Research Topics

Alternative protein source for a sustainable and healthy nutrition

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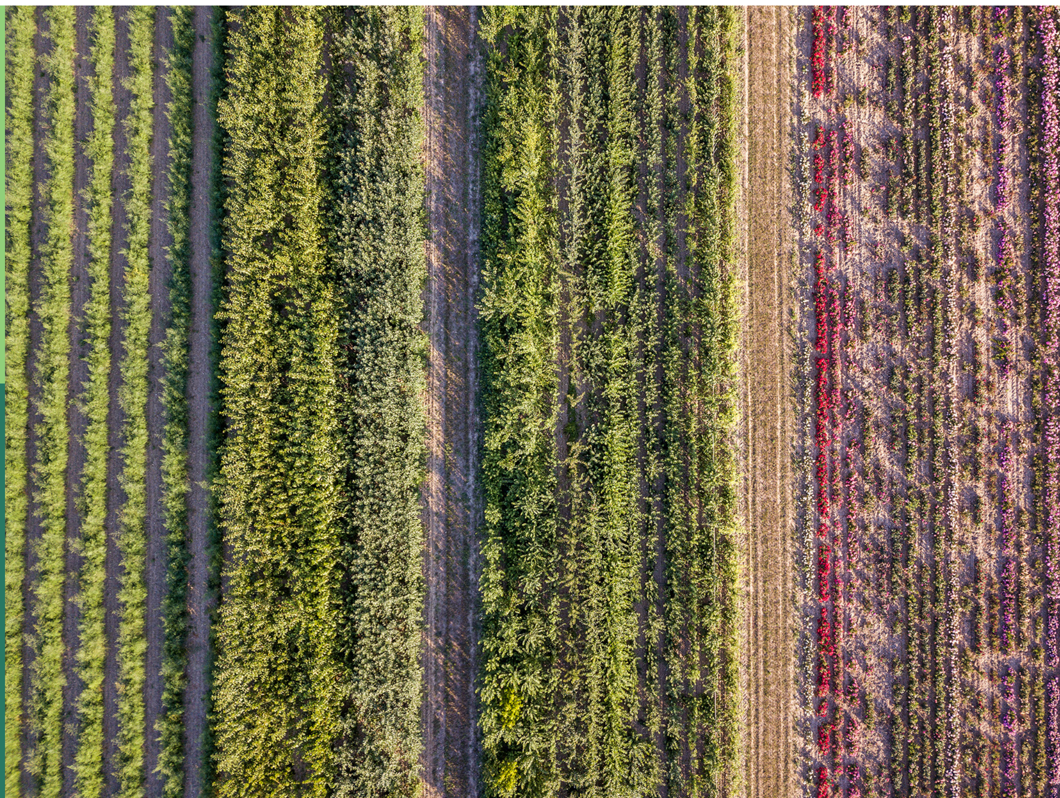

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Alternative protein source for a sustainable and healthy nutrition

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Editorial: Alternative protein source for a sustainable and healthy nutrition

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Editorial on the Research Topic
Alternative protein source for a sustainable and healthy nutrition

Introduction

The global food supply faces significant challenges in providing everyone with an adequate amount of nutritional ingredients without causing substantial harm to the planet. In this context, alternative proteins are increasingly discussed due to the costs and benefits associated with their production and consumption.

The exact definition of alternative proteins is itself a subject of debate. Grossmann and Weiss (2021) define them as "proteins produced from sources that have low environmental impact to replace established protein sources. They can also be obtained from animal husbandry with good animal welfare." Hence, this definition encompasses both animal and non-animal sources, spanning from insects and cultured meat to plant-based alternatives, which can even include some invasive plants. For instance, the study by Iyer et al. assessed the potential of Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia as alternative protein sources.

Economically, alternative proteins are gaining traction, with projections indicating an annual growth rate exceeding 36% (Joseph et al., 2020). Yet, several aspects need to be discussed to determine whether alternative proteins are a substantial tool for improving consumer welfare and limiting the use of the planet's resources. These include their actual environmental sustainability, their influence on improving animal welfare, providing consumers with more nutritional foods, and the impact on diets. Other aspects complete the picture, such as consumer acceptance, technology availability, and accessibility. These aspects will be briefly discussed below.

Environmental sustainability

Alternative proteins are increasingly viewed as a more sustainable option compared to traditional animal husbandry proteins. While there is compelling evidence indicating that plant-based foods, such as legumes, boast lower environmental footprints (see Ferreira et al.), the sustainability of certain alternative proteins, particularly those derived from animals, remains a Research Topic of debate. As well-pointed out by Santo et al., novel products like cultured meat lack comprehensive data to assess their environmental impact accurately, primarily due to limited data availability at scale. Nevertheless, early findings from a life cycle assessment (LCA) using real company data suggest that cultured meat may offer greater sustainability compared to conventional chicken and beef production methods (Onwezen et al., 2021).

In the case of insect-based proteins, research indicates that scale insect production may have a comparable environmental impact to chicken farming (Green et al., 2022). However, further research is needed to fully elucidate the environmental implications of alternative animal proteins and optimize their production processes for maximal sustainability.

In this context, is of primary importance to focus on sustainability calculation methods. Although LCA provides several evidences about the impact that a process can have on Earth's resources, it poses some limits that can be better challenged by new calculation methods, as shown in Francis et al., where the environmental impact has been calculated with new weights that take into account the total or specific production impacts at the country level.

Animal welfare

The adoption of alternative proteins is expected to mitigate the negative impact of traditional animal husbandry on animal welfare. However, the discourse surrounding alternative proteins and animal welfare warrants nuanced analysis. For instance, the welfare of insects seems to be currently perceived as less significant than that of vertebrates, and there is ongoing debate regarding the consciousness and ability to experience pain in insects (Delvendahl et al., 2022). It could be argued that in the present landscape, the emphasis of animal alternative proteins is more on reducing animal suffering rather than eliminating it entirely. This is exemplified in cultured meat production, where animals are not slaughtered but still undergo biopsy procedures. Cultured meat has drawn the bulk of the research focus in cellular agriculture, while precision fermentation—a technology that allows for the creation of individual components of animal products, such as milk or egg proteins—remains relatively underexplored. The study by Zollman Thomas et al. illustrates the potential of precision fermentation-made eggs in Germany, Singapore, and the USA in terms of consumer acceptance. Their findings suggest that such products are likely to find a willing market, especially amongst vegetarians and vegans.

Consumers' health

The adoption of alternative proteins is expected to yield primarily beneficial outcomes for consumers in terms of health and nutritional intake. These proteins typically contribute to a higher fiber content and lower cholesterol levels, thereby aiding in the prevention of non-communicable diseases. For instance, in the study by Sistia et al., plant-based diets have been linked to a reduced risk of obesity among women of reproductive age. However, it is important to note that alternative protein diets may contain lower levels of protein, zinc, and vitamin B12 compared to traditional diets (Green et al., 2022). Additionally, there is a growing concern regarding the higher incidence of allergies associated with plant-based foods, which could adversely affect the quality of life and increase healthcare usage among susceptible individuals (Kopko et al., 2022). Therefore, it is of primary importance of improving the quality of proteins, as shown in the discussion provided by the article of Pikosky et al..

Given these considerations, the shift in diet toward alternative proteins may not be optimal for all consumer categories, especially in the context of personalized nutrition. A transition phase, exemplified by a flexitarian diet, presents an opportunity to assess the feasibility of transitioning to a different protein source from both physiological and psychological perspectives (Banach et al., 2022).

New technologies

A critical point concerning alternative proteins is their substantial energy use. This characteristic hampers their environmental sustainability and limits the acquisition of economies of scale. It is expected that improvements in renewable energy production will foster the production and innovation of alternative protein production and related technology (Green et al., 2022). This Research Topic can be even more challenging for rural or marginalized communities that struggle to access basic foods like lentils or tofu (Green et al., 2022).

Alternative proteins can broaden their appeal to consumers through research that expands their choice sets. This can be done by introducing new protein sources, as explored by Craine et al. with a new legume from sainfoins, or by adding further benefits to known products, as demonstrated by Mudgil et al. in their work on improving probiotics.

Economic sustainability

The affordability of alternative proteins represents a major challenge. A healthy diet has already been proven to be more expensive than others with less healthy food (Hirvonen et al., 2020), which may hinder the adoption of alternative proteins even with more mature technology and economies of scale (Green et al., 2022). People with less education may also find it harder to be fully informed about alternative options to meat and may be reluctant to adopt them without proper and accessible information. Additionally, in wealthier populations, there may be more challenges due to the luxury halo characterizing meat consumption (Green et al., 2022).

Acceptance

Current evidence suggests that the adoption of alternative proteins varies among different populations. The study by Huang and Uehara indicates a growing willingness among consumers in China and Japan to embrace alternative proteins in the near future, while other evidence suggests that only a minority of consumers in the US express a readiness to try these foods (Joseph et al., 2020). In Europe, acceptance appears higher due to the widespread availability of meat substitutes in mainstream retailers and food services (Mylan et al., 2023), although their capacity to fully replace meat and its derivatives remains uncertain.

In this context, the influence of flexitarian diets is worth considering. Although lacking a precise definition, flexitarian diets allow for occasional consumption of meat and animal-based foods within a predominantly plant-based framework (Green et al., 2022). They are gaining importance due to increasing awareness of environmental, nutritional, and animal welfare concerns, particularly among those finding it challenging to adhere to strict vegan or vegetarian diets long-term.

Healthiness, taste, and environmental attributes are identified as primary drivers for alternative protein consumption. Additionally, individual traits such as neophilia-neophobia—a propensity to embrace or reject novel foods—and personal dietary preferences influence acceptance, with vegans and vegetarians being more receptive to plant-based options (Pliner and Hobden, 1992). However, insect-based foods face unique challenges related to consumer perceptions of appropriateness and food safety (Onwezen et al., 2021).

On the product side, the acceptance of alternative proteins may be enhanced by their resemblance to meat derivatives, possibly due to familiarity. Texture plays a crucial role, with consumers generally preferring a smooth, tender, meat-like texture, particularly younger consumers (Aaslyng and Højer, 2021). Color and appearance, resembling meat, are also significant factors (Joseph et al., 2020). Improving the textural properties of meat analogs, for example using mung bean and pumpkin seed proteins, could enhance consumer acceptance of meat alternatives, as suggested by Baig et al. Therefore, efforts to make alternative proteins more akin to traditional meat products could facilitate their adoption among diverse consumer groups.

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Considering Plant-Based Meat Substitutes and Cell-Based Meats: A Public Health and Food Systems Perspective

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Over the past decade, there has been growing interest in the development and production of plant-based and cell-based alternatives to farmed meat. Although promoted for their capacity to avoid or reduce the environmental, animal welfare, and, in some cases, public health problems associated with farmed meat production and consumption, little research has critically evaluated the broader potential public health and food systems implications associated with meat alternatives. This review explores key public health, environmental, animal welfare, economic, and policy implications related to the production and consumption of plant-based meat substitutes and cell-based meats, and how they compare to those associated with farmed meat production. Based on the limited evidence to date, it is unknown whether replacing farmed meats with plant-based substitutes would offer comparable nutritional or chronic disease reduction benefits as replacing meats with whole legumes. Production of plant-based substitutes, however, may involve smaller environmental impacts compared to the production of farmed meats, though the relative impacts differ significantly depending on the type of products under comparison. Research to date suggests that many of the purported environmental and health benefits of cell-based meat are largely speculative. Demand for both plant-based substitutes and cell-based meats may significantly reduce dependence on livestock to be raised and slaughtered for meat production, although cell-based meats will require further technological developments to completely remove animal-based inputs. The broader socioeconomic and political implications of replacing farmed meat with meat alternatives merit further research. An additional factor to consider is that much of the existing research on plant-based substitutes and cell-based meats has been funded or commissioned by companies developing these products, or by other organizations promoting these products. This review has revealed a number of research gaps that merit further exploration, ideally with independently funded peer-reviewed studies, to further inform the conversation around the development and commercialization of plant-based substitutes and cell-based meats.

Keywords: meat alternative, meat substitute, meat analog, cellular meat, seafood alternative, greenhouse gas emissions, land use, water footprint

INTRODUCTION

Interest in plant-based substitutes and cell-based meats—collectively referred to as meat alternatives hereafter—has grown rapidly over the past decade. While some consumers choose to avoid meat from farmed animals (hereafter "farmed meat") or animal foods altogether, a growing number of people are replacing a share of their meat intake with "plant-based substitutes" that seek to approximate the texture, flavor, and/or nutrient profiles of farmed meat using ingredients derived from pulses, grains, oils, and other plants and/or fungi. These products may soon be joined by "cell-based meats" (also referred to as "cultured meat," "*in-vitro* meat," "lab-grown meat," "cellular meat," "cultivated meat," or "clean meat") grown from animal stem cells using tissue engineering techniques, which currently remain for the most part in the prototype stage of development.

The global market for plant-based substitutes is projected to reach $85 billion (USD) by 2030, up from $4.6 billion (USD) in 2018 (Gordon et al., 2019). At the same time, while cell-based meat is not yet commercially available, research and development are proceeding rapidly. One think tank estimates that demand for beef and dairy products in the U.S. will shrink by 80–90% by 2035, driven largely by a projection that the cost of "modern protein foods" (including certain plant-based substitutes and cell-based meats) will be five times cheaper than existing animal proteins (Tubb and Seba, 2019). Although these estimates are speculative, and not necessarily supported by other industry experts, they emphasize the disruptive potential of meat alternatives on the animal agriculture sector.

Meat alternatives are often promoted as a means of mitigating the environmental, animal welfare, and, in some cases, public health problems associated with farmed meat production and consumption while appealing to mainstream consumers through existing supply chains. Growing scientific consensus has established that substantial shifts toward plant-forward diets, particularly in high meat-consuming countries, are essential for meeting climate change mitigation targets (Bajželj et al., 2014; Hedenus et al., 2014; Bryngelsson et al., 2016) and remaining within planetary boundaries (Willett et al., 2019). At the same time, there has been increased attention to the negative public health (Casey et al., 2015; Godfray et al., 2018) and animal welfare [Pew Commission on Industrial Animal Farm Production (PCIAFP), 2008] impacts of industrial food animal production, the prevailing model of meat production in the U.S. and increasingly in other parts of the world (Lam et al., 2019). A growing body of evidence has also associated red and processed meat consumption with certain chronic diseases and early mortality (Micha et al., 2012; Pan et al., 2012). Taken together, these concerns have driven efforts to reduce consumption of meat from farmed animals. Acknowledging that farmed meat production is not homogenous, in cases where the bulk of evidence is applicable only to meat from industrial food animal production, we use the term "conventional meat" to exclude more agroecological alternatives.

Seafood alternatives are also being developed to address concerns about the depletion of many of the world's wild fisheries [Food and Agriculture Organization of the United Nations (FAO), 2014] and the environmental impacts and constraints associated with many forms of farmed fish (i.e., aquaculture) production (Fry et al., 2016). There is almost no research examining the production or consumption of seafood alternatives, but we assume that many of the implications may be inferred from research on terrestrial meat alternatives since they are derived from similar ingredients. Thus, unless otherwise indicated, the terms "meat alternatives," "plant-based substitutes," and "cell-based meats" include seafood alternatives for simplicity of reading.

To date, few studies have critically evaluated the purported benefits of meat alternatives. To address this gap, this review explores the potential public health, environmental, animal welfare, economic, and policy implications associated with the production and consumption of plant-based substitutes and cell-based meats, and how they compare to those associated with farmed meat. Our findings are based on the best available evidence in the peer-reviewed academic literature, and in some cases, selected reports and other gray literature. We note limitations and research debates whenever possible.

The subsequent sections are laid out as follows: overview of concerns and considerations regarding farmed meat and seafood; discussion of the promises of meat and seafood alternatives; public health, environmental, animal welfare, economic, and policy implications associated with meat alternatives; conclusion; and suggested steps for further research. Appendix A (**Supplementary Material**) provides detailed methods of the literature search used for this review.

BACKGROUND: CONCERNS AND CONSIDERATIONS REGARDING FARMED MEAT AND SEAFOOD

Below we summarize some of the key public health and food systems concerns and considerations associated with farmed meat and seafood production and consumption in order to inform the evaluations of meat alternatives that purportedly attempt to mitigate some of these concerns in the subsequent sections. Livestock production systems have the potential for both positive (e.g., nutrient recycling) and negative (e.g., nutrient pollution) outcomes; the former include contributions of grazing systems to protein security and ecosystem services, which we discuss below, as well as to landscape aesthetic, gastronomic heritage, and other social and cultural factors (Ryschawy et al., 2019) that are beyond the scope of this review.

Public Health

Epidemiologic studies have linked Western dietary patterns that are high in the consumption of animal products, processed foods, refined sugars, and fats with escalating rates of chronic diseases. Red and processed meat consumption, in particular, has been associated with increased risks of heart disease and type 2 diabetes (Micha et al., 2012), stroke (Kaluza et al., 2012), certain cancers (particularly colorectal) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2018), and all-cause mortality (Pan et al., 2012; Larsson and Orsini, 2014; Zheng et al., 2019). A nascent body of exploratory literature suggests that the consumption of certain compounds in animal foods (e.g., L-carnitine, found primarily in red meat) may promote the growth of intestinal microbiota that produce metabolites associated with an increased risk for cardiovascular disease and inflammatory bowel disease (Koeth et al., 2013, 2019).

In these studies, the term "red meat" includes beef, pork, lamb, and veal, and "processed meat" includes meats preserved using high levels of salt and/or chemical preservatives (e.g., bacon, hot dogs, sausage); these health risks are not necessarily documented for unprocessed versions of white meats such as chicken and turkey (Micha et al., 2012). While several studies have modeled potential population-level health benefits of reduced red and processed meat consumption (Smed et al., 2016; Springmann et al., 2016, 2018), it is important to recognize that animal-source foods, including meat, can be a valuable source of protein and bioavailable micronutrients, especially for young children and in the absence of accessible plant-based alternatives (Semba, 2016).

In contrast to the health concerns associated with red and processed meat consumption, regular consumption of seafood—particularly "oily" fish and certain mollusks rich in omega-3 fatty acids—has been associated with many health benefits, including a reduced risk of cardiovascular disease in adults and improved cognitive development during gestation and infancy (Mozaffarian and Rimm, 2006). That said, there is not enough seafood available globally for everyone to consume at the recommended levels to reap the noted health benefits, even accounting for the growth of aquaculture (Thurstan and Roberts, 2014).

While food safety concerns are not exclusively tied to animal foods, many of the bacterial pathogens responsible for foodborne illness—such as *Salmonella, Escherichia coli*, *Campylobacter,* and *Listeria*—live in the guts of animals. Pathogens of animal origin can enter the food supply via multiple pathways, such as if manure is transported via runoff onto nearby produce fields or contaminates water sources used for irrigation (Solomon et al., 2002; Erickson and Doyle, 2012). More directly, if animals' digestive tracts are accidentally severed during processing and slaughtering, the spilled contents may contaminate meat with the potential for widespread cross-contamination. These concerns are heightened by the potential presence of antibiotic-resistant pathogens on meat (Waters et al., 2011), a hazard linked to the misuse of antibiotics in industrial food animal production (Silbergeld et al., 2008; Haskell et al., 2018).

Beyond risks to consumers, workers in industrial food animal production operations may be exposed to zoonotic pathogens—including antibiotic-resistant strains—and wide a range of airborne hazards (Fitch et al., 2017); an estimated one in four workers in indoor confinement operations suffer from some form of respiratory illness (Donham et al., 2007). Aquaculture workers may similarly contend with bacterial, respiratory, injury and other occupational hazards (Myers, 2010). Although not exclusive to the farmed meat and seafood industries, animal slaughtering and meat processing workers are often required to perform strenuous labor for long hours under hazardous conditions, and face high rates of injury and illness (Fitch et al., 2017).

Neighbors living close to industrial food animal production operations face elevated risks of respiratory outcomes, stress, negative moods, and infection with zoonotic pathogens, including methicillin-resistant *Staphylococcus aureus* (Casey et al., 2015). More than just an unpleasant smell, strong odors from industrial operations can interfere with daily activities, social gatherings, and overall quality of life, and have been implicated in adverse physical and mental health outcomes (Horton et al., 2009; Wing et al., 2013). Communities may additionally face health risks associated with waterborne bacterial and chemical hazards originating from nearby operations (Burkholder et al., 2007).

Environmental

Livestock production accounts for an estimated 14.5 percent of global greenhouse gas (GHG) emissions from human activities (Gerber et al., 2013). Meat and dairy from ruminant animals (e.g., cattle, goats), farmed crustaceans (e.g., shrimp, prawns), and trawled lobster are particularly GHG-intensive (Clune et al., 2017; Poore and Nemecek, 2018; Kim et al., 2019). Some research suggests that under specific soil, climate, and animal density conditions, well-managed grazing livestock may sequester carbon, thus lowering the GHG footprints of ruminant products (Tichenor et al., 2017); however, other research contends that this effect is time-limited, reversible, and potentially outweighed by other GHGs generated by grazing systems (Garnett et al., 2017).

The estimated amount of land devoted to livestock production ranges from 2.5 (Mottet et al., 2017) to 3.7 billion ha (Foley et al., 2011)—roughly half to three-quarters of global agricultural land—while animal foods account for only 18% of calories and 25% of protein in the global food supply (Mottet et al., 2017). This is in part due to the amount of forage and feed required to produce an equivalent amount of calories and protein from meat as could be provided directly from plants grown for human consumption, with the caveat that animal proteins generally are more bioavailable to humans and have all essential amino acids in sufficient amounts (Cassidy et al., 2013). Beef is particularly land-intensive compared to other meats (Poore and Nemecek, 2018), in part because cattle have a slower reproductive cycle and are less efficient at converting feed to meat (Nijdam et al., 2012).

Despite the relatively large land footprint of farmed animals, there are two important and related considerations regarding the contributions of grazing ruminants to land use and protein security. First, in contrast to poultry, pork, and increasingly farmed fish (Fry et al., 2016)—which are fed crops grown on land that could otherwise be used to grow crops for direct human consumption—ruminant animals can graze on land that is unsuitable, e.g., too rocky or too hilly, for crop production. Of the 2.5 billion ha devoted to livestock production, 1.3 billion ha are non-arable grasslands (Mottet et al., 2017). Thus, reducing beef production and consumption would not necessarily free up a proportional amount of land to feed people or other livestock (Peters et al., 2016). Second, farmed animals, particularly grazing ruminants, can convert plants that are inedible to humans into human-edible proteins. Grassland-based systems in the United Kingdom, for example, were found to provide 1.1 kg protein from beef and 1.4 kg protein from milk per kg of human-edible plant protein from feed and forages. By contrast, poultry, pork, and grain-fed beef provided only 0.5, 0.4, and 0.3 kg protein, respectively, per kg human-edible plant protein (Wilkinson, 2011; Peyraud and Peeters, 2016). Grassland production systems thus present an opportunity to contribute to protein security; grain-fed systems, however, remain the predominant model of livestock production in industrialized countries. Within the US, for example, only 1% of the current beef supply comes from exclusively pasture-based systems, though the potential exists to produce up to 27–35% of the current beef supply using exclusively pasture (Hayek and Garrett, 2018). On average globally, ruminant meat currently relies on cropland to the same extent per unit of protein as pork and poultry (Herrero et al., 2015).

With a few exceptions, more inputs into feed production (e.g., water, pesticides, fertilizers) are needed to produce the same amounts of calories and protein in meat compared to plant foods intended for direct human consumption (Marlow et al., 2009). Livestock production as an industry also contributes more to biodiversity loss (Machovina et al., 2015) and disruptions in nutrient cycles that exacerbate groundwater pollution and eutrophication (Bouwman et al., 2013) than the production of crops for human consumption. Eutrophication occurs when excess nutrient levels (primarily nitrogen and phosphorus) cause toxic algae blooms that deplete oxygen levels in the water and kill fish, plants, and other aquatic life. Resource inputs and the associated impacts may be reduced with agroecological approaches such as integrated crop-livestock and/or multi-species farming, and well-managed pasture-based livestock production systems in general; these approaches can also provide other ecological services including reducing dependence on synthetic fertilizers through nutrient recycling, fostering soil health, and sustaining biodiversity of grassland ecosystems (Janzen, 2011; Röös et al., 2017; Martin et al., 2020).

Animal Welfare

Over 9.5 billion terrestrial animals were slaughtered for meat in the US in 2017 [U.S. Department of Agriculture (USDA), 2019], with global estimates at around 75 billion terrestrial animals [Food and Agriculture Organization of the United Nations (FAO), 2020]. Global meat production (in tonnage) has increased over 4.5-fold from 1961 to 2018, nearly twice the rate of population growth [Food and Agriculture Organization of the United Nations (FAO), 2020]. Industrial food animal production is designed to produce abundant amounts of meat, eggs, or milk rapidly and at minimal cost. Most operations raise animals in crowded facilities, often in confined crates or cages, without outdoor access or the ability to exhibit their natural behaviors [Pew Commission on Industrial Animal Farm Production (PCIAFP), 2008]. Animals in many cases are subject to painful bodily alterations (e.g., debeaking, dehorning, castration), often without pain relief [Pew Commission on Industrial Animal Farm Production (PCIAFP), 2008]. Animal welfare problems may exist on small-scale, organic, or pasture-based farms, too; such operations do not necessarily have higher animal welfare standards [Pew Commission on Industrial Animal Farm Production (PCIAFP), 2008].

Economic

In much of the industrialized world, traditionally diversified farms have been replaced over the past century with operations that specialize in producing specific crops or animals at a large scale, buoyed by mechanization, standardization, and increased off-farm inputs (e.g., pesticides, pharmaceuticals) (Ikerd, 2008). Large multi-national corporations have consolidated small businesses and other corporations to control multiple stages along the food supply chain, including in the meat processing and marketing industry (Weis, 2013; Howard, 2016). Such systems are credited with improving efficiency, reducing costs, and lowering consumer prices, but are also implicated in the decline in workers' wages (Oxfam America, 2015); the loss of farmers' and public autonomy over the food system (Ikerd, 2008; IPES-Food, 2017); and the deterioration of rural communities and economies (Lobao, 1990; Stofferahn, 2006), including local property values (Keeney, 2008).

THE PROMISES OF MEAT ALTERNATIVES

A variety of alternatives exist to approximate or even replicate certain aspects of meat's texture, flavor, and/or nutrient profile. These range from natural foods that resemble certain characteristics—not necessarily nutritional—of meat (e.g., pulses, mushrooms, jackfruit), to products that are not designed to mimic meat but can be used in similar ways (e.g., tofu, tempeh, seitan, bean burgers), to more processed products that are designed to imitate the experience of eating certain meat products (e.g., meat-like burgers, hot dogs, fish filets) (Lagally et al., 2017).

Products in the last category have been gaining particular momentum over the past decade, with new technological advances aimed at replicating selected characteristics of meat down to the molecular level. Several products are designed to be "viscerally equivalent" to farmed meats in order to appeal to those who enjoy meat (Stephens et al., 2018). Most of these plant-based substitutes use soy, wheat, or pea protein isolates or concentrates as their primary protein source, though products derived from fungi (i.e., mycoprotein) and lupin beans also exist. Examples of common plant-based substitute brands and products include Gardein Meatless Meatballs, Morningstar Farms Original Chik Patties, Beyond Meat's Beyond Burger and Impossible Foods' Impossible Burger (see **Table S3**). A rapidly growing number of companies are also aspiring to produce cell-based meats that are not only viscerally equivalent but also "biologically equivalent" to farmed meat through cultivation of animal cells (Stephens et al., 2018). The technological feasibility of replicating the exact structure, texture, color, flavor, and nutritional composition of farmed meat, however, remains in question. Replicating these characteristics for fresh, unprocessed meat would require several particularly complex technical feats, including simulating the role of blood in delivering oxygen and nutrients throughout thicker pieces of tissue, as well as co-culturing fat, muscle, and connective tissues (Fraeye et al., 2020).

Meat alternatives are promoted for their environmental, animal welfare, and in some cases, public health benefits. "Eat Meat. Save Earth," is the mission proclaimed on Impossible Foods' website (Impossible Foods, 2020), accompanied with statistics comparing the land, water and GHG emissions associated with an Impossible Burger and a conventional beef burger. Popular press echoes these messages about how "Fake Meat Will Save Us" (Egan, 2019). As one journalist states: "Farmfree food will allow us to hand back vast areas of land and sea to nature, permitting rewilding and carbon drawdown on a massive scale. It means an end to the exploitation of animals, an end to most deforestation, a massive reduction in the use of pesticides and fertilizer, the end of trawlers and longliners" (Monbiot, 2020). Cell-based meat is also purported to be "healthier, safer, and disease-free" compared to farmed meat (Arshad et al., 2017). Notably, these claims are most often compared to beef, which generally has the largest environmental impacts among animal products.

The extent to which meat alternatives achieve these purported benefits depends in part on several factors, including the specific ingredients or inputs used to produce them (**Figures 1**, **2**), the extent to which consumers accept and incorporate these products into their diets, and which farmed meats they are replacing (e.g., beef vs. poultry, conventional meat vs. meat from agroecological production systems), if any. Thus, in the following sections, we compare the impacts of meat alternatives to a variety of farmed meats. Although several literature reviews have examined trends in consumer perceptions about and theoretical willingness to try meat alternatives (Hartmann and Siegrist, 2017; Bryant and Barnett, 2018; Weinrich, 2019), the studies underlying these reviews may be outdated given the influx of new plant-based substitutes into the market and demonstrated consumer acceptance in the past few years [International Food Information Council (IFIC), 2020; McCarthy and DeKoster, 2020]. As cell-based meats enter the marketplace, consumer perceptions and acceptance may also change. We also recognize that potential public health, environmental, and animal welfare benefits associated with meat alternatives would only occur if demand for those products offsets a share of farmed meat production, rather than simply adding to the combined total production of farmed meat and meat alternatives (Stephens et al., 2018). Given the importance of consumption patterns on the potential benefits associated with meat alternatives, we call for additional research in Appendix B (**Supplementary Material**) to better understand how consumers are incorporating these products into their diets.

It is worth mentioning that since cell-based meat has not yet been commercialized, existing research about its production is based on a few anticipatory life cycle assessments (LCAs) which assumed hypothetical inputs, production processes, and technological advances (Tuomisto and Teixeira de Mattos, 2011; Tuomisto et al., 2014[1]; Mattick et al., 2015b). Some researchers have noted that several assumptions and simplifications made in these LCAs are not supported by existing scientific evidence and should be interpreted carefully (Lynch and Pierrehumbert, 2019; Thorrez and Vandenburgh, 2019). For instance, the presented LCAs covered in this review assumed that the cell-based meat would be grown without fetal bovine serum, a reality that remains one of the industry's biggest (see Inputs). Nevertheless, we include those studies' results, since it is the most detailed information about the potential inputs and implications of cell-based meat production. Given the limitations of existing research, it is of critical importance that ongoing, independent, and comprehensive multi-product environmental analyses are conducted as the technologies and commercial operations for meat alternatives develop and scale (Mattick et al., 2015a).

Many plant-based seafood substitutes use soy, wheat, or pea protein isolates as their primary protein source (**Table S4**) and are comparable to plant-based terrestrial meat substitutes. Some products on the market are not designed to mimic seafood exactly but can be used in similar ways (e.g., products made from carrots, eggplant, or tomatoes); these are not examined in this review. Additionally, while the term "seafood" includes sea vegetables (e.g., seaweed, algae)—some of which may have high concentrations of protein and micronutrients (Fleurence et al., 2012)—their impacts are not assessed here. Cell-based seafood products are also in development, though the regulatory pathways and markets will likely be different than those of cell-based terrestrial meats.

Lastly, while this review primarily compares meat alternatives to the farmed meats for which they are intended to substitute, meeting dietary protein needs does not necessarily require consumption of either group of products. Producing and consuming other protein-rich foods, such as minimally processed legumes (including soybeans, lentils, beans, and peas) and insects, should be considered as part of the path forward for sustainable food systems.

PUBLIC HEALTH IMPLICATIONS

Here we review the array of public health implications associated with meat alternatives, exploring the nutrition, chronic disease,

[1]Tuomisto et al. (2014) is not a new LCA, but includes updates on some numbers reported in a previous study (Tuomisto and Teixeira de Mattos, 2011).

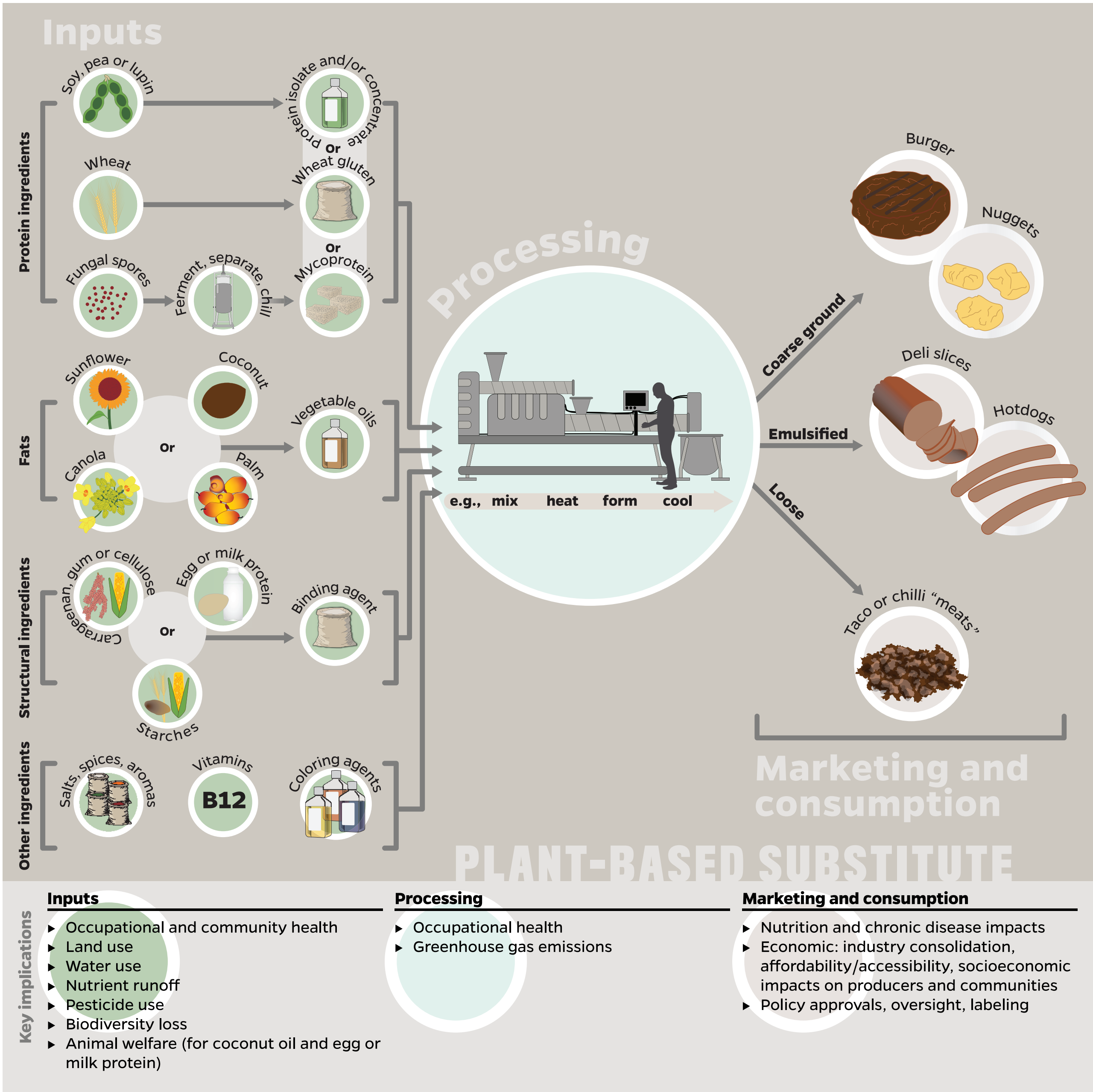

FIGURE 1 | Potential inputs, processes, and final product(s) to be marketed and consumed as plant-based substitutes, and how these stages correspond to key implications explored in this paper. Many of the implications listed here are applicable to multiple stages, e.g., GHG emissions occur in the production of inputs, processing, and retail/consumer stages; however, we listed each implication only with the stage to which it is most relevant or has the greatest impact. The inputs represent a compilation of ingredients included in plant-based substitutes; most products do not contain all of these ingredients at once. This figure was designed by the authors using information reported in Joshi and Kumar (2015), Bohrer (2019), and Kyriakopoulou et al. (2019).

and food safety implications associated with consuming them and the occupational and community health impacts associated with their production.

