | 83.8 |
| Employee Health and Safety | - Sick employee records | 81.3 |
| | - Preventing contamination | 81.3 |
| General Infrastructure | - Lighting and ventilation | 100 |
| | - Safe reheating of food | 100 |

## Overall compliance

Figure 2 shows the distribution of compliance status among participants. The "Yes" section reflects the total percentage of compliance across all questions, while the "No" section highlights areas of non-compliance among the participants from the old age homes study.

# Discussion

## Hygiene and sanitation practices

The researchers found that, while most old age homes in Mangaung and Lejweleputswa meet basic hygiene standards, certain gaps exist that could potentially affect food safety for elderly residents. Most study participants (83.8%, N = 67) reported having essential handwashing facilities, including basins with hot and cold water, soap, and hygienic hand-drying methods, which are important for preventing the spread of microbes. However, 16.3% (N = 13) lacked these facilities, making it harder to maintain proper hand hygiene and increasing the risk of foodborne illnesses. The food safety checklist also highlighted concerns in personal hygiene, with 60% compliance observed in handwashing practices, and only 20% of food handlers demonstrating proper handwashing. This suggests that, despite the presence of handwashing facilities, there may be gaps in the training and reinforcement of proper hand hygiene practices. The findings from the systematic review by Insfran-Rivarola et al. (2020) indicate that while training interventions have positive effects on knowledge and practices, specific aspects such as handwashing might require more focused or practical training to ensure consistent application, as seen in the low compliance rates here. This highlights the need for targeted interventions to improve hand hygiene behaviors across the facilities. In line with the findings of Johnstone et al. (2022), who identified gaps in hand hygiene practices in a community survey in Soweto, targeted interventions should focus on specific groups, like food handlers. Their study showed that only 42% of respondents practiced proper hand hygiene, highlighting the need for focused educational efforts.

1 Compliance Overview refers to the key practices and standards assessed within each theme to ensure adherence to food safety and hygiene regulations. Compliance Percentage indicates the proportion of facilities that fully meet the specified standards for each practice, expressed as a percentage of the total number of facilities surveyed. A higher percentage reflects greater adherence to the required practices within that area.

TABLE 2 Summary of non-compliance ("no" responses)[2].

| Theme | Key practice | Number of study participants responses on non-compliant facilities (N) | Percentage non-compliant (%) |
|---|---|---|---|
| Hygiene and Sanitation | Handwash basins with essentials | 13 | 16.3 |
| | Hygienic drying method | 13 | 16.3 |
| | Changing and storage facilities | 14 | 17.5 |
| | Designated restroom with wash basin | 14 | 17.5 |
| Food Safety Practices | Temperature checks upon food delivery | 32 | 40.0 |
| | Temperature checks on chillers/ freezers | 23 | 28.7 |
| Food Handling and Contamination Prevention | Reheating of food undertaken on-site | 14 | 17.5 |
| | Monitoring temperature of reheated food | 14 | 17.5 |
| | Risk of contamination of foods on display | 44 | 55.0 |
| Employee Health and Safety | Sick employee records | 15 | 18.8 |
| Cleaning and Maintenance | Food surfaces well-constructed and easy to clean | 20 | 25.0 |
| | Cleaning schedule | 17 | 21.3 |
| Waste and Pest Management | Adequate facilities for refuse disposal | 17 | 21.3 |
| | Adequate facilities for waste oil disposal | 17 | 21.3 |
| | Pest control and formal contracts | 13 | 16.3 |

Similarly, emphasizing the importance of soap for hand hygiene and considering the social context could help improve hand hygiene practices in food safety. The checklist findings are consistent with the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638 of 2018), which require facilities to have hot and cold water, soap, and hygienic drying methods for effective hygiene practices.

Resource limitations and gaps in monitoring are factors that may contribute to these challenges (Mphaga et al., 2024). For instance, although most study participants reported having proper handwashing facilities, those without these facilities often faced budget constraints, limited time, or a shortage of EHPs to provide the necessary training and consistent monitoring. This lack of resources and capacity made it difficult for some of these facilities to meet the required standards. Similarly, Aljasir (2023) found that inadequate food handling practices, due to insufficient training and resources, lead to foodborne illnesses in Gulf countries. Similarly, Pakdel et al. (2023) highlighted how inadequate food safety management, including improper design of food processing facilities and lack of hygienic monitoring, can lead to food contamination risks, especially in settings that use open food processing equipment. Their findings emphasize the importance of proper monitoring and intervention strategies, which align with the challenges faced by old age homes in ensuring food safety. Additionally, since EHPs are mandated to inspect the premises only twice a year, as outlined in the National Environmental Health Norms and Standards for Premises and Acceptable Monitoring Standards for Environmental Health Practitioners in terms of the National Health Act (61 of 2003), Section 4: 2 (2.1), the findings of the study indicate this as a gap due to insufficient opportunities for ongoing support and follow-up inspections, which has hindered compliance at some facilities.

The results show that 82.5% (N = 66) of the study participants reported that their old age homes have changing and storage areas, as well as restrooms with wash basins. However, 17.5% (N = 14) indicated a lack at their facilities. Changing and storage areas are essential for ensuring food handlers can change into clean clothing, reducing the risk of contaminating food with external dirt or bacteria. These areas also help maintain cleanliness and prevent

2 Data derived from the study on food safety practices in old age homes, specifically focusing on non-compliant responses from participants. The number of non-compliant facilities (N) and the corresponding percentages (%), as indicated for each theme and key practice, reflect the observed gaps in adherence to established food safety standards within the study's scope.

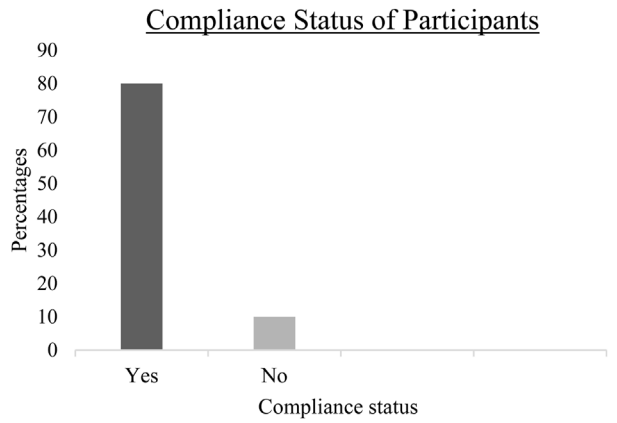


FIGURE 2
Overall compliance of food safety and hygiene practices[3].

cross-contamination, which is critical for food safety, as emphasized by Pakdel et al. (2023). Proper hygiene facilities, such as restrooms with wash basins, are fundamental to preventing contamination, as their absence puts both food handlers and elderly residents at risk. According to Putri and Susanna, (2021), the availability of wash basins plays a role in promoting regular handwashing among food handlers, a practice that is particularly important after activities such as using the restroom or handling raw food. Therefore, regular handwashing reduces the risk of transferring harmful microorganisms to food, thereby enhancing food safety.

The food safety checklist revealed gaps in cleanliness and adherence to hygiene standards in food storage and preparation areas, with 60% compliance in areas like food storage and refrigeration cleanliness. As noted by Lorenzo et al. (2018) and Ehuwa et al. (2021), maintaining cleanliness in food storage and refrigeration areas is vital, as it prevents the growth of harmful microorganisms and helps ensure food is stored at safe temperatures. Improving these facilities is vital to protect the health and safety of everyone involved.

## Food safety practices

The researchers' study results show that while most food safety practices are being followed, there is room for improvement in some areas. The control of food temperature in the facilities is generally managed using food thermometers, which allow food handlers to regularly check and maintain proper cooking and holding temperatures. However, non-compliance is evident in areas such as the failure to check temperatures upon food delivery (40%, N = 32) and in chillers/freezers (28.7%, N = 23), which can jeopardize food safety if not addressed, as reported by study participants. Hot holding units, such as warming ovens or food warmers, are commonly used in most facilities to keep food above 65°C, ensuring it remains safe for consumption until served. All participants (100%, N = 80) reported practicing key measures such as stock rotation, keeping raw and cooked foods separate, and cooking food to temperatures above 65°C. They also reported adhering to the minimum time-temperature requirements for food safety, which include ensuring that food is not kept at room temperature for longer than 2 h. This practice is crucial to prevent the rapid growth and multiplication of pathogenic bacteria, which can compromise food safety. Furthermore, maintaining a minimum temperature of 65°C complies with the standards set out in the Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972), specifically the standards and requirements for food on display, storage, and temperature under regulation 8 (4) (a) (i) [Annexure E: Food Temperatures]. According to column 1 under the category "Heated Products" and column 3 of this section, the Act specifies the required core temperatures for food to ensure safety.

Food safety practices extend beyond temperature control. Bhagwat (2019) emphasizes that the quality of water used in various food production processes, such as processing, cleaning, and storage, is crucial in ensuring food safety. Contaminated water can introduce harmful microorganisms that jeopardize food safety, highlighting the importance of effective water quality management as a critical component of food safety practices within facilities. Therefore, ensuring that water used in food preparation meets safe drinking standards and is properly treated is as critical as maintaining proper food temperatures. This is especially important for cleaning processes, where water is often used for sanitizing surfaces, utensils, and equipment. The quality of water, free from pathogens and contaminants, directly affects the effectiveness of cleaning procedures, which are vital to preventing cross-contamination and the spread of foodborne illnesses.

For hot drinks, temperature control is also important to prevent foodborne illness. Beverages like tea or coffee are typically heated to temperatures above 65°C; however, the duration for which they are maintained at this temperature is not always monitored as closely as it is for cooked food. To ensure safety and quality, it would be beneficial for food handlers to serve hot beverages at a consistent and safe temperature and to serve them immediately after preparation.

There is room for improvement in monitoring food storage and temperature control. While 71.3% (N = 57) of study participants reported regularly checking the temperatures of chillers and freezers, 28.7% (N = 23) do not. This lack of monitoring is concerning, as improper storage temperatures can allow bacteria like *Salmonella* and *E. coli* (*E. coli*) to grow, increasing the risk of foodborne illnesses, especially among elderly residents (Adhikari et al., 2018). Moghnia et al. (2021) emphasize that improper storage conditions are a significant risk factor in healthcare settings, and this issue remains pertinent in old age homes as well. Facilities typically rely on their suppliers to ensure that food is free from harmful bacteria before it is received. According to Mphaga et al. (2024), to further mitigate risks, it is crucial for facilities to adopt measures to test for the presence of harmful bacteria in food before its reception. This can be done by implementing random sampling, which should be carried out by a staff member who has been trained by an EHP in food safety practices. The samples would then be sent to accredited

3 The majority (84.6%) reported compliance, while 15.4% indicated non-compliance. This suggests that most participants adhered to the required protocols, though a smaller proportion did not. This data is important for assessing overall adherence to the study guidelines.

laboratories for microbial testing to detect common pathogens like *Salmonella*, *E. coli*, and *Listeria*. This practice aligns with the guidelines set out in the Codex Alimentarius Commission (2003), which advises that food facilities should take necessary steps to ensure that the food they receive meets microbiological safety standards. In addition, facilities should verify that their suppliers comply with food safety standards, including those outlined in the Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972), and maintain proper documentation of regular bacterial testing for food products. Regular audits and tests are also consistent with the Hazard Analysis and Critical Control Points (HACCP) system, which requires that food safety hazards be identified and controlled at critical points in the supply chain, including the receipt of food products (HACCP, 2018). These proactive steps play a role in ensuring that all food entering the facility is thoroughly inspected and deemed safe for consumption. By reducing the risk of contamination, these measures contribute to protecting the health and wellbeing of vulnerable residents.