Nutrition and Chronic Disease

In general, many plant-based substitutes contain comparable amounts of calories, protein, and iron as the meats they are intended to replace (Bohrer, 2019). As ultra-processed foods, plant-based substitutes have relatively high amounts of sodium compared to unprocessed meats and may contain ingredients and additives including flavoring, coloring, and binding agents (Bohrer, 2019; Curtain and Grafenauer, 2019). **Tables S3, S4** highlight key ingredients in plant-based burgers and seafood substitutes, respectively, from some top retail brands in the

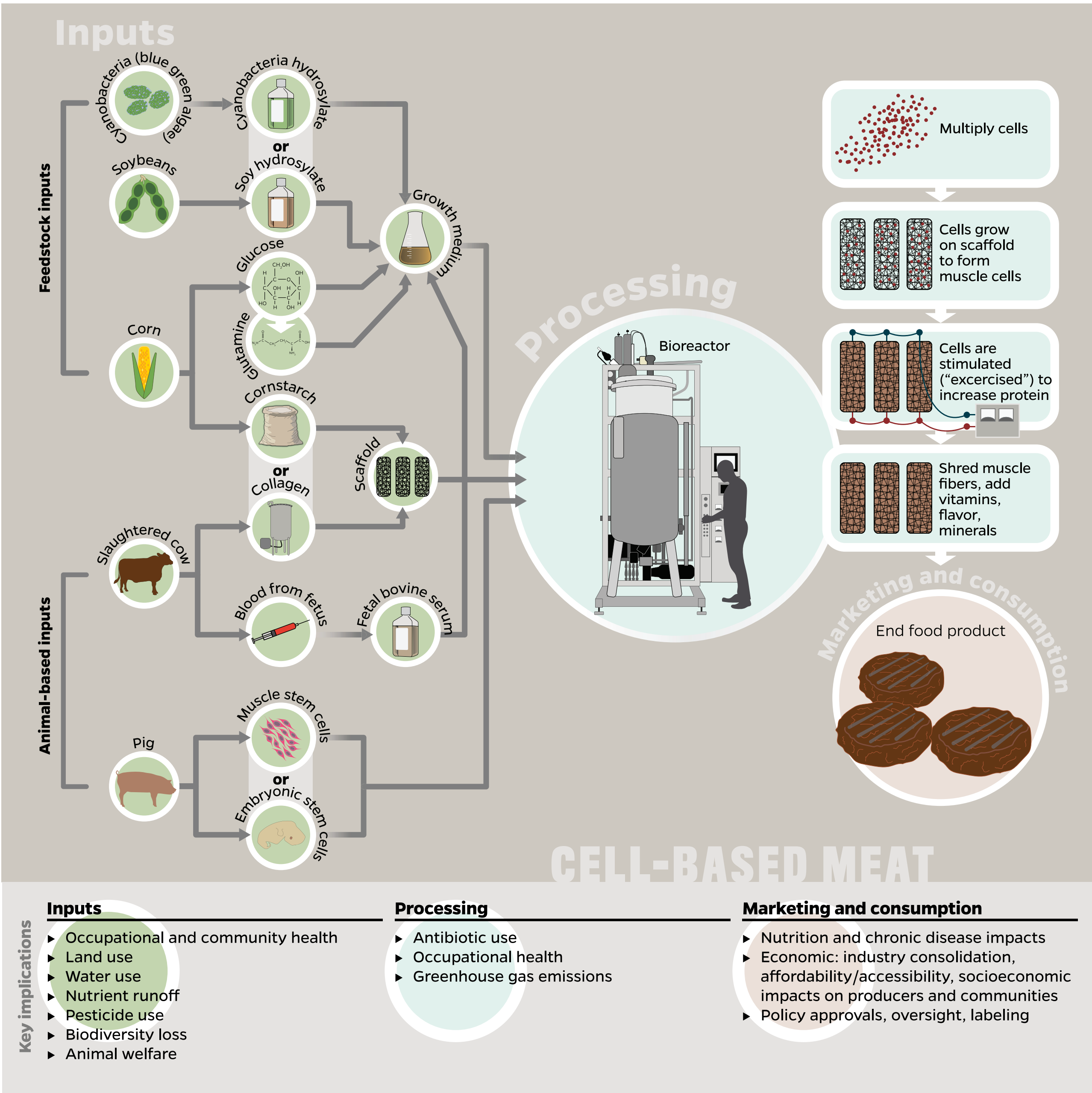

FIGURE 2 | The potential inputs, processes, and final product(s) to be marketed and consumed as cell-based meats, and how these stages correspond to key implications explored in this paper. Many of the implications listed here are applicable to multiple stages, e.g., GHG emissions occur in the production of inputs, processing, and retail/consumer stages; however, we listed each implication only with the stage to which it is most relevant or has the greatest potential impact. Since no products are currently available on the market, the figure was designed by the authors using hypothetical inputs proposed by Tuomisto and Teixeira de Mattos (2011) and Mattick et al. (2015b) as well as currently required animal-based inputs (e.g., fetal bovine serum, collagen-based scaffolds) (Stephens et al., 2018; Thorrez and Vandenburgh, 2019), although we recognize the goal to eliminate the latter eventually.

U.S. market. Several products contain coconut oil; among those in **Table S3** that contain coconut oil, their saturated fat levels are lower than that of beef, but comparable to or higher than those of poultry and pork. These ingredients and additives are not necessarily beneficial or harmful to human health from a nutrition perspective. For example, despite consumer perceptions of coconut oil as a health-promoting food, evidence of its health benefits is lacking, though more robust research is merited (Lockyer and Stanner, 2016). At the same time, although consumer desires for "clean labels" have prompted concerns about the use of certain binding agents and gums in plant-based substitutes, research has shown methylcellulose and guar gum to have similar cholesterol and glucose-lowering effects as other dietary fibers (Mudgil et al., 2014; Kuczora, 2015; Bohrer, 2019). See also Food Safety for potential food safety concerns associated with additives.

Although they may contain similar macronutrient profiles, replacing meat with a plant-based substitute does not necessarily reflect a healthy dietary pattern (Hu et al., 2019). A plant-based burger or hot dog may be served with a refined bun, few vegetables, and nutrient-poor sides such as fries or chips. Similarly, seafood substitutes could theoretically be fortified with omega-3 fatty acids, but it is unknown whether doing so would provide comparable health benefits to eating whole unprocessed fish. Furthermore, consumption of ultra-processed foods is associated with greater caloric intake and weight gain (Hall et al., 2019) and a range of adverse long-term health outcomes (Lawrence and Baker, 2019). Further research is needed to determine whether plant-based substitutes are replacing processed or unprocessed foods in people's diets, and if they can ultimately lead to healthier dietary patterns.

By contrast, dietary patterns rich in whole plant-based foods such as legumes, whole grains, vegetables and nuts have been associated with a reduced risk for chronic diseases and adverse health outcomes (Nelson et al., 2016). While plant-based substitutes are primarily derived from legumes, it is unknown whether substitutes derived from plant protein isolates offer similar nutritional benefits or chronic disease reductions as whole legumes (Hu et al., 2019). Soy protein isolates (containing >90% soy protein) or concentrates (70–90% soy protein), for instance, are primary ingredients in many plant-based substitutes (Malav et al., 2015). Whole soy foods (e.g., edamame, tempeh) and minimally processed soy foods (e.g., full-fat tofu and soymilk) are complex foods rich in protein (including all the essential amino acids), omega-3 fatty acids, and many biologically active components (most notably isoflavones) (Omoni and Aluko, 2005). Soy food and/or protein consumption, either in comparison to animal protein intake or in the form of supplementation, has been associated with improved blood lipid levels (Anderson et al., 1995; Reynolds et al., 2006), moderately improved measures of bone health (Zhang et al., 2005; Bawa, 2010), reduced menopausal symptoms (Franco et al., 2016), reduced risk of type 2 diabetes (Tang et al., 2020), and modestly decreased breast cancer risk (Fritz et al., 2013). While some benefits associated with soy consumption were studied using soy protein isolates or extracts, these lack some of the beneficial nutritional components found in whole soybeans (Messina and Messina, 2010), partly as a result of the manufacturing processes used to extract protein (Erdman, 2000). For that reason, consuming whole soy foods is generally recommended over consuming isolated soy components (Michelfelder, 2009; Messina and Messina, 2010). Further research comparing the health effects of whole soy foods to plant-based substitutes made with isolated soy proteins—and likewise, whole peas to plant-based substitutes made with isolated pea proteins—is merited.

Plant-based diets have also been associated with more diverse gut microbiomes than omnivorous diets, though this may largely be due to compounds and characteristics of whole plants consumed in plant-based diets (Tomova et al., 2019). It is unclear if or how meat alternatives would impact the gut microbiome and associated health outcomes (Hu et al., 2019).

An additional consideration specific to Impossible Foods' plant-based substitutes relates to the heme iron (in the form of soy leghemoglobin) content, the ingredient that imparts the product's "meaty" flavor and aroma. High levels of heme iron intake from red and processed meat consumption have been associated with elevated risk for type 2 diabetes (Bao et al., 2012), cardiovascular disease (Fang et al., 2015), colorectal cancer (Bastide et al., 2011; Fonseca-Nunes et al., 2014; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2018) and lung cancer (Tasevska et al., 2009; Fonseca-Nunes et al., 2014). Impossible Foods has reported that the heme in its beef substitute is comparable in amount and, once cooked and digested, identical molecularly to that found in farmed beef [GRAS Notice (GRN) No. 737, 2017], suggesting that consumption of this plant-based substitute may be associated with similar chronic disease risks as red and processed meat consumption. That said, consumption of heme iron—the most easily absorbed form of iron—is associated with a reduced risk for iron deficiency, a prevalent nutritional concern for women of childbearing age and pregnant and lactating women globally (Zimmermann and Hurrell, 2007).

As cell-based meats are not yet commercially available, there is little information available about their nutritional content. On the one hand, while developers aspire to replicate the nutrition profile of farmed meat, many unanswered questions remain about the technological feasibility of achieving this *in vitro*, particularly with regard to the quality and composition of proteins, amino acids, vitamins, minerals, fatty acids, and compounds such as taurine and creatine (Fraeye et al., 2020). On the other hand, some of these attributes could be leveraged to enhance the nutritional value over that of farmed meat; proponents have claimed that the quantity and type of fat could be controlled, and that other functional ingredients, such as vitamin C or omega 3 fatty acids, could be added to the growth medium (Post, 2012; Bhat et al., 2019).

Food Safety

Most plant-based substitutes contain at least one major food allergen among their ingredients, with wheat and soy being the most common [Food Drug Administration (FDA), 2004]. Individuals allergic to peanuts and soy may also experience reactions to pea and lupin protein, though this is rare (Lavine and Ben-Shoshan, 2019). Allergic and gastrointestinal reactions to mycoprotein-based plant-based substitutes (e.g., Quorn) have also been reported; though rare, the incidence of adverse reactions to mycoprotein in the general population is debated (Jacobson and DePorter, 2018; Finnigan et al., 2019). Individuals with intolerances to certain food additives and gums must also be careful given their prevalence in plant-based substitutes.

Carrageenan, for example, is a structural ingredient derived from seaweed that is commonly used in plant-based substitutes and other processed foods for purposes of thickening, gelling, or stabilizing. The safety of carrageenan has long been debated, with attention being focused on its potential to elicit gastrointestinal inflammation, alterations to intestinal microflora, and other related outcomes such as irritable bowel syndrome and colon cancer (Bixler, 2017; David et al., 2018). Additionally, because carrageenan is grown in seawater, it has the potential to accumulate significant concentrations of heavy metals (Almela et al., 2002; Besada et al., 2009), though no research has characterized exposures to arsenic, cadmium, lead, and mercury that result from consumption of carrageenan-containing foods.

Some concerns have also been raised about the safety of new additives present in some plant-based substitutes, such as mycoprotein used in Quorn products and soy leghemoglobin used in Impossible Foods products. See Policy Implications for a discussion of the approval processes and regulatory debates.

Some propose that if cell-based meat were produced under sterile conditions, it could reduce the incidence of foodborne illness (Bhat and Bhat, 2011). By not involving the processing of whole animal carcasses, cell-based meats would likely reduce the potential for contamination that exists in farmed meat handling and processing, such as *Escherichia coli* contamination from contact with digestive organs and feces. However, fully sterile conditions would be near impossible to achieve and thus antibiotics would likely be required as inputs for the tissue culture medium in order to inhibit the growth of bacterial pathogens (Stephens et al., 2018; Thorrez and Vandenburgh, 2019). The exact nature of antibiotic use in this context is not yet known, though the quantities and regularity of use would likely be lower than in industrial livestock operations. Transmission of zoonotic diseases may decline if cell-based meat production reduced human-livestock interactions (Bhat and Bhat, 2011; Arshad et al., 2017), though more research on this potential is merited.

Occupational Health

There is little known about occupational exposure risks incurred by workers in plant-based substitute manufacturing, though they are likely less hazardous than those faced by farmed meat processing workers (see Public Health). One consumer advocacy group has raised concerns about the use of hexane in processing soy protein isolates used in plant-based substitutes (Vallaeys et al., 2010). It may also be used to process pea protein isolates, though less information on this is available (Tömösközi et al., 2001; Holt, 2018). Hexane is a neurotoxic and highly explosive solvent and also a hazardous air pollutant [Environmental Protection Agency (EPA), 2000]. To our knowledge, no specific information is available on the amount of hexane used in the production of soy and pea protein isolates, and on the extent of measures to protect workers, prevent environmental releases, and monitor exposures.

Given the level of uncertainty regarding the specific laboratory processes and regulatory landscape that will emerge for cell-based meat production (see Regulatory Oversight of Cell-Based Meat), occupational health and safety implications for cell-based meat workers remain unclear.

Community Health

Both plant-based substitutes and (hypothetically) cell-based meats rely on crops that are already significant parts of the agricultural system, including soybeans, wheat, and corn (**Figures 1**, **2**). In addition to contributing to nutrient runoff that can contaminate local groundwater sources, the production of these crops often involves pesticides associated with long-term chronic health problems for people who work on and live near farms (Harrison, 2011). Concerns have also been raised that the use of low levels of some herbicides in soybean production, including dicamba; 2,4-D; and glyphosate may induce multiple-antibiotic resistance in pathogens, compromising the effectiveness of life-saving medicines (Kurenbach et al., 2015). Additionally, the heavy use of agricultural fungicides, such as in the production of peas and soybeans, has been implicated in the rise of resistance to anti-fungal medicines, which has particularly serious consequences for immunosuppressed individuals (Revie et al., 2018). All of this said, because it takes more soy used as animal feed to produce one conventional meat burger compared to the amount of soy used as an ingredient in a plant-based burger, conventional meat often requires more pesticides to produce than plant-based substitutes (see Pesticide Use). The relative risks for community health associated with plant-based substitutes and cell-based meats compared to conventional meats, and also more agroecologically produced meats, should be more thoroughly evaluated.

It also remains to be seen whether potential antibiotic use and waste management practices associated with cell-based meat production will impact people who work on or live near production facilities, as they do with industrial food animal production.

ENVIRONMENTAL IMPLICATIONS

The environmental impacts of meat alternatives depend largely on two stages of production: (1) the agricultural production of inputs and (2) the processing of inputs into final products. For plant-based substitutes, these inputs include primary ingredients, e.g., soybeans, wheat, peas, fungi, and lupins (**Figure 1**). For cell-based meat, inputs provide energy or nutrients to the cell medium; the specific inputs that will be used for commercial production are unknown largely because of their proprietary nature. Hypothetical studies of cell-based meat development have modeled production using cyanobacteria (i.e., blue-green algae) or compounds derived from soybeans and corn as inputs, neither of which are necessarily viable (Thorrez and Vandenburgh, 2019) but provide a basis for initial analysis (**Figure 2**). The following section will review the GHG, land, water, pesticide use, eutrophication, and biodiversity implications associated with the production of meat alternatives compared to farmed meat production.

Greenhouse Gas Emissions

Based on our review of the literature (**Figure 3**; see **Supplementary Data** for details), the median GHG footprint of plant-based substitutes was 34, 43, 63, 72, 87, and 93% smaller than those of farmed fish, poultry meat, pig meat, farmed crustaceans, beef from dairy herds, and beef from beef herds, respectively, per 100 grams protein. Among the animal foods considered in our review, only wild tuna and insects were less GHG-intensive than plant-based substitutes. Plant-based substitutes were 1.6, 4.6, and 7.0 times more

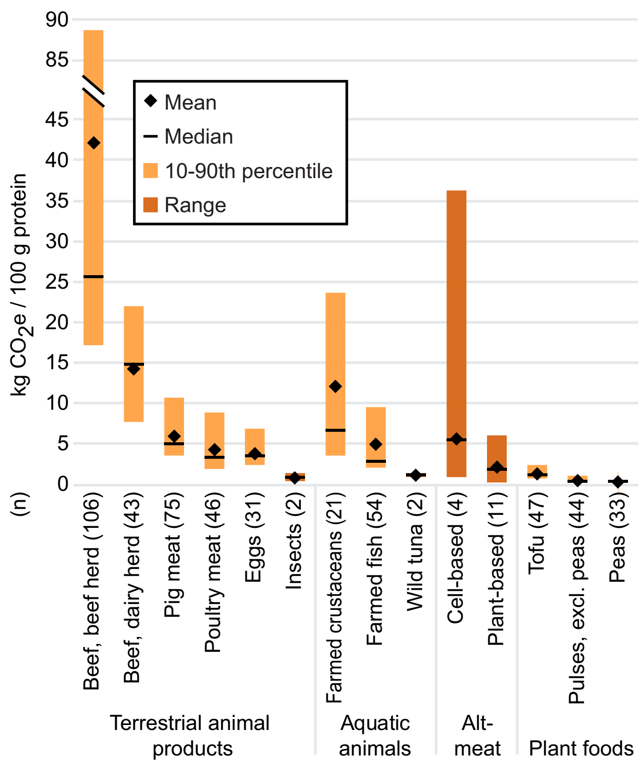

FIGURE 3 | Cradle-to-processing gate GHG footprints (wherever possible) per 100 g protein. For cell-based meat, plant-based substitutes, insects, and wild tuna, the mean and median were calculated using the mean value from each individual study (*n*) to avoid over-representing results from studies that included more products than other studies. The minimum and maximum values of the range were derived from individual product footprints. For data sources and individual product footprints, see **Supplementary Data**. Data for all other foods are from Poore and Nemecek (2018); for these *n* represents the number of observations.

GHG-intensive than the less-processed plant proteins in this review, i.e., tofu, pulses (excluding peas), and peas, respectively. Only one study has quantified any environmental impacts associated with plant-based seafood substitutes (Quorn Foods, 2019); the GHG footprints were comparable to those of plant-based terrestrial meat substitutes made from similar ingredients, so we included those figures in the aggregate data for plant-based substitutes.

The hypothetical GHG footprint of cell-based meat varied significantly more than that of plant-based substitutes, from 0.9 to 36.3 kg CO_2e/100 g protein (median: 5.6 kg CO_2e/100 g protein). This variation was due to different assumptions embedded in the projections, such as the cell-based meat facility's size and the potential density and proliferation rates of cells (Mattick et al., 2015b). The median GHG footprint per 100 grams protein of cell-based meat was 17, 62, and 78% lower than those of farmed crustaceans, beef from dairy herds, and beef from beef herds, respectively, but 1.1 to 6.1 times higher than those of other animal products and 4.8, 13.4, and 20.6 times higher than those of tofu, pulses, and peas, respectively. The hypothetical GHG emissions associated with cell-based seafood products have not been explored, but would likely be similar to projections for terrestrial cell-based meat. The industrial energy requirements for cell-based meat were also higher than poultry meat in Mattick et al. (2015a) and poultry meat, pig meat, and beef in Tuomisto et al. (2014).

Given that a large proportion of the GHG footprint of plant-based substitutes and cell-based meat comes from the energy required to manufacture the products, these footprints could theoretically decrease if the energy grid were decarbonized. By contrast, significant reductions in the GHG-intensity of livestock production seem unlikely (Goldstein et al., 2017), with the caveat that emerging technologies to reduce methane from enteric fermentation may address a share of beef's emissions (Maia et al., 2016; Vyas et al., 2018).

Comparing the climate impacts of plant-based substitutes, cell-based meats, farmed meats, and seafood is complicated by varying atmospheric lifespans and global warming potentials among different GHGs. For example, methane remains in the atmosphere for a shorter period but has a more potent warming effect than carbon dioxide. Life cycle assessments generally standardize the warming potential of different GHGs (including carbon dioxide, methane, and nitrous oxide) in terms of carbon dioxide equivalents (CO_2e), usually over a 100-year period. This metric, however, obscures the fact that a significant proportion of the GHG footprint of farmed beef is comprised of methane from enteric fermentation and manure decomposition, whereas the GHG footprints of meat alternatives are largely comprised of CO_2 from electricity use, resulting in a more persistent but less intensive warming effect (Lynch and Pierrehumbert, 2019). The use of CO_2e has raised debates in the academic and policy world, as the choice of metric and timeframe under consideration could result in very different policy priorities for reducing GHG emissions (Garnett, 2011; Allen, 2015). Over a 100-year time frame, for example, the GHG footprint of cell-based meat was found to be between 51 and 97% smaller compared to conventional beef produced in the Midwest U.S., whereas it was between 92% smaller to 9% larger using a 500-year time frame (Lynch and Pierrehumbert, 2019). The long atmospheric lifespan of carbon dioxide highlights the urgency of decarbonizing the energy grid, whether for cell-based meat production or any other energy-intensive activity.

Land Use

The median land use footprint of plant-based substitutes was 41, 77, 82, 89, and 98% smaller than that of farmed fish, poultry meat, pig meat, beef from dairy herds, and beef from beef herds, respectively, per 100 grams protein (**Figure 4**; see **Supplementary Data**). Thus replacing a share of farmed meat in the diet with plant-based substitutes could theoretically free up cropland to feed more people or provide other ecological services such as reforestation for carbon sequestration (Albanito et al., 2016) or the preservation of pasture-based livestock production systems that provide biodiversity benefits in certain landscapes (Röös et al., 2017). The median land use footprint of plant-based substitutes was 32, 52, and 75% smaller than that of

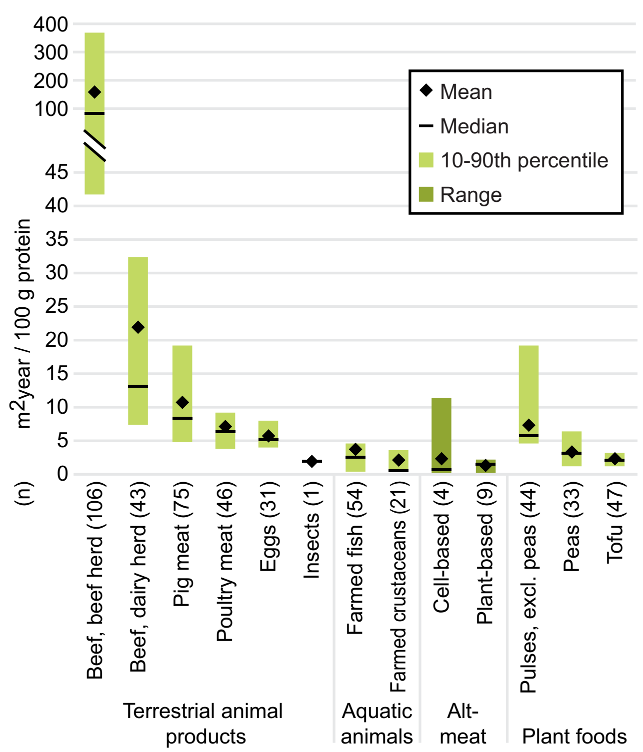

FIGURE 4 | Land use per 100 g protein. For cell-based meat, plant-based substitutes, and insects, the mean and median were calculated using the mean value from each individual study (*n*) to avoid over-representing results from studies that included more products than other studies. The minimum and maximum values of the range were derived from individual product footprints. For data sources and individual product footprints, see **Supplementary Data**. Data for all other foods are from Poore and Nemecek (2018); for these *n* represents the number of observations.

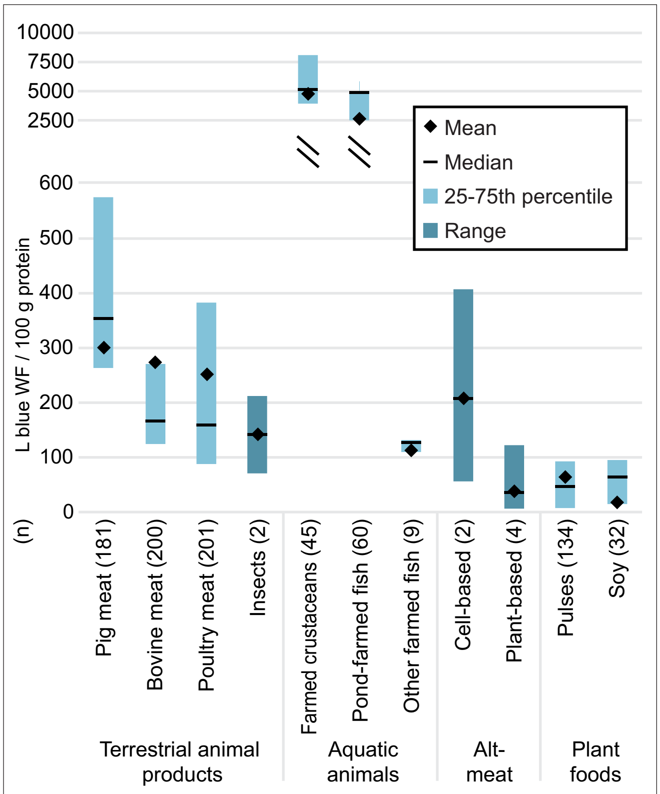

FIGURE 5 | Blue water footprints per 100 g protein. For cell-based meat, plant-based substitutes, and insects, the mean and median were calculated using the mean value from each individual study (*n*) to avoid over-representing results from studies that included more products than other studies. The minimum and maximum values of the range were derived from individual product footprints. For data sources and individual product footprints, see **Supplementary Data**. Data for all other foods came from Kim et al. (2019); for these *n* represents the number of data points included.

tofu, peas and other pulses, respectively. These comparisons are skewed, however, by the fact that the values for less-processed plant proteins reflect global averages that include data from low-yielding countries (Poore and Nemecek, 2018), whereas the LCAs for plant-based substitutes likely assumed ingredients were sourced from more efficient production systems in industrialized countries.

The estimated land required to produce the ingredients for cell-based meat varied widely (0.1–11.5 m^2 per year per 100 g protein; median: 0.6), depending largely on the choice of feedstock and inputs for the cell cultivation. Models of cell-based meat production using cyanobacteria (i.e., blue-green algae) hydrolysate (Tuomisto and Teixeira de Mattos, 2011; Tuomisto et al., 2014; Smetana et al., 2015) had the smallest land use requirements. Comparisons which modeled conventional livestock feeds (Alexander et al., 2017) or soy and corn-derived inputs (Mattick et al., 2015b) as the nutrients for the cell culture medium found that the land use requirements for cell-based meat were comparable to those of poultry when comparing based on protein content.

The land-sparing possibilities associated with meat alternatives would not necessarily occur with shifts away from farmed meat production. If farmed meat consumption were only reduced in industrialized countries, exports of feed crops could simply increase—though this could reduce demand on land clearing for agricultural use in other environmentally sensitive regions such as South America, where deforestation is a leading driver of climate change and biodiversity loss (Tilman et al., 2001; Machovina et al., 2015). If environmental land-sparing options were desired, it would be essential to have adequate policies preventing newly available land from being developed or used for other industrial purposes. It is also worth considering how significant changes in land use could impact rural communities where agriculture is often the economic driver (see Socio-Economic Implications).

Water Use

Fewer studies have quantified the amount of blue water (i.e., freshwater from ground or surface sources) consumed to produce meat alternatives. Based on our review of the available literature (**Figure 5**), per 100 grams protein, the median blue water footprint of plant-based substitutes was 21 and 42% smaller than those of pulses and soy; 76, 77, and 89% smaller than those of farmed poultry meat, bovine meat, and pig meat; and two orders of magnitude smaller than those of aquatic animals raised in ponds, e.g., farmed shrimp and tilapia. The values for pulses and soy were likely larger than those of plant-based substitutes in part because the former reflect global averages that include data from low-yielding countries (Kim et al., 2019), whereas the LCAs for plant-based substitutes likely assumed ingredients were sourced from more efficient production systems in industrialized countries. By contrast, the median blue water footprint of cell-based meat was larger than those of all other foods considered in our review except for those of farmed pig meat and pond-raised aquatic animals. See **Supplementary Materials** and **Data** for details.

Eutrophication

As highlighted in **Table S3**, many popular plant-based substitutes are derived from legumes, which in addition to their food value, are noted for their ability to improve soil fertility through fixing atmospheric nitrogen into a form that is usable by plants (Voisin et al., 2014). Incorporating legumes into crop rotations can diversify farmers' production systems and sources of income and reduce their dependency on synthetic nitrogen fertilizer (Voisin et al., 2014).

As with fertilized fields, nitrogen can leach from legume-based cropping systems into surface or ground water, which can contribute to eutrophication. Limited data exists on how much plant-based substitutes exacerbate eutrophication, but existing research suggests they provide significant benefits over conventional meats. One study found that the average freshwater eutrophication potential of plant-based substitutes was an order of magnitude smaller than that of conventional pork sausage patties, and two orders of magnitude smaller than those of beef and chicken patties (Fresán et al., 2019). Another study found that conventional pork production resulted in six times greater eutrophication potential and required 3.4 times more fertilizer per unit of protein compared to a pea-based plant-based substitute (Zhu and van Ierland, 2004). These findings are comparable to those of other studies that have found that growing pulses releases 85–94% less reactive nitrogen per unit of protein than producing seafood or conventional meat (Leach et al., 2016).

One study that modeled the hypothetical eutrophication potential of cell-based meat, based on inputs of soy hydrosylate and glucose and glutamine (both derived from corn), found it comparable to, or slightly lower than that of, conventional poultry production (Mattick et al., 2015b). This is expected given the inputs for the modeled cell culture were similar to those in poultry feed (e.g., corn, soy), and likely used in similar quantities based on the fact that poultry and cell-based meat production required roughly the same amount of land (Mattick et al., 2015b). For cell-based meat production systems that grow cyanobacteria as the primary input instead of corn or soy, nitrogen-fixing species of cyanobacteria could be selected to reduce the use of synthetic nitrogen fertilizer (Tuomisto and Teixeira de Mattos, 2011).

Pesticide Use

Limited research has explored the pesticide use associated with the production of meat alternatives. One study found that conventional pork production involved 1.6 times more pesticide use per unit of protein compared to the production of a pea-based plant-based substitute (Zhu and van Ierland, 2004). Another study found that conventional meat protein (an average of different animals) required six times more biocides (pesticides and disinfectants) to produce than the same amount of a soy-based plant-based substitute (Reijnders and Soret, 2003).

Conventionally grown soybeans, a common ingredient in plant-based substitutes as well as conventional animal feed, are among the most common crops genetically modified to be tolerant to herbicides such as glyphosate (i.e., "Roundup"); 2,4-D; and dicamba [U.S. Department of Agriculture Economic Research Service (USDA, ERS), 2019; U.S. Department of Agriculture (USDA), 2019]. Soybeans were the leading driver behind the growth in herbicide use in the U.S. from 1996 to 2011 and have contributed to the rise of herbicide-resistant "superweeds" (Benbrook, 2012). While the carcinogenicity of glyphosate to humans has been intensely debated (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2015; Williams et al., 2016), growing resistance has trapped farmers in a costly treadmill requiring them to apply more and multiple herbicides to control weeds (Benbrook, 2012).

The pesticide use involved in cell-based meat production depends largely on the inputs used in the culture medium. If soy and corn-based inputs were used as inputs, as modeled by Mattick et al. (2015b), it could be assumed that pesticide use in cell-based meat production would be comparable to that associated with conventional poultry production (since they required comparable amounts of land).

Biodiversity and Ecosystem Function

Producing legumes—the primary protein ingredient in most plant-based substitutes—can improve soil biodiversity and above-ground vegetative and invertebrate biodiversity, although the extent depends on management practices including tilling, chemical pest control, and fertilizer inputs (Williams et al., 2014). Soil biodiversity in turn promotes resistance and resilience against disturbance and stress, improves water and nutrient use efficiencies in crop production, and suppresses soil-borne disease (Brussaard et al., 2007).

Declining biodiversity of agricultural systems is also a concern for long-term food security and resilience, threatened in part by monoculture production systems and genetic uniformity in crop varieties and livestock breeds in conventional livestock production (Thrupp, 2000; Jackson et al., 2007). To the extent to which meat alternatives integrate ingredients other than soybeans and wheat (which are among the most produced crops worldwide, for both human foods and livestock feed), such as peas and lupins from which several plant-based substitutes are now derived, this could help diversify diets and foster agrobiodiversity.

Many plant-based substitutes include coconut or palm oil among their ingredients. Both of these plant-based lipids are grown in tropical regions rich in biodiversity, which is threatened by deforestation and anthropogenic forest disturbance (Barlow et al., 2016). Oil palm plantations have been a significant driver of deforestation and the associated biodiversity loss in Southeast Asia and South America (Vijay et al., 2016). While coconut plantations have not been implicated in significant demand-driven deforestation thus far, a massive scaling up of the plant-based substitute industry could pose biodiversity and sustainability concerns (Goldstein et al., 2017). That said, these concerns attributed to plant-based substitutes would also need to be evaluated in light of existing deforestation for pasture and feed crop production associated with conventional meat production (Goldstein et al., 2017).

ANIMAL WELFARE IMPLICATIONS

Meat alternatives, if widely adopted as a replacement for farmed meat, may greatly reduce dependence on livestock to be raised and slaughtered for meat production. That said, several technological challenges remain before animals can be completely removed from the supply chain of cell-based meat, including the source of the animal cell line and inputs used.

Source of Animal Cells

The first challenge relates to the source of the animal cell line. Cell-based meat production can occur in two ways, one of which requires only one animal and the other which requires a continuous stream of animals. In the first example, unfertilized eggs are obtained from a female animal and then fertilized by sperm in a petri dish (similar to *in vitro* fertilization) (Welin, 2013). If treated correctly, the embryonic stem cell line can be used indefinitely (Specht et al., 2018). While recent research suggests that these pluripotent cells could be manipulated into muscle fibers, it is a new technology and meat derived from them would require significant long-term safety testing, would have to be labeled as a genetically modified organism (i.e., GMO), and could undergo genetic mutations that might pose safety concerns or logistical challenges (Bhat et al., 2019; Thorrez and Vandenburgh, 2019).

Given these factors, the other source of animal cells—obtaining adult muscle stem cells from a biopsy of a living or dead animal—is currently the industry standard (Welin, 2013). Adult muscle stem cells can only replicate about 50–60 times before they reach their capacity to multiply and would need to be replaced (Kadim et al., 2015). A biopsy would also be required each time a new line of meat cells is produced (e.g., for each product a company develops). Additionally, while in theory substantially higher amounts of meat could be obtained per animal compared to animal slaughter for farmed meat (Stephens et al., 2018), the number or even magnitude of animals that would be implicated in each scenario has not, to our knowledge, been estimated in the literature. Furthermore, some have claimed that cell-based meat production could support the propagation of traditional native livestock breeds for cell harvesting in slaughter-free herds (Stephens et al., 2018), though it will depend upon the choices of the companies that commercialize cell-based meat. Comprehensive animal welfare assessments exploring the well-being of animals who are raised and undergo biopsies for the production of cell-based meat should be conducted.

No information is currently available on the extraction of cells from fish or shellfish to make cell-based seafood, such as whether they will come from wild or farmed fish, dead or alive. As these products develop, their animal welfare implications will have to be considered in the context of the debate over the extent to which fish or shellfish have the capacity to suffer and feel pain, and how animal welfare regulations established for terrestrial animals apply to commercial fishing and aquaculture operations (Huntingford et al., 2006; Browman et al., 2019).

Inputs

The second animal welfare challenge for cell-based meat concerns several inputs used that are still animal-derived due to technological or financial limitations. These include fetal bovine serum, scaffolds on which to grow the muscle tissue into thick pieces, and animal-derived hydrogels that are used to mimic natural tissue (Stephens et al., 2018). Fetal bovine serum (FBS), for instance, is a universal growth supplement for cell and tissue culture media extracted from the blood of a live cow fetus after the mother is slaughtered for meat processing (Gstraunthaler et al., 2013). While it is a byproduct of the meat industry (animals are not raised and slaughtered solely to produce FBS), its use means that cell-based meat production still hinges on farmed livestock production and raises several animal welfare concerns. The amount of serum obtained depends on the age of the fetus, but one 2002 study estimated that 800,000 liters of FBS were produced annually worldwide for use in culture media, corresponding to about 2 million bovine fetuses (Jochems et al., 2002). Demand for FBS has steadily increased worldwide, primarily due to use in drug and vaccine production and tissue engineering (Brunner et al., 2010). Serum-free growth media do exist and extensive research is dedicated to advance the field (van der Valk et al., 2018; Zhang et al., 2020). While they are currently prohibitively expensive (Specht et al., 2018; Thorrez and Vandenburgh, 2019), some prototypes have been shown to be able to effectively replace FBS, albeit less efficiently (Kolkmann et al., 2020). Until serum-free media becomes a viable option, more research into how many animals would be required to produce enough FBS for cell-based meat production is merited, though it will likely be far less than the volume of animals slaughtered for farmed meat production.

While most plant-based substitutes in theory do not contain animal products, the use of coconut oil in many plant-based substitutes raises animal welfare concerns. Many large coconut plantations in Thailand rely on monkeys, either stolen from the wild or bred on farm to harvest the coconuts. While there are some coconut oil producers that are "monkey free," the continued employment of these highly intelligent animals in chained working conditions raises ethical dilemmas for the continued expansion of the coconut industry without specific standards on this issue (Barclay, 2015; Moyer, 2015). A small number of plant-based substitutes contain egg or milk protein, raising concerns about the welfare of laying hens and dairy cows, though the companies selling these products have recently been adding (e.g., Quorn Foods) and/or transitioning to 100% plant-based products (e.g., Morningstar Farms) (Blythman, 2018; Forgrieve, 2019).

ECONOMIC IMPLICATIONS

The following section explores how current trends in the development and production of meat alternatives may affect industry consolidation; consumer prices; and the economic well-being of small and mid-sized producers, rural communities, and less industrialized countries.

Industry Consolidation

Over the past decade, there has been significant investment in the research and development of meat alternatives (Mouat et al., 2019). Several of the leading meat processing and aggregation companies have announced they are developing their own plant-based substitutes (e.g., Tyson Foods, JBS, Nestle, Cargill, Hormel Foods, Perdue) or investing in existing ones; Tyson Foods, for example, was an early investor in Beyond Meat before starting its own product (Henderson, 2019). Other companies have been buying up existing plant-based substitute brands, e.g., Kellogg's owns Morningstar Farms, and Unilever acquired The Vegetarian Butcher (Lucas, 2019). Although cell-based meat production was initially developed by university-based researchers and in a few cases (e.g., Singapore Food Agency, 2020) driven by public-private investments, it is now primarily driven by venture-capital backed companies, some of which have received investment from large meat processing companies (Stephens et al., 2019).

The investment of agribusiness into the research and development of meat alternatives raises questions about who will benefit from the growth of this industry. Some have suggested that cell-based meat production could provide a new market opportunity for small businesses—akin to micro-brewery labs (van der Weele and Driessen, 2013; van der Weele and Tramper, 2014; Stephens et al., 2018). It is unclear, however, the extent to which smaller-scale "producers" will have access to government subsidies and grants or the technical information needed to produce cell-based meat, especially after it required so much capital for research and development (Stephens et al., 2018). The extent of intellectual property rights that will emerge around cell-based meat is also unclear, though analogous debates over seed patenting may be relevant (Barton and Berger, 2001). Some concerns have been raised that cell-based meats will allow multinational meat companies to assume more power in the food value chain (van der Weele and Driessen, 2013). Others have pointed out that since the vast majority of cell-based meat companies—as well as several plant-based substitute companies—are owned by agribusinesses or biotech startups headquartered in industrialized countries (Mouat et al., 2019), meat alternative industries could perpetuate economic and political power disparities between the Global North and South (Hocquette, 2016). It could be argued, however, that since attitudes toward and expectations about freshness in meats might be relaxed for meat alternatives (e.g., facilitating acceptance of frozen products), they could theoretically be produced further away from consumers than farmed meats and thus their production could potentially serve as an economic driver in less industrialized countries. It is also worth noting that proponents of meat alternatives would likely not disagree with such critiques, but argue that such products are not intended to address problems associated with agribusiness consolidation or globalization.

Socio-Economic Implications

If meat alternatives were to significantly replace farmed meat production, as some speculate (Tubb and Seba, 2019), it could have far-reaching socioeconomic effects on producers, workers, and rural communities. A rapid transformation of the agricultural marketplace from farmed to cell-based meat production—and, to a lesser extent, plant-based substitute production—could entail a significant overhaul in the labor workforce involved in protein production, from one largely based on farmers, farmworkers, meat processors, and veterinarians, to one based on chemists, cell biologists, engineers, and factory and warehouse workers (Mouat and Prince, 2018; Stephens et al., 2018). Although farmers and farmworkers would still need to produce raw ingredients or inputs for meat alternatives, a significant reduction in livestock production could contribute to massive layoffs and unemployment in the livestock farming and meat processing sectors. One report speculates that half of the 1.2 million jobs in U.S. beef and dairy production alone could be lost by 2030 and that farmland values could collapse by 40–80% due largely to its projection that "modern protein foods" will be five times cheaper than existing animal proteins (Tubb and Seba, 2019). It is unclear how many new jobs would be created by either plant-based substitute or cell-based meat industries. The status of trade agreements and tariffs, which remain a source of instability in both meat (Keefe, 2018) and crop (CoBANK Knowledge Exchange, 2019) markets, will also heavily influence whether livestock and feed crop farmers continue to produce their products and simply export more to other countries or industries, raise different animals, grow different crops, or sell their operations.

The implications of such drastic economic transitions should be further explored, especially for the well-being of farmers and farmworkers, who already experience poor mental health outcomes compared to other professions due to a variety of factors including financial stress, pesticide exposure, and climate variabilities (Daghagh et al., 2019). This is especially pertinent given recent media attention (the scientific literature has not caught up yet) to a looming economic and suicide crisis among American farmers considering the persistent agricultural recession, diminished farm income, rapidly increasing debts, and extreme flooding events (Harvie, 2017; Weingarten, 2018; Simpson, 2019). Moreover, if cell-based meat were produced in cities, it could also further perpetuate rural population loss and the associated disintegration of rural economies, which are largely dependent on agriculture (Tuomisto and Teixeira de Mattos, 2011; Johnson and Lichter, 2019; Pender et al., 2019). On the other hand, the potential for plant-based substitute and cell-based meat production to create new jobs in urban areas and in locations that do not have large agricultural workforces (e.g., Singapore) and in STEM fields in both the public and private sector is also worth analyzing. Although the rise of meat alternatives is but one of many factors affecting the agricultural marketplace, research and policies to support farmers in transitioning their farms to meet new market demands and to assist workers in relevant job retraining programs is merited.

Affordability/Accessibility

Price remains one of the most significant barriers for widespread adoption of plant-based substitutes and, especially, cell-based meats. While plant-based substitutes are becoming a competitive force in the marketplace, they comprise only a small overall market share and prices for most products are higher than those of farmed meats. Some proponents claim that plant-based substitutes will become cost-competitive with farmed meats as research and development costs are recouped, farmed meat processing companies enter the meat alternatives marketplace, manufacturing operations achieve economies of scale, and raw material varieties and prices are optimized (Specht, 2019). If plant-based substitutes do achieve price parity or eventually prove less expensive than farmed meats, it has been suggested that widespread market uptake could eventually make farmed meat a premium product, based on the assumption that plant-based substitutes would likely continue to replace lower quality products such as burgers (Bonny et al., 2015).

It also remains to be seen whether cell-based meat will be able to reach price parity with farmed meats. While the first cell-based meat burger for human consumption was produced in 2013 for an estimated $280,000 USD, one company (Biotech Foods) claims that they have now reduced the cost to 100 euros per kilogram, while another (Mosa Meat) projects it could be as low as $10 USD per burger by 2021 (González and Koltrowitz, 2019). The cost of animal-free growth medium is still around 50 times higher than what it would need to be cost-competitive with farmed meat, and that is only considering the cost of the growth medium (van der Weele and Tramper, 2014). If it does not achieve price parity, some have suggested cell-based meat could remain a niche product for wealthier consumers to avoid the guilt of consuming animal products (Cole and Morgan, 2013).

Depending on how the farmed meat production market is affected by the rise of meat alternatives, there could also be significant impacts on other industries that rely on the byproducts of farmed meat production, potentially affecting the cost of vaccines and other therapeutic substances as well as wool, cosmetics, and some pet food (Mattick et al., 2015a), unless these products are also replaced by cellular or acellular alternatives. That said, the costs of therapeutic and biomedical technologies relying on cell tissue engineering could also be decreased if affordable large-scale cell-based meat production were attained (Specht et al., 2018).

POLICY IMPLICATIONS

The introduction of meat alternatives to the U.S. consumer market has fueled debates at the state and federal policy level. These debates center on food safety approvals, how to label plant-based substitute products, and in the case of cell-based meat, which agency will be responsible for overseeing production and marketing. Some of these debates stem from limitations in the existing regulatory framework around food production, inspection, and marketing, and are not unique to meat alternatives; however, critics have highlighted their importance in the context of these products.

Product Approvals

Concerns have been raised about how new ingredients in the food supply and food production processes are approved (IPES-Food, 2017), concerns which are relevant for the manufacturing of meat alternatives. Many food ingredient approval processes established by the US Food and Drug Administration (FDA) are voluntary and industry-led. For example, food companies can declare new substances they plan to use in food products to be generally recognized as safe (GRAS) based on their own risk assessments. Companies can voluntarily seek input from the FDA on their GRAS filings, but FDA notice or pre-market approval is not required (unlike the typical food additive safety review process) [Food and Drug Administration (FDA), 2016]. Some plant-based substitutes include novel food ingredients that have been introduced to the food supply through the GRAS process, including mycoprotein in Quorn products (Marlow Foods Ltd., 2001) and soy leghemoglobin in Impossible Foods products [GRAS Notice (GRN) No. 737, 2017]. In the case of soy leghemoglobin, a heme protein derived from genetically engineered yeast, Impossible Foods voluntarily submitted its GRAS determination to the FDA in 2017 and went through several rounds of questions and responses with the agency. During these exchanges, the agency determined that the reddish-brown color that soy leghemoglobin imparts in the company's uncooked beef substitutes qualified the ingredient as a color additive and thus required FDA approval before it could be sold to consumers in uncooked forms [Food and Drug Administration (FDA), 2019a]. In July 2019, the agency sent a notice saying it had no further questions about the ingredient's GRAS status and approved the company's petition to use soy leghemoglobin as a color additive [Food and Drug Administration (FDA), 2019b]. While some commentators have raised concerns about the process and decision (Storm, 2017; Lefferts, 2019), others find it sufficient to suggest that soy leghemoglobin is unlikely to pose a risk for consumers (Clinton, 2017; Johnson, 2017), though, to our knowledge, this has yet to be assessed in the academic literature. Consumer advocacy organizations are similarly concerned about the GRAS process in the context of cell-based meat (Hansen, 2018). A lawsuit was filed in 2017 challenging the GRAS self-certification process (Case 1:17-cv-03833, 2017).

Regulatory Oversight of Cell-Based Meat

The ontological challenges of deciding whether cell-based meat is considered "meat" or not have also posed practical questions in terms of which federal agency will be responsible for regulating production and inspection of cell-based meat in the U.S. After much deliberation, the FDA and USDA Food Safety and Inspection Service (USDA-FSIS) agreed to jointly regulate "human food products derived from the cultured cells of livestock and poultry" in March 2019 [Food and Drug Administration (FDA), 2019c]. The FDA will oversee the cell culturing stages of production from initial cell collection up to cell harvesting, at which point oversight will transition to the USDA-FSIS for meat production and labeling (Sancar, 2019). This division of responsibilities has been a longstanding challenge affecting other food products, but few as blatantly as cell-based meat. This division will not apply to cell-based seafood, which falls under the remit of FDA (Greene and Angadjivand, 2018).

While the joint agreement between FDA and USDA clarified many of the regulatory responsibilities between the agencies, some questions remain, stemming in part from the nature of how regulatory programs are established and funded. Congress enacts legislation that enables executive agencies to establish certain programs, creates budgets and appropriates federal funding for those programs (and the government generally), and provides oversight to ensure programs are running efficiently and funding is being spent appropriately. The development of regulations and programs within agencies through the enabling responsibility does not always align perfectly with Congressional appropriations, however, and conflicts may arise as a result. In the case of the joint agreement between FDA and USDA, the agreement does not empower either agency to spend additional resources on regulating cell-based animal products [Food and Drug Administration (FDA), 2019c]. Thus, the agencies' capacities to oversee these new industries may be limited by funding and personnel constraints, unless Congress authorizes additional funding for them.

Given the rapidly evolving technology involved in cell-based meat production, the regulatory frameworks surrounding it will likely change (Schneider, 2012; Stephens et al., 2018). As different types of cell-based meat products, production methods, and production facilities develop, they may require different regulatory approaches (Stephens et al., 2018). Other policy issues have yet to be addressed, including whether bioreactors will be considered agricultural facilities (which will affect zoning laws), whether waste will be regulated as an animal byproduct, the possibility for food fraud and mislabeling of cell-based and farmed meat products, and how to regulate cell-based meat produced using animal species not typically used for food (Stephens et al., 2018). Regulatory pathways and challenges will also differ between states and countries, depending in part on the strength and influence of the traditional agriculture lobby.

Labeling

There has been considerable debate around how meat alternatives should be labeled, with some livestock industry representatives concerned that consumers could be misled to think they are purchasing farmed meat. Federal legislation was introduced in October 2019 with the intention of limiting the widespread utilization of the term "meat" for products that are plant-based (U.S. House, 2019). The legislation would require labeling any product that does not contain "real" meat to bear the label "imitation" immediately before or after the name of the food, and enhance mislabeling enforcement provisions. This act comes after at least 25 states (O'Connor, 2019) have passed laws restricting use of the term "meat" or "beef" on plant-based substitutes or cell-based meat products. Given the negative connotations of the word "imitation," in terms of both quality and flavor, these laws are likely intended to undermine the marketing of these products, rather than inform consumers who likely already know the nature of the meat alternatives they are buying. It is also worth mentioning that under FDA guidelines, "imitation" applies to any product that "resembles another food but is nutritionally inferior to that food," such as imitation crab used in some sushi [Food and Drug Administration (FDA), 2019d]. Since some plant-based substitutes (and theoretically cell-based meat) contain comparable amounts of all essential nutrients of their farmed counterparts, it could be legally argued that these products are not "imitations." This semantic and legal debate illustrates an interesting schism in the "meat industry": the lobbyists advocating for these laws represent producers who are most likely to be affected by the rise of meat alternatives, rather than processing and manufacturing companies who stand to profit from the new products.

CONCLUSION

Plant-based substitutes and cell-based meats are gaining a foothold in global markets. This review of the evidence explores the extent to which the production and consumption of meat alternatives can mitigate some of the environmental, animal welfare, and public health problems associated with farmed meats, per how these products are often promoted. In doing so, we highlight the complexity of the issues at hand and the need for cautionary approaches to the rapid adoption of these products.

From an environmental perspective, plant-based substitutes can provide substantial benefits over farmed beef, to which they are most often compared by industry and media. Cell-based meat could provide benefits as well for most environmental concerns, with a few caveats: the GHG footprint, blue water footprint, and industrial energy use could be higher than those of farmed beef in some cases. Compared to farmed pork, poultry, eggs, and some types of seafood, the environmental benefits of meat alternatives are generally less pronounced (in the case of plant-based substitutes) or potentially non-existent (in the case of cell-based meat), a nuance that should be more transparent in discussions around meat alternatives.

From an animal welfare perspective, if meat alternatives replace even a small share of farmed meat production, this could substantially reduce the number of animals raised and killed for human protein consumption, demonstrating the ethical appeal of these products. Cell-based meat will, however, require further technological developments to remove all animal-based inputs including fetal bovine serum.

From a public health perspective, there has been limited research on nutrition, chronic disease, and food safety implications associated with consuming meat alternatives,

TABLE 1 | Level of characterization of public health, environmental, and animal welfare implications in research to date on meat alternatives.

Implication category	Topic	Plant-based substitutes	Cell-based meat
Public health	Nutrients	Moderate	None
	Chronic disease risk	Limited	None
	Food safety	Limited	None
	Occupational health	None	None
	Community health	None	None
Environmental	GHG emissions	Moderate	Limited
	Water use	Limited	Limited
	Land use	Moderate	Limited
	Nutrient runoff	Limited	Limited
	Pesticide use	Limited	None
	Biodiversity	Limited	None
Animal welfare		Limited	Limited

This table classifies the extent to which key public health, environmental, and animal welfare implications explored in this paper have been characterized in existing academic literature on meat alternatives. Recognizing that many studies report or review secondary data, and that a significant proportion of the available environmental impact research on meat alternatives is from gray literature, designations were made based both on counting of available studies related to a specific product and implication as well as a qualitative judgment of the depth and quality to which these topics have been covered in the literature. Topics were classified based on the following criteria: none (no specific research on this product and implication), limited (covered by only a few studies), moderate (some research; more is needed); high (thoroughly researched).

and occupational and community health implications associated with their production. For example, it is unknown whether replacing farmed meat with plant-based substitutes would offer similar nutritional and health benefits as less-processed plant foods; the relative benefits would depend on the extent to which plant-based substitutes are replacing red and processed meat. Meanwhile, many of the purported health benefits of cell-based meat are largely speculative at this time, given the level of uncertainty around macro- and micronutrient content, the scope and nature of antibiotic use, and waste management practices.

The broader socioeconomic and political implications of widescale replacement of farmed meat with meat alternatives are also critical to consider, despite their frequent omission from most existing research. Meat alternatives are not intended to address concerns associated with industry consolidation, or the loss of farmers' and public autonomy over the food system, but as products to be offered within existing protein supply chains that appeal to those who enjoy meat but seek to reduce environmental, animal welfare, and public health harms. That said, these products illuminate important economic and political tensions between livestock producers and processing/marketing companies, between the workers who may benefit and those who may lose opportunities from their rise in popularity, and between consumers who may or may not be able to access them.

There is no silver bullet solution to addressing the myriad public health, environmental, and animal welfare challenges associated with protein consumption. While plant-based substitutes and cell-based meats may offer many benefits over some farmed meats, it is critical to remain cautious and nuanced in discussing their merits rather than assuming that they will solve our current challenges without any drawbacks. By the same token, these products should not be dismissed out of hand as fringe developments in the food system or as simply "imitations," but should be approached with the same nuance as other foods. At the same time, the role of shifting toward more diverse, unprocessed whole foods (including pulses) while providing economic support for more agroecological producers—more than just substituting processed foods within otherwise unhealthy dietary patterns and inequitable supply chains—should not be overlooked. Mitigating the systemic problems of our food system will likely require the food processing and service industries, producers, and consumers to think beyond simply replacing one "meat" on a plate with another.

FUTURE RESEARCH

This literature review has revealed a number of gaps in the research around plant-based substitutes and cell-based meats. **Table 1** portrays the extent to which key public health, environmental, and animal welfare implications explored in this paper have been characterized in existing academic literature on meat alternatives. It is also worth mentioning that much of the existing environmental research on plant-based substitutes and cell-based meats has been funded or commissioned by companies developing these products, or by other non-profit organizations promoting these products (see Appendix in **Supplementary Material** Appendix A.2.1). In Appendix B (**Supplementary Material**), we highlight a list of specific research needs that should be further explored, ideally with independently funded peer-reviewed studies.

AUTHOR CONTRIBUTIONS

SG and RS designed search protocol for academic databases. RS wrote the first draft of the manuscript. RS, SG, EB, and BK wrote sections of the manuscript. RS and BK compiled and standardized data for the figures and supplementary information provided. BK, JD, RN, and MB provided expertise on sections of the manuscript. KN provided guidance on all facets of and supervised the project. All authors reviewed and contributed to manuscript drafts and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Invasive Plants Are a Valuable Alternate Protein Source and Can Contribute to Meeting Climate Change Targets

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Agriculture has come under pressure to meet global food demands, whilst having to meet economic and ecological targets. This has opened newer avenues for investigation in unconventional protein sources. Current agricultural practises manage marginal lands mostly through animal husbandry, which; although effective in land utilisation for food production, largely contributes to global green-house gas (GHG) emissions. Assessing the revalorisation potential of invasive plant species growing on these lands may help encourage their utilisation as an alternate protein source and partially shift the burden from livestock production; the current dominant source of dietary protein, and offer alternate means of income from such lands. Six globally recognised invasive plant species found extensively on marginal lands; Gorse (*Ulex europaeus*), Vetch (*Vicia sativa*), Broom (*Cytisus scoparius*), Fireweed (*Chamaenerion angustifolium*), Bracken (*Pteridium aquilinum*), and Buddleia (*Buddleja davidii*) were collected and characterised to assess their potential as alternate protein sources. Amino acid profiling revealed appreciable levels of essential amino acids totalling 33.05 ± 0.04 41.43 ± 0.05, 33.05 ± 0.11, 32.63 ± 0.04, 48.71 ± 0.02 and 21.48 ± 0.05 mg/g dry plant mass for Gorse, Vetch, Broom Fireweed, Bracken, and Buddleia, respectively. The availability of essential amino acids was limited by protein solubility, and Gorse was found to have the highest soluble protein content. It was also high in bioactive phenolic compounds including cinnamic- phenyl-, pyruvic-, and benzoic acid derivatives. Databases generated using satellite imagery were used to locate the spread of invasive plants. Total biomass was estimated to be roughly 52 Tg with a protein content of 5.2 Tg with a total essential amino acid content of 1.25 Tg (~24%). Globally, Fabaceae was the second most abundant family of invasive plants. Much of the spread was found within marginal lands and shrublands. Analysis of intrinsic agricultural factors revealed economic status as the emergent factor, driven predominantly by land use allocation, with shrublands playing a pivotal role in the model. Diverting resources from invasive plant removal through herbicides and burning to leaf protein extraction may contribute toward sustainable protein, effective land use, and achieving emission targets, while simultaneously maintaining conservation of native plant species.

Keywords: plant protein, marginal lands, sustainable agriculture, net zero emissions, nutritional characterisation, invasive plants, essential amino acids, cyclic economy

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INTRODUCTION

Existing mainstream food supply systems are capable of surpassing global nutritional requirements and yet, we find about 12.3% of the human population living with chronic malnutrition (FAO et al., 2020). Increased atmospheric CO_2 and malnutrition can be causally attributed to inefficiencies along the food supply chain primarily through wastage and poor resource allocation (Bajželj et al., 2014; Sabaté et al., 2015; Xue et al., 2017). The high-energy input required for modern agriculture is mostly sourced from fossil fuels, which directly leads to greenhouse gas emissions. This in turn contributes to climate change, increasing crop vulnerability due to altered pest behaviour, temperature eccentricities, and altered seasonal patterns. Cultivation of crops and plants, which is essentially an anabolic carbon storing process, is rendered ineffective owing to such intensive cultivation practises to meet global food demand. One of the major sources of food-based carbon emission is animal husbandry. Most of the global protein produced is directed toward animal feed. Currently, animal husbandry enjoys the single largest resource allocation; especially land, in global food production and is the dominant source of protein for most people (Day, 2013; FAO, 2019). Justification for the present scale of animal husbandry enumerates reasons such as employment, superior essential amino acid profile of animal products, useful functional properties and gastronomic preferences. However, protein turnover from this enterprise is low, while associated greenhouse gas (GHG) emissions are high, making it the single largest contributing factor to agricultural emissions (Pragna et al., 2018).

In the case of Scotland, large parts of the Scottish Highlands are ill suited for arable farming and are primarily used for rough grazing. Plants such as Gorse *(Ulex europaeus)*, Vetch (*Vicia sativa*), Broom (*Cytisus scoparius*), Fireweed (*Chamaenerion angustifolium*), Bracken (*Pteridium aquilinum*), and Buddleia (*Buddleja davidii*) are able to quickly grow in urban as well as marginal lands owing to their robust physiologies and quicker germination times, allowing them to dominate available land and often encroach into farmlands (Eldridgea et al., 2011). Since they can grow in low nutrient conditions, they are known to colonise lands left fallow for rejuvenation. This necessitates the investment of resources to remove such plants. While historically, the excised mass was used for animal fodder during times of famine (Łuczaj, 2010; Pinela et al., 2017), they are currently dried and burnt, dug into the soil for increased organic matter or discarded into the landfill which contributes to carbon emissions. Investigation into their nutritive value and developing efficient protein extraction processes may help solve the problem of resource waste and add potential means of compensation for the investment made toward their active removal. Numerous large-scale leaf protein extraction designs have been proposed, although commercial impetus is lacking owing to efforts being diverted to crop variety selection and design. However, adopting such revalorization strategies may mitigate costs generally incurred from raw material sourcing while simultaneously dealing with downstream waste-management. Currently, the resources dedicated to unwanted plant removal is substantial and costs are borne entirely by local farmers, impinging on their income margins. Additionally, these plants pose a threat to local diversity if not kept in check (Parker, 2000; Dassonville et al., 2008; Shiferaw et al., 2019).

Characterisation of these plants as a potential source of nutrition may help provide impetus for their effective utilisation and limit or decrease their spread in their non-native locations or stimulate interest for their conservation in native lands. Extraction methods for obtaining leaf proteins have been well-studied; particularly by Pirie (1977) who pioneered the use of industrial pulping mechanisms to express plant juices. However, contemporary methods for removal of pungent and strong-tasting phenolics were not cost effective and subsequent advances made in crop genetics, and development of novel cultivars overshadowed the idea. Recent years, however, have seen an increase in the pursuit of leaf protein recovery, particularly from agricultural waste. Understanding the total protein content and the amino acid composition of native Scottish plants may help open newer avenues for protein sourcing and allow for more robust food systems resilient to global policies and environment.

Improved utilisation and reallocation of land use for alternate and sustainable protein production is required to help reduce dependence on animal husbandry and offset part of the associated emissions. Along with other non-conventional sources such as insects (Henchion et al., 2017), fungi (Bano et al., 1963), and seaweed (Tamayo Tenorio et al., 2018), revalorisation of invasive plants appears to be an abundant and a potentially lucrative route of obtaining protein (Pirie, 1932, 1969a,b; Nagy et al., 1978). The work described here assesses the revalorisation potential of invasive plants through comprehensive nutritional investigation. It considers potential health benefits, and the predicted impact on land resource allocation and associated GHG emissions.

MATERIALS AND METHODS

World data on land use and green-house-gas emissions were obtained from FAOSTAT (FAO, 2019).

All chemicals and kits were purchased from Merck (Darmstadt, Germany), and used without further purification unless stated otherwise.

All experiments and measurements were performed in triplicate.

All colorimetric assays were carried out in 96-well-plates and incubated with film cover (Greiner Bio-One, Kremsmuenster, Austria). Absorption values were obtained using SpectraMax 190 (Molecular Devices, San Jose, USA).

Plant Samples

All plant samples were collected between March and July in the North-East of Scotland at the following GPS co-ordinates. Gorse *(Ulex europaeus)*, Broom (*Cytisus scoparius*), and Bracken (*Pteridium aquilinum*); 57.257, −2.483, Fireweed (*Chamaenerion angustifolium*); 57.157, −2.086, Vetch (*Vicia sativa*); 57.157, −2.139, and Buddleia (*Buddleja davidii*); 57.158, −2.099. Collection procedure was in accordance to guidelines by the Ministry of Forest, CA (British Columbia Ministry of Forest, 1996). The sampling design aimed to gather biomass across numerous plant samples over a unit land area. For each species at the site of invasion, leaves were harvested from a random number of multiple plants in one-metre-squared plot until one kilogramme of leaf mass was obtained. This was performed in triplicate across adequately spaced one-metre plots. The leaf samples were separated from stalks and debris and pooled, and freeze-dried (Labconco, UK). Samples were freezer-milled (Centramex, USA), and stored at 4°C under vacuum. The whole leaf samples were used to profile amino acids, phenolics, and total NSP (Non-starch Polysaccharide).

Plant Homogenisation and Extraction

The leaf samples (10 mg) were vortexed in phosphate buffer (10 mL; 10 mM; pH 7.5) at 25°C and kept in an ultrasonic bath (Branzen, B12) for 2 min. The supernatant was separated by centrifugation (4°C; 15 min; 4,000 g). A subsample of the aqueous supernatant extract was used for colorimetric estimation of soluble phenolics, sugar and protein content. Remainder of the supernatant and precipitate were collected and freeze dried. Freeze dried precipitate was used to estimate bound proteins.

Total Soluble Phenolics

Fast Blue assay was performed to estimate total soluble phenolics as described by Lester et al. (2012). Sample or Standard (100 μL) was incubated with 2 μL Fast Blue (0.1% w/v) for 30 s on a shaker. Sodium hydroxide (NaOH; 2 μL; 5% w/v) was added and incubated for 90 min in the dark. Absorbance was measured at 420 nm.

Total Soluble Sugars

Lever's assay was used to estimate soluble sugars as previously described by Lever (1973, 1977). Lever reagent comprised of 4-hydroxybenzoic acid hydrazide (PAHBAH, 0.76% w/v), bismuth (III) nitrate pentahydrate (0.48% w/v), potassium sodium tartrate (0.28% w/v), NaOH (2% w/v) in ultrapure Milli-Q water. Sample or standard (5 μL) was incubated with 200 μL of Lever reagent at 70°C for 30 min. The samples were then allowed to cool for 10 min and absorbance was checked at 415 nm.

Protein Estimation

Sample hydrolysis was carried out using HCl (6 N) with phenol (2% w/v) to aid removal of oxygen radicals (Muramoto et al., 1987). Freeze-dried supernatant and retentate (10 mg) were digested in HCl (6 N; 10 mL, prepared as described above) and digested using MARS 6 (CEM, US) microwave digester with a temperature ramp to 150°C for 20 min. The temperature was maintained for 60 min and then cooled overnight.

Protein content was determined by amino acid estimation using the Ninhydrin Assay based on the work described by Harding and MacLean (1916). An aliquot (10 μL) of the supernatant was dried at 60°C in a microtiter plate and re-suspended in phosphate buffer (10 mM, 100 μL, pH 7.5) to which ninhydrin reagent (75 μL) was added and incubated at 60°C for 60 min. The absorbance was measured at 570 nm.

Amino Acid Estimation

Hydrolysis for amino acid profiling was performed as previously described for protein estimation with the addition of 10 mg of tryptamine. Essential amino acids were profiled and quantified using Gas Chromatography in tandem with Mass Spectrometry (GC-MS) with U_{13}-C amino acids as internal standards as described previously in Calder et al. (1999).

Phenolic Characterisation

Phenolics were characterised in a three-stage extraction process as previously described (Russell et al., 2007). Briefly, 0.1 g of freeze-dried, freeze-milled leaf samples were sequentially extracted under acidic, alkali, and finally neutral pH conditions using biphasic solvent extraction with ethyl acetate as the organic layer. The aqueous layer was adjusted to the required pH condition using 1 M HCl or 1 N NaOH.

Quantification was performed using LC-MS as previously described without modifications (Neacsu et al., 2015). Internal standard used were ^{13}C benzoic acid; 2 ng/μL in 0.02% acetic acid in 75% methanol for negative mode MS and 2-amino-3,4,7,8-tetramethylimidazo[4,5-f] quinoxaline; 0.5 ng/μL in acetic acid (0.02% v/v) in methanol (75% v/v) for positive mode MS.

Non-starch Polysaccharide (NSP) Determination

Non-starch polysaccharides were estimated as described by Englyst et al. using GC with inositol as an internal standard (Englyst and John, 1984; Englyst and Hudson, 1996; Bach Knudsen et al., 1997). Briefly, plant samples were hydrolysed in H_2SO_4 (7 M) at 100°C for 1 h. Monosaccharides were analysed by Gas Chromatography with Flame Ionised Detection (GC-FID) using Inositol as internal standard.

Lignin Content

Lignin content was assayed using the Acetyl Bromide method (Barnes and Anderson, 2017). The protocol was modified to improve carbohydrate removal using enzymes (Hatfield et al., 1999; Fukushima and Hatfield, 2001; Hatfield and Fukushima, 2005; Hojilla-Evangelista et al., 2017). Briefly, the freeze-dried original plant samples (100 mg) were washed with phosphate buffer (10 mM; 10 mL, pH 7.5) at 35°C twice and suspended in citric acid buffer (50 mM, 20 mL, pH 4.9) with 20 units each of cellulase, pectinase and xylanase (all three from Sigma-Aldrich) at 40°C for 4 h. Samples were centrifuged at 4,000 g for 15 min. Retentate was recovered and resuspended in citric acid buffer and incubated with Visozyme® (Novozymes, Denmark) overnight at 32°C. Samples were centrifuged again under the same condition and the supernatant was discarded. Retentate was washed twice with 5 mL ethanol at 30°C, followed by 10 mL of 90% aq. DMSO at 40°C. Sample was finally washed with 10 mL acetone at 30°C and allowed to dry overnight *in vacuo*. Dried sample (5 mg) was suspended in acetyl bromide (5% v/v) prepared in glacial acetic acid and incubated for 2 h at 70°C. Samples were left overnight in the dark under ambient temperature. The absorbance of the supernatant was measured at 280 nm. Absorption coefficient was assumed to be 23.6 g/cm/mL.

Statistics and Data Analysis

Statistical analysis was performed using R (Version 4.0.2) and RStudio (Version 1.2.1335). Biological values were tested using one-way (OW)-ANOVA followed by the Tukey-HSD *post-hoc* test at 99% confidence interval. Significance is expressed as *F(degrees of freedom, residuals) = F-value, p-value* as recommended by Field et al. (2012) unless stated otherwise. All graphs and visualisation have been made using ggplot2 (Wickham, 2016).

GPS location was downloaded from the GBIF database. Identifier of the dataset used to generate maps and analysis can be found at https://doi.org/10.15468/dl.tgk8j9. Packages used in R were: "dplyr" (Wickham et al., 2020), "ggspatial" (Dunnington, 2020), "ggrepel" (Slowikowski, 2020), "sf" (Pebesma, 2018), and "rnaturalearth" (South, 2017).

Principal Component Analysis (PCA) of the phenolic composition was performed on logarithmically transformed data using packages factoextra (Kassambara and Mundt, 2019) and FactoMineR (Lê et al., 2008). Diagnostics of loadings and contribution to the PCA analysis was performed with the help of the package corrplot (Wei and Simko, 2017).

The database on world agricultural values, obtained from FAOSTAT was subjected to exploratory factor analysis using the package psych (Revelle, 2020). Further confirmatory analysis was performed using lavaan (Rosseel, 2012) and lavaanPlot (Lishinski, 2018). Univariant PCA was performed using factoextra, to visualise overarching trends across countries under reduced dimensions. From the database, general regions such as "OECD," "South-east Asia," and similar classifications in the database were removed. Finally, all measures of land use were made intrinsic for a country by calculating its percentage coverage relative to its geopolitical land area. A naïve approach was adopted toward factor selection where percentage deviation was used as the metric of choice which showed high variation across individual countries. Factors with a percentage deviation value >100 were chosen for further exploratory factor analysis using maximum likelihood (ML) factor reduction to understand correlation to the latent factors. Finally, a System Equation Mode (SEM) was constructed based on the correlation using confirmatory analysis. Income group classification was performed according to data from World Bank (2020).

RESULTS

Nutritional Characterisation of Six Invasive Plant Species

Six invasive plants (Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia) were characterised for total protein content, amino acid profile, non-starch polysaccharide (NSP) composition, and phenolic composition to assess their potential for revalorisation.

Protein and Amino Acids

Across the plant samples, Bracken was found to have total protein content significantly greater than other plant samples as shown in **Table 1**. A one way (OW)-ANOVA test at a 99% confidence interval across plant samples for total protein content was found to be significant [$F_{(5,12)} = 725.3$; $p = 1.88 \times 10^4$], but similarities were revealed using Tukey-HSD *post-hoc* test between Gorse and Broom ($p = 0.10$), and Vetch and Fireweed ($p = 0.69$). Soluble protein represents the protein fraction extractable using mechanical grinding and buffer solubilisation which ANOVA deemed to have significant overall distinction [$F_{(5,12)} = 1{,}208$; $p = 8.9 \times 10^{-16}$]. A *post-hoc* test, however, could not distinguish between Gorse and Bracken at a 99% confidence interval ($p = 0.03$). Gorse had 47.3 ± 1.2 mg/g soluble protein (40.8% total protein), while Bracken had 45.2 ± 0.5 mg/g soluble protein (31.6% of total protein). The insoluble protein indicates proteins which remain bound to the cell wall and require further effort for extraction to efficiently use the biomass. ANOVA test found significant difference across the plants [$F_{(5,12)} = 1{,}356$; $p = 4.45 \times 10^{-16}$], although a *post-hoc* test could not distinguish between Buddleia and Fireweed ($p = 0.96$).

TABLE 1 | Total, soluble, and bound protein in Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia.

Plant	Total	Soluble	Insoluble
Gorse	115.9 ± 0.8^b	47.3 ± 1.2^c	63.3 ± 0.3
Vetch	106.7 ± 0.3^a	16.9 ± 0.8	99.1 ± 0.4
Broom	112.3 ± 1.5^b	40.3 ± 0.1	90.5 ± 0.8
Fireweed	105.2 ± 1.4^a	24.0 ± 1.0	78.9 ± 1.1^d
Bracken	142.8 ± 0.9	45.2 ± 0.5^c	107.9 ± 0.6
Buddleia	81.4 ± 0.5	7.1 ± 1.1	78.1 ± 1.4^d

Data is expressed as mean ± standard deviation in mg/g dry plant mass. Values with same superscript were statistically similar for a category across plant species using OW-ANOVA at 99% confidence interval with Tukey-HSD post-hoc test.

In **Table 2**, the amino acid composition of the plants is shown. The difference in amino acid profile was found to be statistically significant [$F_{(5,318)} = 4.645$; $p = 4.17 \times 10^{-4}$] across plants, but a *post-hoc* test found Buddleia as the influencing component with a difference which was significant at a 99% confidence interval from Broom ($p = 0.005$) and Bracken ($p = 2.1 \times 10^{-5}$) and at a 95% confidence interval from Fireweed ($p = 0.040$) and Gorse ($p = 0.010$). Statistical difference was insignificant between the other five plants. Similarities in individual essential amino acid content at 99% confidence interval is also shown in **Table 2**. Total essential amino acid content of the leaf mass was found to be similar between Gorse and Broom ($p = 0.99$).

The total essential amino acid content of Gorse, Vetch, Broom, Fireweed, Bracken and Buddleia are 33.05 ± 0.04, 41.43 ± 0.05, 33.05 ± 0.11, 32.63 ± 0.04, 48.71 ± 0.02, and 21.48 ± 0.05 mg/g dry plant mass. When this is presented for % of total protein, the essential amino acid content was the highest for Vetch and appeared to be unique across the plants [$F_{(5,12)} = 259.1$, $p = 8.67 \times 10^{-12}$] using OW-ANOVA. No statistical difference was found between Bracken and Buddleia ($p = 0.015$), and Gorse and Bracken ($p = 0.013$) at 99% confidence interval.

Non-protein Components

Additional plant components such as carbohydrate and non-nutrient plant bio-actives have potential use as food or feed and could provide health benefits or wider uses as biomaterials contributing to an effective cyclic economy. **Table 3**

TABLE 2 | Amino acid content of Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia.

	Gorse	Vetch	Broom	Fireweed	Bracken	Buddleia
His	1.54 ± 0.12	1.85 ± 0.04[a]	2.24 ± 0.01	1.84 ± 0.02[a]	2.64 ± 0.03	1.06 ± 0.11
Ile	2.64 ± 0.01	3.69 ± 0.02	1.92 ± 0.01	1.61 ± 0.00[b]	3.42 ± 0.00	1.72 ± 0.01[b]
Leu	7.84 ± 0.01[c]	10.18 ± 0.01[c]	8.03 ± 0.01[c]	8.48 ± 0.00	11.61 ± 0.01	5.39 ± 0.00
Lys	6.80 ± 0.01[d]	6.83 ± 0.02[d]	6.97 ± 0.01[d]	6.54 ± 0.00	9.22 ± 0.01	3.96 ± 0.01
Met	1.36 ± 0.03[e]	1.80 ± 0.02	1.14 ± 0.00[f,g]	1.25 ± 0.01[e,f]	2.21 ± 0.02	1.09 ± 0.02[g]
Phe	4.53 ± 0.02	6.10 ± 0.03	4.86 ± 0.01	5.27 ± 0.00	7.02 ± 0.02	3.07 ± 0.01
Thr	4.17 ± 0.02	5.34 ± 0.03	3.45 ± 0.01	3.28 ± 0.02	6.20 ± 0.02	2.66 ± 0.01
Trp	0.42 ± 0.03[h]	0.59 ± 0.15[h]	1.71 ± 0.33[i]	1.65 ± 0.11[i]	1.64 ± 0.02[i]	0.21 ± 0.10[h]
Val	3.75 ± 0.01	5.05 ± 0.00	2.73 ± 0.00[j]	2.71 ± 0.00[j]	4.75 ± 0.00	2.32 ± 0.01
Ala	7.69 ± 0.02	7.98 ± 0.01	8.53 ± 0.0	9.45 ± 0.01	11.19 ± 0.02	4.53 ± 0.01
Arg	6.13 ± 0.02	6.81 ± 0.02	5.84 ± 0.02	6.27 ± 0.00	8.02 ± 0.01	3.73 ± 0.07
Asp	20.24 ± 0.11	11.04 ± 0.01	21.75 ± 0.1	11.77 ± 0.05	20.21 ± 0.09	7.32 ± 0.3
Cys	0.45 ± 0.00	0.24 ± 0.00	0.46 ± 0.01	0.45 ± 0.01	0.54 ± 0.00	0.19 ± 0.00
Glu	11.54 ± 0.06	14.90 ± 0.04	12.47 ± 0.04	13.28 ± 0.04	18.68 ± 0.02	7.67 ± 0.12
Gly	8.13 ± 0.08	7.66 ± 0.06	9.96 ± 0.02	10.47 ± 0.01	12.36 ± 0.02	4.72 ± 0.02
Pro	7.57 ± 0.01	6.24 ± 0.01	5.90 ± 0.00	5.50 ± 0.01	8.42 ± 0.01	3.61 ± 0.01
Ser	8.16 ± 0.05	6.93 ± 0.10	7.82 ± 0.02	7.06 ± 0.07	10.51 ± 0.03	3.85 ± 0.03
Tyr	4.55 ± 0.01	5.30 ± 0.01	4.54 ± 0.01	4.48 ± 0.01	7.22 ± 0.01	2.85 ± 0.01
Total	107.51 ± 0.21	108.54 ± 0.21	110.32 ± 0.35	101.35 ± 0.15	145.86 ± 0.11	59.93 ± 0.37*

*Data is expressed as mean ± standard deviation in mg/g dry plant mass. Histidine to Valine represent essential dietary amino acids where values with same superscript were statistically similar using OW-ANOVA at 99% confidence interval with Tukey-HSD post-hoc test. The * mark represents the significantly lower total amino acid value obtained for Buddleia compared to other plants.*

TABLE 3 | Summary of proximate values for phenolic and carbohydrate content.