The food safety checklist found that 60%–100% compliance in refrigeration practices was common, however only 60% of facilities adhered to cleanliness and contamination prevention standards in refrigerators, raising concerns about cross-contamination despite proper temperature control. Additionally, 40.0% (N = 32) of study participants do not check the temperatures of food deliveries, exposing food to potential temperature abuse during transportation, which poses another critical food safety risk. The food safety checklist also revealed that all participants in the study (100%, N = 80) strictly follow proper cooking and food preparation practices. However, 80% compliance was observed in temperature documentation and utensil cleaning for food preparation, indicating room for improvement in monitoring practices. This is important because proper documentation and temperature monitoring are crucial for food safety, especially in high-risk environments like old age homes.

## Cleaning and maintenance

The researchers found that 75% (N = 60) of study participants noted that their homes had food preparation surfaces that were easy to clean. The surfaces were made of non-porous, smooth materials such as stainless steel, which resists the absorption of liquids and is free of cracks, crevices, or joints that could harbor bacteria, ensuring proper hygiene is maintained. These surfaces are in compliance with the Foodstuffs, Cosmetics and Disinfectants Act (54 of 1972), under the Standards and Requirements for Facilities on Food Premises, Section 6(1) regulations. However, 25% (N = 20) of the study participants reported that their homes had surfaces that were harder to clean, such as wooden countertops, tiles with unsealed grout, or damaged stainless steel with scratches or dents. These types of surfaces often have cracks or porous areas that can absorb moisture and trap bacteria, making cleaning and disinfecting difficult. As a result, harmful bacteria can build up, as pointed out in the Guidelines for Environmental Infection Control in Healthcare Facilities (2003). The importance of clean surfaces in preventing contamination is also highlighted in studies by Little and Sirsat (2024); Ehuwa et al (2021) and Kirchner et al. (2021), who found that surfaces in food preparation areas that are not properly maintained are more likely to harbor bacteria. The study also found that food safety practices in the facilities showed 50%–80% compliance, especially in keeping thermometers clean. However, it was concerning that only 50% of the facilities cleaned thermometers between uses, which raises the risk of contamination during temperature checks.

In the study, 78.8% (N = 63) of study participants followed a cleaning schedule, which helps ensure important areas are cleaned regularly. However, 21.3% (N = 17) did not follow a cleaning schedule, which may lead to areas being missed and a higher risk of contamination. The food safety checklist confirmed that 60%–90% compliance was observed in dishwashing and waste management, with some gaps noted in waste storage and pest control, especially in facilities with only 60% compliance in maintaining clean waste bins. Regular cleaning and sanitization, especially of surfaces that come into contact with food, are essential to minimize the risk of foodborne illnesses (Little and Sirsat, 2024; Kirchner et al., 2021; Codex Alimentarius Commission, 2003).

## Pest control and waste management

Pest control is an important part of food safety, as discussed in previous studies. In this study, 83.8% (N = 67) of study participants reported that their facilities had pest control measures in place, including contracts with pest control companies, bait stations, routine inspections, and waste management systems. These facilities also sealed potential entry points, such as cracks in walls and floors, to prevent pests. Similarly, Bingham and Hagstrum (2023) highlighted that sanitation is a key element in pest management, removing insects and food residues that may serve as shelter for pests. Inadequate sanitation can reduce the efficacy of pest control measures, highlighting the need for comprehensive pest management strategies, a point also emphasized by Morrison et al. (2019), who found that decreased sanitation negatively affected the efficacy of pest control tactics, highlighting the importance of proper sanitation in maintaining effective pest management. However, 16.3% (N = 13) of the study participants indicated that their facilities in this study lacked pest control systems, which increases the risk of food contamination from pests like rodents and insects. According to Donkor (2020), pests such as rodents, flies, cockroaches, and stored-product insects can carry and spread diseases. For instance, rodents are known to carry diseases like *Salmonella* and can contaminate food and surfaces through their droppings, urine, or saliva. Cockroaches are also known to spread pathogens, such as *Salmonella* and *E. coli* by crawling on food surfaces and transferring bacteria. Flies, another common vector for foodborne diseases, can land on decaying matter and then transfer harmful bacteria to food (Yin et al., 2022). Similarly, stored-product insects, like beetles and moths, can contaminate food by feeding on it, leaving behind faeces, shed skin, and other waste products. The food safety checklist showed 60%–90% compliance with pest control and waste management practices, but only 60% of facilities were fully compliant with waste storage cleanliness and pest prevention. This indicates that pest control measures need to be more rigorously enforced in all facilities, as the presence of pests can compromise food safety and the health of residents.

In terms of waste management, 78.8% (N = 63) of the study participants stated they had proper systems for storing and disposing of waste, including waste oil. However, 21.3% (N = 17) of the study participants stated that their homes did not have proper waste disposal systems, which raises concerns about hygiene and the risk of pests.

## Non-compliance and areas for improvement

The researchers identified several key issues related to food safety, focusing on food handling and temperature control. It was found that 55.0% (N = 44) of the study participants did not have enough measures to protect food on display from contamination. This means some of the food was left exposed to dust, airborne germs, and pests, which could easily make the food unsafe. The lack of protective coverings, such as plastic wraps, increased the risk of contamination from improper handling by staff. Byrd-Bredbenner et al. (2013) discuss a similar problem in their study, pointing out that many consumers do not recognize the risks of foodborne illness at home, which leads to unsafe food handling practices. Their research suggests that food safety programs aimed at changing consumer attitudes and behaviors are necessary to reduce the risk of foodborne illness. Both studies highlight the need to raise awareness about food contamination risks and take proper steps to prevent it, whether in food facilities or at home. This is further supported by the findings of Siddiky et al. (2024), who emphasize that food handlers in institutional settings would benefit from enhanced exposure to food safety interventions, active participation in training sessions, and strict adherence to food hygiene regulations to improve their knowledge and practices. Their study indicates that food handlers who were more knowledgeable about food safety had better food safety practices, especially regarding hand hygiene and food separation, which aligns with the need for better food safety education in various environments.

In addition, the food safety checklist showed that 60%–80% of facilities followed proper food storage and contamination prevention practices. However, some facilities did not fully comply with food safety rules for storage. This means that many food items were not kept in the right containers or at the correct temperatures, which can lead to spoilage or bacterial growth, as explained by Nkosi and Tabit (2021). To improve this, it is essential to ensure food on display is properly covered, stored in suitable containers, and regularly checked to maintain safe temperatures.

The current study findings also revealed that 17.5% (N = 14) of the study participants stated that they did not check the temperature of reheated food, which creates a serious health risk. Reheated food that is not brought to the correct temperature can allow harmful bacteria, such as *Salmonella* or *E. coli*, to grow, as discussed by Ehuwa et al. (2021). This highlights how important it is to monitor food temperatures during reheating to prevent foodborne illnesses. Additionally, the food safety checklist showed that only 50% of facilities cleaned their thermometers between uses. Since thermometers are crucial for ensuring food is reheated safely, poor cleaning practices increase the risk of contamination.

Furthermore, 81.3% (N = 65) of study participants stated that their facilities kept records of sick employees, but 18.8% (N = 15) did not. The checklist findings suggest that keeping these records is important for stopping the spread of foodborne illnesses caused by sick food handlers. These results show the need for stronger monitoring and cleaning processes, as well as consistent record-keeping, to improve food safety and protect public health.

## Resource limitations and systemic challenges in food safety practices

While the discussion effectively highlights gaps and compliance, resource limitations likely play a key role in non-compliance with food safety standards. From the observations made, financial constraints prevent some facilities from investing in essential infrastructure, such as proper handwashing stations or maintenance of food safety equipment, which contributes to gaps in hygiene and food safety practices. Moreover, systemic challenges in enforcement and monitoring may exacerbate these issues. The study revealed that some facilities experienced delays in follow-up inspections after discrepancies were noted. According to Section 4 of the National Norms and Standards Relating to Environmental Health in Terms of the National Health Act (61 of 2003), under the heading "Homes for the Aged," Section 2 (2.1), EHPs are required to inspect food-handling facilities, including those in old age homes, at least twice a year to ensure compliance with food safety standards. However, delays in conducting follow-up inspections may lead to gaps in maintaining consistent compliance.

The National Environmental Health Strategy (2016–2020) states that there should be one EHP for every 10,000 individuals within a population. Despite this guideline, many municipalities face challenges in meeting the recommended staffing levels due to resource constraints. As a result, the current status reveals a shortage of EHPs, which further exacerbates delays in inspections and follow-up actions. These challenges are compounded by the lack of records for second inspections after initial violations, raising concerns about the enforcement of regulations. Insufficient staffing levels contribute to the delays, as fewer EHPs mean a reduced capacity to inspect all facilities on time and thoroughly. This highlights the need to address staffing shortages within regulatory bodies to ensure that food safety standards are consistently upheld. The combination of resource constraints and enforcement challenges underscores the importance of improving monitoring systems and implementing measures that ensure timely follow-up actions to correct identified issues, as outlined by the existing legislative framework.

## Legislative and regulatory recommendations

To address the gaps in food safety and hygiene practices observed in the study, several legislative and regulatory measures could be introduced.

### Stricter enforcement of hygiene standards

More frequent and unannounced inspections would help ensure that facilities consistently follow food safety rules. A system of penalties for not following the rules, particularly for issues like

not having proper handwashing stations or pest control, could encourage better compliance with regulations.

### Infrastructure requirements and resource allocation

Legislation should require that all facilities have basic infrastructure, like handwashing stations, temperature monitoring systems, and pest control measures, in place before they can receive a CoA after their first inspection. These measures are essential to prevent contamination and protect the health of both food handlers and residents. For facilities struggling to meet these requirements, financial assistance or subsidies should be available. Government grants could help support food safety improvements in underfunded facilities, especially in rural or low-income areas. Private companies may also offer financial support, equipment, or training programs. Additionally, non-governmental organizations (NGOs) that focus on health or food security could assist with funding or expertise. These forms of support will help ensure that all facilities, regardless of their financial situation, can maintain a safe and hygienic environment, ensuring the health and safety of vulnerable populations, such as the elderly.

### Mandatory food safety management systems (FSMS)

All old age homes should have a complete Food Safety Management System (FSMS) in place, which includes mandatory health checks for food handlers, regular training on proper food safety practices, and accurate record-keeping of food safety activities. These measures help ensure that food is safe and that the risk of foodborne illnesses is minimized. The Foodstuffs, Cosmetics, and Disinfectants Act (54 of 1972) mandates these practices to protect public health, particularly in environments like old age homes, where residents are more vulnerable to foodborne illnesses. Additionally, the Occupational Health and Safety Act (85 of 1993) supports these measures by requiring employers to protect the health and safety of their workers, including ensuring that food handlers are healthy and trained in proper food safety practices.

### Training and continuous education

Mandatory training programs for food handlers, along with regular refresher courses, are important to ensure food safety knowledge is consistently applied. According to the National Environmental Health Norms and Standards for Premises and Acceptable Monitoring Standards for Environmental Health Practitioners in terms of the National Health Act (61 of 2003), ongoing food safety training must be provided to all staff working in food service settings in old age homes, and this training should be carried out EHPs. However, the regulations only require inspections of old age homes to be conducted twice a year. While this sets a minimum standard, the findings suggest that this frequency is not enough to address recurring food safety and hygiene issues. Therefore, more training sessions should be held, and the frequency of inspections should be increased.

## Limitations of the study

This study has several limitations. First, the sample size of 14 old age homes and the geographic focus on Mangaung and Lejweleputswa may limit the generalizability of the findings to other regions or a broader population of old age homes. Additionally, the reliance on self-reported data from food handlers and staff introduces the potential for bias, despite efforts to complement this with observational data. The study's cross-sectional design only provides a snapshot of food safety practices at a single point in time, and no formal statistical tests for significance were performed, limiting the ability to draw conclusions about causal relationships. Finally, while the study assessed compliance, it did not measure the actual outcomes, such as foodborne illness rates, which would provide a more comprehensive evaluation of the effectiveness of these practices.

## Conclusion

This study highlights gaps in food safety practices and compliance with food safety regulations in old age homes in the Free State, South Africa. Among the fourteen facilities assessed, nine demonstrated compliance with most food safety regulations, while five did not meet these standards; three located in the Mangaung Metropolitan area and two in the Lejweleputswa District Municipality. While food handlers demonstrated a high level of knowledge about food safety, inconsistencies in practice, particularly related to hand hygiene and the provision of personal protective equipment, were observed. Additionally, non-compliance with basic hygiene standards in these five homes stresses the need for stricter enforcement of regulations. These gaps contravene the main act that governs all food safety regulations, the National Health Act (61 of 2003), particularly the Foodstuffs, Cosmetics and Disinfectants Act (54 of 1972) and the Regulations Governing General Hygiene Requirements for Food Premises, the Transport of Food and Related Matters (R638) stipulated under it, as well as the National Environmental Health Norms and Standards for Premises and Acceptable Monitoring Standards for Environmental Health Practitioners. The study recommends improving hygiene facilities, such as providing designated handwashing stations and access to necessary personal protective equipment (e.g., aprons, gloves, hairnets), more frequent inspections to ensure ongoing compliance, enhanced food safety training for all food handlers, and stricter adherence to standard operating procedures (SOPs) to ensure the protection of elderly residents and reduce the risk of foodborne illnesses.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by the University of the Free State's Research Ethics Committees (UFS-HSD2023/0476 and UFS-ESD2023/0104). The studies were conducted in accordance with the local legislation and institutional

requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

TN: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. NM: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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# Comparative nutrient and sensory analysis of eight different commercial *Chlorella* powders

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A growing global population and climate change challenge conventional agriculture and global food safety. Microalgae are an emerging sustainable nutrient food resource, increasingly regarded as an important component for the human diet. The chlorophyte *Chlorella* sp. is regulated and commercialized for food applications. Commercial *Chlorella* preparations are either produced by heterotrophic fermentation or photoautotrophic cultivation in bioreactors or outdoor ponds. Products can differ significantly with regard to taxonomy of the production strain, changed genetic properties due to strain development, cultivation conditions, and downstream biomass processing methods. Notably, heterotrophic strains may be adapted to generate little or no photosynthetic pigments, resulting in yellow or white variant. In this study, eight different commercial *Chlorella* products from different EU suppliers were analyzed and compared regarding identity of the production strain, macro-and micronutrient profiles, sensory properties, as well as microbial load. The production processes featured different cultivation methods, production strains, genetic variants (yellow and white) and downstream processing methods. Data indicates significant variations between the *Chlorella* products. White and yellow *Chlorella* products showed different protein and taste profiles compared to green, photosynthetically competent wild type strains. We confirmed relatively high and stable protein concentrations but could detect variations in sugar and specifically lipid and vitamin profiles depending on strain, cultivation and downstream processing methods. The microbial load varied strongly between closed and open cultivation systems but was compliant with legislative (EFSA) specifications for all samples. The comparative nutrient- and sensory data set

presented in this study will aid in selecting algae products for the development of innovative foods, thereby accelerating adoption and differentiation of algae food products.

KEYWORDS

sensory analysis, nutritional profile, Chlorella powder, microalgae, innovative food

# 1 Introduction

Because of a rapidly growing global population global food demand will increase by 50% until 2050 (Nations, 2022a; Nations, 2022b). While food production is responsible for about 34% of all human-induced greenhouse gas emissions it also faces challenges because of climate change and the scarcity of arable land. To address these issues and to ensure food security for the world population, it is essential to explore more sustainable alternatives to conventional agriculture and livestock breeding (Crippa et al., 2021; Mirón et al., 2023). Microalgae build up biomass much faster than terrestrial crops and represent a rich source of high-quality protein, essential fatty acids, vitamins, minerals, and bioactive compounds supporting a healthy human diet (Andrade et al., 2018; Bhuvana et al., 2019; Yang et al., 2023). *Chlorella vulgaris*, for example, shows a projected market value of USD 413.3 million by 2028 with an annual biomass production of about 5,000 tons and has been recognized as the most valuable commercially produced green microalga (Levasseur et al., 2020). This species is regulated for food and feed applications from the food and drug administration (FDA) as well as European food safety authority (EFSA) and is generally recognized as safe (GRAS) (Additives and Food, 2015; Food and Administration, 2016). The reported intracellular protein content of *Chlorella* sp. biomass is between 40% and 70% and complies with the requirements of a high quality protein according to the essential amino acid index (EAAI), which is used to evaluate protein quality for human nutrition (Becker, 2007; Bito et al., 2020; Fu et al., 2021; Markou et al., 2012; Matard-Mann et al., 2017; Muys et al., 2019; Qin et al., 2020; Yang et al., 2023). In addition to its high protein content, *Chlorella* sp. biomass contains a variety of vitamins and other bioactive and potentially health promoting compounds (Bito et al., 2020; Markou et al., 2012; Matard-Mann et al., 2017; Qin et al., 2020).

The composition of the commercialized biomass is mainly dependent on the cultivation method, the production species/strain, their genetic variation and downstream processing (Hosseinizand et al., 2018; Kolackova et al., 2023; Muys et al., 2019; Nwoba et al., 2019; Silva et al., 2021; Vilatte et al., 2023). *Chlorella* sp. can be cultivated photoautotrophically in illuminated, closed photobioreactors or open ponds, with carbon dioxide as carbon source (phototrophic/autotrophic). Alternatively, algae biomass can be cultivated heterotrophically in conventional stirred tank fermenters using monomeric sugars (e.g., glucose) as a carbon source. Moreover, both photoautotrophic and heterotrophic cultivation modes can be combined in mixotrophic cultivation modes using photobioreactor systems (Barros et al., 2019; Patel et al., 2021). The ability to grow *Chlorella* heterotrophically enables the development of genetically adapted strains with less or devoid of photosynthetic pigments. These yellow or white algae products have altered sensory profiles (Cabrol et al., 2023; Schüler et al., 2020), which offers new options for the development of food products. Nevertheless, microalgae biomass is currently predominantly used as an additive to products to increase their nutritional value, or as a dietary supplement due to its potentially health-promoting properties. Therefore, in addition to the contained nutrients color, taste, and odor can play a major role in the general consumer acceptance of microalgae in food. In this context, product quality and shelf life of microalgae biomass products is highly dependent on the microbial loads of microalgae preparations, which are significantly affected by the individual cultivation and downstream processing methods.

There is sporadic information on production strains, cultivation methods and nutritional profiles mainly provided by manufacturers of microalgae biomass. However, detailed and standardized analysis of micro-and macronutrient composition as well as sensory profiles are typically not reported. Moreover, data on microbial loads pertaining to these products are commonly not disclosed. This study used standardized analysis methods to compare macro- and micronutrient composition, sensory profiles, as well as microbial load of eight different dried commercial *Chlorella* sp. powders. Highlighting the impacts of different ways of microalgae production and processing may be of interest to food technologists to evaluate the potential for food development of different *Chlorella* products.

# 2 Materials and methods

## 2.1 *Chlorella* powders

Eight different types of *Chlorella* powders covering different suppliers, strains, cultivation methods, and downstream processing wer analyzed for their nutrient and sensory profiles (Table 1).

## 2.2 Qualitative pigment analysis

For the pigment analysis 10–15 mg of the different biomass samples were extracted with 4 mL HPLC-grade acetone in 10 mL glass tubes with solvent-proof screw-top lids according to Paper et al. (Paper et al., 2022). and directly analyzed using a Shimadzu Prominence LC-20A HPLC-system (Shimadzu, Duisburg, Germany) equipped with an SPD-M20A diode array detector on

**Abbreviations:** FFA, free fatty acid; FAME, fatty acid methyl ester; EU, European Union; RT, room temperature; EDTA, Ethylenediaminetetraacetic acid; FA, folic acid, H4-THF, tetrahydrofolic acid, 5-CH3-H4-THF, 5-methyl-tetrahydrofolic acid, 5-CHO-H4-THF, 5-formyl-tetrahydrofolic acid, 10-CHO-FA, 10-formyl-folic acid; Fru, fructose, Xyl, xylose, Gal, galactose, Man, Mannose.

a Phenomenex Luna C8 LC column (250 mm × 4.6 mm I.D; particle size 5 µm, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany) according to (Paper et al., 2022). Pigment standards were purchased from CaroteNature (Lupsingen, Germany) and Sigma-Aldrich Chemie (Weinheim, Germany) in HPLC grade.

## 2.3 Microbiological load

2.4. Microbiological analysis was performed according to standard ISO methodology in an ISO17025 accredited laboratory (STS 0093). All media were purchased from HiMedia (Lucerna-Chem AG, Lucerne, Switzerland). All media were performance tested according to ISO 11133:2014 before use. 10 g of powdered algae samples were reconstituted in 90 mL of Sodium Chloride Peptone Broth (SCPB; Millipore, Zug, Switzerland). Serial dilutions (1:10) were prepared for duplicate plating of chosen dilutions. Aerobe mesophilic microorganisms (ISO 4833-1:2013) were analyzed using Plate Count Agar (PCA) incubated at 30°C for 72 h. Total yeast and molds were enumerated following ISO 7954:1987, where samples were incubated for up to 5 days at 25°C on Yeast Extract Glucose Chloramphenicol Agar (YGC-A). Quantitative determination of presumptive *Bacillus cereus* was performed using Mannitol Egg Yolk Polymyxin Agar (MYP-A) as described in ISO 7932:2004/Amd 1:2020. Samples were incubated for 24 h at 30°C. On the medium, typical colonies of *B. cereus* appear rough and dry, set against a bright pink background with an egg yolk precipitate. Colonies exhibiting this characteristic appearance were examined microscopically for the typical morphology of *B. cereus*, which is characterized by large cells (3.5 µm) that sometimes form short chains or long strands, featuring central to terminal ellipsoidal spores that do not cause the cells to swell. For the enumeration of *Enterobacteriaceae*, Violet Red Bile Glucose Agar (VRBG-A) was used according to ISO 21528-2:2017. An overlay of VRBG-A was used to prevent swarming colonies and to provide semi-anaerobic conditions. Samples were incubated at 37°C for 24 h. Small (1–2 mm in diameter), round and violet to pink colonies with a halo surrounding the colony and a confirmed oxidase negative test were counted. After all ISO accredited methods were performed, the 1 g of powdered sample was reconstituted in 9 mL of SCPB and heated to 80°C for 12 min to eliminate all viable microorganisms (McHugh et al., 2017). The heat-treated samples were analyzed for mesophilic aerobic spores using Tryptic Soy Agar (TSA) incubated at 37°C for 48 h (Kyrylenko et al., 2023). The presence of total anaerobic spores was estimated by plating the heat-treated samples on Brain Heart Infusion Agar (BHI-A) and incubating at 37°C for 24 h in an anaerobic workstation (Whitley A85 Workstation, Meintrup DWS Laborgeräte, Herzlake, Germany) (Hu et al., 2021)

## 2.4 Determination of water content and water activity

The water content of the samples was determined by drying oven method according to the MEBAK method R-110.40.020 [2016-03] (Mebak, 2016a). The water activity (aw) of the samples was measured using a Novasina TH 500/RTD-502 AW SPRINT (Novasina, Lachen, Switzerland) with the measurement chamber set to 25°C.

## 2.5 Determination of raw Protein content

The raw protein content of the samples was determined by Kjeldahl method according to the MEBAK method R-110.41.030 [2016-03] (Mebak, 2016b) taking into account the nitrogen-to-protein conversion factor ($k_p$) of 4.78 (Sägesser et al., 2023).