Plant	Carbohydrate		Phenolic	
	Soluble	NSP	Soluble	Lignin
Gorse	26.1 ± 0.7[c]	172.3 ± 9.0	25.5 ± 6.8[b]	83.7 ± 4.5[a]
Vetch	53.9 ± 2.0[d]	129.5 ± 3.2	29.4 ± 4.8[b]	11.7 ± 1.8
Broom	32.5 ± 3.2[c]	163.3 ± 6.5	96.8 ± 18.1	64.4 ± 1.3
Fireweed	54.2 ± 2.3[d]	82.6 ± 7.5	28.9 ± 3.0[b]	35.7 ± 1.2
Bracken	28.4 ± 3.9[c]	172.1 ± 6.3	26.9 ± 5.1[b]	78.8 ± 2.8[a]
Buddleia	50.0 ± 2.0[d]	112.3 ± 5.3	54.1 ± 5.7	48.1 ± 7.1

Results are presented in mg/g dry plant mass. Values with the same superscript are statistically similar using OW-ANOVA at 99% confidence interval with Tukey-HSD post-hoc test. NSP, non-starch polysaccharide.

summarises proximate values of non-protein components and their distribution in the aqueous soluble and non-soluble fractions measured across the six leaf samples.

Correlation analysis across all measurements given in **Tables 1** and **3** revealed a strong relation between soluble carbohydrate and lignin ($r^2 = -0.89$, $p = 5.52 \times 10^{-7}$), soluble protein and lignin ($r^2 = 0.78$, $p = 1.43 \times 10^{-4}$), lignin and NSP ($r^2 = 0.70$, $p = 1.15 \times 10^{-3}$), soluble carbohydrate and NSP ($r^2 = -0.87$, $p = 3.64 \times 10^{-6}$), soluble carbohydrate and soluble protein ($r^2 = -0.89$, $p = 5.57 \times 10^{-7}$), and of particular interest, soluble protein and NSP ($r^2 = 0.77$, $p = 1.58 \times 10^{-7}$). No significant associations were found with insoluble protein bound to the cell wall with any measured proximate components.

Two-way ANOVA test on a model expressing soluble protein content (**Table 1**) as a function of constituent NSP monomers (**Table 4**) showed significant association with Arabinose [$F_{(1,11)} = 437.74$, $p = 3.29 \times 10^{-10}$], Fucose [$F_{(1,22)} = 269.72$, $p = 4.37 \times 10^{-9}$], Rhamnose [$F_{(1,22)} = 20.60$ $p = 8.4 \times 10^{-3}$], and Galactose [$F_{(1,22)} = 10.66$ $p = 0.008$]. A multiple regression analysis revealed Xylose and Rhamnose content in the leaf mass could serve as predictors for soluble protein content which was significant at [$F_{(1,15)} = 402.16$, $p = 3.035 \times 10^{-12}$] and [$F_{(1,15)} = 34.69$, $p = 2.97 \times 10^{-5}$], respectively, with values expressed in mg/g dry plant mass in the equation:

$$Soluble\ Protein = 0.98 \cdot Xylose - 3.07 \cdot Rhamnose + 22.15$$

Goodness of fit of the linear model is shown in **Supplementary Figure 1**.

From the perspective of process development, such associations are particularly important as plants such as Gorse, Broom and Bracken could yield higher dividends with simple mechanical extraction, leaving behind substrates rich in NSP and Lignin. It further highlights the association between protein and cell wall and the possible treatment; particularly enzymatic, which may be employed to efficiently utilise the biomass. Furthermore, the comparable Lignin and NSP values of Gorse, Broom and Bracken to de-pithed bagasse (Hajiha and Sain, 2015) warrants further investigation into their potential use in paper-making or other higher value processing (Rainey and Covey, 2016).

TABLE 4 | Neutral sugar composition of non-starch polysaccharide in Gorse, Broom, Fireweed, and Buddleia. Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia.

Plant	Type	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
Gorse	Insoluble	4.7 ± 0.5[a]	1.4 ± 0.1[c]	23.0 ± 0.3	41.1 ± 0.8	4.6 ± 0.4[e,f]	11.7 ± 0.2[g]	85.8 ± 1.5
	Soluble	–	0.1 ± 0.0	0.2 ± 0.1	–	–	0.2 ± 0.1	0.1 ± 0.0
Vetch	Insoluble	4.6 ± 0.1[a]	1.1 ± 0.2	10.1 ± 0.1	10.0 ± 0.1[d]	16.0 ± 0.2	16.4 ± 0.2	71.3 ± 0.4[i]
	Soluble	–	0.1 ± 0.0	0.1 ± 0.0	–	–	0.1 ± 0.0	0.1 ± 0.0
Broom	Insoluble	4.0 ± 0.1[b]	1.0 ± 0.0	28.4 ± 0.5	34.9 ± 1.0	5.2 ± 0.1[e]	14.4 ± 0.4[h]	75.4 ± 2.2[i]
	Soluble	–	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	–	0.3 ± 0.0	0.1 ± 0.0
Fireweed	Insoluble	2.6 ± 0.2	1.4 ± 0.0[c]	11.2 ± 0.3	9.8 ± 0.2[d]	4.1 ± 0.1[f]	17.7 ± 0.4	35.8 ± 2.3
	Soluble	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
Bracken	Insoluble	4.2 ± 0.1[a,b]	1.5 ± 0.0[c]	18.3 ± 0.2	31.9 ± 0.4	6.3 ± 0.1	12.4 ± 0.0[g]	97.5 ± 2.9
	Soluble	–	0.1 ± 0.0	0.2 ± 0.0	–	–	0.2 ± 0.1	0.1 ± 0.0
Buddleia	Insoluble	7.3 ± 0.2	0.7 ± 0.1	13.0 ± 0.2	7.7 ± 0.2	8.7 ± 0.3	14.9 ± 0.3[h]	60.0 ± 3.0
	Soluble	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	–	–	0.3 ± 0.0	0.1 ± 0.0

Data is presented at mean ± standard deviation in mg/g dry plant mass. Values with the same superscript are statistically similar using OW-ANOVA at 99% confidence interval with Tukey-HSD post-hoc test. Values with the same superscript indicate similar statistical similarity across corresponding plant samples.

The free sugars measured in the aqueous extract reflect the moieties undergoing translocation in the leaf fated for catabolism or storage (Goldschmidt and Huber, 1992). The soluble phenolics were highest for Broom although, Gorse was found to have the highest overall phenolic content of 109.2 ± 8.1 mg/g dry plant mass with 76.6% of it bound to the plant cell wall components. Syringaresinol was the most abundant lignan across the plants and was particularly high in Gorse (47.4 ± 8.1 mg/Kg), Broom (43.3 ± 6.4 mg/Kg), and Bracken (28.7 ± 2.7 mg/Kg) (**Supplementary Table 1**). Gorse was rich in chlorogenic acid (1.5 ± 0.1 g/Kg) and in general showed a higher content of all phenolic groups.

Among the 112 phenolic compounds analysed (**Supplementary Table 1**), 25 compounds accounted for 90% of total variance observed across all plants and largely influenced their characterisation. Correlation and contribution of each component plant and phenolic compound, respectively, to the composite PCA axes is given in **Supplementary Tables 2, 3**. The plot (**Figure 1**) revealed that flavonoids as well as cinnamic- and phenylpyruvic acid derivatives largely influenced the profile diversity. Relative position of plants as function of their phenolic profiles is provided in the **Supplementary Figure 2** which shows a loose recapitulation of their phylogenetic grouping. Gorse, Broom, and Vetch, which belong to the Fabaceae family are expected to plot closer while distancing themselves from Bracken, Fireweed, and Buddleia. However, Gorse and Broom appeared to have a rather unique profile while Vetch plots closer to Bracken. Fireweed and Buddleia plot separately, reflecting their distinctive phenolic profiles.

Pearson's test on cinnamic derivatives against phenylalanine (**Table 2**) reveals a strong positive correlation to cinnamic acid and 3,4,5-trimethoxycinnamic acid content across the six investigated plants ($R^2 = 0.92$ and 0.94, respectively). Larger samples sizes may be required to corroborate this observation, although established biochemical pathways give some support to this observation (Hyun et al., 2011; Vargas-Tah and Gosset, 2015).

Global Distribution of Investigated Invasive Plants

Based on the hits for GPS points classified under a given invasive plant family, their global and Scottish abundance are shown in **Figure 2**. In the global list of family abundance, among 635 families identified, Fabaceae featured second while Onagraceae ranked 39, Dennstaedtiaceae ranked 76, and Buddlejaceae ranked 245. The Scottish family abundance identified the presence of 131 families with Fabaceae ranking fourth, Onagraceae ranked 19 Dennstaedtiaceae ranked 52, and Buddlejaceae ranked 62.

The expected resolution for such broad, satellite-based vegetation survey is between 120–250 m. The details are provided in the GPS uncertainty margins available in the GIBF database and an account of uncertainties and challenges in remote measurement is provided by Lawrence et al. (2006); Niphadkar and Nagendra (2016); Baron et al. (2018). At a shrub density between 45 and 70 Kg/m^2 (Passioura, 1991), the mean biomass held in invasive plants is roughly 52.2 Tg, which translates to 26.1 Tg of carbon and 5.2 Tg of protein. Globally, annual protein requirement is 215 Tg. This could potentially supply about 2.5% of global annual protein requirements, assuming 1.2 g/Kg body weight of daily protein intake as recommended by the National Institutes of Health (NIH) (Phillips et al., 2016). In Scotland, the biomass held in invasive plants is roughly 448.27 Gg, which translates to 224.14 Gg of carbon and 45 Gg of protein. Scottish annual protein requirements are approximately 118.4 Gg, indicating that invasive plants could supply about 25% of annual protein requirements. Interestingly, in terms of minimum essential amino acids recommended by the World Health Organisation (WHO) (Joint WHO/FAO/UNU Expert Consultation, 2007), the estimated protein present in the invasive plant biomass could potentially satisfy about 3.5% of global annual requirements. Comparison of essential amino acids produced from existing agricultural products to the estimated values in the invasive plant mass is given in **Figure 3** using global and Scottish datapoints.

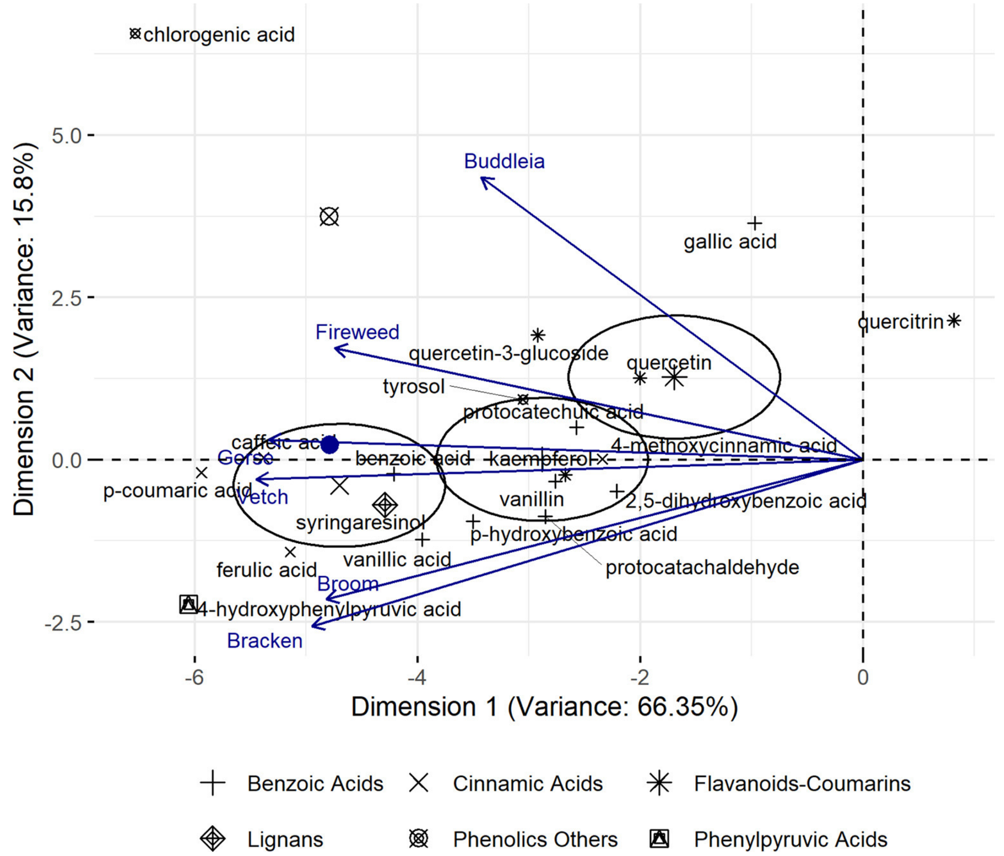

FIGURE 1 | Principal Component Analysis (PCA, univariant-scaled) profile of Gorse, Vetch, Broom, Fireweed, Bracken, and Buddleia across six phenolic groups. Ellipses represent 95% confidence intervals beyond which loadings significantly driving plant characterisation are expected to plot. Relative similarity of plants represented as resultant vectors of contributing phenolic profile is indicated by their distance from the mean which is indicated as a filled blue circle along Dimension 1.

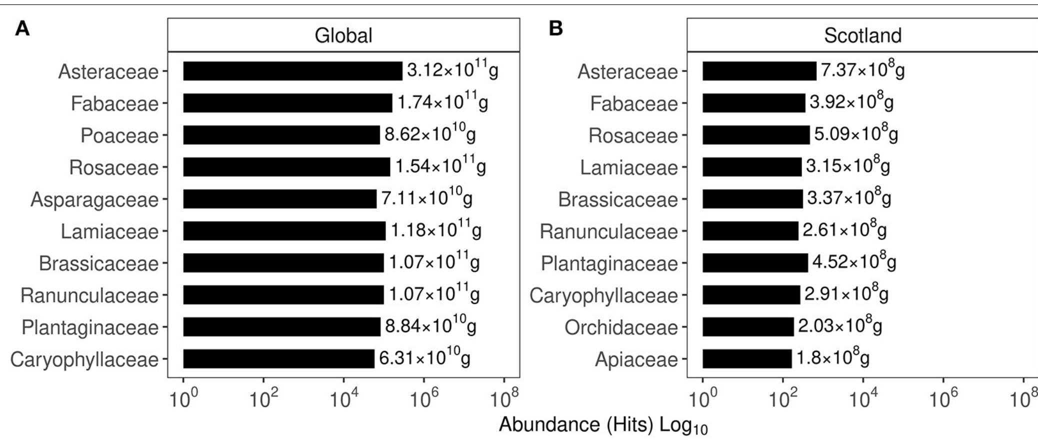

FIGURE 2 | Abundance of the top 10 plant families based on their occurrence in the GIBF database. **(A)** Family abundance globally. **(B)** Family abundance in Scotland. Values express the protein content estimated in the biomass of these plants.

It was observed that the essential amino acid content of all invasive plants could satisfy partial global and Scottish dietary requirements from locally sourced invasive plants. Estimates of the individual essential amino acid content of commercial agricultural produce as well as the invasive plant biomass is provided in **Supplementary Figure 4**. The essential amino acid content in families to which the six plants investigated is shown in **Figure 4** and the GPS locations were plotted on a map to understand their spread and identify areas most susceptible to their invasion in **Figures 5, 6**.

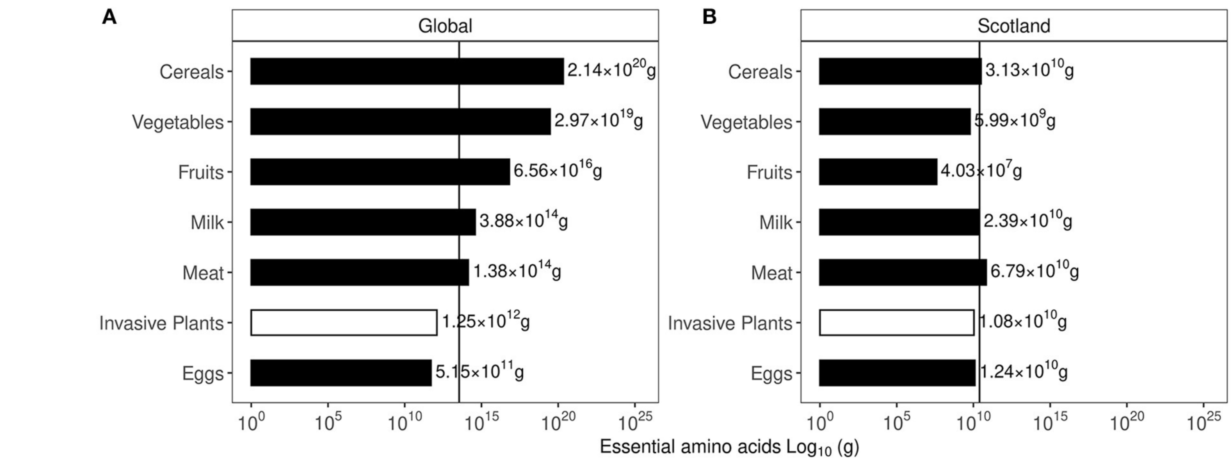

FIGURE 3 | Estimated content of essential amino acid present in invasive plant biomass. **(A)** Values for global essential amino acid content of agricultural produce and invasive plant mass. **(B)** Values for Scottish essential amino acid content of agricultural produce and invasive plant mass.

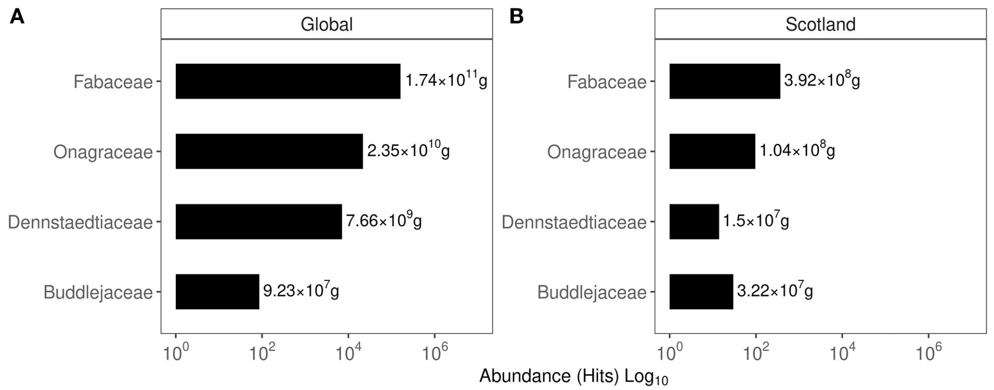

FIGURE 4 | The relative abundance of the families of the plants investigated in this work based on their occurrence in the GIBF database. **(A, B)** Values represent the protein estimated in the invasive biomass.

Implications for Land Allocation

Allocation of fertile land for food production and forestry has a major impact on global carbon storage (Smith et al., 2008). Of the available fertile land used for economic agriculture and forestry, 25.1% is used exclusively for animal husbandry, predominantly as meadows and pastureland (**Figure 7**). It is the single largest allocation in agricultural land use surpassing the combined value of all other actively developed land such as irrigated, planted, and organic and permanent crop land.

Despite the landmass available for plant growth shown in **Figure 7**, emissions associated to high-energy agriculture, potentially offset any anabolic carbon storage. Animal husbandry contributes to 22% of total agricultural emissions through direct enteric fermentation via ruminants and a further 15% through manure management. This involves soil conditioning of crop and pastureland (**Figure 8**) or disposal, making it the single largest contributor of agricultural emission. Burning of organic matter, either as a means of crop/plant waste disposal or fresh land acquisition through "slash-and-burn" cultivation (Uhl, 1987; Kleinman et al., 1995; Brady, 1996) accounts for 17% of agricultural emissions.

Effect of increased resource allocation to animal husbandry and its use as the dominant source of dietary protein can be observed in the carbon footprint to produce yield. **Figure 9** illustrates the carbon-cost of common agricultural raw produce. Cumulative effect of enteric emissions and manure handling on the carbon footprint can be observed for ruminant meat such as cattle, goats, and buffalo, which is about 40 times higher than plant based or non-ruminant chicken produce.

The initial database from FAOSTAT contained factors that were converted to intrinsic percentages where applicable. For example agricultural land area was converted to an intrinsic value relative to the individual country size. Each subset of agriculture-associated emissions was converted to a percentage of total agricultural emissions. The initial set of factors were: Bovine Meat (g/day), Livestock (LSU/ha), Pesticide (Kg/ha), Fertiliser (Kg/ha), per capita Emission (t), Agri Employment (% labour

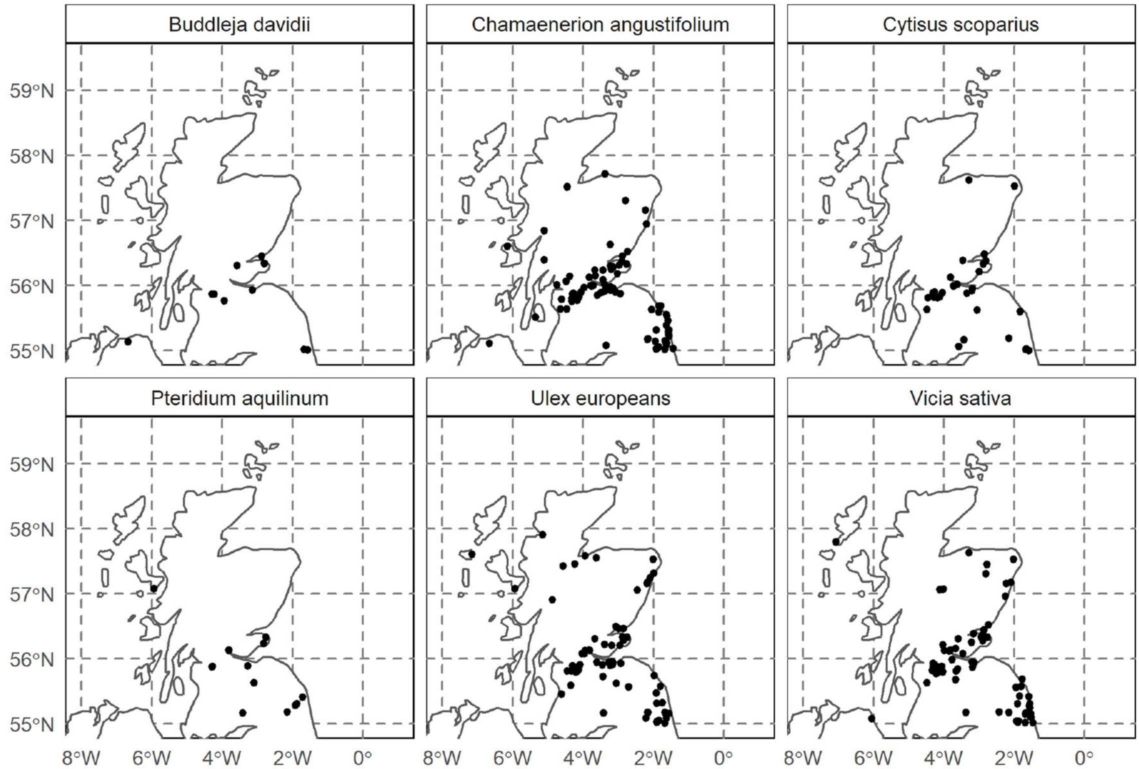

FIGURE 5 | Invasive plant distribution in Scotland, identified using GPS-Satellite imagery.

force), Enteric Fermentation (% agricultural emission), Crop Residue Burning (% emission), Agricultural Soils (% emission), Soil Manure (% emission), Pasture Manure (% emission), Manure Management (% emission), Synthetic Fertilisers (% emission), Animal Origin Emission (% emission), Grassland (% land area), Agricultural land (% land area), Arable Land (% land area), Cropland (% land area), Forest land (% land area), Permanent Pasture (% land area), Permanent Cropland (% land area), Shrubland (% land area), and Urbanisation (% land area).

Among the available factors, only those with a percentage deviation >100 were chosen as shown in **Supplementary Figure 5**. Factor analysis is optimal across independent factors with normally distributed data. Within a strict maximum cut-off value of $|R^2| > 0.4$ and at a 99% confidence interval, correlation analysis found Livestock to have strong and significant associations with Urbanisation and Pesticide use ($R^2 = 0.79$ and 0.45, p: 7.54×10^{-15} and 1.88×10^{-4}, respectively). Since Livestock could be expressed as an equation of Urbanisation and Pesticide, it was filtered out. Lastly, the values for plant protein as a percentage of total protein produced (Plant.Production) failed the Royston test for normality and was filtered out. The final dataset contained six factors, namely, Permanent Cropland (Perm.Cropland), Shrubland, Urbanisation, Labour force in agriculture (Agri.employment), Pesticide use, and Permanent Pasture.

An exploratory factor analysis (EFA) using maximum likelihood (ML) suggested an optimal number of two latent factors (shown in **Supplementary Figure 3**) with corresponding correlations shown in **Supplementary Table 4**. The RMSR (root mean square of residuals) was 0.06. The RMSEA (root mean square of approximation) index was found to be 0.055 with a 90% confidence interval ranging from 0 to 0.205 and a BIC at −11.85. The Tucker-Lewis Index (TLI) was 0.88.

Based on the suggestions provided by EFA, a confirmatory analysis (CA) of a system equation model (SEM) was performed where the first latent factor (ML1) was expressed as an additive, non-interactive model comprised of Permanent Cropland, Permanent Pasture, and Shrubland, while the second latent factor was expressed as an additive, non-interactive model comprised of Employment, Pesticide use, and Urbanisation. The resultant model is depicted in **Figure 10** below. The RMSEA of the model was 0.064 (10% confidence range: 0.00–0.167) and a comparative fit index (CFI) of 0.925. The standardised root mean square residual was 0.061.

As a complementary assessment, the PCA analysis of the selected high-variant intrinsic agricultural factors is shown in **Figure 11** below. The plot accounts for 56.44% of total observed variance and it is interesting to note that countries, particularly the high and upper-middle income countries plot distinctly compared to the low and lowering middle income groups. This suggests that the emergent factor which can be elucidated over

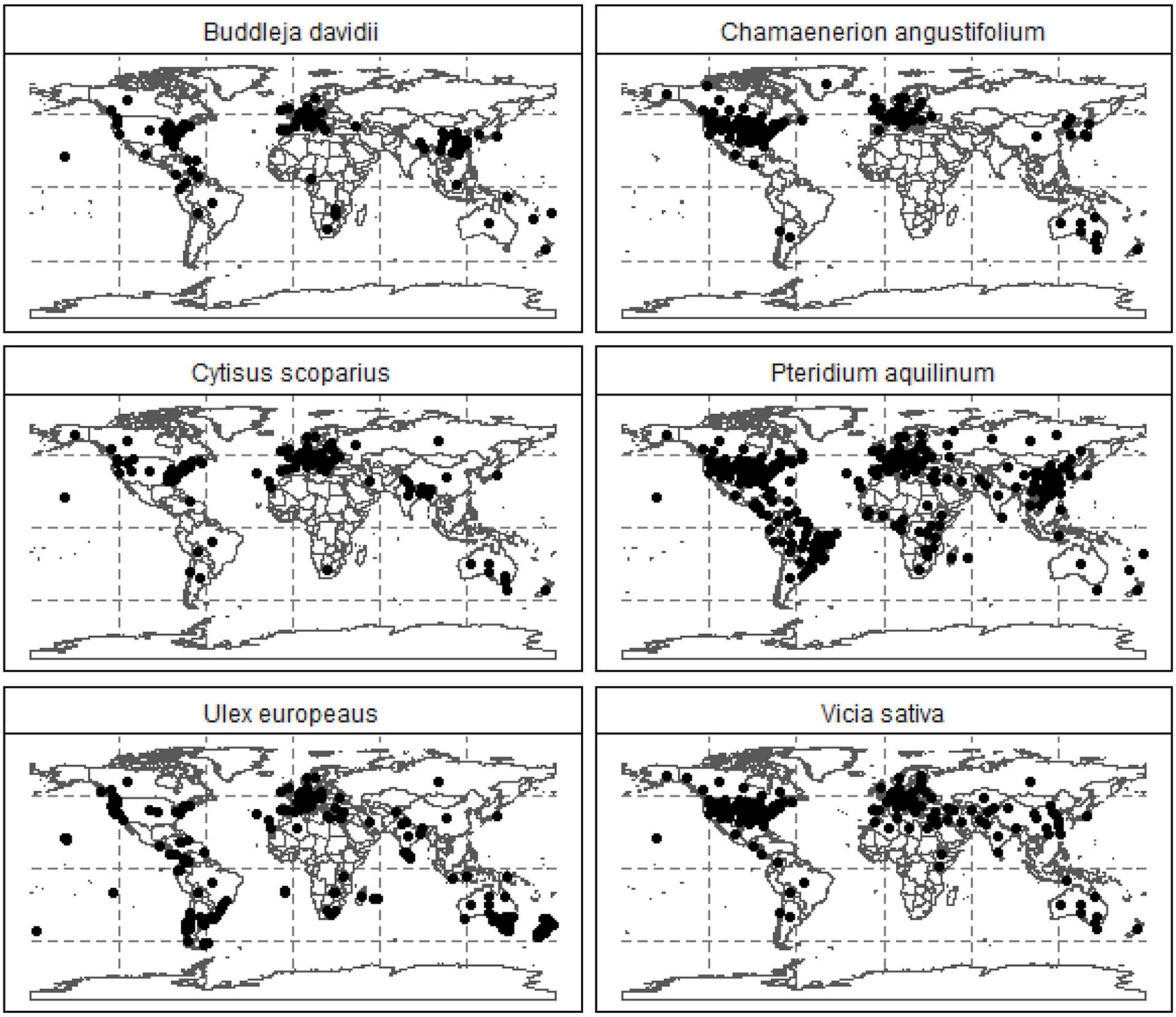

FIGURE 6 | Global distribution of the six invasive plants investigated here.

the factor analysis is the overall economic status of a country although no direct economic parameter such as GDP or per-capita income was fed into the factor analysis steps. Correlation and contribution of the factors and variables, respectively, to the composite axes is given in **Supplementary Tables 5, 6**.

DISCUSSION

The strong association between land use and CO_2 emissions was previously demonstrated by Smith et al. (2013) who suggested the reallocation of agricultural resources toward more climate-friendly plant-based cultivation. A detailed account of the carbon flow through the agricultural system was reported by Molotoks et al. (2018), which provides strong quantitative evidence of the contribution of fertiliser use and forest land reclamation toward emissions associated to food production. Complementary to their work, global agricultural data from FAOSTAT was used to identify trends that arise when high variable factors are assessed. The principle underpinning the factor analysis described was that the dynamics of a multicomponent system was a function of its most variable factors. In the principal component analysis (PCA) plot shown in **Figure 11**, vectors (high variant factors) drove the relative positioning of individual countries; revealing characteristic clustering (the emergent factor), which in this case reflected economic status. Diagonally opposing vectors function antithetically while those of similar orient amplify effect. A more defined relation between the factors is provided by the structural equation model (SEM) depicted in **Figure 10** which, in conjunction with PCA revealed a strong association between the economy and land use and by extension, the emissions associated to agriculture. Shrubland was used a proxy for the marginal lands where native and introduced invasive plants are found. The opposing influence between shrubland and permanent cropland in the PCA plot reveals their contrasting effects in the characterisation of countries and their role in economic output as well as local ecology.

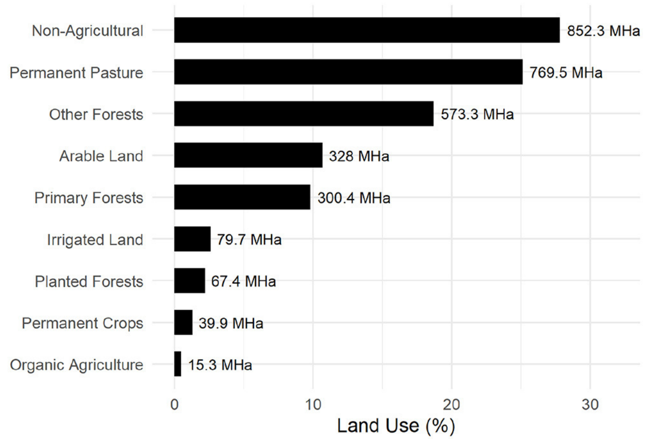

FIGURE 7 | Global land use for Agricultural and Forestry (FAO, 2019).

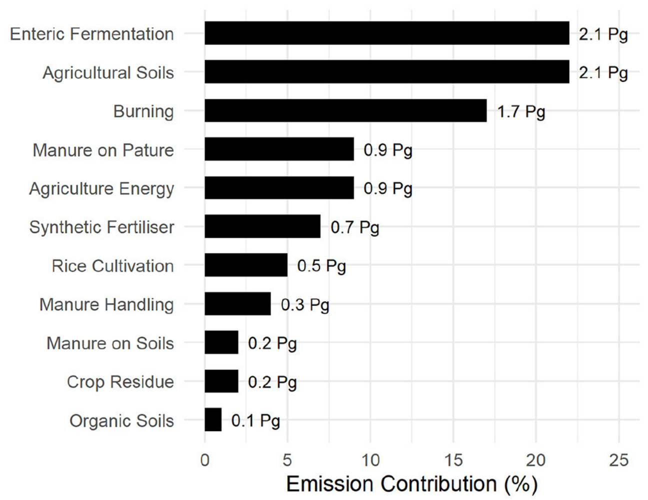

FIGURE 8 | Global emissions from agriculture and associated land use compiled from FAOSTAT (FAO, 2019). Emission values are expressed in Petagram (Pg).

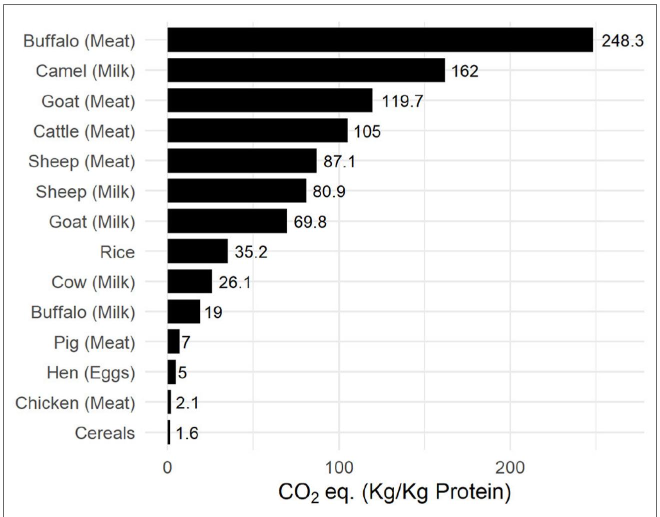

FIGURE 9 | Carbon-impact of common agricultural produce. Data was compiled from FAOSTAT (FAO, 2019).

Maps showing the confirmed locations of invasive plants chosen for this study were identified using GPS-satellite imagery as shown in **Figures 5** and **6**. Among the families of the invasive plants chosen for this study, Fabaceae was highly represented in Scottish and global regions followed by Onagraceae, Dennstaedtiaceae, and Buddlejaceae. These plant families are in abundance in the North-East of Scotland. While satellite-imagery has improved over the decades and is invaluable in identifying endangered plant species and contributing toward conservation efforts, the work toward understanding the spread of invasive species remains relative nascent. The GPS locations from where the plant samples were obtained (see Materials and Methods) were not registered in the year 2020 database despite the plants having a sizeable coverage within those locations. Such errors decrease overall plant count and may underreport the severity of the problem faced on account of their spread. Molotoks et al. (2018) estimated agricultural carbon losses faced by invasive plant encroachment to 1 Pg, while according to estimates here based on satellite surveys, the total biomass present across all invasive plants is about 52 Tg. It is uncertain to what extent the biomass has been underestimated. It may be that recurring loss of agricultural biomass is caused by regular encroachment of invasive plants from the presently identified locations, which act as their natural reservoirs. Improved data collection is required to ameliorate uncertainties and help give a better estimate of the invasive biomass. Despite the possible underestimation, it was observed that the essential amino acid content of these plants may be sufficient to supplement Scottish and to some extent, global requirements.

Among the invasive plants investigated, Bracken was found to have the highest protein content (**Table 1**) and Vetch to contain the highest essential amino acid content in its constituent proteins (**Table 2**). However, in terms of protein accessibility easily achieved from mechanical grinding, Bracken and Gorse were found to have the highest soluble protein content (**Table 1**). However, the presence of potent carcinogens, namely, ptaquiloside (Niwa et al., 1983; O'Driscoll et al., 2016) found in Bracken makes it an unsuitable candidate until purification methods capable of removing these anti-nutrients are developed. In Vetch, only 15% of total protein was accessible, which was less than half that of Gorse, Broom, or Bracken. This may require further enzymatic or chemical extraction methods to fully realise the nutritional potential, but processes such as this may result in loss of essential amino acids depending on the nature of treatment. Traditional alkali-based extraction followed by neutralisation can result in severe loss of lysine and tryptophan (Jung et al., 2006) and generate significant effluent volumes, while the enzymatic procedures would substantially increase process cost (Sari et al., 2015).

Physical features differentiating leaves such as their hardness, affect the design used to effectively extract proteins, which is primarily governed by the plant cell-wall composition. It is interesting to note that Gorse, Broom, and Bracken are often

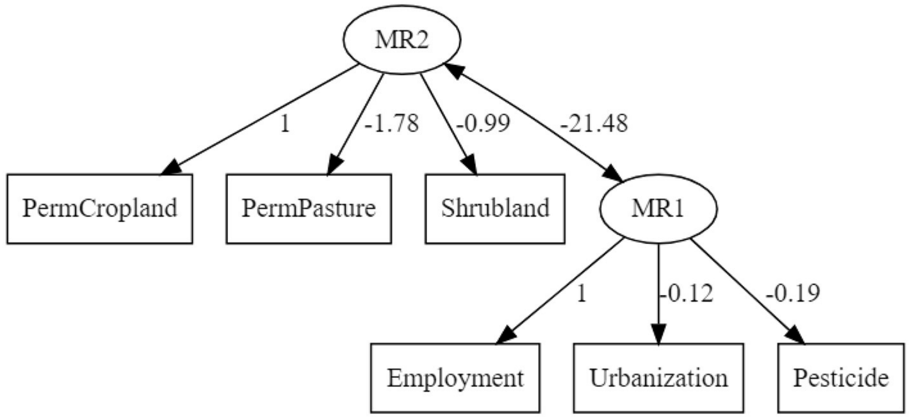

FIGURE 10 | SEM-model constructed using intrinsic agricultural factors. Pesticide, pesticide used in tonnes; Urbanisation, percentage land area converted to artificial surfaces; Employment, percentage labour force in agriculture; PermCropland, percentage land area used as cropland; PermPasture, percentage land used as permanent pasture; and Shrubland, percentage marginal land.