## 2.6 Determination of ash content

About 1 g of the different *Chlorella* powders ashed at 650°C in pre-weight crucibles for 4h. Afterwards, the crucibles were placed in a vacuum desiccator for cooling and weight again to determine the ash content. All experiments were carried out in biological triplicates.

## 2.7 Amino acid analysis

The amino acid composition after acidic hydrolysis was determined according to the literature (Wollmann and Hofmann, 2013). Aliquots (5 mg) of the *Chlorella* powders were suspended in aqueous hydrochloric acid (6 M; 3 mL) and incubated for 24 h at 110°C. Cooled and neutralized (NaOH; 12 M) solutions were further diluted to 5 mL with water. Aliquots (990 µL) thereof were then spiked with the internal standard (10 µL) and quantified by means of SIDA-HPLC-MS/MS (Hillmann et al., 2012).

## 2.8 Fatty acid analysis

The fatty acid profile was determined according to Engelhart-Straub et al. using 10 mg of the different *Chlorella* powders. The sample preparation, namely, the extraction and transesterification of the lipids, was performed using a Multi-Purpose Sampler MPS robotic (Gerstel, Linthicum Heights, MD, United States) with C12:0 TAG as internal standard for quantification (Engelhart-Straub et al., 2022). The samples were measured at an GC-2025 coupled to an AOC-20i Auto Injector and AOC-20s Auto Sampler (Shimadzu, Duisburg, Germany). Marine oil fatty acid methyl ester (FAME) mix (20 components from C14:0 to C24:1; Restek GmbH, Bad Homburg, Germany) was used as standard for the quantification of the fatty acid methyl esters (FAMEs).

## 2.9 Sugar analysis

The *Chlorella* powders were chemically hydrolyzed using 2% sulfuric acid. For this, 50 mg of each powder was suspended in 19.6 mL water and incubated at 95°C for 1 h in a water bath. Afterwards, the solids were separated from the water phase by centrifugation (19,000 rcf, 10 min, RT). The supernatant was acidified to a final sulfuric acid concentration of 2% (v/v) with

concentrated acid and the pellet was resuspended in 2% (v/v) sulfuric acid. After autoclaving (60 min, 121°C) both acidic liquid phases were neutralized with solid calcium carbonate. 2 mL of each supernatant were frozen overnight, thawed, and filtered through a 0.2 µm Phenex-NY syringe filter (Phenomenex, Torrance, CA, United States). 3 µL of 0.5 M EDTA was then added to 197 µL of sample. The samples were analyzed in a Agilent HPLC according to Jurkowski et al. with 60 min runtime per sample (Jurkowski et al., 2022).

## 2.10 Determination of vitamins C, D, and E

Vitamin C concentrations were quantified using the Ascorbic Acid Assay Kit MAK074 (Sigma-Aldrich, St. Louis, MO, United States). By means of a coupled enzyme reaction, the resulting colorimetric product was measured at 570 nm, which forms proportionally to the ascorbic acid content in the sample. Vitamin D2 and D3 contents were quantified by means of the ELISA Kit E-EL-0012, purchased from Elabscience Biotechnology Inc. (Wuhan, China). The measurements were conducted at 450 nm. Vitamin E contents were quantified by the Colorimetric Assay Kit E-BC-K033 (Elabscience Biotechnology Inc.), measuring the reduction of $Fe^{3+}$ to $Fe^{2+}$ by Vitamin E and further colorimetric reaction with phenanthroline at 533 nm. All sample preparations were conducted completely according to the respective guidelines and spectrophotometric measurements were performed with a microplate reader (Infinite 200, Tecan Group AG, Männedorf, Switzerland) at individual wavelengths specified for each assay.

## 2.11 Folate quantification

### 2.11.1 Extraction

The algae samples were analyzed in triplicate according to Striegel et al. with regards of the five folate vitamers PteGlu, $H_4$folate, 5-$CH_3$-$H_4$folate, 5-CHO-$H_4$folate, and 10-CHO-PteGlu (Striegel et al., 2018). The complete information about chemicals, standards, and preparations of solutions as well as validation of the method can be obtained from this previous publication (Striegel et al., 2018). 5–10 mg of freeze-dried, ground algae samples were used for quantification. After equilibration for 15 min with 10 mL buffer, the respective internal standards [$^{13}C_5$]-PteGlu, [$^{13}C_5$]-$H_4$folate, [$^{13}C_5$]-5-$CH^{3}$-$H_4$folate, [$^{13}C_5$]-5-CHO-$H_4$folate, and [$^{13}C_5$]-10-CHO-PteGlu were added in equal amounts of the expected concentration of the unlabeled analytes in the sample. Following 15 min equilibration time and boiling for 10 min, 900 mL chicken pancreas solution and 400 mL rat serum were added for deconjugation. After overnight incubation at 37°C, the samples were boiled for 10 min and 10 mL acetonitrile was added to the cooled sample. The supernatant of each sample was used for purification by solid phase extraction (SPE), filtered and measured by LC-MS/MS. All given results are based on dry biomass.

### 2.11.2 Instrumentation

The LC-MS/MS analysis was carried out as described in a previous publication with slight modifications (Striegel et al., 2018). The quantification was performed on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) with a Raptor ARC 18 column (2.7 µm, 100 × 2.1 mm, Restek, Bad Homburg, Germany) and a Raptor ARC-18 precolumn (2.7 µm, 5 × 2.1 mm, Restek) as a stationary phase held at 30°C. The mobile phase for the binary gradient consisted of (A) distilled water with 0.1% (v/v) formic acid and (B) acetonitrile with 0.1% (v/v) formic acid at a flow rate of 0.4 mL/min. The LC system was coupled with a triple quadrupole mass spectrometer (LCMS-8050, Shimadzu, Kyoto, Japan), operated in positive ESI mode.

## 2.12 Metal ion analysis

For the measurement of metal content in algal biomass, between 0.5 g and 1 g of dry algal biomass were incubated in 10 mL of demineralized water under constant shaking. Afterwards, the samples were centrifuged at 10.000 rcf for 10 min and the supernatant was analyzed. ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) (Agilent 725 Series ICP Optical Emission Spectrometer, Agilent Technologies Inc., United States) was used to determine the metal content in the investigated solutions. As a calibration standard, a Certipur® ICP multi-element standard solution IV from Merck (Merck KGaA, Darmstadt, Germany) comprising 23 elements was used. The ICP Expert II Agilent 725-ES Instrument Software Version 2.0 (Agilent Technologies Inc., United States) was used to analyze the data.

## 2.13 Sensory analysis

### 2.13.1 General conditions and panel training

Sensory analysis was carried out by fifteen trained panelists (ten women and five men, age 21–45 years) from the Chair of Food Chemistry and Molecular Sensory Science at the Technical University of Munich using a quantitative descriptive analysis. Panelists had no history of known disorders and were trained weekly for a minimum of at least 2 years to be able to distinguish precisely between different aroma and taste qualities (Utz et al., 2022). Before the quantitative descriptive analyses (QDA) began, the panelists agreed upon a consistent panel language according to Stone and Sidel and available sensory lexicons (de Godoy et al., 2020; Stone et al., 2012; Stone et al., 2004). Thus, for orthonasal aroma evaluation, the following attributes including their reference compounds in brackets (20 mL; 100-fold odor thresholds in water) were chosen: metallic (Iron (II) sulfate), green (hexanal), hay-like (3-methyl-2,4-nonanedione), malty (3-methylbutanal), earthy (2,3,5-trimethyl-pyrazine), citrus-like (octanal), and sea breeze-like (without a reference substance) (Fischer and Schieberle, 2009; Kreissl et al., 2022; Mall and Schieberle, 2017). For taste evaluation, the panelists continuously wore nose clips to avoid cross-modal interactions with odorants. For training, aqueous solutions of sucrose for sweet taste, lactic acid for sour taste, monosodium glutamate for umami taste, sodium chloride for salty taste, caffeine for bitter taste, and tannic acid for astringency were used according to earlier investigations (Utz et al., 2021). For kokumi sensation, a reduced glutathione solution in model broth was utilized, respectively (Brehm et al., 2019). Before and between each test, the panelists rinsed their mouth

TABLE 1 Overview of tested *Chlorella* powders showing species, cultivation method, downstream processing and supplier according to the supplier's information.

| Name | Species | Cultivation | Downstream | Supplier |
|---|---|---|---|---|
| A | *Chlorella vulgaris* (green) | Autotrophic, tubular photobioreactor, Germany | Spray dried | Algenfarm Klötze GmbH and Co. KG |
| B | *Chlorella sorokiniana* (white) | Heterotrophic, stainless steel bioreactor, Netherlands | Washed, spray dried | Aliga microalgae – Aliga ApS |
| C | *Chlorella sorokiniana* (white) | Heterotrophic, stainless steel bioreactor, Netherlands | Washed, high-pressure homogenized, spray dried | Aliga microalgae – Aliga ApS |
| D | *Chlorella sorokiniana* (white) | Heterotrophic, stainless steel bioreactor, Netherlands | Spray dried | Aliga microalgae – Aliga ApS |
| E | *Chlorella sorokiniana* (green) | Heterotrophic, stainless steel bioreactor, Netherlands | Spray dried | Aliga microalgae – Aliga ApS |
| F | *Chlorella pyrenoidosa* (green) | Autotrophic, freshwater pool, People's Republic of China | Mechanical cell disruption, spray dried | BIONUTRA - BTG Berlin Trade GmbH |
| G | *Chlorella vulgaris* (yellow) | Heterotrophic, stainless steel bioreactor, Portugal | Spray dried | ALLMICROALGAE - Natural Products, SA (Allma) |
| H | *Chlorella vulgaris* (white) | Heterotrophic, stainless steel bioreactor, Portugal | Spray dried | ALLMICROALGAE - Natural Products, SA (Allma) |

for about 20 s with Evian water (Évian-les-Bains, France). All sensory data was collected in special sensory cabins with air-conditioning at 20–25°C.

### 2.13.2 Flavor profile analysis (FPA)

According to Caul (1957) a flavor profile analysis was performed (Caul, 1957). Thereby, each FPA of different *Chlorella* powders was divided into orthonasal aroma and taste experiments. 100 g/L *Chlorella* powder was suspended in Evian water (pH 5.5) and presented in closed sensory vials (45 mL) to the panelists, who evaluated the described aroma and taste qualities from 0 (not detectable) to 5 (very intense). Principal Component Analysis (PCA) was performed on the average scaled sensory data using the PCA() function from FactoMineR in R (R Core Team, Vienna, Austria). The first two principal components were visualized in a combined plot using the ggplot2 and ggrepelR packages for both individuals and variables.

## 2.14 Statistical analysis

Differences in biomass composition were statistically evaluated with a one-way ANOVA followed by a *post hoc* analysis according to Scheffé. A level of $p \leq 0.05$ was considered significant. Calculations were performed with Microsoft Excel® (365 MSO, Version 2501, Microsoft Corp., Redmond, United States).

# 3 Results

## 3.1 Pigment analysis

The qualitative HPLC-based pigment analysis showed signals for chlorophylls (e.g., chlorophyll A and B) as well as lutein in all green *Chlorella* powders (A, E, F). In addition, β-carotene was detected in samples E and F, while neoxanthin was just present in sample E. The yellow *Chlorella* powder G showed a signal for lutein as dominant pigment, but zeaxanthin was also detected for this sample. For the white *Chlorella* powders there were no pigments detectable at all (Supplementary Figure S2).

## 3.2 Microbiological load

The photoautotrophically generated sample A (Algomed, *C. vulgaris* (green)) and F (Bionutra, *Chlorella pyrenoidosa* (green)), which are cultivated in closed photobioreactors or open ponds, had elevated counts of aerobic mesophilic microorganisms, aerobe mesophilic spores, anaerobe mesophilic spores, and *B. cereus* (Table 2). However, food is considered potentially hazardous only if levels emetic toxin producing *B. cereus* strain(s) exceed $10^5$ cfu/g in foods (Authority, 2005). Furthermore, as all samples exhibited a water activity ($a_w$) below 0.4 sporulation and growth of all spores in the samples is unlikely. All tested *Chlorella* powders fulfilled the requirements for food applications in microbiological contaminations (EFSA).

## 3.3 Biomass composition

Protein content was calculated with Kjeldahl measurements using a conversion factor of 4.78 and was overall lower for the chlorophyll-deficient strains B-D and G-H (Sägesser et al., 2023). The carbohydrate content, determined by HPLC analysis after chemical hydrolysis to convert carbohydrates like cell wall components and starch to monomeric sugars, was higher for all heterotrophic cultivations (B-E, G-H) independent of the chlorophyll content. The lipid quantification according to FAME analysis resulted in lipid contents lower than 10% for all samples, with slightly lower results for the *Chlorella*

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TABLE 2 Microbiological profiles of the tested *Chlorella* powders; A: Algomed, *Chlorella vulgaris* (green), phototrophic; B: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed, HPH; C: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed; D: Aliga, *Chlorella sorokiniana* (white), heterotrophic; E: Aliga, *Chlorella sorokiniana* (green), heterotrophic; F: Bionutra, *Chlorella pyrenoidosa* (green), phototrophic; G: Allma, *Chlorella vulgaris* (yellow), heterotrophic; H: Allma, *Chlorella vulgaris* (white), heterotrophic. All experiments were carried out in biological duplicates.

| Product | Aerobe mesophilic microorganisms cfu/g | Aerobe mesophilic spores cfu/g | Anaerobe mesophilic spores cfu/g | *Bacillus cereus* Cfu/g | *Entero-bacteriaceae* Cfu/g | Yeasts (Y)/ Molds (M) Cfu/g |
|---|---|---|---|---|---|---|
| A | $8.0 \times 10^6$ | $3.7 \times 10^5$ | $1.6 \times 10^5$ | $3.4 \times 10^3$ | <10 | <100 |
| B | $2.0 \times 10^3$ | $1.3 \times 10^2$ | 10 | $4.0 \times 10^2$ | <10 | <100 |
| C | <1,000 | <10 | <10 | $1.0 \times 10^2$ | <10 | <100 |
| D | <1,000 | 30 | <10 | $6.0 \times 10^2$ | <10 | <100 |
| E | <1,000 | <10 | <10 | <100 | <10 | <100 |
| F | $3.5 \times 10^4$ | $2.0 \times 10^3$ | $2.5 \times 10^2$ | $1.3 \times 10^3$ | <10 | 50 (M) |
| G | <1,000 | 50 | 15 | <100 | <10 | <100 |
| H | <1,000 | 30 | <10 | <100 | <10 | <100 |
| Method | ISO 4833 | Kyrylenko et al. (2023) | Hu et al. (2021) | ISO 7932 | ISO 21528 | ISO 7954 |

*sorokiniana* samples (B-D) (Table 3). In addition, the ash content was in a comparable range for all samples without washing step in the downstream processing (A, D-H) and lower with washing step (B, C). Proteins, carbohydrates, lipids, ash, and moisture did not sum up to 100 g, the difference (A: 23.7, B: 17.5, C: 7.0, D: 9.9, E: 2.7, F: 20.8, G: 15.4, H: 19.6) is mostly referred as fiber content.

### 3.3.1 Amino acid profile

Independent of the species, cultivation, or downstream processing all *Chlorella* powders had a comparable amino acid profile containing all essential amino acids except L-methionine and L-tryptophan. The highest fluctuation between samples was found in L-arginine ranging from 2.1% to 10.4% of the total amino acids. Also, the E/N-ration ranging from 0.65 to 0.89 (A: 0.89, B: 0.76, C: 0.68, D: 0.69, E: 0.65, F: 0.80, G: 0.75, H: 0.75) is comparable over all tested samples (Figure 1A; Supplementary Table S1).

### 3.3.2 Carbohydrate composition

The sugar profile of the tested *Chlorella* powders mainly consists of glucose, rhamnose and a mixed peak of fructose, xylose, galactose, and mannose, representing the monomers of cellulose, pectin, and hemicellulose. While there is no significant difference in the sugar profile between the different species, heterotrophic cultivation resulted in a higher amount of glucose (A, F) compared to the photoautotrophically cultivated biomass (Figure 1B; Supplementary Table S2)

### 3.3.3 Fatty acid composition

In contrast to the amino acid profile, the fatty acid profile showed more pronounced differences between the products. While the amount of C16:0 is comparable for most samples, sample B showed a twofold higher C16:0 content. In general, the green powders (A, E, F) contained more C18:3 compared to the chlorophyll deficient strains (Figure 1C; Supplementary Table S3).

### 3.3.4 Vitamins C, D, and E

The photoautotrophic samples (A, F) showed a higher vitamin E content, but a lower vitamin C content compared to the heterotrophic samples (B-E, G-H). Among the *Chlorella sorokiniana* (B-E) strains, vitamin E was determined for the chlorophyll-containing sample E in the highest amount and at low quantities for the white *Chlorella* powders (B-D). In addition, the mechanical cell disruption using high-pressure homogenization led to an increase of detectable vitamin D and reduced vitamin C (samples B compared to C and D) (Figure 2A; Supplementary Table S4).

### 3.3.5 Folates

Folates were present in all tested samples, but the amount and proportion of the vitamers was different. While the folate content was higher for the *Chlorella vulgaris* strains (A, G, H) as well as *Chlorella pyrenoidosa* (F), it was much lower for the *C. sorokiniana* strains (B-E) (Figure 2B; Supplementary Table S5).

### 3.3.6 Metals

Potassium was the most prominent metal ion found in all samples. The samples from Aliga (B-E) also showed a significant amount of sodium in the powder, which is most probably a residue from the cultivation medium. Magnesium was present in all samples, while calcium was just found in traces in the Aliga samples (B-E). Washing, or washing combined with mechanical cell disruption led to a decrease in metal ion content. (B, C compared to D) (Figure 2C; Supplementary Table S6).

## 3.4 Sensory analysis

The quantitative descriptive analysis of the orthonasal aroma evaluation of the microalgae samples revealed similar aroma profiles among the different products (Figure 3A). For all samples except B-E (Aliga), hay-like was the most dominant aroma. Sample A (Algomed) exhibited the highest intensity of the green and hay-like aromas, scoring

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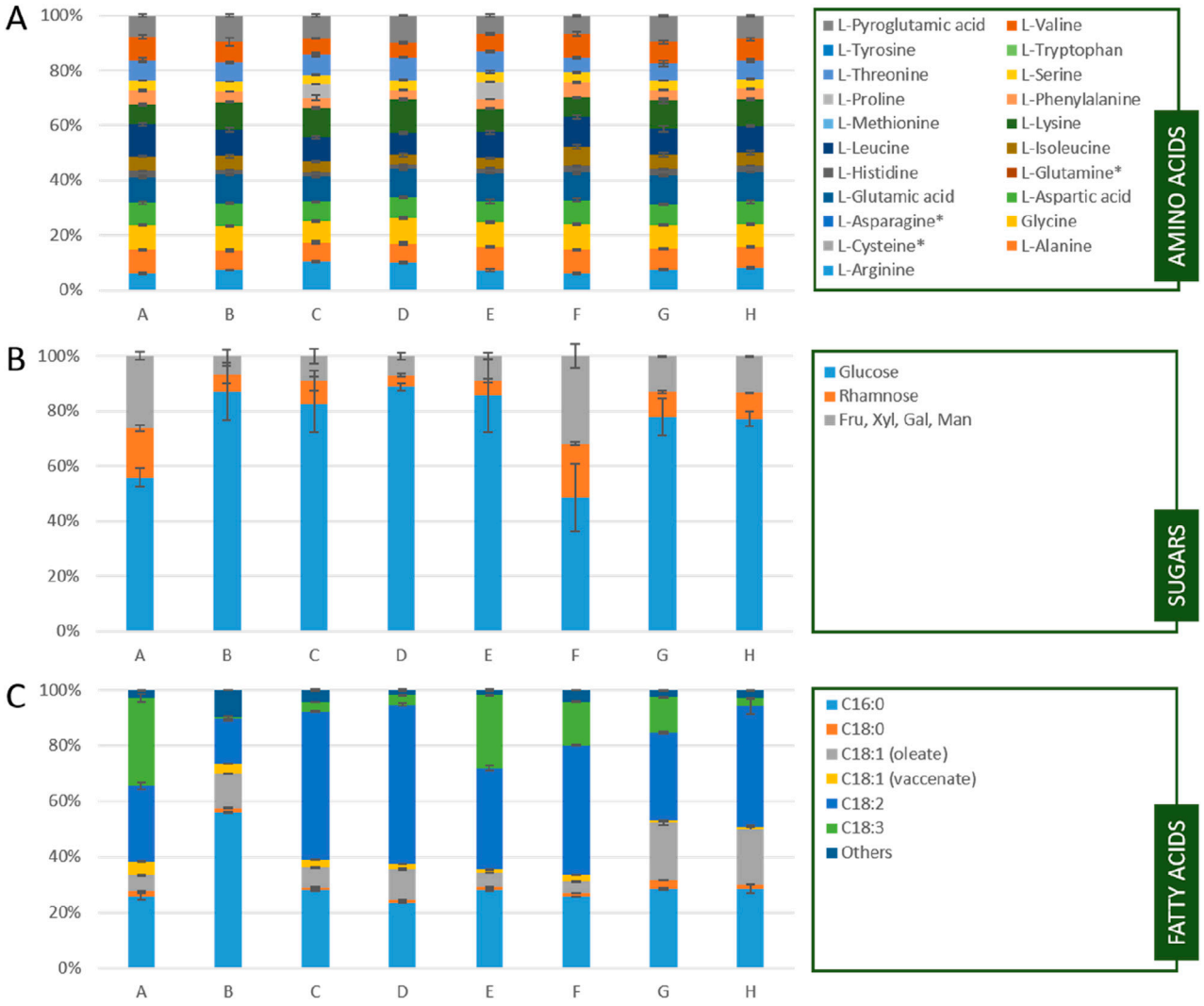


FIGURE 1
**(A)** Amino acid profile **(B)**sugar profile and **(C)** fatty acid profile of the tested *Chlorella* powders; A: Algomed, *Chlorella vulgaris* (green), phototrophic; B: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed, HPH; C: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed; D: Aliga, *Chlorella sorokiniana* (white), heterotrophic; E: Aliga, *Chlorella sorokiniana* (green), heterotrophic; F: Bionutra, *Chlorella pyrenoidosa* (green), phototrophic; G: Allma, *Chlorella vulgaris* (yellow), heterotrophic; H: Allma, *Chlorella vulgaris* (white), heterotrophic; * L-Cysteine is not analyzable without derivatization due to its high reactivity, L-Asparagine and L-Glutamine are oxidized to L-Aspartic or L-Glutamic acid by acidic hydrolysis. nd = not detectable; Fru: fructose, Xyl: xylose, Gal: galactose, Man: Mannose; In A bars and error bars represent the means and standard deviation of biological duplicates (n = 2) and technical triplicates (n = 3) for B and C bars and error bars represent the means and standard deviation of biological triplicates (n = 3).