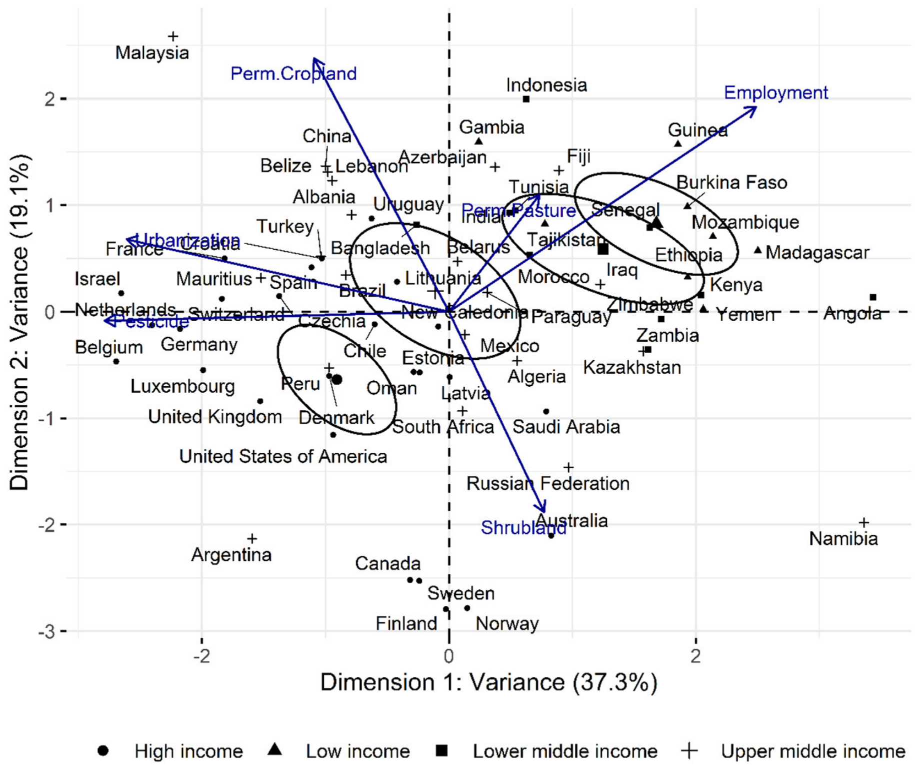

FIGURE 11 | Principal component analysis (univariant-PCA) of countries according to permanent pasture (% of total land area), Shrubland/marginal land (% of total land area), LSU/ha, Plant protein production (plant protein produced as a percentage fraction of gross national protein produced), pesticide use (t), Urbanised land (% of total land area), permanent cropland (% of total land area), and Agriculture employment in agriculture (% labour force).

subject to herbivory (Tempel, 1981; Broadfield and McHenry, 2019) and values of high lignin and NSP (**Tables 3**, **4**) may reflect investment toward defensive mechanisms by toughening leaf mass or employing anti-nutrients; as in the case of Bracken (Niwa et al., 1983; O'Driscoll et al., 2016). The particularly woody nature of Gorse may be attributed to the high xylose content capable of producing branched, interlocking structures in conjunction with the lignin (Grantham et al., 2017; Gericke et al., 2018; Wierzbicki et al., 2019). When leaf samples are dried, this manifests as a brittle leaf structure making it amenable to crushing and juicing for protein extraction. Bracken and Broom leaf samples on the other hand show similar, but lower xylose content compared to Gorse, which in conjunction with a relatively lower lignin content and may have resulted in a more flexible leaf structures (Arola et al., 2013; Busse-Wicher et al., 2014).

Gorse has been prolific in more than 16 countries (Broadfield and McHenry, 2019); particularly in Oceania where it was introduced as an ornamental plant as well as to build natural barriers around private properties (Hoffmann and Broadhurst, 2016; **Figure 6**). Downstream process development for protein extraction using leaves from Gorse appears to be a lucrative solution of deriving nutrition as it had the highest protein recovery using economical methods such as mechanical grinding and buffer extraction. Although leaf proteins have previously faced disregard owing to low essential amino acids such as lysine, tryptophan and methionine, which were lost during traditional alkali extractions (Cuq et al., 1983; Zhang et al., 2017), improvements in throughput and reliability of ultrafiltration technology has since rendered the concern moot, as amino acid preservation is greater given the absence of any chemical treatment. All six plants studied plants exhibit a valuable essential amino acid profile (**Table 1**) suggesting that the final nutrient yield is a function of protein extractability.

The non-protein residue post-aqueous extraction appears to be rich in polysaccharides and lignin which could potentially be revalorised for use in other industries examples being; enzyme assisted ethanol production (Cheng et al., 2017) or use in paper industry. Left over marc has previously been demonstrated as an alternate substrate for edible mushrooms (Mintesnot et al., 2014; Kaszynski, 2016). Overall, the harvest index of such plants is high, as organs such as leaves and parenchyma-rich portions constitute about 48–55% of the plant mass (corresponding to Harvest Index of 0.48–0.55) compared to cereals with indices of about 0.3–0.45 (Singh and Stoskopf, 1971).

Gorse is also rich in soluble phenolics, particularly chlorogenic acid and major bioactive compounds such as phenylpyruvic-, benzoic-, and cinnamic acids derivatives. These compounds are commonly associated with health benefits owing to their anti-inflammatory and free radical-quenching properties (Ozcan et al., 2014; Shahidi and Ambigaipalan, 2015) and their role in maintaining gut health (Ozdal et al., 2016). The nutraceutical market is expected to grow to a value of around $10 billion by the year 2026 (Childs, 1999; Bröring and Cloutier, 2008; Research Markets, 2019) and with growing consumer demands and research investment in the plant-based "well-ness" industry, identification of viable and sustainable sources of bioactives is essential.

Invasive plants tend to have robust physiologies and faster germination times, which increase turnover and reduce income latency. Plants such as Gorse and Bracken grow rapidly during periods of spring, allowing harvest in early summer (Conway, 1957; Bowman et al., 2008). Harvesting and exploiting the nutritional value of such plants may help alleviate some of the burden on the existing agricultural systems to produce protein. Current terrestrial carbon held in vegetation amounts to 302.4 Pg (FAO, 2019) which is almost completely offset by animal husbandry industry. Since agricultural emission is a direct result of land use (Smith et al., 2008; Molotoks et al., 2018; Porter et al., 2019), land improvement and maintenance of pasture lands adds to the carbon cost of animal products as seen in **Figure 8**. Although classification of grazing land into rough grazing and maintained meadows is not available for all countries of the world, a significant portion is expected to be repurposed marginal land incapable of high-intensity farming (Asner et al., 2004; Peco et al., 2006). These sites tend to retain a considerable part of plant biodiversity and the native plants found in these sites come under enormous survival pressures from climate change, herbivory, and invasive plants and in many cases have been pushed to extinction (Duncan et al., 2004; Truscott et al., 2008; Lankau, 2012).

Given the average rise in global temperatures was recorded to be 1.96°C (2018–2019) (FAO, 2019; Roe et al., 2019), vulnerable economies, which are primarily agrarian are likely to face greater impact of adverse climatic conditions which could pivot them further into distress. A combination of novel, sustainable nutrition sources introduced through minimum disruption is essential to ensure continued livelihood and nutrition supply. The effect of conscious food choice on global carbon emission has been explored by Bajželj (2014) and Bajželj et al. (2014) and the comparison of scenarios across different food and production choices is quantitatively summarised by the EAT-Lancet Commission (Willett et al., 2019). This study strongly indicates the importance of reduction in the consumption of animal sources, while simultaneously moving away from intensive cultivation practises and instead focusing on food nutrition quality, rather than production yields. Leaf protein extraction technology could help harness the potential in revalorising the biomass of invasive plants, which is currently disposed of inefficiently and help toward production of alternate and more sustainable protein sources to complement existing food production.

CONCLUSIONS

Agriculture in our current economic system carries the burden of being a critical occupation for human survival, while having to maintain economic viability and now, faces the brunt of being a major contributor of anthropogenic GHG emissions. Based on the relationship between land resource allocation, emission and fiscal patterns observed in global agricultural data, the case is presented for revalorising invasive plants to ameliorate food insecurities. Marginal lands tend to serve as reservoirs for native plant species, which have now come under threat from excessive herbivory from unattended grazing, climate change and added competition from invasive plant species. Using databases cataloguing the location and spread of invasive plants through satellite imagery, Fabaceae species were found to be the second most abundant family globally, with the three most invasive species in Scotland belonging to this family. Among the six invasive plants investigated, Gorse (*Ulex europaeus*) was identified as a good candidate for implementation owing to its wide geographical spread, high protein extractability and potential of the co-products for further revalorisation efforts.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AI performed the experiments, data collection, and manuscript preparation. SD, CB, and WR supervised the work, helped with data interpretation, and preparation of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.575056/full#supplementary-material

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REVIEW
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Legumes as a Cornerstone of the Transition Toward More Sustainable Agri-Food Systems and Diets in Europe

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Legume grains are important sources of nutrients in human and animal diets and have been so for millennia. Their history as part of traditional diets dates to the origins of agriculture when their benefits for soil health and agricultural productivity started to be realized, mostly empirically, by farmers. In time, legumes have lost their popularity as human food, either because of a negative connotation as "poor man's meat," occasional gastrointestinal side effects, or habitually longer preparation times when compared to other types of plant foods. Also, the steep rise in the consumption of meat derived foods in the last half of a century has taken a toll on replacing legumes as a major protein source. Alongside this negative trend in consumption, a negative drift in production was also observed, especially in Europe, where legumes currently occupy a minimal fraction of agricultural land. One of the main factors is a loss in competitive edge amongst farmers due to sustained lower public and private investments in breeding programs and legume adapted technology for planting, managing, harvesting, processing, and storing, when compared to cereals. Recently, increased awareness of the need to move to sustainable food systems is revitalizing legume production and consumption in Europe, leading to a compilation of policies and initiatives that aim to put legumes again as foundations for this transition. Legumes have been reinvented in a multitude of products (drinks, cereal bars, bread, meat replacers, snacks, flours, and several others) and included in farming systems of conservation agriculture, organic production, intercropping, and crop rotation, combining ancient traditions of legume production "with a spin," incorporating new legume technological knowledge in farming systems. However, the transition has been slow and hampered by many cultural, societal, political, and economic impediments. This paper summarizes initiatives that aim to enable the comeback of legumes and their placement in a more prominent position in human diets and agricultural fields and highlights strategies that aim at overcoming the obstacles that impede achieving the development of more sustainable agri-food systems and sustainable diets in Europe.

Keywords: climate change, food systems, legumes, sustainable development, sustainable diet

INTRODUCTION

In the context of the UN *2030 Sustainable Development Goals*, there is growing recognition of the need for profound transformations in the way we produce, process, and eat our food. This means creating agri-food systems which deliver "*food security and nutrition for all in such a way that the economic, social, and environmental bases to generate food security and nutrition for future generations are not compromised*" (FAO, 2018). Such changes in the realm of production should be accompanied by a transition to more sustainable food consumption patterns as well (Berners-Lee et al., 2018). A global dietary shift toward more plant-based diets has been identified as a critical necessity in the fight against malnutrition and sustainability-related issues (Willett et al., 2019). In the past few years, the search for alternatives to animal-based food has brought legumes into the spotlight as one of the best options given their multiple positive social, economic, and environmental assets (Stagnari et al., 2017), features that have long been empirically appreciated (Phillips, 1993). Legumes are plants belonging to the *Leguminosae* family, comprising about 800 genera and 20.000 species (Stagnari et al., 2017). Given their protein-rich profile, this paper will focus on grain legumes that are used for human food purposes within European countries—such as beans, peas, chickpeas, lentils, lupin, soybean, and exclude forage legumes used just for animal feed production, such as alfalfa and clover.

Literature reports legumes as one of the earliest domesticated plants (Ahmed and Hasan 2014), believed to have marked the transition from a hunting-gathering way of life to agricultural practices (Phillips, 1993). In fact, legume cultivation was widespread where agriculture was practiced, also evidenced by archaeological signs of the simultaneous existence of legumes and cereals (Phillips, 1993). It appears lentils were already present within cropping systems of ancient Egyptian civilizations and carbonized seeds dating back 7000 to 8,000 years B. C. have been found in Turkey (Ahmed and Hasan, 2014). Peas and dwarf field beans seem to have been cultivated in Switzerland between 4000 and 5000 B.C. (Ahmed and Hasan, 2014). It is believed that the cultivation of soybean in China began between 2000 and 3000 B. C. (Ahmed and Hasan, 2014). Archaeological sites revealed signs of domestication of bean crops as early as 10,000 years ago in Mexico and Peru (Gomes and Vasconcelos, 2014). Over 3,000 years ago, beans, soybean, and staple crops started being domesticated in America and Asia (Ahmed and Hasan, 2014). The use of legumes in pastures and for soil improvement purposes was already acknowledged by the Romans in 37 B.C., reflecting the intuitive use of legumes' nitrogen-fixing abilities (Gomes and Vasconcelos, 2014). Nonetheless, recognition of the value of such practices seems to have faded over the course of history, not least in Europe too.

Around the 1960s, the main grain legume production in Europe (chickpea, cowpea, groundnut, lentil, and common bean) destined for human consumption, occupied 67% of total production area, dropping to 27% by 2013 (Watson et al., 2017). Such decline appeared mostly driven by rising competition from cheaper imports, especially from Canada and the substitution of legumes intake by meat products within Mediterranean countries, the highest legume consumer populations in Europe (European Commission, 2018b). In the 1980s, pea and soybean became the two most widely grown protein crops for animal feed (Watson et al., 2017). Currently, soybean, field peas, and broad beans are the dominant grain legumes across Europe (Watson et al., 2017). Soybean alone has had a remarkable increase over the last decades, particularly driven by the great demand for high-protein materials for livestock feed in Europe (Watson et al., 2017). In 2018/2019 943,000 ha was under soy cultivation in Europe (European Commission, 2018a) and a further 44% increase (to about 1.3 million ha) is anticipated until 2030, expressing the highest growth of all European crops (European Commission, 2018a). In relation to field peas and broad beans, their combined production reached 4.4 million tons in 2018 (European Commission, 2018a). Around two-thirds of production is directed to animal feed purposes whereas just about 20% is for human consumption (European Commission, 2018a). Lupins, lentils, and chickpeas are not so abundant in the European Union (EU): most lentil and chickpea production is destined for human consumption, but lupin is mainly directed to livestock feeding (European Commission, 2018a). In 2018, grain legumes occupied only 1.4% of the total crop area in Europe (European Commission, 2018a), that is around 10% of their average role in cropping systems worldwide (Watson et al., 2017). Moreover, only 43% of the food legumes consumed in Europe are produced on European farmland (Watson et al., 2017). Europe's domestic production expresses a deficit of about 70% of high-protein materials, 87% of which rely on imported soybean and soymeal (Watson et al., 2017). Indeed, evidence suggests a continuing decline in legume production in Europe (Stagnari et al., 2017), probably explained by a relative economic un-competitiveness compared to more profitable crops, such as cereals, which account for 31% of the total utilized agriculture area in Europe (European Commission, 2018a).

The decline in grain legume production over the last decades in Europe contrasts with increases in other regions such as Australia and Canada (Stagnari et al., 2017). Concordant policy action areas are needed to offer guidance to further develop pathways for legume-based food- and feed-systems and to trigger change, at least for the European context. To this end, stakeholders, and experts from a series of regional legume-oriented value chain workshops were invited to contribute to an online Delphi exercise. Based on this, policy recommendations were identified for the alleviation of barriers, and the development of favorable policies and transition pathways, which are capable of promoting the production of legumes, and creation of legume-based products in the EU. These included: (1) investment in agri-food and -feed research and knowledge transfer; (2) preventing the use of inorganic nitrogen fertilizer; (3) nutrition, diet, and health policies and public campaigns that promote the inclusion of legumes in the human diet (Balázs, et al., 2019). This narrative review includes scientific papers and technical reports and summarizes initiatives that aim to enable the comeback of legumes and their placement in a more prominent position in human diets and agricultural fields and highlights strategies that aim at overcoming the obstacles that impede achieving the development of more sustainable agri-food systems and sustainable diets in Europe.

LEGUMES AND SUSTAINABILITY

Food Security, Health, and Nutrition

Food Security

Food security is recognized as a universal human right with a central role in human development. However, promoting food security is a complex mission with political, economic, environmental, social, and cultural dimensions. The number of people with insufficient food worldwide is estimated at more than 820 million and many more consume an unhealthy diet that contributes to premature death and morbidity (Willett et al., 2019). In Europe in 2017, almost 12% of the population expressed an inability to afford a good-quality meal every second day (European Environment Agency, 2019b). Also, at the end of 2019 around 0.5 million people in Europe, were classified as suffering from acute food insecurity (FSIN, 2020). Hence, it is urgent to optimize food production in a sustainable way, so as to contribute to reductions in hunger, to improve life expectancy, to reduce infant and child mortality rates, and to decrease global poverty (Willett et al., 2019). Legumes, being more affordable high protein nutrient-dense foods, could contribute significantly to the eradication of hunger and malnutrition (Bessada et al., 2019).

Health and Nutrition

According to *The Lancet,* a healthy diet has appropriate caloric intake and is composed by a diversity of plant-based foods, low amounts of animal source foods, unsaturated rather than saturated fats, and small amounts of refined grains, highly processed foods, and added sugars (Willett et al., 2019). However, modern diets are characterized by a high intake in calories and heavily processed and animal source foods. In fact, in the last 50 years, the intake of animal proteins among European adults, essentially meat and dairy products, has doubled and currently remains twice the global average (64 kg/year) (European Environment Agency, 2019b). Also, the consumption of sugar and sugar products per person per year (13 kg/year) seems higher than other foods, such as fish and seafood (10 kg/year) (European Environment Agency, 2019b). For sustainability and health reasons, the transformation to healthy diets by 2050 will require important dietary changes, namely a >50% reduction in global consumption of unhealthy foods, such as red meat, and sugar, and a >100% increase in consumption of healthy foods, such as nuts, fruits, vegetables, and legumes. However, the changes needed differ greatly by region (Willett et al., 2019). During the last decade, legumes have re-emerged as an interesting and balanced source of nutrients. They are nutrient-dense foods, namely of protein, fiber, and diverse minerals, like iron, zinc, and potassium (Grela and Samoli, 2017) and vitamins, such as thiamine, niacin, folate, riboflavin, pyridoxine, vitamin E, and A (Mudryj et al., 2014). Moreover, legumes provide important dietary bioactive compounds to the diet (e.g., phenolic acids, tannins, and flavonoids) known for their antioxidant potential, amongst other health-protective effects (Singh et al., 2017). Evidence suggests that legume consumption is associated with positive outcomes on cardiovascular risk factors, such as, blood lipid profile, glycaemic control, inflammatory status, oxidative stress, as well as gut microbiota composition, and activity. They also favor the control of body weight, probably because they give greater satiety (Ferreira et al., 2020).

Natural Resources and Climate Change

Land and Water Resources

Expected future higher demand for food will require not only larger areas of crop cultivation and yield increases but most worryingly, under business-as-usual projections, greater livestock production. Indeed, recent predictions suggest that global meat intake will increase by about 76% by mid-century (Godfray et al., 2018). This means that over time, if consumption patterns do not change, pressure will build upon earth's limited resources, as livestock production requires significant land areas and freshwater supplies.

Presently, both grazing land, and animal feed crops account for 80% of all agricultural land (Giovannucci et al., 2012). Also, about 29% of the water footprint of the global agricultural sector is related to the production of animal products (Mekonnen and Hoekstra, 2012). In Europe, livestock production systems represented 28% of land use in 2016 (European Environment Agency, 2019a). Also, feed and animal production require around 25% of total water extraction within the agriculture sector in the EU (European Commission, 2019a). Hence, humans and livestock will ultimately have to compete for nature's resources, as well as the same sources of food. In this context, protein-rich plant crops, such as grain legumes, could help reduce the need for animal-based protein food sources with huge environmental advantages (Stagnari et al., 2017). Lesser animal-based foods intake, and therefore lower livestock production, would allow feed crops to be converted into human food, and thereby not compromise long term food security (Giovannucci et al., 2012). This would result in better natural resources management as well, since plant-based protein agroecosystems require far less resources and energy inputs (Clark et al., 2019).

GHG's Emissions

The nutrient richness of animal-based foods, especially meat, and their significant protein content has served to justify their presence within most diets (Wood, 2017). However, there is growing evidence that meat-rich dietary patterns are closely linked to serious environmental constraints, and most significantly to global warming aggravation (Willett et al., 2019). Livestock production produces important amounts of the three main greenhouse gases, namely, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (Godfray et al., 2018). Meat production alone is the single most significant source of CH_4, which is a gas with a global warming potential equivalent to 28 times CO_2. Ruminant livestock particularly, generate approximately 80 million tons of CH_4 per year. This represents around one-third of all anthropogenic emissions of CH_4 and almost 80% of agriculture emissions (Peoples et al., 2019). In Europe, the agriculture sector accounts for almost 10% of all GHGs emissions in the EU. According to the literature, a dietary shift toward more plant-based protein food sources, like grain legumes, is a must to help mitigate global warming and therefore lighten climate changes (Willett et al., 2019). In fact, it is estimated that the production of plant-based foods can produce 25–150 times less GHGs emissions than ruminant's meat production (Clark et al., 2019). Also, substituting meat for grain legumes could actually lead to a reduction up to 74% in GHGs emissions enabling the achievement of the 2020 target for the US (Harwatt et al., 2017). Such dietary shifts among Europeans would lead as well to a 6% (22 million t CO_2 eq) reduction of the carbon footprint of the EU agricultural sector by 2030, compared to the baseline (European Commission, 2019a).

Artificial Fertilizers

Nitrogen (N) is a basic element for the formation of plants' biologic structures (Leghari et al., 2016). Hence, sufficient N supply is crucial for plant growth and development, ultimately defining the yield quality, including the nutritional composition of plant-based food products used for both animal and human feed (Peoples et al., 2019). Plants can acquire N either by root assimilation (Kiba and Krapp, 2016) or, in the case of legumes, by atmospheric fixation (Sulieman and Tran, 2015). However, the pressure to increase production has led farmers to resort to increasing use of synthetic N, in the form of fossil fuels-derived chemical fertilizers, to support crop productivity and guarantee profitable harvests. However, the vast use of N-rich artificial fertilizers over time has raised important environmental concerns particularly due to the multiple loss processes that labile reactive forms of N may suffer. Volatile losses as ammonia (NH_3) or nitrous oxide (N_2O), as well as leaching of both nitrate (NO_3^-) and organic N threatens the quality of air, soil, and water resources, affecting global ecosystems (Sutton et al., 2011). It was estimated that synthetic N fertilizers directly account for approximately 12% of the annual average 5180 million tons of CO_2 equivalent GHG emissions associated with agriculture activities between the year of 2010 and 2014 (Peoples et al., 2019) and it has been estimated that nitrogen pollution can cost the EU up to €485 billion per year (Sutton et al., 2017). The ability of legumes to biologically fix atmospheric N_2 in a symbiotic association with soil bacteria rhizobia creates a continuous N supply within agroecosystems without the use of additional artificial fertilizers (Clúa et al., 2018) and the presence of legumes within mixed croplands could ultimately stimulate soil fertility and enhance yields, all with less environmental impact. Grain legumes also release high-quality organic matter into the soil and facilitate nutrients' circulation by promoting water retention (Stagnari et al., 2017). The potential yield gains can even occur without compromising the nutritional composition of the harvest, namely its protein content (Plaza-Bonilla et al., 2017). The adoption of crop rotation systems including legumes is expected to increase not only overall crop's profitability but also reduce total production costs (Preissel et al., 2015; Mahmood et al., 2018). On the one hand, it has been demonstrated in Kenya that legume-cereal rotations have gross margins equal to or higher than cereal rotations alone (Rao and Mathuva, 2000). Indeed, the inclusion of peas in five-year rotations with 80% cereals within French territories was able to lift the gross margin by 11.0%, that is 29€/ha (Von Richthofen G. L. Pro Partners, 2006). The total production costs could be minimized as well, by 50€/ha, if legume crops were to be incorporated within continuous cereals rotations (Von Richthofen G. L. Pro Partners, 2006). Notwithstanding, the maximum economic benefits from legumes will only be achieved after long-periods of crop rotations, when tangible monetary profits start to become apparent (Mahmood et al., 2018).

Biodiversity

Legumes have also an important role in protecting natures' biodiversity. Over the years, the intensification of agriculture production has led to the wide dissemination of the most profitable crops at the expense of landscapes' diversity, as well as, natural habitats of different species (Everwand et al., 2017). Excessive N inputs from massive use of artificial fertilizers may be one major contributor, causing soil acidification and direct toxicity, among other negative consequences (European Commission, 2018a). Globally, ecosystems are losing the ability to provide basic needs and natural resources such as crop pollination, clean air, and water, and control of floods or soil erosion (European Commission, 2011). In this way the world's biodiversity is becoming greatly jeopardized, including across the EU (European Commission, 2011). Literature suggests that the presence of legumes within current intensive cropping and cereal-dominated agri-food systems promotes the conservation of habitat heterogeneity and ensures the continuity of multiannual habitats for species considered critical for nature conservation, such as arthropods, bird populations, and small mammals (Peoples et al., 2019). Also, legume crops offer vital floral resources that guarantee the survival of populations of pollinators which in turn benefit food production and plant breeding (Marzinzig et al., 2018). Ultimately, the beneficial effects of legumes in increasing biodiversity should be more widely promoted and used as an incentive to promote their production.

MAIN OBSTACLES TO INCREASE LEGUME PRODUCTION AND CONSUMPTION

Breeding Programs

The world has witnessed an astonishing period of food crop output growth over the past 60 years, especially for cereals, even in the face of increasing land shortage and increasing land prices. In this time period populations more than doubled, and the production of cereal crops tripled, with only a 30% increase in land area cultivated (Pingali, 2012). Cereals have been the focus of plant breeding programs prior to and since the Green Revolution, including the development of N-responsive varieties that deepened dependence on chemical fertilizers, all of which has overshadowed the contribution of legumes and their traditional synergy with cereals. The yearly productivity gains from 1960 to 2000 for cereal germplasm improvement alone averaged 1.0% for wheat, 0.8% for rice, 0.7% for maize, and 0.5, and 0.6% for sorghum and millets, respectively (Evenson and Gollin, 2003). On the contrary, legumes breeding has lacked investment and research has been identifying possible ways to turn them into more economically attractive crops (Watson et al., 2017). Indeed, important advances in yield and yield stability could be performed considering unique traits of each crop, in order to favor species with higher resistance to biotic and abiotic stresses (Watson et al., 2017). Pea crops suffer from poor standing ability, poor ground coverage, low competitive ability against weeds and general low productivity on many soil types (Watson et al., 2017). Faba bean is adapted to heavy or clay-rich soils but expresses high sensitivity to water deficit on sandy soils (Watson et al., 2017). Only the white lupin expresses good ground coverage whereas the yellow lupin is the most drought tolerant and suitable for the sandiest soils (Watson et al., 2017). Frost tolerance is limited in soybean and appears unknown in common bean (Watson et al., 2017). Specific tolerance to insect pests and diseases are also important priorities to consider in future breeding programs (Watson et al., 2017).

Farming Policies and Technologic Barriers

Alongside these breeding effort inequalities, policy and structural barriers restricted their supply responsiveness. Policies that promoted staple crop production, such as fertilizer and credit subsidies, price supports, and irrigation infrastructure (particularly for rice), tended to exclude the production of traditional non-staple crops, such as legumes (Welch and Graham, 2000). In fact, the evolution of agricultural practices has been based on the adoption of most widely used and highest profitable techniques (Magrini et al., 2018) and adopting crops. This has led to a technologic lock-in, favoring crop specialization and marginalizing less cost-responsive species (Watson et al., 2017). As a consequence, competition within the agri-food sector caused producers to resort to more lucrative crops, namely cereals, at the expense of crops with lower and more variable yields, such as grain legumes (Magrini et al., 2016). In this context, the promotion and use of agrochemicals became the dominant model with evident disregard for potential environmental hazards (Therond et al., 2017). The lack of technical advice about the use of nitrogen-fixing plants like grain legumes, which reduce the need for synthetic inputs, has been a significant part of these practices (Watson et al., 2017). Thus, research has shown that legumes' low profitability for farmers may be attributed to three important reasons, that is, (i) lack of appreciation for legumes' benefits because margins and yields calculations do not consider the scale of crop rotations; (ii) lack of interest from the agro-industrial supply chain that jeopardizes legumes' added value, and (iii) low profits cause insufficient compensation for the associated reduction in artificial fertilizers use (Magrini et al., 2016). Thus, at the present time cereals dominate agricultural food and feed production around the world and Europe alone is the top producer of wheat (Magrini et al., 2018). Growing consumption of cereal-based food products has also contributed to create such scenario within food sectors (Magrini et al., 2018). According to the EU *Agricultural Outlook 2019-2030*, the EU market for cereals is expected to continue growing, reaching about 320 million tons by 2030 (European Commission, 2019a).

Challenges exist when a farmer or business is to define the optimal route to market for a legume crop. This will have to start by identifying the value network structure, collaborative partners and grasp a deep understanding of the market drivers and barriers which are defined by the specific contexts for operation (Hamann et al., 2019). Once a link with the market has been established, the business will be looking for options to both maintain and expand the business and this requires links to the upstream and downstream "value network." A successful legume commercialization strategy should ideally allow for "scaling up" (or "scaling out"), which means that the strategy must also consider the value network capacities (Hamann et al., 2019), and in the case of legumes, a lack of economy of scale is often pointed out as one of the major barriers for a wider legume adoption. The low levels of legume production do not allow for cost advantages that are often available when growing other more productive crops. Hence, increasing production, would address the economy of scale obstacle and promoting demand and consumption of (locally grown) legumes could work as direct drivers to stimulate farmers to produce more legumes.

Consumers

It has been acknowledged that the adoption of healthier food habits may be impaired by consumers' low health/nutrition literacy (Magrini et al., 2018). This situation is aggravated by the fact that there seems to exist great variability among food intake recommendations in general, and particularly regarding grain legumes (Marinangeli et al., 2017) which can be extended to cooking skills. In fact, lack of know-how as well as, time-related constraints (e.g., long soaking or cooking time) have been pointed out as significant barriers to regular consumption of legumes (Havemeier et al., 2017). Also, misconceptions surrounding potential gastrointestinal discomfort following legumes' intake, may have led to the over exclusion of these foods by many consumers (Hall et al., 2017). Last, but not least, the choice for animal products' alternatives such as legumes may be compromised by a reduced environmental awareness which remains very common across society (Hartmann and Siegrist, 2017). Indeed, it appears consumers underestimate the environmental impact of meat consumption/production and demonstrate rather low willingness to change meat intake habits (Hartmann and Siegrist, 2017).

Food Industry

Nevertheless, globally the food industry has been increasingly orienting its activity in order to reflect contemporary dietary trends (e.g., flexitarian, vegetarian, "gluten-free") and to increase the incorporation of legumes and legume-based ingredients, thereby creating healthier and more sustainable food products (Lascialfari et al., 2019). Even during the 2000s many soybean and wheat protein-based food products were developed to meet such trends (Lascialfari et al., 2019). In 2013, these kind of products still represented 90% of all plant-based foodstuffs innovations (European Commission, 2018b). However, since 2010 the development of new products containing pulses, such as chickpea, pea, bean or lentil has boosted (European Commission, 2018b). Indeed, the demand for lentils and chickpea for human consumption in Europe has increased 24 and 20%, respectively, since 2014 (European Commission, 2018a).

A review performed by The Canadian Ministry of Agriculture and Agri-Food revealed that between 2010 and 2014, more than 3.500 new pulse-based food products were launched in the EU food market (European Commission, 2018b). The vast majority of the products represent highly processed foods based on legume ingredients, containing, chickpea (35%), pea (34%), bean (25%) and lentil (14%) (European Commission, 2018b). Such products have been mostly promoted based on nutrition-related claims, namely the nutrient-dense high-protein quality of legumes (European Commission, 2018b). In fact, animal protein substitutes appear one of the key market drivers which express an annual growth rate of 14% (European Commission, 2018c). Convenience and environmentally friendly aspects seem important assets as well (European Commission, 2018b). Today a wide range of legume-based products can be found on the European markets, including flours, pastas, and all kinds of plant-based snacks (European Commission, 2018b). Still, there is room for critical technological improvements in order to produce fully satisfying products and broaden their public reach, especially where factors like taste, texture, anti-nutrient management and convenience are concerned (Sozer et al., 2016). The downside is that Europe's grain legume production is not sufficient to meet such increasing demand (European Commission, 2018a) with just 69% self-sufficiency in tradable plant protein (Watson et al., 2017) and, consequently, supplies rely heavily on imports from other countries such as the USA, Latin America and Canada (European Commission, 2018a).

DISCUSSION

It is widely recognized that legume crop production has lacked public investment over the years (Magrini et al., 2018). Food markets express a preference for crops like cereals, hence the worth of legumes has been neglected for quite some time (Magrini et al., 2016). However, the mitigation of the environmental consequences caused by the agri-food sector has been a hot topic on the agenda of diverse political entities in the past few years, especially within the EC (European Commission, 2018a). The need to invest in alternative plant protein food sources has been widely acknowledged and strongly advocated. Indeed, the EC in a recent report recommends that more investment should be applied to the development of plant proteins among European countries, reaching as far as consumer behavior (European Commission, 2018c). Taking the example of Portugal, in 2017 Portugal's government approved a new law demanding the inclusion of a vegetarian plate in every public canteen (Assembleia da República, 2017). This has created momentum for the inclusion of legumes across the wider community and has the potential to have a positive impact for the increase in legume consumption. Also, few countries have opted to have legume grains in a separate category of their national food guides (as is the case of Portugal), which may showcase legumes in a more positive and higher profile role. On a more general level, however, it is still apparent that legume-focused policies are confusing and scattered when looking at different local, regional, national, and international scales.

The reintroduction of legumes within present-day agriculture and food practices has been extensively discussed especially during the last 8 years. The designation of 2016 as the *International Year of Pulses* by FAO (in 2013) and the creation of a World Pulses Day every 10th February (since 2019) has paved the way for several other international campaigns for the promotion of more sustainable food production systems where grain legumes appear as key contributors, especially as potential dietary protein sources (Calles et al., 2019). Since then, several joint initiatives from both political parties and food companies have been put into practice (Global Pulse Confederation Pulses, n.d.). From Canada (Pulse Canada, n.d.) to Australia (Pulse Australia, n.d.), a global movement has spread to raise awareness about the need to increase production, as well as, consumption of legumes instead of relying on animal products for dietary protein sources. In Portugal, for instance, the Portuguese Nutrition Association has created a unique campaign to promote the intake of one portion (80 g cooked; 25 g raw) of grain legumes per day (Portuguese Nutrition Association, n.d.). Global intake recommendations are not consensual though and grain legumes are still underrepresented in most official food guides. While there is a growing number of legumes promotion initiatives, information regarding the actual impact of such campaigns, including within the Portuguese population, is missing making it difficult to plan future interventions (Calles et al., 2019).

Yet given the complexity of the food sector, major transformations of current procedures in order to increase the role of legumes throughout food supply chains will not occur overnight. Moreover, such changes will require both upstream and downstream approaches, involving all kinds of stakeholders (Magrini et al., 2018). Some examples are summarized in **Table 1**. Ultimately, collaborations between public research and small companies should be stimulated to help disseminate new understanding regarding both legume production and processing (Lascialfari et al., 2019). Also, companies should nurture close relationships with their agricultural growers or cooperatives, assuring locally produced legume supplies so that the desired higher legume consumption can rely on European legume cultivation rather than on higher levels of imports (Lascialfari et al., 2019). Research institutions could also be strong allies in this grand transition, particularly stimulating research and providing technologic support (Magrini et al., 2016). In this context, new tools and cropping systems designs could be used, such as, field on-farm experiments where farmers test new practices on their farms and cropping system planning tools (Watson et al., 2017).

In terms of food policies, the EC has set up a wider and stronger plan to be put into action from 2020: The *European Green Deal* aims to transform the EU *"into a fair and prosperous society, with a modern, resource-efficient and competitive economy where there are no net emissions of greenhouse gases in 2050 and where economic growth is decoupled from resource use"* (European Commission, 2019b). The climate-neutrality target was transposed into law in March 2020, through the *European Climate Law*. It has proposed a reduction of about 50%-55% of EU's GHG's emissions by 2030, compared with 1990 levels. Such

TABLE 1 | Summary of main actions to promote legumes within sustainable agri-food systems.

Governments/Institutions	Agriculture production	Food industry	Consumers
- Foster information dissemination - Promote educational programs for farmers - Increase incentives for legume crops and legume specific extension services - Improve legume promotion campaigns	- Increase locally grown legumes as feeds and reduce soybean importation dependency - Increased adoption of intercropping, crop mixtures and crop rotations with legumes - Increase farmers know-how on legume production - Increase the share of organic farming including legumes - Adopt biological-regulated production models - Breeding and selection of locally adapted legume varieties - Adapt logistics—harvesting/storage firms and market organizations	- Advance research on legume health claims - Improve legume technologic traits (e.g., reduce cooking time) - Design new foods with legumes, especially targeting young audiences (e.g., children) - Improve legumes promotion campaigns	- Dietary shift toward more plant-based diets - Increase legume intake - Improve nutritional education - Improve cooking skills - Improve environmental information

a plan will embrace all kinds of stakeholders, including regions, local communities, civil society, industry and schools. In relation to the food sector itself, a *Farm to Fork* strategy has been created within the *European Green Deal* objectives, "*designing a fair, healthy and environmentally-friendly food system,*" able to support sustainable food production and consumption chains. Again, the selection of alternative protein food sources is reinforced, in light of EC's previous reports (European Commission, 2018c).

CONCLUSIONS

The increase in legume production, and therefore intake, turn out as a major contribution to help mitigate current health and environmental-related global crises. Dried legumes are excellent sources of protein/amino acids, fatty acids, fibers, carbohydrates, and phytochemicals, also possessing a low glycaemic index. Legumes are also ecosystem service providers and environmental "guardians", as they reduce the need for synthetic N fertilization, promote soil conservation, and create more diversified and biodiverse agricultural systems. The acknowledgment of these facts by public authorities and decision-makers may be one important step at overcoming the obstacles that impede the return of legumes to their rightful place within agri-food systems in Europe.

Still, changes in the European food sector will demand high efforts from all stakeholders from both up and downstream the food supply chain. Close collaborations between governments, academic institutions, industries, and farmers are needed in order to facilitate the transition process. Currently, legume production demands more public financial and academic/technologic support, as well as changes in consumers dietary habits. Thus, more research considering legumes cultivation methods and techniques (e.g., genetic trait selection) is highly advisable, together with improvements in farmer's knowledge, especially as far as crop rotation and fertilizers use are concerned. Also, more legumes promotion campaigns are needed and their impact on consumer's behavior needs to be carefully assessed. Recent legal documents published by the EC may have open the way to a more favorable scenario regarding the reintroduction of legumes within European agri-food systems, yet there is still much to be done. In this context, one major future challenge arises regarding the best strategies to successfully accomplish the desirable transformations, particularly considering knowledge dissemination from farmers down to consumer level.