2.8 and 3.2, respectively, suggesting a pronounced grassy and straw-like note. In contrast, sample D (Aliga) had the lowest green aroma score (1.1). Samples G and H both received similar scores in most aroma categories, suggesting a comparable sensory profile between the two Allma samples. Regarding other aroma attributes, sample D (Aliga) stood out with the highest malty aroma score of 2.1. Allma samples (G, H) received lower scores for earthy aroma than the other samples. In general, all the samples were attributed with lower and similar values for metallic, citrus-like, and sea breeze-like aromas. The prevailing scents in photoautotrophic samples (A, F) were green, hay-like, and earthy, whereas in heterotrophic samples, especially Allma (G, H), the dominant aromas were hay-like and malty. Overall, only smaller differences were found between the samples. The biggest distinction was observed for the green and hay-like aromas. The sensory analysis of taste attributes among the different microalgae samples revealed some differences (Figure 3). In terms of sweetness, sample B (Aliga) exhibited the lowest score among the samples, the other ones being in a similar range as for sourness, samples B to D exhibited the most pronounced characteristics compared to the others. Concerning saltiness, sample D displayed notably higher levels than the other samples, which presented similar scores. Sample G exhibited the lowest score for bitterness. For umami, astringency, and kokumi, all the samples presented similar scores. Umami also was the dominant taste in both Allma samples (G, H), which were also characterized by reduced bitterness.

The principal component analysis (PCA) of sensory data revealed further clustering patterns among the *Chlorella* powders in the multivariate space, which are partially connected to cultivation location (along with supplier) and color (Figure 3B). The first two principal components explained 40.9% (PC1) and 31.3% (PC2) of the

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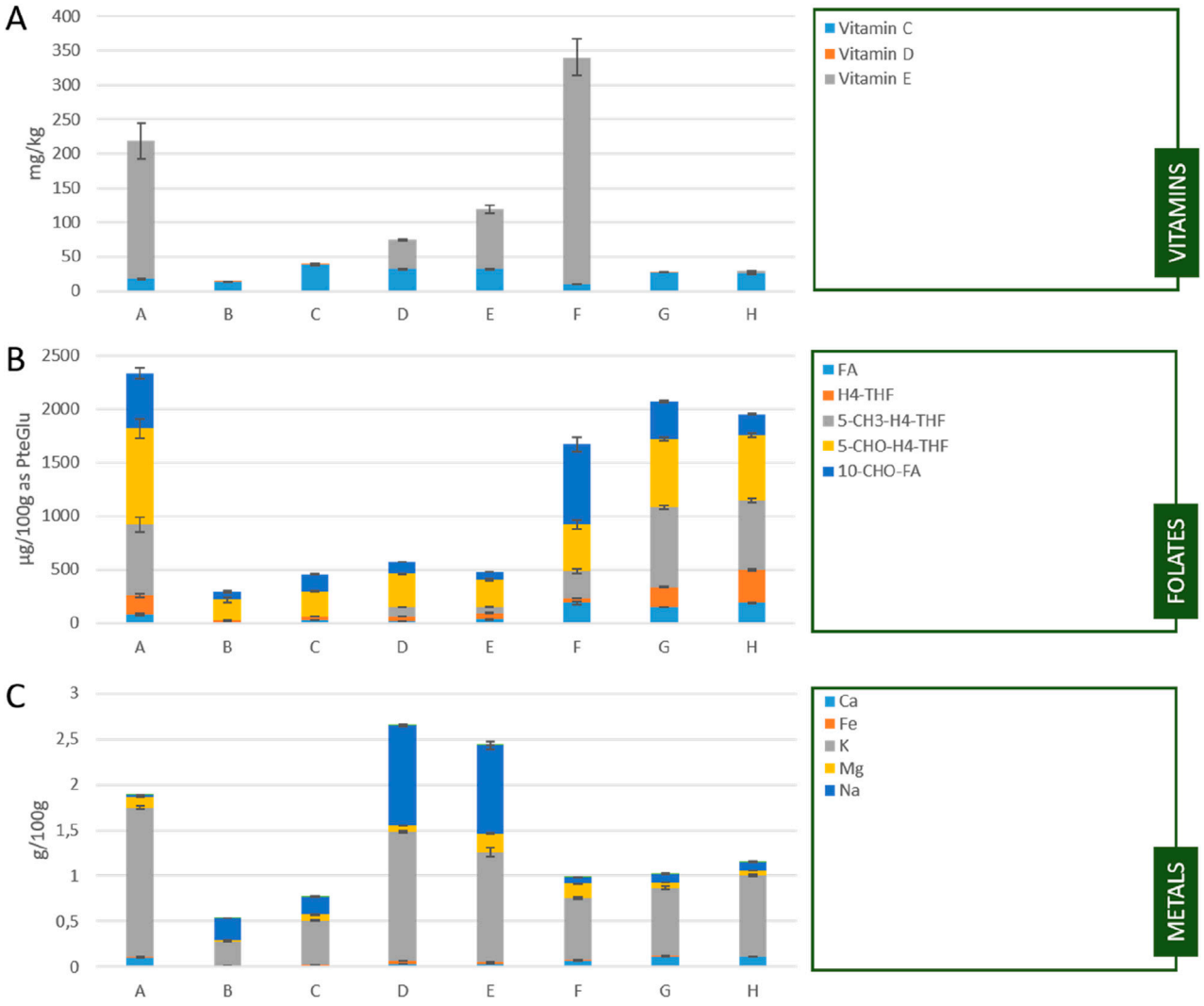


FIGURE 2
**(A)** Vitamin C, D, and E content **(B)** folate content and **(C)** metal content of the tested *Chlorella* powders; A: Algomed, *Chlorella vulgaris* (green), phototrophic; B: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed, HPH; C: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed; D: Aliga, *Chlorella sorokiniana* (white), heterotrophic; E: Aliga, *Chlorella sorokiniana* (green), heterotrophic; F: Bionutra, *Chlorella pyrenoidosa* (green), phototrophic; G: Allma, *Chlorella vulgaris* (yellow), heterotrophic; H: Allma, *Chlorella vulgaris* (white), heterotrophic; FA: folic acid, H4-THF: tetrahydrofolic acid, 5-CH3-H4-THF: 5-methyl-tetrahydrofolic acid, 5-CHO-H4-THF: 5-formyl-tetrahydrofolic acid, 10-CHO-FA: 10-formyl-folic acid; Bars and error bars in **(A)** represent the means of biological (n = 2) and technical replicates (n = 2) as well as relative standard deviation. Bars and error bars in **(B,C)** represent the means and standard deviation of biological triplicates (n = 3).

variance, suggesting differences in sensory perception across the samples. An influence of sample color (green, white, yellow) was observed in PCA clustering (Figure 3B). Green-colored samples (A, E, F) clustered in the positive PC1/PC2 region, suggesting a shared sensory profile which is associated with the sensory variables green, hay-like, and sea-breeze-like attributes (Figure 3C). In contrast, some white samples (B, C, D) are positioned in the negative PC1 region and associated with sour, earthy, and bitter notes (Figures 3B,C). The yellow sample (G) and the white sample H positioned towards the positive PC1 and negative PC2 regions were associated with sweet, umami, and kokumi flavors (Figures 3B,C). These two samples cluster differently from white samples B, C and D and from green samples F, E, and A. This difference suggests that other variables may be playing a role in addition to color. In this case, samples G and H are produced by the same supplier in the same geographical region and with the same processing conditions. Samples cultivated under autotrophic conditions (A, F) and heterotrophically cultivated samples (B, C, D, E, G, H) exhibited more significant variability along PC1 and PC2. This suggests that cultivation conditions and species differences do not seem to contribute to sensory characteristics uniformly. Among heterotrophic samples, *Chlorella sorokiniana* (B, C, D) formed a subgroup from *Chlorella vulgaris* (G, H). However, *C. sorokiniana* sample E and *C. vulgaris* sample A seem to follow the opposite pattern. Further examination of downstream processing methods indicated that high-pressure homogenization does not seem to explain variance. Sample C resulted in a sensory profile like sample B (same supplier, same color, but no high-pressure homogenization was applied), distinct from other samples. These two samples correlate with bitter, astringent, and earthy notes, which other variables may explain in addition to those presented in Table 1.

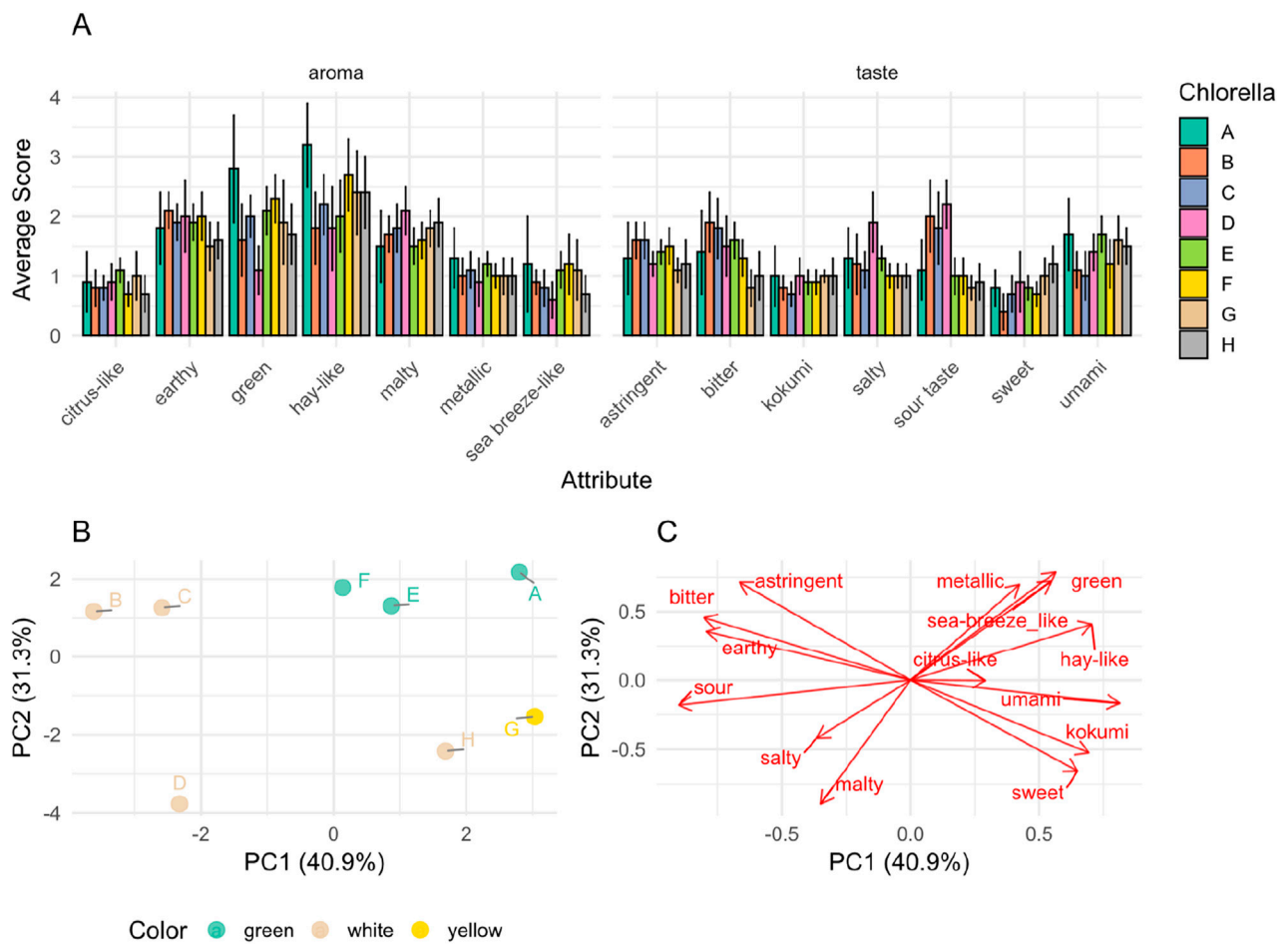


FIGURE 3
Multi-panel visualization of the sensory data **(A)** Comparative flavor profile analysis of the tested *Chlorella* powders (10% in Evian water, pH 5.5) in comparison to the reference "A" (mean ± CI; α = 0.05; n = 12); A: Algomed, *Chlorella vulgaris* (green), phototrophic; B: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed, HPH; C: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed; D: Aliga, *Chlorella sorokiniana* (white), heterotrophic; E: Aliga, *Chlorella sorokiniana* (green), heterotrophic; F: Bionutra, *Chlorella pyrenoidosa* (green), phototrophic; G: Allma, *Chlorella vulgaris* (yellow), heterotrophic; H: Allma, *Chlorella vulgaris* (white), heterotrophic **(B)** PCA individuals plot displaying the distribution of *Chlorella* samples based on sensory attributes, colored by microalgae color. **(C)** PCA variables plot illustrating the contribution of sensory attributes to the principal components.

# 4 Discussion

## 4.1 Pigment analysis

The analysis of photosynthetic pigments gave results that correlated well with the color of the respective products. In accordance to the literature dominant pigments in all green samples were chlorophyll A and B followed by one of the most prominent carotenoids in *Chlorophyceae* lutein, while in the yellow sample chlorophylls were lacking and lutein was the main remaining pigment (Batista et al., 2013; Khairunnisa et al., 2024). Moreover, all white products did not show any photosynthetic pigments correlating with the sample color.

## 4.2 Microbiological load

While all samples had microbial loads below the food grade certification limits, there were significant differences between the autotrophic and heterotrophically cultivated samples. Generally, the autotrophic samples had a higher microbial load than the heterotrophically gown *Chlorella* powders, as latter were generated by precision fermentation using axenic starter cultures in sterilized stainless steel bioreactors. The detected microbial load will most likely be due to the post-harvesting processes that are not conducted under sterile conditions. In this context, washing steps tend to reduce the microbial load, while additional mechanical processing, such as high-pressure homogenization tends to increase microbial loads. The latter may be due to remaining biomass between processing batches, which adds to carry over in the following processing steps. By contrast, in the autotrophic systems the algae cultures contain a symbiotic microbial flora, which is propagated together with the algae biomass in the photobioreactor or open ponds (Almalki et al., 2024). In this context, the autotrophically generated products can be compared to conventional agricultural food products, which all contain a microbial accompanying flora. Biomass processing can significantly impact microbial contamination levels and hence product quality and shelf life.

TABLE 3 Protein, carbohydrate, lipid, ash and moisture content of the tested *Chlorella* powders; A: Algomed, *Chlorella vulgaris* (green), phototrophic; B: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed, HPH; C: Aliga, *Chlorella sorokiniana* (white), heterotrophic, washed; D: Aliga, *Chlorella sorokiniana* (white), heterotrophic; E: Aliga, *Chlorella sorokiniana* (green), heterotrophic; F: Bionutra, *Chlorella pyrenoidosa* (green), phototrophic; G: Allma, *Chlorella vulgaris* (yellow), heterotrophic; H: Allma, *Chlorella vulgaris* (white), heterotrophic; Numbers represent the means and standard deviation of biological triplicates (n = 3). Statistically significant differences in biomass composition were determined in a one-way ANOVA ($p \leq 0.05$), followed a Scheffé *post hoc* test. Different superscript letters (a-f) in the one row correspond to significant differences ($p < 0.05$). For detailed significant differences see Supplementary Tables S7, S13.

| Biomass composition [g/100g] | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| Proteins | $40.1^{a}$ | $16.8^{b}$ | $21.7^{c}$ | $30.2^{d}$ | $43.1^{e}$ | $44.1^{e}$ | $27.2^{f}$ | $27.1^{f}$ |
| | ±0.3 | ±0.1 | ±0.1 | ±1.1 | ±0.1 | ±0.2 | ±0.3 | ±0.1 |
| Carbohydrates | $18.6^{a}$ | $52.8^{bc}$ | $57.7^{b}$ | $43.7^{cd}$ | $39.6^{d}$ | $15.3^{a}$ | $38.7^{d}$ | $37.8^{d}$ |
| | ±0.4 | ±1.4 | ±8.7 | ±1.9 | ±2.4 | ±0.5 | ±1.2 | ±0.4 |
| Lipids | $6.0^{a}$ | $1.8^{b}$ | $2.6^{c}$ | $2.4^{bc}$ | $2.8^{c}$ | $7.8^{d}$ | $7.2^{d}$ | $5.7^{a}$ |
| | ±0.3 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.4 |
| Ash | $6.9^{a}$ | $2.6^{b}$ | $3.8^{c}$ | $6.4^{d}$ | $6.3^{d}$ | $6.5^{d}$ | $6.0^{e}$ | $5.1^{f}$ |
| | ±0.0 | ±0.1 | ±0.0 | ±0.0 | ±0.0 | ±0.1 | ±0.2 | ±0.1 |
| Moisture | $4.7^{a}$ | $8.5^{b}$ | $7.2^{c}$ | $7.4^{c}$ | $5.5^{d}$ | 5.5d | $5.5^{d}$ | $4.7^{a}$ |
| | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 | ±0.1 |

## 4.3 Biomass composition

The determined biomass compositions align with the information provided by the suppliers as well as the ranges reported in the literature. The deviations between the tested samples in proteins, carbohydrates, and lipids might be explained by different batches of biomass and different biomass cultivation and processing methods. Specifically, the composition of *Chlorella* biomass may vary significantly even within the same strain depending on the cultivation conditions, including light intensity, temperature, pH value, nutrient availability, and salinity (Kolackova et al., 2023; Muys et al., 2019). In addition to the cultivation parameters, the downstream processing can affect biomass composition depending on parameters like temperature, drying time, light, oxidation, and drying load (Hosseinizand et al., 2018; Silva et al., 2021; Vilatte et al., 2023). The detailed individual process parameters used for the biomass processing were not available.

The protein content was calculated from the nitrogen content determined by the Kjeldahl method using a nitrogen-to-protein conversion factor ($k_p$) of 4.78. This is a more conservative conversion factor compared to other reports, resulting in the calculation of lower protein contents (Sägesser et al., 2023). The Kjeldahl method is a general quantification method for the determination of organic and inorganic nitrogen including non-protein components, such as photosynthetic pigments (i.e., chlorophyll), cell wall components (i.e., glucosamine) as well as nitrogen-containing media components used for algae cultivation. This non-protein related nitrogen has to be considered when defining a conversion factor, next to the amino acid composition of the contained protein, otherwise protein content may be overestimated. For microalgal biomass different factors ranging from 3.59 to 6.35 are used, (López et al., 2010; Safi et al., 2013). The Kjeldahl method is the commonly used method for raw protein quantification in food and feed, but for a better comparison between different types of biomasses, especially between chlorophyll containing and deficient strains, individual nitrogen-to-protein conversion factors for each sample are needed for accurate protein quantification. In addition, another *Chlorella vulgaris* powder from Allma showed a higher protein content calculated with a conversion factor of 6.25 but with a high *in-vitro* digestibility of the biomass including the protein fraction (Niccolai et al., 2019).

The amino acid profile is quite comparable between all tested samples independent of strain, cultivation condition or downstream processing. *Chlorella* generally has more leucine, lysine, and valine compared to meat like beef, mutton or pork. Reportedly, animal proteins tend to have higher levels of methionine and cysteine as well as glycine. Compared to soybean and egg, *Chlorella* contains lower levels of glutamic acid, lysine and Isoleucine. Overall, the data highlights the nutritional value of *Chlorella* sp. as a valuable protein source. Its amino acid profile makes it a promising candidate for supplementing human diets, particularly for individuals seeking plant-based protein alternatives (Table 4).

The carbohydrate content as well as the sugar profile of the tested *Chlorella* powders was comparable between the photoautotrophic (A, F) and the heterotrophic samples. In general, the heterotrophic samples showed a higher carbohydrate content and a higher share of glucose. The species, detailed cultivation conditions, and downstream processing seem to have a minor effect on the carbohydrate content or sugar profile. *C. sorokiniana* and *C. vulgaris* belong to the glucosamine group of the *Chlorella sp*., which means that the rigid cell is mostly composed of glucosamine together with glucose, mannose, rhamnose, and galactose (Takeda, 1988; Takeda, 1991). Therefore, the mixed peak of fructose, xylose, mannose, and galactose should be mainly composed of mannose and galactose. The higher share of glucose in the heterotrophic samples might also be due to the chemical hydrolysis of intracellular starch used as carbon storage in *Chlorella* (Takeshita et al., 2014). In addition, there are different methods to determine the carbohydrate and/or fiber content in food. While in some cases carbohydrates represent the residual biomass after determination on the other macronutrients, in this study

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TABLE 4 Amino acid composition in *Chlorella vulgaris*, meat samples and legumes. Ala, L-alanine; Gly, glycine; Asp, L-aspartic acid; Glu, L-glutamic acid; His, L-histidine; Ile, L-isoleucine; Leu, L-leucine; Lys, L-lysine; Phe, L-phenylalanine; Pro, L-proline; Ser, L-serine; Thr, L-threonine; Val, L-valine; Pyr, L-pyroglutamic acid, Met, methionine; Tyr, tyrosine; Trp, tryptophan, Cys, cysteine. References: A: average of all *Chlorella* powders tested in this study; B: (Koyande et al., 2019); C: (Jorfi et al., 2012); D: (Joint, 1977); E (Becker, 2007).

| Amino acids | *Chlorella* powders | *C. vulgaris* | Beef | Mutton | Chevon | Pork | FAO/WHO | Egg | Soybean |
|---|---|---|---|---|---|---|---|---|---|
| Ile | 4.8% | 3.9% | 3.5% | 3.1% | 3.6% | 3.4% | 4.0% | 6.6% | 5.3% |
| Leu | 9.7% | 9.1% | 7.1% | 5.8% | 7.1% | 7.3% | 7.0% | 8.8% | 7.7% |
| Val | 7.3% | 5.7% | 4.3% | 5.6% | 4.5% | 4.1% | 5.0% | 7.2% | 5.3% |
| Lys | 9.5% | 8.7% | 8.1% | 8.4% | 8.4% | 8.2% | 5.5% | 5.3% | 6.4% |
| Phe + Tyr | 4.1% | 8.7% | 5.2% | 5.4% | 5.7% | 5.4% | 6.0% | 10.0% | 8.7% |
| Met + Cys | — | 3.7% | 4.3% | 4.8% | 5.0% | 4.8% | 3.5% | 5.5% | 3.2% |
| Trp | — | 2.2% | — | — | — | — | 1.0% | 1.7% | 1.4% |
| Thr | 7.0% | 4.9% | 3.0% | 2.9% | 3.4% | 2.8% | — | 5.0% | 4.0% |
| Ala | 7.7% | 8.1% | 6.9% | 6.5% | 6.6% | 6.7% | — | — | 5.0% |
| Arg | 7.8% | 6.6% | 8.7% | 10.0% | 8.4% | 8.5% | — | 6.2% | 7.4% |
| Asp | 7.9% | 9.3% | 2.8% | 2.9% | 3.0% | 2.9% | — | 11.0% | 1.3% |
| Glu | 10.1% | 12.0% | 7.8% | 7.7% | 8.3% | 7.7% | — | 12.6% | 19.0% |
| Gly | 8.8% | 6.0% | 20.2% | 18.0% | 18.5% | 18.9% | — | 4.2% | 4.5% |
| His | 2.0% | 2.1% | 5.9% | 6.2% | 5.3% | 6.7% | — | 2.4% | 2.6% |
| Pro | 5.6% | 4.9% | 8.2% | 8.7% | 8.5% | 8.2% | — | 4.2% | 5.3% |
| Ser | 3.5% | 4.2% | 4.1% | 4.2% | 4.2% | 4.2% | — | 6.9% | 5.8% |
| Reference | A | B | C | C | C | C | D | E | E |

carbohydrates like starch and most cell wall components were chemically hydrolyzed and quantified by HPLC (Grácio et al., 2024). The residual biomass after determination of the macronutrients including carbohydrates is commonly considered as fiber content. Depending of the methods used for quantification of total dietary fibers there is a wide range in literature covering the non-defined amount of the tested *Chlorella* powders between 7.0 and 23.7 (Gao et al., 2024; Niccolai et al., 2019; Nova et al., 2019)

The cultivation conditions play a crucial role in shaping the fatty acid profile of microalgae. For instance, using hetero- or photoautotrophic cultivation, nutrient availability, light intensity, temperature, as well as pH can affect the synthesis of specific fatty acids (Alkhamis et al., 2022; Bajwa et al., 2019; Cheirsilp et al., 2023; Gómez-De la Torre et al., 2023; Yun et al., 2020). Interestingly, the fatty acid profile of sample B was quite different compared to sample C and D, even though they are the same strain and only underwent different downstream processing methods. While the washing step did not affect the fatty acid profile significantly, the high-pressure homogenization step led to a decrease in unsaturated fatty acid and in total lipid content. This could be explained by strong mixing as well as an increase in temperature during the process leading to oxidation of unsaturated fatty acids. This effect was analyzed by Canelli et al. observing higher lipid oxidation with the mechanical cell disruption (Canelli et al., 2021).