AUTHOR CONTRIBUTIONS

HF is the first author of the manuscript and was responsible for the main scientific search as well as the final elaboration of the present review. EP and MV were equally senior contributors to this manuscript providing expert advice on areas directly related to their research fields, namely, nutrition and sustainable food production, respectively. Also, both helped put together the final manuscript suggesting meaningful and thorough corrections and improvements. All authors contributed to the article and approved the submitted version.

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Recognizing the importance of protein quality in an era of food systems transformation

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A transformation of current food systems is needed to nourish the growing global population in more sustainable ways. To support this, some are advocating for a shift to plant-based or -exclusive diets. These recommendations – typically borne out of concerns for the environment – often fail to account for unintended nutritional consequences, which could be particularly pronounced for protein intake. While there is enough protein to meet current global needs, the issue of protein quality is often overlooked and oversimplified. High-quality protein, including from animal source foods (ASF), is needed to meet nutritional demands in low- and middle-income countries (LMIC), particularly among vulnerable population groups. In high-income countries (HIC), protein quality is important for at-risk populations who have higher protein requirements and lower energy and/or protein intakes. Further, as the global population increases, driven primarily by population growth in LMIC, it is possible that protein production will need to increase in HIC to support exports to help feed the global population. The global dialogue and resulting dietary recommendations must therefore become more nuanced to consider the interaction between nutritional value and environmental impact to help better reflect trade-offs across multiple domains of sustainability. Nutritional life cycle assessments are one way to help accomplish this nuance and evaluate how all types of food production systems should be refocused to improve their environmental efficiency and nutritional impact.

KEYWORDS
protein, protein quality, food systems, nutrition, sustainability, animal source foods

Introduction

Food systems affect population health, natural resource use, and socioeconomic issues and are in turn vulnerable to environmental changes. This vulnerability – coupled with growing demands to feed a global population of nearly 10 billion by 2050 – has accelerated recommendations to achieve "food systems transformation" (Fanzo et al., 2020). These recommendations take many forms, including national guidance through food-based dietary guidelines (FBDG) or reports from non-governmental organizations.

A prominent theme is a shift to plant-based or -exclusive diets, with emphasis on plant-based proteins, largely for environmental reasons. Cell cultured proteins, insects, and mycoproteins are also being considered as animal protein alternatives, though uncertainties remain regarding their viability on a mass scale. While some recommendations acknowledge the valuable role of both ASF and plant-based foods (PBF) as part of a healthy diet, others argue for PBF to replace ASF (Herforth et al., 2019; Comerford et al., 2021). Among one-third of national FBDG with "protein food" messages, PBF are explicitly presented as substitutes for ASF or are implied to be alternatives through inclusion in the same general message as ASF (Herforth et al., 2019). This perspective over-indexes on the environment and overlooks other domains of sustainable food systems, including nutrition, health, and sociocultural factors (Drewnowski, 2017). This paper focuses on what overlooking other domains of sustainability, particularly nutrition and health, could mean for protein quality.

Protein quality

Protein contains essential amino acids (EAA) that are needed for physiological functions across all life stages (Institute of Medicine, 2003). Foods and dietary patterns differ in protein and amino acid (AA) content and thus in their protein quality (Millward et al., 2008). Protein quality is defined as the ability of a dietary protein to meet the body's metabolic demand for AA and nitrogen. It is based on AA composition, digestibility of the dietary protein, and bioavailability of the AA from that dietary protein (Boye et al., 2012; FAO, 2013). Protein quality is therefore critical when assessing nutrient adequacy of the food supply and dietary intake at the individual- and population-level (Cifelli et al., 2016).

There is significant variation in protein quality across ASF and PBF, which is important to consider when recommending shifts in dietary patterns (Gwin et al., 2021). ASF like dairy, eggs, and meat are highly digestible (>90%); depending on the processing method and/or presence of antinutrients, PBF like maize, oat, bean, and pea typically have lower digestibility (45–80%) (van Vliet et al., 2015). There may also be differences in how the protein is metabolized and utilized by the body. For example, AA from soy and wheat are more readily converted to urea than those from milk, which results in a lower potential of these PBF to stimulate muscle protein synthesis (van Vliet et al., 2015). Dietary patterns that include a diverse mixture of ASF and PBF (including common staple foods and neglected or underutilized crops) often have high protein quality as their AA profiles complement one another. It is possible to consume complete protein through a combination of different types of PBF with complementary AA compositions; however, doing so is challenging among population groups that have higher protein requirements and/or are not accustomed to consuming a diverse variety of PBF.

Protein intake and quality across global contexts

Low- and middle-income countries

Ensuring adequate supply and consumption of high-quality protein is a global issue. Complex interactions between food availability, prices, and market structure in LMIC influence access to and consumption of foods (Turner et al., 2020). Influenced by such factors, total energy and protein intakes are typically low among populations in LMIC, where protein intake is primarily driven by protein derived from PBF (Allen, 2012). An analysis of protein intake among adults across 103 countries in Sub-Saharan Africa and Asia found that after adjusting for protein quality, average daily protein intake was below the requirement in all countries (Moughan, 2021). Additionally, demonstrating the importance of protein quality in LMIC, these data can serve as a model of what might happen in HIC if recommendations to substitute ASF with PBF do not consider how such dietary shifts can impact protein quality, particularly among at-risk populations. Such recommendations should specify the types and quantities of foods that can be swapped without compromising nutrient intakes.

Intake of high-quality protein is critical for children and adolescents with high nutrient needs to support periods of rapid growth. Improving dietary quality during early life has been a challenge in LMIC, where children's diets primarily consist of PBF that lack the required energy and nutrient density (Dewey, 2013). Because ASF are dense in complete protein, essential fatty acids, and multiple bioavailable micronutrients, the inclusion of even small amounts in the diet can be beneficial for the undernourished (Neumann et al., 2001; Allen, 2012).

High-income countries

Intake of protein derived from ASF is higher in HIC compared to LMIC. In the United States (U.S.), average per capita protein intake is ∼1.2 g/kg/d, with ∼65% of the protein consumed coming from ASF (Pasiakos et al., 2015). However, it is important to consider how average protein intake and requirements differ by population sub-groups in HIC. Among older adults, experts have recommended the importance of higher protein intakes with considerations for protein quality due to the "anabolic resistance" of aging and risk of sarcopenia (Bauer et al., 2013; Deutz et al., 2014). Optimal intake of dietary protein may alleviate declines in muscle function, muscle wasting, and frailty, and proteins derived from ASF can support muscle protein synthesis because they contain relatively high amounts of EAA that are more digestible and bioavailable (Sahni et al., 2015; Tessari et al., 2016).

Although consuming more total protein from PBF or a combination of complementary plant-based proteins may result in a similar amount of digestible and bioavailable EAA compared to ASF, doing so would require additional calorie consumption that may not be advisable in certain populations. A modeling exercise matching total protein from a vegan dietary pattern to recommended protein intakes illustrated that higher total energy intake would be needed to meet EAA requirements in older women compared to a dietary pattern incorporating ASF due to the lower EAA density (EAA/100 kcal) of most PBF (Fussell et al., 2021). The study did not consider digestibility or bioavailability, which may have further impacted the observed differences.

Further, few studies have assessed the impact of consuming plant-based or -exclusive diets on skeletal muscle mass and strength among older adults. This highlights the need for further research as the loss of muscle mass and strength that occurs with aging is a public health problem (Fussell et al., 2021; Domi et al., 2022). Some evidence has shown the benefits of including sources of high-quality protein in older adults' diets. An intervention providing dairy foods resulted in improved intakes of protein and calcium and a reduced risk of falls and fractures among older women (Iuliano et al., 2021). A systematic review concluded that higher-quality protein was beneficial for muscle protein synthesis at rest and following resistance exercise in older and young adults, and that it was associated with greater gains in strength when combined with resistance exercise training (Morgan et al., 2021). Most studies included in the review used isolated protein ingredients or whole foods that are of high protein quality (e.g., milk, whey, soy). Studies that employ a broader range of protein quality in the context of mixed dietary patterns are needed, as well as in situations with low protein intake (Morgan et al., 2021).

The trend in overlooking and oversimplifying the importance of protein quality

Although evidence demonstrates the importance of considering protein quality and quantity when designing dietary recommendations, the topic has often been overlooked or oversimplified (Millward et al., 2008; Burd et al., 2019; Comerford et al., 2021). For example, driven by concerns for the environmental impact of current dietary practices, several countries have adapted FBDG that promote increased consumption of PBF and decreased consumption of ASF, either directly or indirectly, while not acknowledging a consideration for protein quality (Brink et al., 2019; Meltzer et al., 2019).

Typical recommended PBF to address protein intake include legumes, nuts, and seeds. Achieving transformation to sustainable healthy diets as defined by the EAT-*Lancet* Commission would require >100% increase in the global consumption of foods like legumes and nuts (Willett et al., 2019). Yet, intake of these foods is low – average per capita consumption of legumes is 21 g/day globally and 9.3 g/day in the U.S., which is below the recommendation in the Dietary Guidelines for Americans (DGA) (Dry Bean Council US, 2021; Semba et al., 2021a). Shifting dietary patterns toward higher legume, nut, and seed consumption requires significant changes in behavior, knowledge, and food preparation skills. A question therefore remains on how feasible such shifts would be given current dietary practices (Semba et al., 2021b).

Some research indicates recommendations to swap PBF for ASF can negatively impact intakes of protein and select micronutrients, particularly when modeled based on current consumption patterns. A study modeling different dietary scenarios using NHANES data found that increased intake of PBF resulted in an increased percentage of children (2–18 years) and adults (≥19 years) not meeting the Estimated Average Requirement (EAR) for protein, vitamins A and D, and calcium, which are nutrients of concern in the U.S. (Cifelli et al., 2016). Another modeling study found that doubling the intake of PBF led to a decrease in protein intake by about 22% among women and men aged ≥51 years. Additionally, protein intake among women ≥71 years decreased below the RDA and the percentage not meeting the EAR increased to 33% (Houchins et al., 2017). This demonstrates the potential detrimental effect on dietary outcomes if population groups increase PBF intake while decreasing ASF intake.

ASF's nutritional contributions to the protein quality debate

ASF are recognized for contributing to the overall quality of diet but have also received negative attention for their environmental impact. Early assessments of the effect of macrobiotic diets (i.e., diets based on whole-grain cereals, pulses, and vegetables) on infant and child growth and development in the Netherlands demonstrated the importance of including ASF in the diet. Results from these studies observed markedly lower intakes of energy and protein among children receiving macrobiotic diets compared to those receiving omnivorous diets, which was linked with linear growth faltering, fat and muscle wasting, and delayed development (Dagnelie and van Staveren, 1994).

ASF are rich sources of essential fatty acids and multiple micronutrients that are commonly lacking in the diets in LMIC, including vitamin A, vitamin B12, vitamin D, iron, zinc, and calcium (Neumann et al., 2001). They are particularly important for infants, young children, adolescents, and pregnant and lactating women who are undergoing physiological changes and have higher nutrient requirements (Nordhagen et al., 2020). Micronutrients in ASF have high bioavailability and enhance the absorption of nutrients from PBF with high phytate and fiber content that may inhibit the absorption of minerals (Gibson et al., 2003).

Animal products differ in their nutrient composition. Using dairy as an example, milk and milk products contain 13 essential nutrients, including high-quality protein, vitamin A, vitamin B12, vitamin D, riboflavin, folate, and calcium (Allen and Dror, 2011). Studies have consistently shown a positive association between dairy intake and linear growth in children aged 12–60 months. Further, the elimination of cow's milk from the diet has been found to be associated with a reduction in height and an increased risk of bone fractures among children (Goulding et al., 2004; Clark et al., 2020).

In the U.S., dairy is under-consumed relative to recommendations in the DGA (Krebs-Smith et al., 2010). Few people reach the recommended intakes of several key nutrients without consuming the recommended amounts of dairy foods (Weaver, 2014). A trend toward decreasing ASF intake could further reduce the intake of this food group.

In terms of plant-based milk alternatives, it is important to consider the variation in their nutritional profiles and that most do not provide the same nutrients as cow's milk. A study comparing the nutrient composition and carbon footprint of cow's milk and plant-based beverages (e.g., soy, oat, almond, coconut, and rice beverages) found that the protein and EAA content of cow's milk was higher. Although the carbon footprint of cow's milk was higher compared to plant-based beverages when expressed per serving, when expressed based on index of nutritional value (i.e., ability to contribute to meeting EAA requirements), the carbon footprint of cow's milk was lower than that of all plant-based drinks examined, except for soy beverage (Singh-Povel et al., 2022). These findings reflect the importance of considering the nutritional value of food choices when reporting environmental impact and making broader conclusions regarding sustainability.

Achieving the nuance needed through nutritional-based functional units in life cycle assessments

The sustainability of food systems can be measured across four domains: health, environmental, economic, and societal (Drewnowski, 2017). Each domain has respective metrics. For example, nutrient profiling models estimate the nutrient density of foods. Life cycle assessments (LCA) evaluate environmental impacts of foods relative to land, water, and energy use. Choices related to dietary protein may be influenced by culture. Assessments of food consumption patterns across populations can be used to understand the cultural and societal importance of such foods (Drewnowski, 2017).

The complexity lies in integrating metrics across domains to capture a holistic impact of food production. A study examining the relationship between the energy and nutrient content of foods and associated greenhouse gas emissions (GHGE) found that many foods with low GHGE had relatively low nutritional value; meat and dairy products, which were more nutrient-dense, had higher GHGE values per 100 g but lower values per 100 kcal. This raises the question as to whether the higher GHGE cost of some foods could be offset by their higher nutritional value (Drewnowski et al., 2015). Another analysis expressed GHGE of ASF and PBF relative to EAA and found the perceived environmental advantage of plant-based protein production to be smaller than previously estimated. Expressing land use relative to EAA also negated some perceived advantages of plant-based proteins (Tessari et al., 2016). When evaluating the environmental impact of animal- and plant-based foods, different conclusions can be drawn between assessments based on protein quantity and those that account for protein quality. For example, GHGE for milk production has been estimated as ∼400% higher than for plant production when expressed as per ton of gross protein consumed. This difference was reduced to 59% when expressed based on kilograms of digestible lysine consumed to account for protein quality. Milk production was also the most efficient production system in terms of water use when expressed on a digestible lysine basis (Moughan, 2021).

Incorporating nutritional-based functional units (FU) in LCAs is one way to harmonize the environmental and nutritional impacts of food production and dietary patterns. They may include nutrient quantity, calories (i.e., per 100 kcal), amount of individual nutrient (i.e., grams of protein), composite scores of several nutrients, and nutrient quality (i.e., Digestible Indispensable Amino Acid Score). One methodology incorporated protein quality and quantity into LCAs to more comprehensively compare ASF and PBF in terms of protein content and quality and environmental impacts (Berardy et al., 2019). Another methodology has introduced an emissions per unit nutrient density metric to examine GHGE from food production to compare different types of food products based on their nutritional value rather than according to a singular nutrient or specific attribute like weight (Doran-Browne et al., 2015). Nutritional-based FU may be helpful in ensuring protein quality is not overlooked in the effort to deliver on healthy diets from sustainable food systems.

Conclusions regarding the environmental impact of food products can vary depending on the metrics used, each of which has strengths and limitations. Deciding which approach to use may depend on context – for example, nutrients of concern differ across populations and countries, as do trade-offs between the nutritional contribution and environmental impact of foods. Utilizing a variety of metrics to make comparisons between findings may allow for more comprehensive assessments to inform public health guidance.

Discussion

It is critical that the dialogue surrounding food systems transformations consider the multiple domains of sustainability

– health, environmental, economic, and societal. Traditionally, assessments of the sustainability of food production and consumption have focused on the environmental dimension. There is a lack of evidence on how shifts in food systems and dietary patterns will impact other dimensions of sustainability, which are all interconnected. Research is needed on the impact of consuming plant-based or -exclusive diets on health outcomes among population sub-groups with unique nutritional needs, such as older adults, so that the most vulnerable can make well-informed dietary choices. Evidence is also lacking on the ability of populations with low intakes of legumes, nuts, and seeds to increase consumption of these foods and on the affordability and availability of such PBF across regions and population groups of different socioeconomic and cultural backgrounds. Changes in the food market, including development of ultra-processed foods, lab-grown meat, plant-based beverages, and animal protein alternatives like insects and mycoproteins, require further exploration to evaluate their role. Without such evidence, the feasibility of recommendations that have shifted toward plant-based or -exclusive diets remains unclear.

There is also a need for more robust assessments and standardized metrics for food systems that capture the complexity of sustainability and the trade-offs across the domains. The utilization of a variety of metrics can help address the limitations and constraints of each individual metric and allow for the presentation of a more complete picture. This can provide more comprehensive information for decision-makers and the public seeking to understand how to optimize sustainable production and consumption of both ASF and PBF. Studies focused on evaluating the environmental impact of dietary patterns should consider the nutritional value of food choices and the nutrient requirements of a population, with attention placed on the dietary needs of population sub-groups, particularly those that are at at-risk. While nutritional-based FU can help achieve this nuance, additional questions must be asked to determine which FU would be the best to use, which can vary depending on the overall goals of the study. Further work is needed to expand the use of nutritional-based FU to include more types of dietary and environmental data, and economic considerations like affordability and accessibility.

It is recognized that plant-based diets may be the preferred dietary choice for many. However, it is important to consider how diets can be optimized in terms of meeting intake requirements for protein, AA, and key micronutrients like vitamins A and D, B-vitamins, calcium, iron, and zinc. PBF and ASF contain different quantities and combinations of nutrients and thus play complementary roles in the diet (Comerford et al., 2021). As ASF provide relatively higher quality protein it is important to consider their contribution to optimal health and nutrition outcomes. Moreover, it is critical to take a holistic perspective on the linkages between health, the environment, and socioeconomic factors when assessing the sustainability of food production systems, food choices, and dietary patterns to inform dietary recommendations.

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/s.

Author contributions

MP, JR-C, and GM developed article conceptualization. MP and GM supported the original draft preparation. All authors contributed to the review, editing, and subsequent draft preparation. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

MP, JR-C, and GM are employees of National Dairy Council, Rosemont, IL, United States.

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Mungbean and pumpkin protein isolates as novel ingredients for the development of meat analogs using heat-induced gelation technique

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Mungbean and pumpkin are rich source of proteins and nutrients which could be utilized in novel food formulations. This study involves formulation of meat analog using mungbean protein isolate (MBPI) and pumpkin protein isolates (PPI) through optimization process using Box–Behnken Design (BBD) of response surface methodology (RSM). MBPI and PPI were used as base ingredients for the development of meat alternatives using an innovative heat-induced gelation process. Methylcellulose (MC) and gum Arabic were used as supporting matrices for obtaining desired texture of the meat analog. The emulsifying activity, water-holding capacity, and oil-holding capacity of MBPI and PPI were analyzed. The set of physicochemical response factors used in RSM was moisture content, protein content, color, and textural properties of the formulated meat analogs. The selected independent variables were set at three levels (–1, 0, 1) with protein ratio (20:10, 15:15, and 10:20 of MBPI-PPI), Water (32, 37, and 42%), and MC (5, 6, and 7%). RSM results showed that the model effectively described the correlation between the independent variables (protein ratio, water percentage, and MC percentage) and the response factors. The microstructure of the analog showed porous and fibrous structures. It was observed that the degree of cross-linking between protein molecules could have impacted the textural properties that were associated with viscoelastic characteristics as reflected in the rheological analysis. Overall, the study shows that the mungbean and pumpkin seed proteins could be utilized as a potential ingredient to improve the textural properties of the meat analog, while it is also recommended to explore such proteins with other mechanical processing techniques like extrusion.

KEYWORDS

plant-based meat analogs, plant proteins, physicochemical properties, texture, microstructure, viscoelasticity, mung bean, pumpkin proteins

1. Introduction

Recently, increasing numbers of consumers globally are adopting plant-based diets as a substitute for traditional animal-based foods due to the negative impact of animal-based foods, on human health and the environment (He et al., 2020; Singh et al., 2021). Consuming processed red meat has been connected to health concerns, particularly those related to coronary artery disease, cancer, and cardiovascular disease, with putative processes related to the amount of saturated fat, cholesterol, iron, phosphatidylcholine, and carnitine in the meat (Herz et al., 2021). In addition, apprehensions towards the ethical issues associated with animal welfare is on a rapid rise. Due to these concerns, there have been more studies in recent times on exploring plant-based ingredients as alternative sources to formulate meat alternatives (Yuliarti et al., 2021). Currently, plant proteins are employed most frequently to create meat substitutes, which are typically used as isolates and concentrates in powdered form. According to research by Gu et al. (2022) eating plant-based meals high in polyunsaturated fatty acids, oligosaccharides, and dietary fiber greatly lowers the risk of obesity and cardiovascular illnesses. Additionally, consuming more plant-based proteins (particularly those found in legumes) instead of red meat may lower the chance of developing type II diabetes (Gu et al., 2022). To create a balanced overall amino acid profile, producers of plant-based foods may either employ mixtures of proteins (such as legume and cereal proteins) or may supplement their products with the essential amino acids that are absent in the plant protein. One of the most effective approaches for developing a functional plant-based diet is the fabrication and restructuring of plant proteins to resemble the textural properties of meat, which can greatly reduce the ubiquitous health complications linked with red meat consumption.

Plant-based protein composites known as "meat analogs (MAs)" generally contain ingredients from non-meat sources and are designed into a matrix to imitate the textural and organoleptic characteristics of animal meat. Several studies reported the production of meat analogs using different plant proteins having nutritive and techno-functional properties such as soy (Chiang et al., 2019), pea (Zhu et al., 2021), rice (Lee et al., 2022), oat (De Angelis et al., 2020), peanut (Rehrah et al., 2009). The formation of a fibrous structure within the plant protein matrix is the basis for maintaining the unique juiciness and chewiness often sought after in animal meat. However, restructuring plant protein ingredients is one of the challenges in terms of MAs production (Palanisamy et al., 2018). Interestingly, different approaches including protein ingredient combinations, physical structuring techniques, and inclusion of gelling ingredients can be employed to overcome this challenge (Dekkers et al., 2018; Singh et al., 2021). Heat-induced gelation of proteins involves the linkage of non-polar surface groups through hydrophobic interactions. The heating of proteins causes the globular protein molecules to unfold and exposes the non-polar surface groups (Herz et al., 2021). Plant proteins mostly comprise of globular proteins (Mcclements and Grossmann, 2021). Formation of protein gel matrix in the presence of polysaccharides like pectin (Moll et al., 2023), guar gum (Nanta et al., 2021), and products produced from cellulose such as MC (Bakhsh et al., 2021), could help in the restructuring of proteins. MC is a hydrophilic cellulose derivative that consists of 1,4-β-D-glucan monomers, in which-OH is partially replaced by CH_2COOH groups (Michelin et al., 2020). It is widely utilized due to its structuring, thickening, or gelling ability in the aqueous phase. It can operate as an emulsifier in oil-in-water emulsions (Pirsa and Hafezi, 2022). These characteristics of MC attracted researchers to investigate its potential in creating robust texture and elasticity in MAs, in recent years. Furthermore, the formation of protein-rich gels through the interactions with MC, to provide improved fibrous texture of the MAs is well documented (Bakhsh et al., 2021; Zhao et al., 2021; Taghian Dinani et al., 2023).

Moreover, the appropriate selection of protein source is critical in impacting the desirable textural properties of the MAs which consequently plays an important role in the final structure and mouthfeel of the MAs. Therefore, it is pivotal to select a plant protein with excellent functionality to mimic conventional animal proteins. While soybean meal and wheat gluten have been widely used as the main plant-based protein ingredients for "MAs" because of their high-quality essential amino acids that are bioavailable for human nutrition (Kumar et al., 2022), but these ingredients have certain drawbacks eg. allergic proteins (Ozturk et al., 2023). For this reason, exploring novel proteins that can replace existing sources in the production of high-quality MAs is the utmost.

Mungbean and pumpkin seed proteins are currently being used as preferred protein ingredients in the food industry (Shrestha et al., 2023) due to their functionality, economical, and sustainable features. Hence, proteins from mungbean and pumpkin might have a huge potential in developing meat analogs. Numerous benefits of mungbean protein isolate (MBPI) have been demonstrated in processed foods, in terms of foaming, emulsifying, and water-absorbing properties (Du et al., 2022). However, its potential for meat analogs is scarcely explored. Pumpkin seeds have a protein content between 31.5 and 51%, making them also a potential source for plant-based meat substitutes (Batool et al., 2022). Even though there is a plethora of knowledge regarding the functional aspects of different plant-derived proteins, the functional characteristics of pumpkin seed protein fractions are not well understood and their application in the fabrication of MAs has not been explored so far (Vinayashree and Vasu, 2021). Considering all these factors, the design of this study was centered on the use of mungbean and pumpkin seed protein isolates along with MC to produce potential MAs.

2. Materials and methods

2.1. Materials

Food-grade mungbean protein (Munpein™ with 80% protein content) and pumpkin seed protein (PumpteinX™, with 74% protein content) isolates were obtained from ET protein (Xinping Street, Suzhou, China). The ingredients and chemicals including potato starch, methylcellulose (MC), gum arabic, and calcium chloride were procured from Sigma-Aldrich, Inc., United States. The sunflower oil (refined), salt, and baking powder were procured from the local market of Al Ain, United Arab Emirates (UAE).

2.2. Estimation of functional properties of protein isolates

2.2.1. Emulsifying activity

The methodology described by O'sullivan et al. (2016) was adopted with slight modifications. Five sets of protein solutions were prepared by briefly dissolving 300.0 mg of protein isolates in 30.0 mL deionized water (1% protein equivalent) and the pH was adjusted to 2, 4, 6, 8, and 10 using 1 M HCl and 1 M NaOH. The resulting solutions were blended with 10.0 mL sunflower oil and homogenized using a high-speed homogenizer (ULTRA TURRAX® T 25 digital homogenizer IKA®-Werke GmbH &

Co., Staufen, Germany) at 20,500 rpm for 1 min at room temperature to form emulsions. Subsequently, a 50.0 μL aliquot (avoidance of the supernatant foam) was then taken carefully from the bottom of each tube by a micropipette and mixed with 5 mL of 0.1% SDS solution immediately. The absorbance of the mixture, which indicated the emulsifying ability, was determined at 500 nm (A0) (Multiskan Sky, ThermoFisher Scientific, United States). Each sample was prepared in duplicate and each of these duplicates was measured three times. The emulsion activity index (EAI) was calculated using the following formula stated by Pearce and Kinsella (1978):

$$EAI\left(\frac{m^2}{g}\right) = 2 \times 2.303 \times \frac{A0}{0.25} \times weight\ of\ protein\left(g\right) \quad (1)$$

2.2.2. Water and oil holding capability

The water and oil holding capacity of the protein isolates were established using the following procedure. In brief, 1.0 g of protein ($W0$) was placed in centrifugal tubes and weighed together ($W1$). Then, 10 mL of distilled water or oil was added to the tube and vortexed. The solution was shaken at room temperature for 1 h. After standing at ambient temperature for 30 min, the tube was centrifuged at 5000× g for 20 min. The supernatant was decanted and the tube with sediment was weighed ($W2$). Water holding capability (WHC) and oil holding capability (OHC) were calculated as:

$$WHC(\%) = 100 \times \frac{(W2 - W1)}{W0} \quad (2)$$

$$OHC(\%) = 100 \times \frac{(W2 - W1)}{W0} \quad (3)$$

2.3. Experimental design and optimization of meat analog preparation

Design expert software (version 13.0, Stat Ease Inc., Minneapolis, United States) was applied to determine the optimum ingredients for the preparation of meat analogs. The key ingredients namely protein, water, and polysaccharides concentrations can significantly affect the structure and final quality of meat analogs. Thus, the effect of protein ratio [mung bean protein isolate (MBPI): pumpkin protein isolate (PPI)], water, and MC concentrations to obtain a desired texture is important to explore. The selected independent variables were set at three levels (−1, 0, 1) with protein ratio (20:10, 15:15, and 10:20 of MBPI-PPI), Water (32, 37, and 42%), and MC (5, 6, and 7%). In total, 15 experimental runs identified as low (−1), medium (0), and high (1) including three central points were carried out to optimize the three independent variables as shown in Table 1. For this experiment, color, hardness, springiness, chewiness, moisture, and protein content of the meat analogs were used as dependent variables or response factors. Multiple linear regression analysis of the runs done in triplicate was performed to obtain the regression coefficients following a second-order polynomial model.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 + \beta_{22} X_2 + \beta_{33} X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (4)$$

Where Y represents the dependent variables, β_0 is the intercept, β_1, β_2, and β_3 are the linear regression coefficient, β_{12}, β_{13}, and β_{23} represent the 2-way interactions, whereas β_{11}, β_{22}, and β_{33}, represent the quadratic coefficients. The generated 3D surface plots from the polynomial equation were used to interpret the correlation between the dependent variables and each independent variable, i.e., protein, water, and MC.

2.4. Preparation of meat analog (MAs)

The MAs were prepared according to a modified protocol described by Yuliarti et al. (2021). Herein, each formulation of MA (100.0 g) contains ice-cold water, different MBPI-PPI ratios, potato starch, sunflower oil, calcium chloride, salt, baking powder, MC, and gum arabic in different combinations according to BBD (Table 1). The protein and MC emulsions were prepared separately in a food processor (Kenwood Multi-Functional 750 W, FDP03.COWH, China) for 3 min. The protein (MBPI and PPI)-based emulsion was prepared using proteins, baking powder, calcium chloride solution, salt, potato starch, and ice-cold water. Similarly, MC emulsion was prepared by mixing MC powder, soybean oil, and ice-cold water. Thereafter, protein and MC emulsions were combined and thoroughly homogenized for an additional 3 min to obtain a uniform emulsion. The obtained batter was then shaped into a mold with the dimensions: 4.0 cm × 3.0 cm × 2.5 cm (L × W × H) and afterward steamed at 100°C for 15 min. The analog was immediately frozen at −20°C for 48 h before further analysis.

2.5. Physicochemical properties of meat analogs (MAs)

2.5.1. Moisture content

The moisture content of all samples was determined using an oven-dry method. Briefly, 3.0 g of sample was sliced, transferred into pans, and placed in an oven at 103°C for at least 16 h until constant weight was attained. The percentage of initial moisture content in terms of wet basis ($\%MC_{initial}$) was calculated after cooling in a desiccator using Eq. 5:

$$\%Moisture\ content\left(MC_{initial}\right) = \left(\frac{W_{initial} - W_{final}}{W_{initial}}\right) \times 100 \quad (5)$$

2.5.2. Total protein content

The total protein content was measured by the Kjeldahl method as described by AOAC International. The amount of total nitrogen in the raw materials was multiplied with a conversion factor of 6.25 to determine the total protein content.

2.5.3. Color

Color measurements of the interior cross-section from the analog were analyzed using a colorimeter (Konica Minolta CR-400, Tokyo,

TABLE 1 Box–Behnken experimental design for optimization of meat analog and the output obtained in the form of different response factors.

Runs	Independent variables			Response factors					
	Protein ratio	Water (%)	MC (%)	Moisture content (%)	Protein (%)	Color (L*)	Hardness (mJ)	Chewiness (mJ)	Springiness (mm)
1	1 (20:10)	0 (37)	1 (7)	51.47 ± 3.15[a]	26.87 ± 2.75[abc]	43.1 ± 0.12[b]	36.02 ± 1.09[ef]	95.56 ± 3.94[ab]	5.86 ± 0.48[ab]
2	0 (15:15)	0 (37)	0 (6)	41.857 ± 2.48[cde]	21.49 ± 1.49[cdef]	36.01 ± 0.24[fg]	46.62 ± 1.41[bc]	83.48 ± 2.78[cdef]	3.25 ± 0.24[ef]
3	−1 (10:20)	−1 (32)	0 (6)	35.29 ± 1.43[fgh]	15.54 ± 1.94[fg]	28.42 ± 0.17[k]	58.88 ± 2.27[a]	75.12 ± 3.07[fgh]	1.72 ± 0.18[gh]
4	0 (15:15)	0 (37)	0 (6)	41.25 ± 1.83[cde]	21.54 ± 2.15[cde]	36.34 ± 0.09[f]	46.91 ± 1.26[bc]	83.57 ± 1.49[cdef]	3.22 ± 0.37[ef]
5	0 (15:15)	−1 (32)	−1 (5)	33.50 ± 0.83[gh]	14.11 ± 0.89[g]	31.42 ± 0.26[i]	60.96 ± 2.83[a]	69.14 ± 5.36[h]	0.70 ± 0.07[h]
6	1 (20:10)	0 (37)	−1 (5)	39.44 ± 1.67[def]	20.56 ± 1.65[def]	40.24 ± 0.18[d]	48.32 ± 0.91[b]	77.37 ± 1.97[efgh]	1.74 ± 0.19[gh]
7	−1 (10:20)	0 (37)	−1 (5)	31.53 ± 1.13[h]	16.19 ± 2.07[efg]	29.68 ± 0.16[j]	56.13 ± 1.28[a]	71.97 ± 2.41[gh]	0.64 ± 0.05[h]
8	−1 (10:20)	1 (42)	0 (6)	39.29 ± 2.47[def]	23.45 ± 1.86[abcd]	35.33 ± 0.20[g]	42.53 ± 0.73[cd]	86.19 ± 4.76[bcde]	3.59 ± 0.45[def]
9	1 (20:10)	1 (42)	0 (6)	47.84 ± 0.63[ab]	27.60 ± 0.63[ab]	45.86 ± 0.49[a]	34.91 ± 1.39[f]	92.76 ± 1.09[abc]	4.63 ± 1.06[bcd]
10	0 (15:15)	0 (37)	0 (6)	42.03 ± 1.74[cde]	21.73 ± 1.05[bcde]	37.81 ± 0.03[e]	46.86 ± 2.16[bc]	83.62 ± 1.43[cdef]	3.17 ± 0.27[ef]
11	0 (15:15)	−1 (32)	1 (7)	46.06 ± 1.22[abc]	20.85 ± 1.75[def]	36.68 ± 0.22[f]	44.02 ± 0.83[bcd]	87.78 ± 3.78[abcde]	4.26 ± 0.36[cde]
12	0 (15:15)	1 (42)	1 (7)	50.49 ± 0.78[a]	28.27 ± 1.37[a]	42.08 ± 0.39[c]	28.18 ± 0.51[g]	97.02 ± 5.73[a]	6.32 ± 0.78[a]
13	0 (15:15)	1 (42)	-1 (5)	38.01 ± 1.89[efg]	22.00 ± 3.63[bcde]	38.42 ± 0.25[e]	40.38 ± 2.66[de]	79.57 ± 2.51[defgh]	2.45 ± 0.17f[g]
14	-1 (10:20)	0 (37)	1 (7)	44.04 ± 2.36[bcd]	22.13 ± 1.85[bcde]	34.08 ± 0.17[h]	40.44 ± 1.15[de]	89.26 ± 3.48[abcd]	5.04 ± 0.62[abc]
15	1 (20:10)	−1 (32)	0 (6)	44.06 ± 1.55[bcd]	19.28 ± 2.48[defg]	40.46 ± 0.08[d]	48.88 ± 2.85[b]	81.13 ± 4.29[defg]	1.15 ± 0.11[gh]

Data represents mean ± SD. Different small alphabets in the same column represents significant difference between the samples.

Japan). The color evaluation was expressed based on the Commission International de l'Eclairage (CIE) system and described as L*, a*, and b*. Measurements were taken at three differing points on the cross-section of each of the MA samples.

2.5.4. Texture profile analysis

The texture profile analysis (TPA) of MAs was determined using a texture analyzer (CT3, Brookfield Engineering Laboratories, Middleboro, USA) according to the protocol described by Yuliarti et al. (2021) with some modifications. Here an analog with dimensions 4.0 cm × 3.0 cm × 2.5 cm (L × W × H) was loaded onto the platform of a texture analyzer. The center of the analog was compressed twice to 40% from the original height using a cylindrical probe (diameter 7 mm) at a speed of 5.0 mms^{-1} at room temperature. TPA parameters including hardness, chewiness, and springiness were recorded using force vs. time plots.

2.6. Rheological properties

According to a previously described procedure, the viscoelasticity attribute was analyzed as per the method described by Zhu et al. (2021). A rheometer (HR-2, TA Instruments, Newcastle, United States) fitted with a parallel plate geometry (diameter: 40.0 mm) and a 1.0 mm gap was used to measure the rheological parameters at a temperature of 25°C. A spatula was used to carefully deposit 2.0 g of the MA sample on the bottom Peltier plate. Frequency sweeps (0.1–100 rad s^{-1}) in the viscoelastic linear domain at a 1.0% strain were conducted to determine the rheological properties of the samples and the storage modulus G′ and loss modulus G″ were recorded.

2.7. Microstructure

Scanning Electron Microscope (SEM) (JEOL scanning electron microscope, model: JSM-6010PLUS/LA, Tokyo, Japan) was used to determine the microstructure of the MAs using the method described by Yuliarti et al. (2021). Specifically, fresh analog was cut into small pieces (2–3 mm in thickness) and then solidified with liquid N_2. Frozen samples were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 12 h. Thereafter, the samples were rinsed with distilled water 3 times consecutively for 15 h followed by dehydration in a serial ethanol solution (50% for 15 min with 2 times, 70% for 15 min with 2 times, 80% for 15 min with 2 times, 90% for 15 min with 2 times, 100% for 30 min). The samples were placed in the vacuum chamber of SEM and images were recorded at a voltage of 20 kV at 100X magnification.

2.8. Statistical analysis

All experiments were carried out in triplicate and average values with standard deviation were reported. The data were subjected to one-way analysis of variance (ANOVA) using SPSS 24.0 software (SPSS INC., Chicago, IL, United States, 2002), and the mean values were compared using Tukey's test ($p < 0.05$). Differences between the different meat analog samples were considered significant at $p < 0.05$. The RSM data was analyzed using design expert software (trial version 13.0, Stat Ease Inc., Minneapolis, United States). Analysis of variance (ANOVA) was applied to determine the linear regression, quadratic coefficients, and interactions. The coefficient of estimation of R^2, the adjusted coefficient of determination (adjusted R^2), and the predicted coefficient of determination (predicted R^2) based on the polynomial equations were estimated at 95% ($p < 0.05$) significant levels.

3. Result and discussion

3.1. Protein functional properties

3.1.1. Emulsifying activity index (EAI)

EAI is often measured to determine the interfacial area emulsified per gram by an emulsifier (Jia et al., 2020). In the present study, the EAI of MBPI and PPI were measured as a function of pH (2.0, 4.0, 6.0, 8.0, and 10.0), and the results are shown in Figures 1A,B. Overall, the EAI of both proteins (MBPI and PPI) displayed similar magnitude as a function of pH, in which the intermediate EAI values were observed at pH 2.0 and 4.0, the lowest value at pH 6.0, and the highest values were recorded at pH 10 for both PPI and MBPI. However, the EAI values recorded for PPI at different pH were significantly lower compared to MBPI. For instance, the EAI of PPI at pH 2.0 and pH 4.0 were 7.23 and 7.7%, respectively– which decreased significantly at pH 6.0 but increased at pH 8.0 and 10.0, respectively.