The vitamin C, D and E content varies in the published range over the tested *Chlorella* powders (Bito et al., 2020; Sandgruber et al., 2023). While the content of vitamin C was in a comparable range for all powders, the vitamin D content was quite low but higher for sample B with mechanical cell disruption. This increase might be caused by a better accessibility of the lipid soluble vitamin after cell disruption. On the other hand, this behavior was not detectable for sample F with mechanical cell disruption but other supplier, species, and cultivation. The highest vitamin E content was determined for the photoautotrophic samples, followed by the chlorophyll containing, heterotrophic sample (E). Interestingly, vitamin E was detected in the chlorophyll-deficient *Chlorella sorokiniana* but only without the washing step in downstream processing.

Notably, *Chlorella* has been recognized for its high folate levels (Bito et al., 2020; Edelmann et al., 2019; Woortman et al., 2020). Folate content and distribution of the different oxidation levels are reported to be dependent on the cultivation conditions as well as downstream processing (Blancquaert et al., 2015; Fitzpatrick et al., 2012; Woortman et al., 2020). The results for the tested *Chlorella* powders indicate that the species seems to have the highest impact on the folate content and distribution. The three different species *C. vulgaris* (A, G, H), *C. sorokiniana* (B-E) and *Chlorella pyrenoidosa* (F) showed similar results within the group independent of the supplier, cultivation, downstream processing or chlorophyll deficiency. The folate content of *C. vulgaris* and *C. pyrenoidosa* was in the published range of *C. vulgaris* between 1,200 and 3,600 µg/100 g (Bito et al., 2020), *C. sorokiniana* showed significant lower levels between 267 and 530 µg/100 g. While the content of vitamin C, D, and E in the tested *Chlorella* powders was quite low, *Chlorella*, especially *C. vulgaris*, seems to be a suitable source of folates in human diet.

Microalgae are known to adsorb and bioaccumulate metals, due to negatively charged functional groups of their cell walls (Kumar et al., 2015; Paper et al., 2023; Sandgruber et al., 2023). The main source of metals in the *Chlorella* powder is most probably the media composition used for cultivation. Washing the cells in water or buffer has demonstrated the effective removal of salts from microalgae (Khaw et al., 2021; Zhu and Lee, 1997). This can be seen by the significant reduction in metal content for samples B and C compared with the unwashed sample D. This reduction in metals aligns with the ash content determined for the tested samples, representing the total mineral amount.

## 4.4 Sensory analysis

The sensory profile of microalgae can be affected by various compositional factors (Van Durme et al., 2013). For instance, the presence of sulfuric compounds correlates with typical seafood flavors (sea breeze like variables) while shorter chain aldehydes contribute to green, fruity, and vegetable flavors (green and hay like attributes). Food manufacturers may use these intrinsic attributes to incorporate microalgae into alternative protein food products as a nutritious and valuable ingredient (Coleman et al., 2022). Our results point to differences among the tested *Chlorella* powders; these differences must be taken into account by the food manufacturer when choosing the raw material ingredient to be integrated into the production process.

Amino acid composition, particularly the content of phenylalanine, can lead to distinctively higher levels of benzaldehyde, impacting the overall aroma. Furthermore, alanine, glycine, proline, serine, and threonine may provide a sweet taste, whereas histidine, allo-isoleucine, isoleucine, leucine, methionine, phenylalanine, tryptophan, and valine are related to a bitter taste; aspartic acid and glutamic acid are correlated to the umami taste (Zamuz et al., 2019). Additionally, the concentration of PUFAs plays a role, where species with higher PUFA concentrations tend to exhibit more linear aldehydes, due to enzymatic processes and fatty acid oxidation. The presence of carotenoids in microalgae can also lead to the production of ionones, well-known for their floral sensory properties (Van Durme et al., 2013). Few studies have explored the sensory traits of *C. vulgaris*. One study by Van Durme focused on evaluating the aroma of phototrophic *C. vulgaris* while another study by Isleten Hosoglu examined a heterotrophic grown *C. vulgaris* (Hosoglu, 2018; Van Durme et al., 2013). They agree that *Chlorella* has a rather bland flavor profile dominated by 'grassy, vegetable, cucumber' aromas (Hosoglu, 2018; Van Durme et al., 2013) with less fishy, shrimpy and marine odors, making *Chlorella* less suitable for alternative seafood solutions (Francezon et al., 2021). Similar results were observed for the eight tested *Chlorella* powders in this study with a decrease in green and hay-like odor for the chlorophyll deficient strains (B-D, G, H) as well as heterotrophically cultivated green *Chlorella* to a smaller extent (E). While the sour taste was higher for all chlorophyll-deficient samples from Aliga (B-D), the salty flavor was reduced by washing in the downstream processing (D compared to B, C). Overall, from the dimensionality reduction of the sensory data, the color difference, alongside production location and supplier, seemed to emerge as relevant variables. Downstream processing, including drying, washing, and high-pressure homogenization, does not seem to influence the sensory profile of the studied samples. This may be due to limited information on the detailed cultivation and processing steps adopted for the studied samples. Green *Chlorella* samples A, E, F which clustered together based on their sensory profile (Figure 3B) were also found to contain high levels of C18:3 fatty acids compared to the other samples (Figure 1C). This sample cluster is associated the most with green, hay-like sensory notes which are, attributes associated with oxidation of unsaturated fatty acids and consequent production of aldehydes and ketones in alternative proteins (Mittermeier-Kleßinger et al., 2021).

These results represent key information for food suppliers to select the most suitable *Chlorella* powder for the specific application. For instance, a vegan replacement for seafood may look at powders with a higher intensity of umami, kokumi, and sea breeze-like sensory notes while minimizing bitter and sour notes. A deeper mechanistic understanding of the variables influencing each descriptor must still be obtained to fine-tune the cultivation and production process. This mechanistic understanding cannot be based on commercial products but has to be studied and controlled from cultivation to storage and consumption (Zhou et al., 2017).

# 5 Summary

The eight different types of *Chlorella* powders covering different suppliers, strains, cultivation methods, and downstream processing showed a comparable biomass composition with slightly lower protein contents for the chlorophyll deficient strains, slightly higher sugar contents for the heterotrophic samples, and slightly lower lipid contents for *C. sorokiniana*. The amino acid profile was comparable between all tested samples, in the sugar profile the glucose concentration was higher for the heterotrophic samples and the highest deviation was shown in the fatty acid profile. The vitamin levels were in a similar range with a higher vitamin D content for sample B, a higher vitamin E content for the phototrophic samples A and F as well as a lower folate content for the *C. sorokiniana* strains (B-E). The presence of metals was assumed to be linked to the media formulation used for cultivation and was overall reduced by washing during downstream processing. The taste and odor profiles of the tested samples were comparable, with a reduced hay-like and green odor for the chlorophyll-deficient strains. In conclusion, the biomass composition showed mostly differences on a micronutrient level. The cellular capacity to do photosynthesis, such as the presence of chlorophyll, seemed to have the highest impact on taste and odor. Notably, the microbial loads of the different *Chlorella* product preparations varied significantly, which may be due to both cultivation and post-harvesting biomass processing methods. Heterotrophically produced biomass under sterile cultivation conditions had the lower loads. Biomass processing can significantly impact microbial contamination levels and hence product quality and shelf life, while all examined products were within EFSA specifications about microbial loads.

In summary, the comparative nutrient and sensory evaluation presented in this study will aid in selection of microalgae products for food development, fostering a broadening of applications in the food space. Our study could confirm the high nutritional density of algae biomass, with the high protein content being beneficial for generation of protein-rich food, including shakes, bakery products, and meat substitutes. While this study provides an overview of the

nutritional value and sensory perception of commercial *Chlorella* preparation, bioavailability of these nutrients for consumers was not assessed. In this context, human intervention studies to explore nutrient accessibility and effects are currently underway. As a first indicator, the balanced amino acid profile being independent from cultivation and processing conditions together with an abundance of vitamins (specifically vitamin C) may potentially aid digestibility and metabolic valorization of algae protein fractions (Said, 2023). Further studies with focus on isolation, concentration, and processing of algae protein fractions are ongoing in the Protein4Singapore consortium addressing urgent market needs and consumer demand.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

## Author contributions

FM: Conceptualization, Formal Analysis, Methodology, Writing – review and editing, Data curation, Investigation, Visualization, Writing – original draft. TP: Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft, Validation, Writing – review and editing. DiG: Formal Analysis, Validation, Investigation, Methodology, Visualization, Writing – original draft, Data curation. FU: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. MP: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing. AS: Data curation, Formal analysis, Methodology, Validation, Visualization, Software, Writing – review and editing. LO: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. JS: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. MK: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. DaG: Funding acquisition, Project administration, Resources, Writing – review and editing, Data curation, Methodology, Supervision. TN: Formal Analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing. TBe: Formal Analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing. MR: Formal Analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing. CD: Formal Analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing. WB: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. NS: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review and editing, Data curation, Methodology. TBr: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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This article has been corrected with minor changes. These changes do not impact the scientific content of the article.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frfst.2025.1534438/full#supplementary-material

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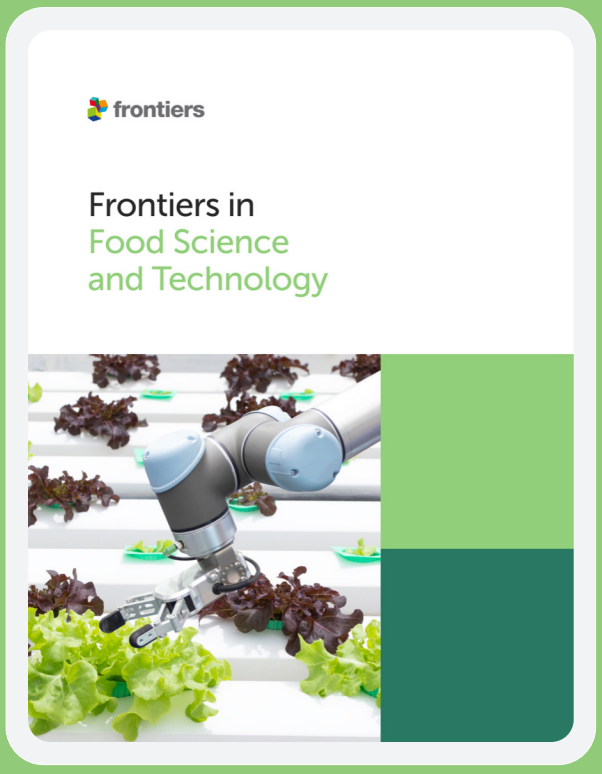


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