Overall, at extreme acidic and alkaline pH, better EAI was demonstrated, while poor EAI was shown at pH 6.0 suggesting that EAI of both MBPI and PPI were pH dependent. Similar results were observed earlier by Shevkani et al. (2015) for the EIA of kidney beans and field pea proteins. Notably, EAI was maximum at pH 10.0 in both substrates, which indicated that alkaline pH improved the emulsifying properties of legume proteins significantly. The highest EAI at pH 10.0 for isolated mung bean and soybean proteins was also reported by Samard and Ryu (2019), and for field pea isolates by Shevkani et al. (2015). As displayed in Figures 1A,B, EAI value considerably declined as the pH increased from 4.0 to 6.0, most likely due to a reduction in electrostatic repulsion between proteins because the pH was close to their isoelectric point (PI); (the pI of MBPI is 4.6 and for PPI is 5) (Zhang et al., 2009). Moreover, the protein aggregation is usually highest at pH near PI, which causes the development of large aggregates that requires more time for migration, thus, decreasing their ability to re-arrange and adsorb at the oil–water interface– resulting in low EAI (Lam and Nickerson, 2015).

It is not surprising that extremely acidic or alkaline pH substantially improved the EAI of MBPI and PPI because, at these pH values, the protein undergoes partial unfolding due to intramolecular repulsions between similar charges which in turn provide greater surface activity to these proteins (Jiang et al., 2018). Findings from this work were consistent with Tan et al. (2021), who showed that the EAI of soy protein isolates could be improved by extreme pH treatments. Such an increase in EAI is attributed to the induced structural changes, enhanced exposure of hydrophobic sites, and peptide chain flexibility initiated by alkaline pH. Noteworthy, substrates (MBPI and PPI) investigated in this study demonstrated significant EAI, making them potential ingredients for various food applications.

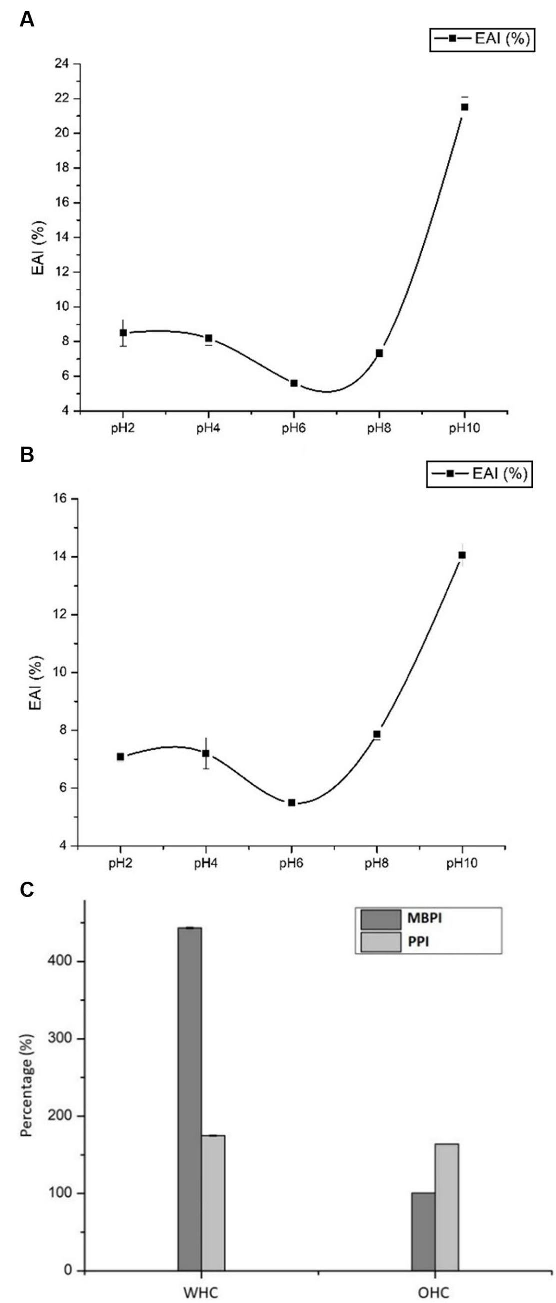

FIGURE 1
EAI of MBPI (Mung bean protein isolate) solutions **(A)** and PPI (Pumpkin protein isolate) solution **(B)** as a function of pH. **(C)** WHC and OHC of MBPI and PPI. Data were obtained in triplicates (*n* = 3) and represented in percentage (%). EAI, Emulsifying Activity Index; WHC, Water Holding Capacity; OHC, Oil Holding Capacity.

3.1.2. Water and oil holding capacity

The water-holding capacity (WHC) and oil-holding capacity (OHC) provide a measure of water/oil interactions with proteins and the water/oil retention capacity of proteins (Ge et al., 2021). Generally, WHC is associated with other functionalities like gelation, solubility, and emulsifying properties. Therefore, WHC could have a substantial impact on creating texture, flavor, and mouthfeel of the products (Ge et al., 2021). The WHC and OHC of MBPI and PPI are expressed as percentages (%), and the obtained data are presented in Figure 1C.

The highest WHC of 443.37% was observed in MBPI, which was almost 2.5 times higher than the WHC of PPI (174.80%). The protein hydrophobicity, conformation, amino acid composition, and the amount of protein present in the isolates could explain the observed differences in the WHC (Vinayashree and Vasu, 2021). Since, MBPIs consisted majorly of vicilin-type 8S, which have low molecular weight proteins and low surface hydrophobicity; thus, they might have displayed better WHC than PPIs that contained legumins-type 11S and 2S albumins. Additionally, vicilin conformation consists of a higher degree of unfolding and flexibility of polypeptide in the tertiary structure (Tang and Sun, 2011), allowing much greater surface area available for the protein-water interaction, and this might have improved the WHC of MBPI. Interestingly, the WHC value recorded for MBPI in this study was higher than pea protein (3.389 g water/g protein), and wheat proteins (1.376 g water/g protein), but less than that reported for soybean protein isolates (5.168 g water/g protein) according to Zhao et al. (2020).

The OHC results revealed that PPI exhibited a significantly higher value (163.93%) than that of MBPI (100.57%) as shown in Figure 1C. Interaction of oil molecules with more exposed hydrophobic groups on the PPI surface due to a conformational change of the protein, could be one possible reason for higher OHC of PPI. Miedzianka et al. (2021) have reported that the exposure of lipophilic groups in pumpkin protein during processing plays a crucial role in enhancing its OHC. Hence, we can postulate that exposure to the hydrophobic site of PPI facilitates the binding and/or interactions with oil molecules. The OHC of both, PPI and MBPI were consistent with those reported for soy, faba, and pea proteins with values ranging between 1.1–1.7 g/g (Ge et al., 2021). Overall, the WHC and OHC of MBPI and PPI indicated that these proteins can bind significant amounts of both water and oil.

3.2. The response of ingredient combinations on developed meat analogs

The effect of different ingredients combinations, protein ratio (**X_1**), water % (**X_2**), and MC % (**X_3**) on response factors (Moisture content (MC), protein content (PC), color, hardness, springiness, and chewiness) of developed MAs are presented in Table 1. Non-linear trends in the responses as a function of ingredient combination were noticed with significant differences.

3.2.1. Changes In moisture content (MC)

As illustrated in Table 1, the moisture content values of different ingredient combinations of MAs were found to vary from 31.53 to 51.47%. The highest moisture content of 51.47% was reported in the MAs when high-level of **X_1** (20:10 of MBPI-PPI), medium level of **X_2** (37%), and high level of **X_3** (7 g) was used, while lowest moisture content was observed with low level of **X_1** (10:20 of MBPI-PPI), medium level of **X_2** (37%), and low level of **X_3** (5 g). A similar moisture content in the formulated MAs was reported by Chiang et al. (2019) soy protein-wheat gluten-based MAs.

Xia et al. (2023) studied the fibrous properties of yeast protein based MAs at different moisture and temperature and suggested the strengthening of fibrous structures at a temperature of 180°C with 55% moisture content. Based on the regression model, the moisture content of MAs in our study was linearly correlated to (Table 2) linear effects of protein ratio, water, and MC, which were found to be significant ($p < 0.05$). Conversely, interactive, and quadratic effects of ingredient combinations did not show a significant effect ($p > 0.05$). In the model, the coefficient of determination value (R^2) presented a higher value than 0.997 which showed that the model was adequate. Also, the predicted R^2 value (0.985) and adjusted R^2 (0.994) were close to unity, suggesting the competence of the developed model to estimate the variation in the experimental test. The relationship between the moisture content of the meat analog and the coded value of the combination of the ingredients is given in the equation (Eq. 6).

$$Y_{MC\%} = 41.711 + 4.081\,X_1 + 2.091\,X_2 + 6.198\,X_3 \quad (6)$$

As shown in Eq. 6, the positive coefficient of all the linear terms of the independent variables indicated that they positively contributed to the moisture content of MAs. The Maximum positive coefficient (6.198) of **X_3** indicates that the amount of methylcellulose (MC) had a comparatively higher contribution to the moisture content than the protein ratio (X_1) and the percentage of water (X_2). Furthermore, the effects of ingredient combinations on moisture content are shown in Figures 2A–C. Notably, moisture content increased correspondingly with an increase in ingredient combinations (i.e., protein ratio, water, and MC). Ferawati et al. (2021) studied the high moisture MAs prepared by faba bean and yellow pea protein isolates and suggested a low moisture requirement of faba bean protein compared to yellow pea protein. It was noticed that the moisture content of MAs displayed a slight increase when the protein ratio and percentage of water were set to high levels while the percentage of MC was fixed at medium level (Figure 2A). However, further increment in the moisture content was found while maintaining medium levels of protein ratio or water percentage as illustrated in Figures 2B,C. This shows that when a high concentration of MC was used in MA formulation, then higher moisture content was retained in the MAs. This observation is attributable to the water-binding capacity of the MC, especially during the heating process. The water-binding capacity of the polysaccharides is due to the abundant hydroxyl groups that can form hydrogen bonds with water molecules (Dekkers et al., 2016).

3.2.2. Protein content (PC)

Proteins are essential for giving MAs their characteristic texture, nutritional value, and organoleptic qualities (Kumar et al., 2022). The combination of different ingredients have an impact on the formation of MAs which depends on the source of protein and processing technique (Kyriakopoulou et al., 2021). Chiang et al. (2019) have reported the protein content of MAs extruded with soy protein-wheat gluten to be in the range of 25.38–26.76%. In this study the PC of MAs in relation to change in X_1, X_2, and X_3 factors ranged from 14.11–28.27% (Table 1). The regression model showed that PC was significantly impacted ($p < 0.05$) by protein ratio, water percentage, and MC percentage. However, the interaction and quadratic effects of ingredient combinations were not statistically significant ($p > 0.05$) as displayed in Table 2. The model demonstrated a non-significant lack-of-fit along with a high coefficient of determination ($R^2 = 0.998$) and predicted R^2 of 0.997, which were practically in agreement with the adjusted coefficient of determination ($R^2 = 0.989$), indicating the accuracy and adequacy of the model.

The regression equation for describing the correlation between PC and ingredients combination (coded) is given below (Eq. 7).

$$Y_{Protein\%} = 21.586 + 2.126\,X_1 + 3.943\,X_2 + 3.157\,X_3 \quad (7)$$

Herein, all three independent factors showed positive coefficients as evident in the equation above, which suggests the positive contribution of these three factors on the PC of MAs. The highest

TABLE 2 Regression coefficients values estimated for ingredients combinations and responses of meat analog.

Term	Moisture %	Protein %	Color	Hardness	Chewiness	Springiness
$\beta 0$	41.71	21.59	36.72	46.797	83.56	3.213
$\beta 1$	4.081*	2.126*	5.269*	−3.731*	3.034*	0.299
$\beta 2$	2.091*	3.943*	3.089*	−8.342*	5.297*	1.145*
$\beta 3$	6.198*	3.157*	2.022*	−7.141*	8.946*	1.993*
$\beta 11$	−0.242	0.005	0.211	0.672	0.204	−0.276
$\beta 22$	0.154	−0.123	0.586	−1.169	0.041	−0.165
$\beta 33$	0.150	−0.155	−0.156	−2.243*	−0.220	0.383
$\beta 12$	−0.055	0.103	−0.377	0.595	0.139	0.403
$\beta 13$	−0.120	0.092	−0.385	0.849	0.227	−0.071
$\beta 23$	−0.017	−0.117	−0.400	1.185	−0.297	0.077
R^2	0.997	0.998	0.987	0.992	0.998	0.984
Adjusted R^2	0.994	0.997	0.976	0.979	0.997	0.956
Predicted R^2	0.985	0.989	0.944	0.879	0.990	0.783
Mean	41.74	21.44	37.06	45.34	83.57	3.182
SD	0.454	0.252	0.793	1.920	0.488	0.141
CV%	1.090	1.182	2.141	4.241	0.584	4.422
Adequate Precision	61.96	77.04	28.86	22.05	79.86	17.37

The star represents the significance of values. Where p-values are below 0.05.

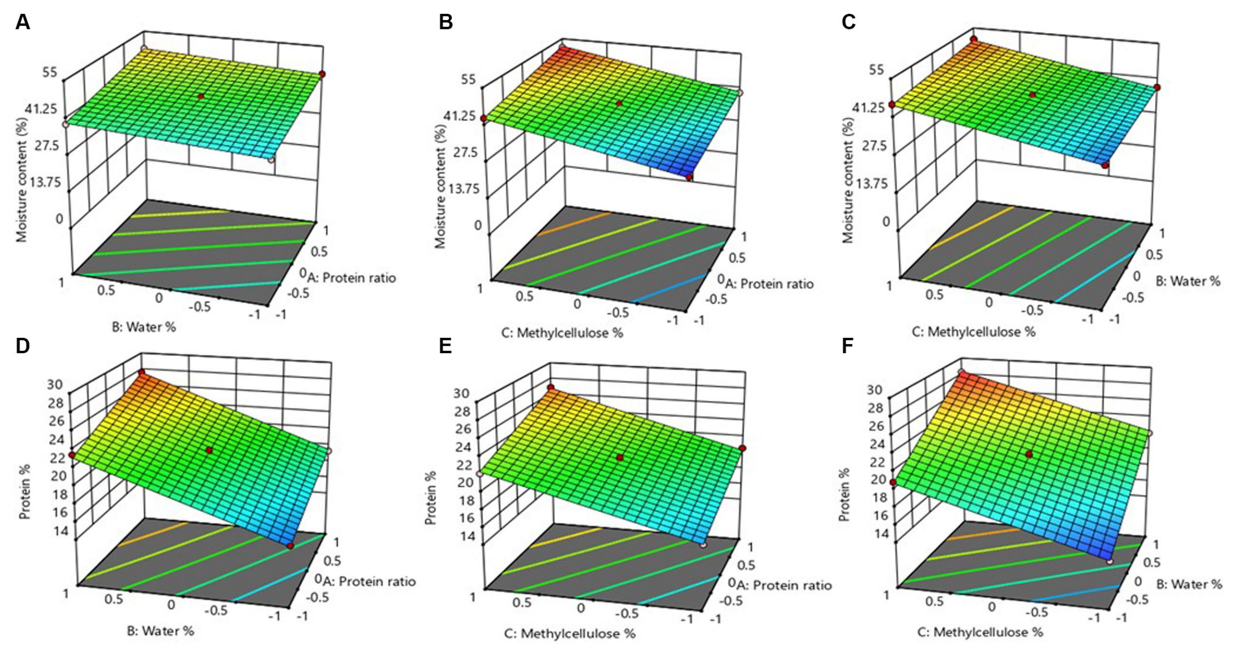

FIGURE 2
Response surface 3D plots showing the combined effect of water % and protein ratio **(A)**, methylcellulose % and protein ratio **(B)**, and methylcellulose % and water % **(C)** on the moisture content of meat analog. While the 3D plots displaying the combined effect of water % and protein ratio **(D)**, methylcellulose % and protein ratio **(E)**, and methylcellulose % and water % **(F)** on the final protein content % of the meat analog.

contribution was shown in the case of factor $\mathbf{X}_2$, followed by $\mathbf{X}_3$, and the lowest contribution on PC was shown by $\mathbf{X}_1$. Response surface plot (Figures 2D–F) shows that at medium-level water and high levels of the other two variables (i.e., protein ratio and percentage of MC), the PC of MAs displayed a declining trend. With the percentage of water set at a maximum level (42%), the PC experienced an increasing trend which can be easily observed in the Figure 2D. Based on the results, high PC was more pronounced in MAs formulated with ingredients combination containing 42% of water, regardless of the protein ratio. It is important to note that the PC for MAs observed in this study was higher than the median PC (14.0%) of all plant-based MAs, reviewed by Cutroneo et al. (2022). Therefore, the combination of the ingredients used in this study resulted in the MAs with sufficient PC, which is a crucial factor when formulating MAs.

3.2.3. Color

The Color attribute of MAs is an essential quality attribute since it mainly influences consumer's perception and acceptance. Combinations of ingredients have an impact on the luminosity function of MAs, which affects how bright or dark the products are as well as how contrast or gradation effects that mimic meat are produced. The luminance function is one of the characteristics that may be utilized to define the color of MAs (De Angelis et al., 2020; Boukid, 2021). Therefore, instrumental color measurement was conducted to quantify the samples using a colorimeter. Purposely, L* values, a luminosity function was measured to reflect the lightness and/or darkness of the MAs (Table 1). The obtained value of the L* of MAs ranged from 28.42–45.86, the maximum value was observed with the combination of the high level of $\mathbf{X_1}$ (20:10 of MBPI-PPI), high level of $\mathbf{X_2}$ (42%), and a medium level of $\mathbf{X_2}$ (6 g), whereas minimum value was noted in a low level of $\mathbf{X_1}$ (10,20 of MBPI-PPI), low level of $\mathbf{X_2}$ (32%), and a medium level of $\mathbf{X_3}$ (6 g). The results generated from the analysis of variance for dependent variables are presented in Table 2. The L* was significantly affected linearly by protein ratio ($p = 0.000$), percentage of water ($p = 0.000$), and percentage of MC ($p = 0.001$). Moreover, there were no statistically significant interactive and quadratic effects between the independent variable ($p > 0.05$).

The results generated from the analysis of variance for dependent variables are presented in Table 2. It showed the model coefficients which confirmed the significance of the model ($p < 0.05$) and that the model is well fitted with a lack of fit *p*-values of 0.741. The non-significant lack of fit p-values indicated that the model effectively described the correlation between the independent variables (protein ratio, water percentage, and MC percentage) and the dependent variables. Moreover, the R^2 of the model was 0.987, and the adjusted R^2 of 0.976 were quite comparable to the predicted $R^2 = 0.944$ generated for a model which further confirmed the significance of the model.

The following equation (Eq. 8) depicts the color (L*) response as influenced by the independent variables (X_1, X_2, and X_3).

$$Y_{Color} = 36.720 + 5.269\,X_1 + 3.089\,X_2 + 2.022\,X_3 \qquad (8)$$

The independent variables show a linear positive relationship with L* values, according to the regression equation. A higher coefficient (5.269) of $\mathbf{X_1}$ indicates that L* depended mainly on the linear effect of variable $\mathbf{X_1}$ whereas $\mathbf{X_3}$ had a lower contribution. This indicates that the color parameters of formulated MAs may be primarily dependent on the plant protein source used for MAs preparation and other ingredients incorporated in the formulation.

The L* increased when higher levels of MBPI were incorporated into the PPI blend (20:10) as shown in Figures 3A,B. Conversely, blending an equal ratio of the two proteins significantly decreased the L*. This suggests that a higher fraction of MBPI could significantly increase the L* (lightness) of the MAs, which is attributable to the innate yellow color of the mung bean protein (Wen et al., 2022). Therefore, the color of the MAs can be tailored by adjusting the amount of MBPI. The values for L* reported in this study were lower than those reported by Chiang et al. (2019) and Yuliarti et al. (2021), who reported L* values above 55 in plant-based MAs. Moreover, the lower L* values observed indicate a reduction in luminosity; the development of dark color in MAs may be advantageous to emulate animal-based meat. In general, dark-brown MAs are often preferred over those with vivid colors (Cho et al., 2020). A study published by Ye et al. (2022) explained that the formation of brown pigments can mimic the color of cooked animal muscle (meat).

3.2.4. Texture

The texture is a vital quality indicator that provides more insight into the surface characteristics of MAs. The texture profile of MAs, which mimics the physical characteristics of meat, such as firmness, juiciness, chewiness, tenderness, and mouthfeel, is significantly influenced by the various ingredient combinations, such as proteins, polysaccharides, oils etc. (Godschalk-Broers et al., 2022). Accordingly, some characteristics of texture including hardness, chewiness, and springiness were examined by a texture analyzer to provide an adequate description of the texture profile of MAs in this study. As shown in Table 1, obtained values ranged from 28.18–60.96 mJ, 69.14–97.02 mJ, and 0.64–6.32 mm for hardness, chewiness, and springiness, respectively (Table 1). The maximum hardness (60.96 mJ) corresponded to the sample containing a medium level of $\mathbf{X_1}$, low level of $\mathbf{X_2}$, and $\mathbf{X_3}$ whereas the minimum hardness value (28.18 mJ) was obtained from an ingredient combination containing a medium level of $\mathbf{X_1}$, high level of $\mathbf{X_2}$ and $\mathbf{X_3}$. The coefficient of determination R^2 and adjusted R^2 were used to verify the robustness of the model. The hardness, chewiness, and springiness R^2 were more than 0.90, which implied the model explained over 90% of all variations in the data. For hardness, significant linear effects ($p < 0.05$) of protein ratio, percentage of water, and percentage of MC were observed. The percentage of MC was found to have significant quadratic ($p < 0.05$) effects on hardness (Table 2).

The regression model (Eq. 9) describes the relationship between hardness and coded value of ingredients after neglecting the non-significant terms.

$$Y_{Hardness} = 46.797 - 3.731\,X_1 - 8.342\,X_2 - 7.141\,X_3 - 2.243\,X_3^2 \quad (9)$$

It is seen from Eq. 9 that the negative coefficient of linear terms of protein ratio ($\mathbf{X_1}$), percentage of water ($\mathbf{X_2}$), and percentage of MC ($\mathbf{X_3}$) had a negative influence on the hardness. A high coefficient of $\mathbf{X_2}$ (8.342) suggested that it had a maximum contribution to hardness. The negative coefficient of the quadratic term of $\mathbf{X_3}$ signified that their interaction was responsible for the decrease in the hardness of MAs. As the percentage of water decreased within the ingredient combinations, hardness increased and vice versa (Figures 3D–F). Previous findings have demonstrated that the addition of high amounts of water could disadvantageously decrease hardness (Chen et al., 2010). Response surface plots showed hardness increased by decreasing the water content (Figure 3D). Similarly, decreasing the amount of MC from 7 to 5 g and increasing the PPI ratio in the protein blends of MBPI-PPI increased the hardness of MAs when the water content was kept constant (Figures 3E,F). It is worth mentioning that reducing the water and

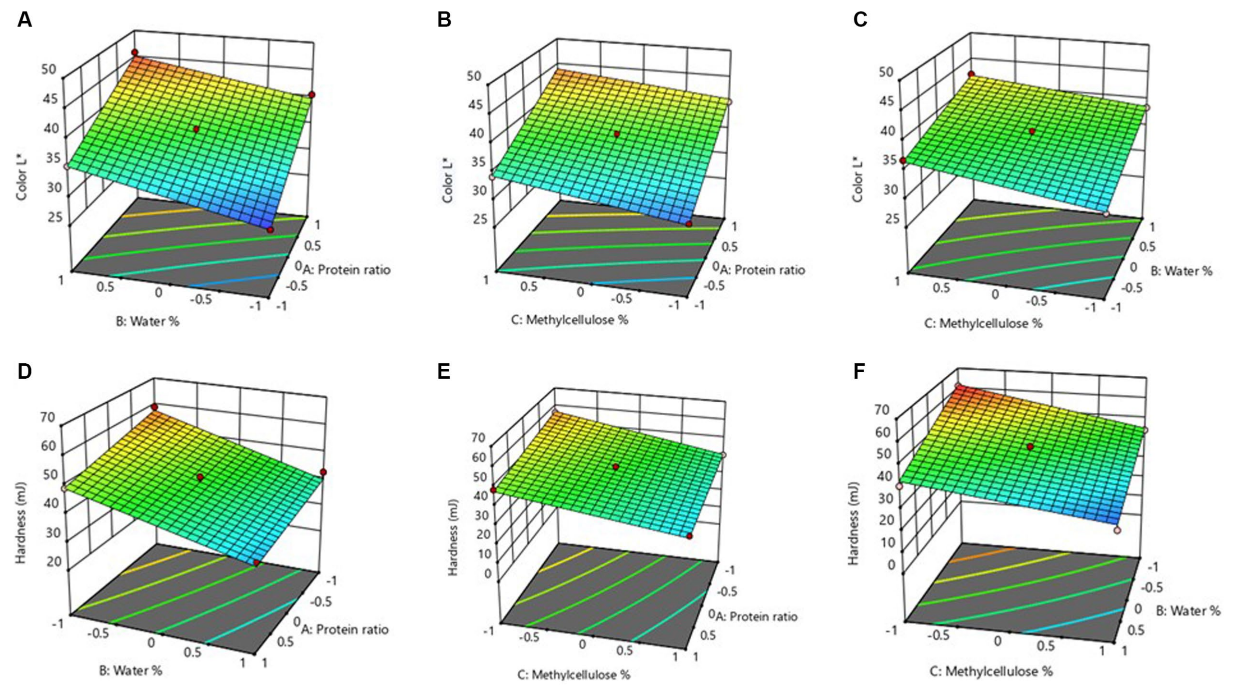

FIGURE 3
Response surface 3D plots showing the combined effect of water % and protein ratic **(A)**, methylcellulose % and protein ratio **(B)**, and methylcellulose % and water % **(C)** on the L* values of the meat analog. While the 3D plots displaying the combined effect of water % and protein ratio **(D)**; methylcellulose % and protein ratio **(E)**, and methylcellulose % and water % **(F)** on the hardness of the meat analog.

the MC levels could make MAs brittle–and hence beneficial to the formation of the intended fibrous structure.

In terms of chewiness and springiness, maximum values of 97.03 mJ and 6.32 mm, respectively were recorded when the ingredient combination containing medium level of $\mathbf{X_1}$, high level of $\mathbf{X_2}$, and $\mathbf{X_3}$ was used. The protein ratio, percentage of water, and percentage of MC showed significant linear effects on chewiness ($p < 0.05$), whereas springiness was not significantly impacted by linear effects of protein, water, and MC ratio ($p > 0.05$) as displayed in Table 2. The protein ratio of equal blends of MBPI-PPI along with increased concentrations of water and MC significantly improved the chewiness (Figures 4A–C), and springiness (Figures 4D–F) of the MAs.

The relationship between the ingredients combination, chewiness, and springiness of MAs are given in Eqs 10, 11, respectively.

$$Y_{\text{Chewiness}} = 83.557 + 3.034\,X_1 + 5.297\,X_2 + 8.946\,X_3 \quad (10)$$

$$Y_{\text{Springiness}} = 3.213 + 0.299\,X_1 + 1.145\,X_2 + 1.993\,X_3 \quad (11)$$

The equations (Eqs 10, 11) describe the positive contribution of $\mathbf{X_1}$, $\mathbf{X_2}$, and $\mathbf{X_3}$ on chewiness and springiness as indicated by the positive coefficients presented. Besides, the coefficient of $\mathbf{X_3}$ demonstrated that its contribution is greatest on both texture attributes (chewiness and springiness). The effect of MA concentrations on chewiness and springiness was further in line with the previous results reported by Bakhsh et al. (2021) supporting the fact that increasing MC concentration could effectively improve the texture parameters of MAs. This was also supported by the study of Arora et al. (2017), where increasing the binding agents led to the formation of harder gels within the formulation, resulting in the improved texture of the mushroom-based sausage analog. In addition, the binding ability of different ingredients used in fabricating the MAs plays a vital role in the product's final structure (Bakhsh et al., 2021).

Therefore, in this study, the amount of water and MC might be the two major ingredients that impacted the textural properties of MAs which imply that appropriate percentages of water and/or MC are necessary for the formation of the fibrous structure. Conversely, the protein ratio of MBPI-PPI showed limited impact on analog texture.

3.3. Optimizing the ingredients for the MAs

The responses were optimized using RSM to attain the MAs with desirable quality, based on the selected variables. Table 3 showed that according to the predicted values, all three independent variables should be set at the highest level to produce MAs with maximum protein content (30.62%), moisture content (53.95%), color L* (46.58), hardness (27.47 mJ), springiness (7.002 mm), and chewiness (100.9 mJ). The generated predicted values were comparable to the actual results with values of protein content (28.27%), moisture content (51.47%), color L* (45.86), hardness (28.18 mJ), springiness (6.317 mm), and chewiness (97.02 mJ), which verify the high reproducibility and reliability of all models evaluated in this study. Furthermore, the desirability function (DF) of optimized models is commonly used to validate the generated models (Mostafa et al., 2022). In this present study, all six models displayed a DF value of 1 which indicates an extremely desirable response and further validated the models.

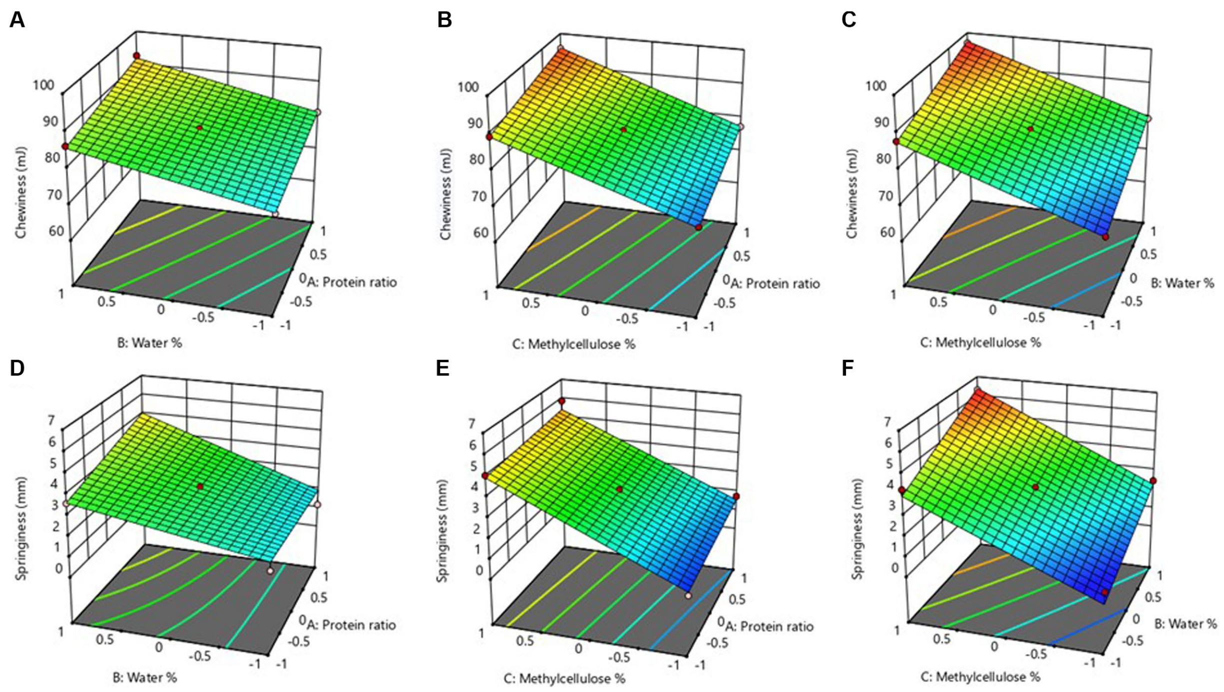

FIGURE 4
Response surface 3D plots showing the combined effect of water % and protein ratio **(A)**, methylcellulose % and protein ratio **(B)**, and methylcellose % and water % **(C)** on the Chewiness of the meat analog. While the 3D plots displaying the combined effect of water and protein ratio **(D)**, methylcellulose 96 and protein ratio **(E)**, and methylcellulose % and water % **(F)** on the Springiness of the meat analog.

TABLE 3 Optimization of meat analog and validation of predicted and experimental values under optimum conditions.

Response factor	Optimum factors levels			Highest values obtained	
	Protein ratio	Water (%)	Methyl-Cellulose (MC) (%)	Response optimizer results	Actual results
Protein %	1 (0)	1 (1)	1 (1)	30.62	28.27
Moisture Content	1 (1)	1 (0)	1 (1)	53.95	51.47
Color	1 (1)	1 (1)	1 (0)	46.58	45.86
Hardness	1 (0)	1 (1)	1 (1)	27.47	28.18
Springiness	1 (0)	1 (1)	1 (1)	7.002	6.317
Chewiness	1 (0)	1 (1)	1 (1)	100.9	97.02

Values presented in brackets denotes the actual process level used in the experimental assay.

3.4. Viscoelastic properties

The rheological properties of optimized MAs (i.e., ingredients combination of high-level protein ratio, high water content, and high MC content) were determined by frequency sweeps to obtain more information on the viscoelastic properties as a response to variation in frequency (Figures 5A,B). The loss modulus *G"* correlated to the viscous nature of the network and the storage modulus *G'* showed elastic properties which are similar to solid-like characteristics (Yuliarti et al., 2021). Figure 5A illustrated that the *G′* and *G″* did not show any significant change at lower amplitude whereas at higher amplitude the *G′* and *G″* cross each other which caused change in the network because *G″* dominated *G′*. Furthermore, frequency sweep measurements showed that both loss and storage moduli increased for the sample as the frequency increased (Figure 5B). Notably, *G′* dominated over *G″* throughout the experiential frequency range, suggesting the dominance of the elastic nature of the analog. Interestingly, this study has shown that the analog developed with higher ratios of MBPI to PPI, high water content, and MC content demonstrated less viscous, but a greater elastic response. This implies that the applied energy was stored in the interior network and not dissipated.

The incorporation of more MBPI fractions in the protein ratio formulation had resulted in increased moduli (*G′* and *G″*), indicating increased protein gel strength, which would expedite a sufficient cross-link network. This indicates that MBPI could contribute more towards strengthening of the structure of the MAs, and it could be attributed to its high protein content which led to a higher ability to easily develop gel, thereby contributing to the strengthening of the analog structure. Similarly, Branch and Maria (2017) earlier showed that mung bean proteins can effortlessly improve gel at the least concentration compared with other proteins, e.g., soy proteins. On the other hand, the elastic behavior exhibited in the MAs can also be attributed to the gelling nature of MC which played an important role in creating an elastic compact network. Our findings infer that the viscoelasticity of MAs can be imparted by increasing the concentrations of MBPI, water, and MC ingredients, to create protein–protein, protein-water, and protein-polysaccharide interactions required to form the right elasticity, strength, and tight networks in the MAs.

3.5. Microstructure

The cross-section micrograph of MAs after freeze drying showed porous and fibrous structures as displayed in Figure 6. The cross-sectional area of the developed analog is shown in Figure 6A. Furthermore, to obtain the effects of ingredients combination on the fibrous structure of analog, SEM images were captured at 100x and 400x (Figures 6B,C) magnification. As observed in Figure 6B, the analog appeared very porous with a rough structure, most probably an aggregated network and some portion of the protein network slightly interconnected. This phenomenon might be explained by protein chains unfolding and aggregating during heating, which resulted in a three-dimensional network. Another possible phenomenon worth mentioning is that MC could form gels that entrap proteins, making proteins cross-link to form strong networks. Moreover, the protein network could be due to the interaction of proteins with polysaccharides mainly through hydrogen bonding (Ran et al., 2022). The incorporation of a high ratio of MBPI to PPI, and high concentrations of MC in the formulation contributed to the MA structure formation. Previous studies have proposed that the blending of two proteins could significantly enhance the formation

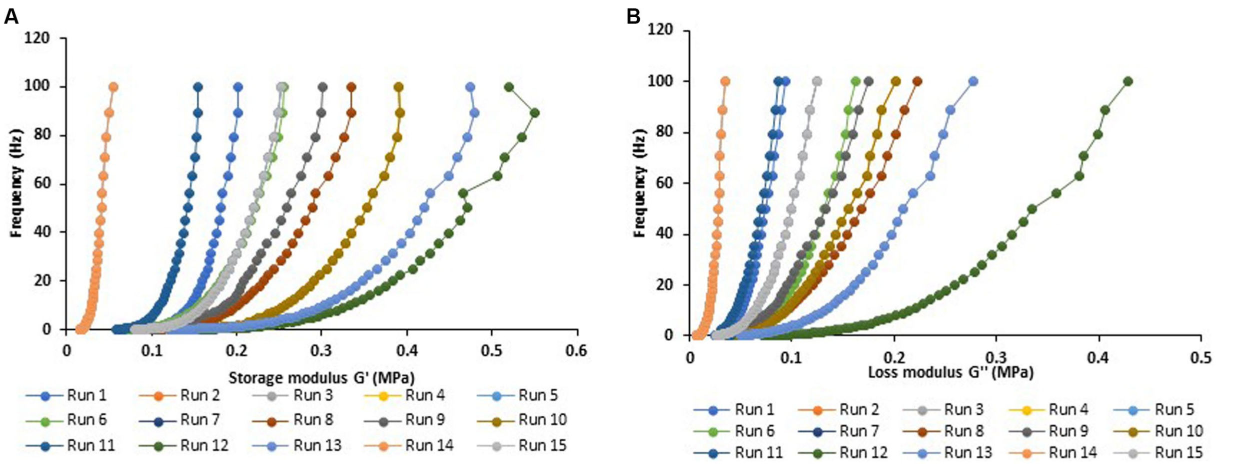

FIGURE 5
The viscoelastic measurements of MBPI + PPI meat analog with frequency sweeps from 0.1 to 100 rad s^{-1} and 1% linear domain **(A)**. Storage modulus *G′* showing the clastic nature of analog and **(B)**. loss modulus *G″* as function of the change in frequency (0 to 100 Hz) Run 1 to Run 15 are according to the Box—Behaken experimental design table (Table 1).

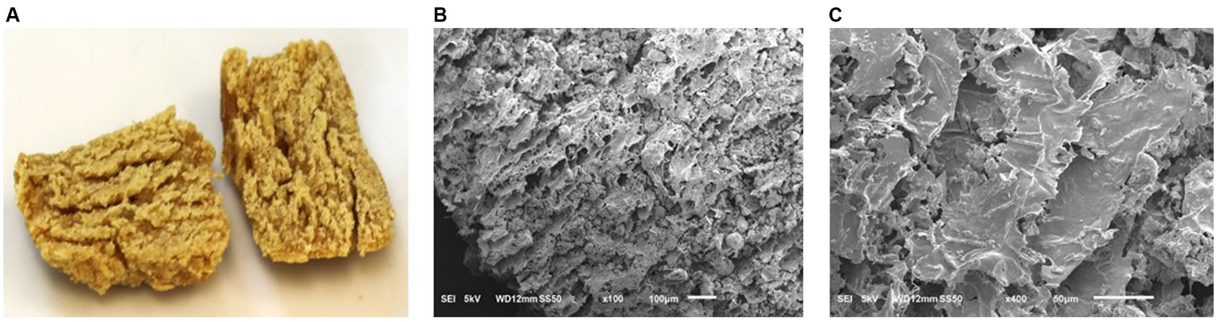

FIGURE 6
(A) Meat analog having MBPI-PPI in a 20:10 ratio, 42% of water, and 7% of MC. **(B)** SEM micrograph of analog showing porous and fibrous network at 100x. **(C)** Micrograph of analog at 400x showing sheet like structures.

of fibrous networks in MAs (Grabowska et al., 2014; Yuliarti et al., 2021). On the other hand, the network structure was loose and partly compact, which showed that the application of the heat-induced gelation technique alone was not sufficient to develop a tight, aligned, and more compact analog. Nonetheless, the network structure observed synchronizes with the viscoelastic results and further indicates the positive effects of mixing polysaccharides with proteins on the creation of structured plant-based MAs.

4. Conclusion

This study investigated the potential of mungbean and pumpkin seed protein isolates as novel ingredients for the formation of plant-based MAs. Developed MAs from ingredients combinations of MBPI-PPI blends, different water content, and MC content using BBD were evaluated for their physicochemical parameters (moisture, protein content, color, texture, visco-elasticity, and microstructure). The results showed that ingredient levels significantly impacted the physicochemical and microstructural qualities of MAs. The ingredients combinations of MBPI-PPI in a 20:10 ratio (high level), 42% of water (high level), and 7% of MC (high level) demonstrated optimum conditions for developing plant-based analog with enhanced quality characteristics. The findings of this study showed that MBPI-PPI blends with polysaccharides played an important role to form an elastic and slightly compact network, to a certain extent. However, the heat-induced gelation technique was insufficient to create a fibrous and layered structure– that emulates the animal-based meat. Nevertheless, the incorporation of MBPI and MC contributed largely to the attainment of texture and visco-elasticity required for the fibrous structure in MAs. Thus, these ingredients could be considered as potential sources to produce plant-based MAs. This study clearly showed that a combination of MBPI: PPI, water, and MC formulated through heat-induced gelation can create MAs. However, further studies warrant attention for creating plant-based MAs that meet consumer acceptance.

Data availability statement

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

Author contributions

MB: investigation, data collection, formal analysis, methodology, and writing-original draft. HM: data curation and data analysis. FA: formal analysis, investigation, and writing-review & editing. NS: writing-review & editing. SM: conceptualization, funding acquisition, supervision, project administration, and writing-reviewing & 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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Not getting laid: consumer acceptance of precision fermentation made egg

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Mounting concern over the negative externalities of industrialized animal agriculture, coupled with falling cost curves of novel food technologies have birthed the field of cellular agriculture: a new category of food technology seeking to reproduce the sensory experiences of animal protein, and promising a cleaner, more ethical way of enjoying animal proteins. This research examines consumer acceptance of precision fermentation (PF) made egg products in Germany, Singapore, and the USA. Using an online survey of 3,006 participants, the study examines demographic and dietary traits that predict willingness to try such products and identifies the reasons why consumers are most attracted to them. The findings suggest that PF made egg products are likely to find a willing market, with a substantial proportion (51–61%) of participants willing to try the product, with vegetarians and vegans displaying the highest enthusiasm. Egg consumption habits and, to a lesser extent, income also predict acceptance. Major reasons for adopting the product were animal welfare in Germany, and health aspects in Singapore and the USA, as well as curiosity in all three countries. Observed differences between the acceptance of PF egg and PF dairy are discussed, as well as comparisons to existing alternative protein (AP) product adoption.

KEYWORDS

precision fermentation, cellular agriculture, fermentation, consumer acceptance, food system

Introduction

Having risen by nearly 70% since the 1960s (FAO, 2023), humanity's consumption of animal protein is becoming an increasingly destabilizing force acting on the planet's climate, and itself a victim of mounting instability. The impacts of rising temperatures and extreme weather events are already impacting the productivity of the agri-food sector (Lesk et al., 2016), with economic volatility, exposed global supply chains and the proliferation of animal-borne diseases providing further threats to the stable supply of animal protein (Sundström et al., 2014).

The livestock industry itself drives much of this instability, producing an estimated 14.5% of global greenhouse gas emissions (Gerber et al., 2013). It is also a leading cause of air and water pollution, deforestation, and water scarcity (Rojas-Downing et al., 2017). Furthermore, the livestock industry is the leading cause of emerging zoonotic diseases such as avian-flu and swine flu (Hayek, 2022), as well as being the leading risk factor for future antibiotic resistance, forecast as one of humanity's greatest emerging threats in the 21st Century (UNEP, 2020). Though public awareness of the severity of the livestock industry's negative aspects has grown recently (Janssen et al., 2016), the critique of our relationship with animals is longstanding, especially from an animal-welfare perspective, with the roots of veganism and vegetarianism laced through religious and philosophical axioms that are millenia-old (Whorton, 1994). As the tools of industrialized, globalized economies blend with humanity's rapidly growing appetite for animal-based protein, increasingly productive, albeit increasingly demeaning conditions for animals have become the global norm (D'Silva, 2006). Hence, there arises a compelling argument for reconsidering our relationship with livestock, diversifying our global protein supply, and heavily reducing our consumption of animal-based proteins.

While not garnering the same focus as meat or dairy (Spain et al., 2018), the humble egg is an optimal vessel to understand the nature of animal protein consumption in the 21st century, along with a corresponding need to change our relationship with it. Driven by selective breeding, optimized feeding and living conditions, and an increasing consumer desire for animal protein, egg production has risen from 1961 levels of 15 million tonnes annually, to over 93 million tonnes in 2020 (FAO, 2020). Though standard chickens now produce around 300 eggs per year (as opposed to the 40 eggs that chickens historically produced in natural settings) (ProVeg International, 2018), a more developed egg production system has, ironically, not insulated consumers from price swings or shortages. Instead, consumers face greater volatility, with global commodity prices, diseases and labor shortages now all directly feeding through to contemporary egg markets (Lorsch, 2023).

While the carbon emissions profile of eggs are less damaging when compared to that of cheese or meat (in part due to the hugely adapted genetic and environmental conditions of modern day chickens), producing around 4.67 kg of CO2-e per 100 g of protein produced (Ritchie et al., 2022), industrial egg production is a leading contributor toward biodiversity loss and localized environmental pollution, with chicken effluent containing high levels of nitrogen and phosphorous (Basitere et al., 2019). This leads to algal superblooms and catastrophic effects for local wildlife populations when running-off from agricultural fields (Han et al., 2017). In the same way, chicken feed accounts for 37% of global soy production (Ritchie and Roser (2021), which acts in turn is a major driver of global biodiversity loss (WWF, 2014).

Recognizing the moral, environmental and practical concerns surrounding animal-product consumption, consumers and policymakers are already examining ways to reduce the consumption of animal-based protein, with the provision and promotion of alternative proteins (APs) earmarked as one of the most feasible means to achieve this (IPCC, 2022). Both plant-based meat and dairy products have made substantial market inroads in the last decade, turning both into multi-billion dollar industries (Good Food Institute, 2023a), though their inability to fully replicate the sensorial experiences and functionality of animal products has left many consumers unwilling to fully remove animal products from their diets.

The emergence of cellular agriculture, a field of research that uses cellular and molecular biology to produce agricultural products from cell cultures, was born from a recognition of this predicament and endures as an attempt to address it. Combining the tools of molecular biology, biochemistry and engineering, cellular agriculture seeks to develop products structurally and functionally identical to those made by animals, yet without animal exploitation.

An emergent pool of research has started to examine the predicted economic and social impacts of cellular agriculture, particularly engaging with the question of whether and how consumers will adopt this new category of products (Bryant and Barnett, 2020) Cultivated meat, meat grown from biopsied animal cells, has drawn the bulk of this research focus, while precision fermentation (PF), a technology allowing for the creation, not of animal flesh, rather the individual components of animal products, such as milk or egg proteins, is comparatively underexamined.

After the alteration of single-celled organisms' DNA, PF is conducted in brewery-like facilities to produce specific compounds—either modeled on those found in nature, or entirely novel compounds. This approach has been in use for some time to manufacture expensive and complex compounds, such as insulin and rennet but the costs of PF are now dropping to a level that means more and more compounds are becoming economically competitive with those produced by animals (BCG, 2022). Several companies, including the co-authors of this paper, Formo, are now applying PF to create functionally identical egg and dairy proteins, blending these with fats, emulsifiers and water to create products without many of the associated environmental, health and ethical concerns associated with conventional animal protein production. Unlike existing plant-based vegan products, PF products exhibit many of the versatile functional properties associated with animal-derived products, significantly improving end-consumer experience. In the context of a PF egg product, this manifests in properties such as coagulation, emulsification, leavening and binding. Just as with conventional liquid egg or dairy storage, PF made products will need to be safely treated and stored, including suitable refrigeration, pasteurization and packaging to avoid premature spoilage. While initial products will likely debut with an associated price premium, especially in food-service settings, scaled production processes, as well as advances in fermentation efficiency will likely deliver products similar in price to premium eggs in the near future.

Initial life-cycle assessments of egg proteins produced via PF (Järviö et al., 2021) show an advantage in terms of most environmental impacts, such as global warming potential and land usage, while also circumventing the localized environmental damage that industrial egg production causes. Likely future sustainability advances will be driven by advances in production efficiency, renewable energy sourcing and effective side stream usage. PF also reduces the need for antibiotic usage, which, when considering that roughly 70% of US antibiotics are fed to chickens, implies a huge step toward a more future of more restrained antibiotic usage (O'Neill, 2015).

Despite a number of companies seeking to produce egg products through PF, as well as compelling grounds for their adoption, no research has examined the extent and dynamics of consumer acceptance for such products. This dynamic will ultimately determine the impact of cellular agriculture and its ability to reorient our relationship with animal protein. Grassian (2020) found that people who are seeking to lessen their intake of animal products were less likely to avoid eggs and dairy in comparison to other animal-based foods. As such, it is probable that eggs are one of the most difficult food groups to avoid for consumers, pointing both to the weaknesses of existing egg substitute products, and the potential for PF made egg to gain a foothold among existing consumer groups.

Some research has examined consumer acceptance of cheese products made via PF, seeing notably higher enthusiasm than for cultivated meat products [with 70.5% of consumers probably or definitely likely to buy such a product (Zollman Thomas and Bryant, 2021)], however, how level of enthusiasm, perceptions, audiences, and rationale varies between PF made products is unclear, especially between countries.

Our research therefore addresses the following three research questions:

1. What is the overall level of consumer interest in PF made egg products in Germany, Singapore and the USA?
2. What are the demographic and dietary traits that most strongly predict a willingness to consume a PF made egg product?
3. For what reasons do consumers consider the adoption of a PF made egg most attractive?

Methods

Participants

Across the three countries, a total of 7,938 participants aged 18 to 75 years old were recruited for an online survey through Dynata, a research panel agency. Four interlocking quotas based on gender and age were implemented, with subsequent weightings applied to the samples to produce results that were nationally representative of the population. 4,011 participants across the three countries were not eligible to complete the survey as the age and gender quotas were full. These participants were redirected back to the panel. To enhance data quality, participants who failed the honesty check ($n = 51$) and two attention check measures ($n = 602$) were excluded. Additionally, responses to open ended questions that were generated by bots were manually identified and removed from the survey ($n = 273$). Replacement participants were provided by Dynata. This resulted in a final sample of 1,000 participants from Germany, 1,000 from USA and 1,001 from Singapore (see Table 1 for participant characteristics).

Procedure

Data was collected via an online survey that was administered on Qualtrics. The study was approved by the Institutional Review Board at Singapore Management University. Participants were briefed that the study examined people's perceptions of new types of food products. After informed consent was obtained, participants were asked to indicate their age and gender to control for balanced response rates and to verify that they have met the specific quota requirements in order to continue with the survey. Participants then read a passage about PF, and its use in creating a new egg product. Careful consideration was put into the development of the passage, formulated to both concisely introduce a complex technology, and simulate the setting with which consumers would be likely to encounter the product 'in the wild'. This process will be elaborated on in the Materials section. A timer validation of 15 s was implemented in Qualtrics to ensure that participants spent sufficient time reading the passage.

In the next section, participants were tested on their comprehension of the passage. The first question asked participants the extent to which they understood the new product and what makes it different from existing products. In an open-ended question, participants were then asked if they had any questions about the product and what makes it different from existing products. Participants then had to answer a multiple-choice question which asked what the actual ingredients of the new egg product were. If participants did not select the option of 'proteins made by microorganisms', a quick reminder was displayed to participants which clarified that What Came Third (the name of the new product) is made using real proteins that are created by microorganisms. This was to ensure that all participants had the same baseline understanding of the PF made product.

The following section consisted of questions pertaining to the acceptance of PF made egg and the reasons that would attract participants to purchase this new product.

The next section required participants to answer questions about their dietary habits including their current diet and their frequency of consumption of various egg products. Participants then answered some demographic questions and were debriefed. They were also given an opportunity to comment or ask questions about the research. At the end of the survey, participants answered an honesty check question which asked them if they had responded to the survey in a reasonably careful and honest manner. Lastly, participants were thanked for their time and were redirected to the panel to receive compensation.

Materials

Consumers' introduction to and framing of PF made food has been shown to impact both the acceptability and desirability of PF produced food products (Broad et al., 2022). For this reason, the text introduction of PF produced egg provided to respondents was constructed with a view to present a simple and transparent overview of the technology, product attributes and a rationale for its introduction. This survey sought to create a description that accurately captured the fundamentals of PF, while also focusing on the qualities of the end product that would be consumed by society.

Due to a lack of consensus within industry and regulatory bodies surrounding PF product nomenclature, and to effectively simulate consumers' initial exposure and assessments of PF made egg products, the product was referred to by a product name: What Came Third.

The passage introducing the PF made egg product are as follows:

> *A company is preparing to launch a new egg product. The product cooks, tastes and behaves identically to a real beaten egg, only it is made without any animals involved.*
>
> *For cooking and nutrition, the most important part of an egg is its protein. Instead of using chickens to make this protein, a process similar to beer or soy-sauce production is used, where microorganisms make the ingredients.*
>
> *By precisely changing the DNA of microorganisms, it is possible to turn them into mini-factories that produce specific proteins with the same function, flavor, nutrition and applications as egg protein. This process is called precision fermentation.*
>
> *These proteins are collected and turned into a product that consumers, chefs and bakers can all use to make diverse dishes like*

TABLE 1 Participants' demographic and dietary characteristics across countries in the weighted sample.

	Germany N=1,001 n (%)	USA N=1,001 n (%)	Singapore N=1,004 n (%)
Gender			
Female	540 (53.9%)	521 (52.0%)	480 (47.9.%)
Male	460 (46.0%)	472 (47.1%)	517 (51.6%)
Gender-queer	1 (0.1%)	9 (0.9%)	6 (0.6%)
Age group[1]			
18–24	97 (9.7%)	126 (12.6%)	101 (10.0%)
25–39	262 (26.2%)	287 (28.6%)	283 (28.3%)
40–59	376 (37.5%)	350 (35.0%)	382 (38.1%)
60–75	266 (26.6%)	238 (23.7%)	237 (23.7%)
Degree of urbanization			
Rural area or village	276 (27.6%)	210 (21.0%)	–
Small or medium sized town	366 (36.6%)	423 (42.3%)	–
A city or large city	359 (35.8%)	368 (36.7%)	–
Educational level			
Less than high school	153 (15.3%)	33 (3.3%)	13 (1.3%)
High school	149 (14.8%)	195 (19.5%)	134 (13.4%)
Some college, no degree	19 (1.9%)	207 (20.7%)	241 (24.0%)
Associate degree	394 (39.3%)	110 (10.9%)	37 (3.7%)
Bachelor degree	135 (13.5%)	276 (27.6%)	484 (48.2%)
Master degree	145 (14.5%)	150 (15.0%)	79 (7.9%)
PhD	6 (0.6%)	30 (3.0%)	15 (1.4%)
Yearly household income			
Low	575 (57.5%)	334 (33.4%)	160 (15.9%)
Middle	366 (36.5%)	403 (40.2%)	462 (46.1%)
High	60 (6.0%)	264 (26.4%)	381 (38.0%)
Current diet			
Omnivore	434 (43.3%)	661 (66.0%)	537 (53.5%)
Flexitarian	440 (44.0%)	250 (24.9%)	371 (36.9%)
Vegetarian	78 (7.8%)	27 (2.7%)	55 (5.5%)
Vegan	34 (3.4%)	19 (1.9%)	18 (1.8%)
Others (e.g., No diet, Pescatarian, Halal, Mediterranean, Gluten-free)	15 (1.5%)	45 (4.5%)	23 (2.3%)

[1]Weights were generated based on age group (Refer to Supplementary material for weight calculations).
Degree of urbanization for Singapore participants was not measured as it was assumed that all participants live in the city.
Low income earners: ≤ €49,999, ≤ SGD35,000, ≤ USD39,999.
Middle income earners: €50,000–€99,999, SGD35,001–SGD100,000, USD40,000–$100,000.
High income earners: ≥ €100,000, ≥ SGD100,001, ≥ USD100,000.

> *scrambles, egg-fried rice, quiches and cakes. Not chicken, not egg, but microorganisms making protein, hence the product name: What Came Third.*
>
> *What Came Third does not involve any animals (nor the antibiotics that animals are often fed), does not contain cholesterol and causes less damage to natural ecosystems than industrial egg production.*

With this introduction, the research sought to simulate market conditions where the availability, awareness and understanding of PF egg products is higher than that of today.

After the passage, participants were shown a picture of a beaten egg and two egg dishes: scrambled eggs and omelet for the German and USA surveys, and scrambled eggs and egg fried rice (a favorite local dish in Asia) for the Singapore survey (Figure 1), in order to communicate the functionality and applications of PF made egg, especially distinguishing it from existing plant-based egg substitutes.

The survey was distributed in English for all three countries, and in German for participants in Germany during the period of January to February 2023. The survey was translated into German through a process of back-translation to ensure brevity without compromising on the survey questions' original meanings. This was carried out by

FIGURE 1
Pictures shown to participants when introducing the precision fermentation made product.

native speakers working at Formo, based in Germany. The questions were the same across all three countries, apart from some demographic questions such as degree of urbanization and income to account for country-specific differences.

Measures

Acceptance of PF egg products

Participants rated their willingness to: try this new product, order a dish from a restaurant/food stall made using this new product, purchase this new product in a supermarket, purchase this new product regularly, and their likelihood of visiting a restaurant where guests had the option to substitute chicken egg for this new product. These items were rated on a five-point scale (1 = *definitely not*, 5 = *definitely yes*). The scores of all five items were aggregated to form a composite measure (mean score of all items), where higher scores indicate a higher acceptance of the PF egg product. The scale demonstrated a high level of internal consistency (Germany: $\alpha = 0.95$; USA: $\alpha = 0.94$; Singapore: $\alpha = 0.93$).

Reasons attracting participant to purchase PF egg products

Participants were given a list of 13 reasons and were asked to select the reason that would most attract them to buy this new product (single-response question). The list of reasons was as follows: Less use of antibiotics, better animal welfare, great taste, less environmental impact, curiosity, fits with a vegan diet, use of new technology, no cholesterol, protein content, price, health, no egg allergens, and others. The reasons were presented in random order (except the 'others' option) to prevent any order effects.

Dietary habits

Current diet

Participants were asked to indicate their current diet. The options are as follows: Omnivore ("I eat animal products unrestrictedly"), flexitarian ("I'm trying to reduce my consumption of animal products"), vegetarian, vegan, and others. If participants indicated "carnivore" under the others option, it was re-coded under "omnivore".

Frequency of consumption of various egg products

Participants rated how frequently they consumed four egg products: Organic eggs, free range eggs, normal eggs, and plant-based egg alternative. These items were rated on a six-point scale (*never, less than once a month, one to three times per month, one to three times per week, four to six times per week, every day*).

Demographic variables

Demographic variables like gender, age, highest educational qualification, yearly household income, and degree of urbanization were included in our analyses. Gender was dummy coded with females as the reference category. Age, highest educational qualification (*less than high school, high school, some college no degree, associate degree, bachelor degree, master degree, PhD*), income and degree of urbanization (*a rural area or village, a small or medium sized town, a city or large city*) were treated as continuous variables. It was assumed

TABLE 2 Frequency of product consumption scores of the weighted sample.

	Range	Germany *N*=1,001 Mean (SD)	USA *N*=1,001 Mean (SD)	Singapore *N*=1,004 Mean (SD)
Frequency of product consumption	1–6			
Organic eggs		2.86 (1.24)	2.26 (1.39)	1.90 (1.23)
Free range eggs		3.09 (1.17)	2.52 (1.37)	2.37 (1.36)
Normal eggs		2.42 (1.32)	3.47 (1.27)	4.05 (1.12)
Plant-based egg alternative		1.58 (1.05)	1.56 (1.13)	1.51 (1.04)

1 = Never; 2 = Less than once a month; 3 = 1–3 times per month; 4 = 1–3 times per week; 5 = 4–6 times per week; 6 = Every day.

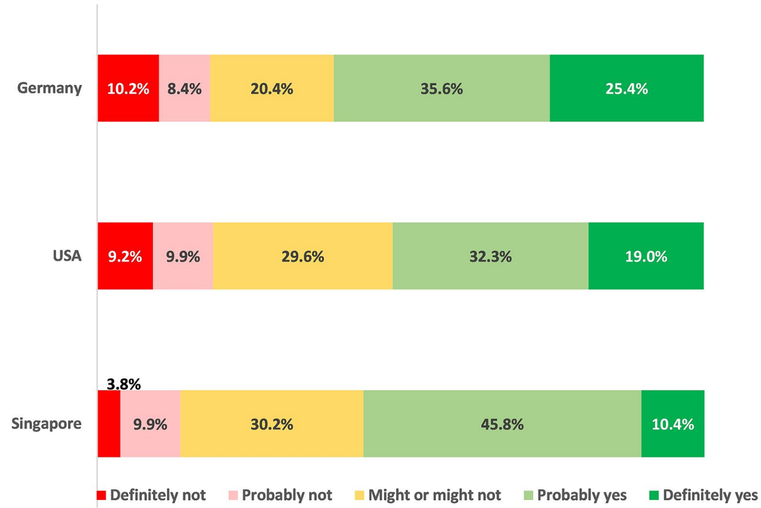

FIGURE 2
Willingness to try precision fermentation egg product across countries.

that all participants from Singapore lived in the city as there are close to no rural areas in the nation. Income was measured on a six-point scale for Germany (1 = *less than €25,000*, 6 = *€125,000 or more*) and USA (1 = *Less than USD20,000*, 6 = *USD125,000 or more*) and on an eight-point scale for Singapore (1 = *SGD15,000 or less*, 8 = *More than SGD150,000*). For our analyses, income was re-coded into three main categories for all three countries: low, middle and high.

Results

Demographic and dietary characteristics

We conducted all analyses with IBM SPSS Statistics 28.0. Samples were weighted to be nationally representative of the population in terms of age groups. Median annual income levels of the weighted samples closely corresponded to that of the population for the three countries (Bundesagentur für Arbeit, 2021; United States Census Bureau, 2022; Singapore Department of Statistics, 2023a). As for education, upper secondary school completion (high school and above) and the proportion of participants living in urban areas (small or medium sized town or a city or large city) closely corresponded to national figures for Germany and USA (World Bank, 2021; OECD Better Life Index, 2023). In Singapore, only 1.3% of the weighted sample did not complete high school education. This was below the national figure of 21% (Singapore Department of Statistics, 2023b). Hence, the post-weighted samples in Germany and USA were broadly representative of society in terms of education and degree of urbanization.

In terms of dietary characteristics, a large number of participants in Germany identified as flexitarians (44%) while the majority of participants identified as omnivores in USA (66%) and Singapore (53.5%). Germany also had the highest consumption frequency of organic (M = 2.86, SD = 1.24), free range (M = 3.09, SD = 1.17) and plant-based eggs (M = 1.58, SD = 1.05), followed by USA and Singapore, while Singapore had the highest consumption frequency of normal eggs (M = 4.05, SD = 1.12), followed by USA (M = 3.47, SD = 1.27) and Germany (M = 2.42, SD = 1.32; Table 2).

Acceptance of PF egg across countries

High acceptance levels of the PF egg product were seen across countries (Figures 2, 3). Around half of the total weighted sample were probably or definitely willing to try (56.1%) and purchase (51%) the product from the supermarket. Germany had the highest levels of willingness to try (61%) and purchase (57.2%) the product, followed by Singapore (try: 56.2%; purchase: 48.1%) and USA (try:

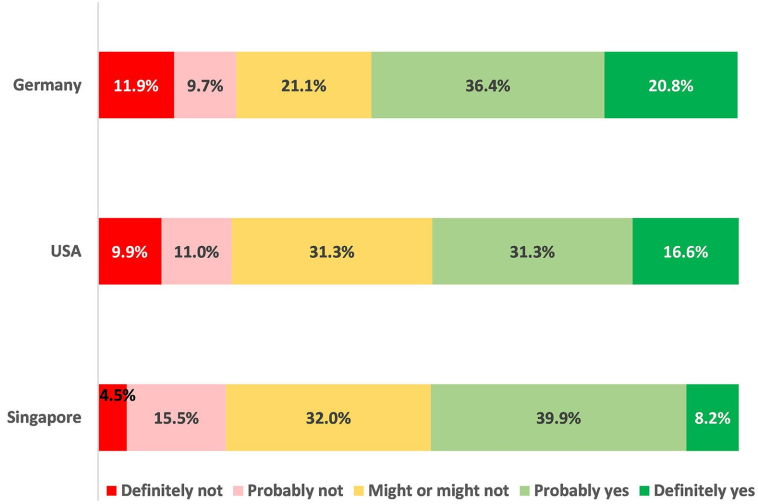

FIGURE 3
Willingness to purchase precision fermentation egg product across countries.

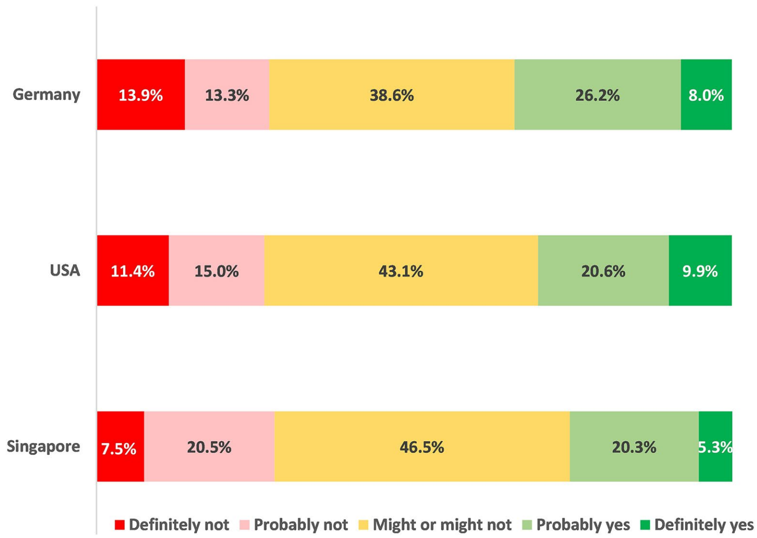

FIGURE 4
Willingness to regularly purchase precision fermentation egg product across countries.

51.3%; purchase: 47.9%). Singapore had lower levels of outright rejection but also lower levels of those who considered themselves definitely willing to try (10.4%) and purchase (8.2%) the product. Levels of willingness to purchase the product regularly were low across the three countries, with a large number of participants from the total weighted sample (42.7%) not knowing whether they might or might not purchase the product regularly (Figure 4).

The next part of product acceptance examined participants' willingness to order a dish from a restaurant/food stall made using this new product and their likelihood of visiting a restaurant where guests had the option to substitute chicken egg for this new product (Figure 5). The pattern of results was similar, with Germany leading in willingness to order a dish from a restaurant/food stall (55.1%), followed by Singapore (53.6%) and USA (46.7%). A higher proportion of Singaporeans were probably or definitely likely to visit a restaurant where guests had the option to substitute for this new product (46.9%), compared to Germany (43.9%) and USA (42%).

The effect of country on product acceptance of PF egg

A one-way ANOVA was conducted to examine the effect of country on product acceptance (Table 3). There were no significant differences between countries on overall product acceptance (composite score; *Welch's* F (2, 1974.13) = 1.24, p = 0.288) and willingness to purchase the product regularly (*Welch's* F (2, 1990.14) = 1.39, p = 0.251) in the weighted sample. There were significant differences between countries

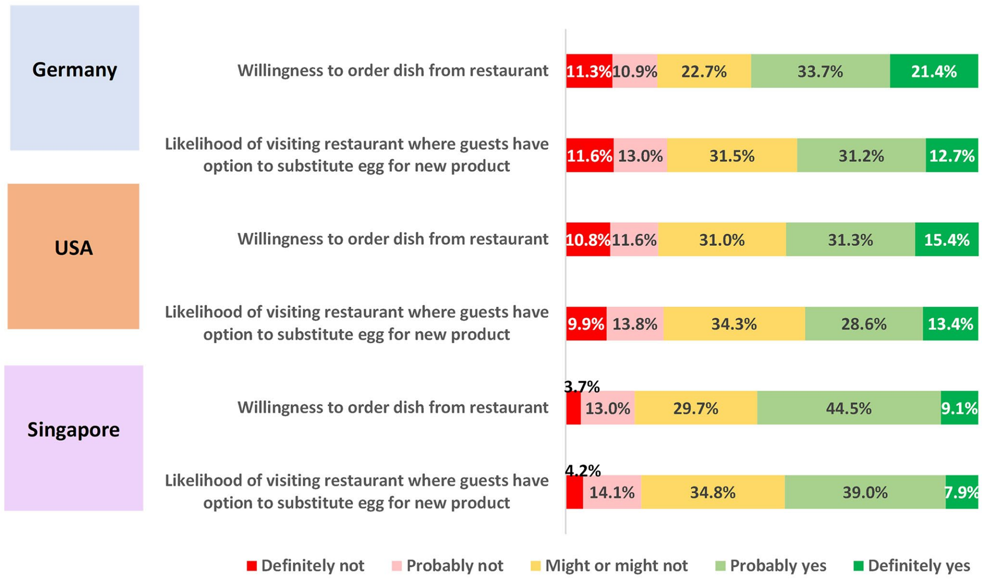

FIGURE 5
Willingness to eat precision fermentation egg product in restaurants across countries.

on willingness to try (*Welch's F* (2, 1969.60) = 4.18, $p = 0.016$), willingness to order the dish from a restaurant/food stall (*Welch's F* (2, 1971.44) = 4.69, $p = 0.009$), willingness to purchase the product from a supermarket (*Welch's F* (2, 1977.97) = 3.42, $p = 0.033$) and the likelihood of visiting a restaurant where guests have the option to substitute egg for the new product (*Welch's F* (2, 1982.79) = 3.99, $p = 0.019$).

Games-Howell post-hoc tests revealed than participants from Germany ($M = 3.57$, $SD = 1.24$) were significantly more willing to try the product as compared to participants from USA ($M = 3.42$, $SD = 1.17$, $p = 0.011$). Participants from USA ($M = 3.29$, $SD = 1.18$) were also significantly less willing to order a dish from a restaurant made using this new product as compared to those in Germany ($M = 3.43$, $SD = 1.25$, $p = 0.028$) and Singapore ($M = 3.42$, $SD = 0.96$, $p = 0.015$). Additionally, participants from Germany were significantly more willing to purchase the product from the supermarket ($M = 3.44$, $SD = 1.25$) as compared to participants from Singapore ($M = 3.32$, $SD = 0.98$, $p = 0.030$). However, participants from Singapore were significantly more likely to visit a restaurant where guests have the option to substitute egg for the new product ($M = 3.32$, $SD = 0.96$) as compared to those in Germany ($M = 3.21$, $SD = 1.17$, $p = 0.035$).

Dietary and demographic predictors of acceptance

To investigate if demographic and dietary variables may explain acceptance of the product across countries, a three-step hierarchical multiple regression was conducted within each country. Demographic variables (i.e., age, gender, income, degree of urbanization,[1] education) were entered in Step 1 of the regression. Current diet[2] (flexitarian, vegetarian, vegan and others) was entered in Step 2 and frequency of consuming various egg products (organic, free range, normal, plant-based eggs) were entered in Step 3 (Table 4). At each step of the model, we assessed the percentage of variance explained by the explanatory variables by calculating the model R^2. We also compared subsequent steps of the model to the previous steps (i.e., Step 2 vs. Step 1, Step 3 vs. Step 2) using ANOVA F-tests to determine if including additional variables significantly improved the explanatory power of the model.

In Step 1, we found that demographic variables predicted a significant amount for variance in product acceptance for all three countries (Germany: $R^2 = 0.045$, F (5, 995) = 9.49, $p < 0.001$; USA: $R^2 = 0.056$, F (5, 995) = 11.84, $p < 0.001$; Singapore: $R^2 = 0.036$, F (4, 999) = 9.29, $p < 0.001$).

Including diet in Step 2 improved the model significantly (Germany; $\Delta R^2 = 0.139$, ΔF (4, 991) = 42.11, $p < 0.001$; USA; $\Delta R^2 = 0.055$, ΔF (4, 991) = 15.34, $p < 0.001$; Singapore; $\Delta R^2 = 0.036$, ΔF (4, 994) = 9.71, $p < 0.001$).

Adding the frequency of consumption of various egg products in Step 3 further improved the model significantly (Germany; $\Delta R^2 = 0.032$, ΔF (4, 987) = 10.04, $p < 0.001$; USA; $\Delta R^2 = 0.075$, ΔF (4, 987) = 22.62, $p < 0.001$; Singapore; $\Delta R^2 = 0.064$, ΔF (4, 990) = 18.27, $p < 0.001$). In combination, the predictor variables explained 21.6% of the variance in product acceptance in Germany, 18.6% in USA and 13.6% in Singapore. We will report the results from Step 3 for concision.

In Germany, older ($B = -0.01$, $SE = 0.00$, $p < 0.001$) and higher educated participants ($B = -0.04$, $SE = 0.02$, $p = 0.032$) predicted lower acceptance of the product. However, higher educated participants in USA were more likely to accept the product ($B = 0.06$, $SE = 0.02$,

1 Degree of urbanization was not included in the regression analyses for Singapore.

2 Current diet was dummy coded with 'omnivore' serving as the reference category.

TABLE 3 One-way ANOVA showing between-country differences in product acceptance of precision fermentation egg for the weighted sample.

	Germany Mean (SD)	USA Mean (SD)	Singapore Mean (SD)	ANOVA
Composite score	3.33 (1.11)	3.26 (1.04)	3.30 (0.85)	*Welch's F* (2, 1974.13) = 1.24, *p* = 0.288
Willingness to try	3.57 (1.24) U	3.42 (1.17) G	3.49 (0.94)	*Welch's F* (2, 1969.60) = 4.18, *p* = 0.016*
Willingness to order dish from restaurant	3.43 (1.25) U	3.29 (1.18) GS	3.42 (0.96) U	*Welch's F* (2, 1971.44) = 4.69, *p* = 0.009**
Willingness to buy	3.44 (1.25) S	3.34 (1.17)	3.32 (0.98) G	*Welch's F* (2, 1977.97) = 3.42, *p* = 0.033*
Willingness to buy regularly	3.01 (1.13)	3.03 (1.10)	2.95 (0.96)	*Welch's F* (2, 1990.14) = 1.39, *p* = 0.251
Likelihood of visiting restaurant where guests have option to substitute egg for new product	3.21 (1.17) S	3.22 (1.15)	3.32 (0.96) G	*Welch's F* (2, 1982.79) = 3.99, *p* = 0.019*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Homogeneity of variance assumption has been violated ($p < 0.001$). Welch test is used instead of *F* test. Games-Howell *post-hoc* tests were used (Equal variances unassumed). Presence of superscript letters (S, U, G) indicate significant differences between particular countries.

$p = 0.012$). In both Germany ($B = 0.12$, $SE = 0.05$, $p = 0.033$) and Singapore ($B = 0.13$, $SE = 0.04$, $p < 0.001$), higher income participants were more likely to accept the product. In Singapore, males were more likely to accept the product as compared to females ($B = 0.23$, $SE = 0.05$, $p < 0.001$). In the USA, living in a more urbanized environment was associated with higher product acceptance ($B = 0.08$, $SE = 0.04$, $p = 0.049$).

As for diet, flexitarians in all three countries were more accepting of the product as compared to omnivores (Germany: $B = 0.71$, $SE = 0.07$, $p < 0.001$; USA: $B = 0.43$, $SE = 0.07$, $p < 0.001$; Singapore: $B = 0.26$, $SE = 0.06$, $p < 0.001$). Vegetarians in Germany ($B = 0.83$, $SE = 0.13$, $p < 0.001$), and vegans in Germany ($B = 1.06$, $SE = 0.19$, $p < 0.001$) and USA ($B = 0.60$, $SE = 0.23$, $p = 0.007$) were more accepting of the product as compared to omnivores.

Additionally, the consumption frequency of organic (Germany: $B = 0.09$, $SE = 0.03$, $p < 0.001$; USA: $B = 0.09$, $SE = 0.03$, $p = 0.003$; Singapore: $B = 0.07$, $SE = 0.03$, $p = 0.014$) and plant-based egg alternatives (Germany: $B = 0.13$, $SE = 0.03$, $p < 0.001$; USA: $B = 0.17$, $SE = 0.03$, $p < 0.001$; Singapore: $B = 0.13$, $SE = 0.03$, $p < 0.001$) positively predicted acceptance of the product in all three countries. Consumption frequency of normal eggs in USA ($B = 0.05$, $SE = 0.02$, $p = 0.040$) and that of free-range eggs ($B = 0.05$, $SE = 0.02$, $p = 0.019$) in Singapore positively predicted acceptance of the product.

Reasons for product acceptance

A Pearson's chi-square test of contingencies was used to investigate whether the top reasons attracting participants to buy PF egg products differed across countries.[3] The chi-square test was statistically significant, χ^2 (26, $N = 3{,}008$) = 325.70, $p < 0.001$. In Germany, the top three reasons were better animal welfare (22%), curiosity (18.2%) and less use of antibiotics (8.8%). In the USA, the top three reasons were curiosity (18.1%), health (11.6%) and no cholesterol (10.1%). In Singapore, the top three reasons were price (17.1%), health (14.8%) and curiosity (13.5%). Curiosity was a popular reason for all three countries, more so for participants in USA. German participants were mostly drawn to the product due to animal welfare reasons while price was the top reason that attracted Singapore participants to the product (Table 5).

3 Total weighted sample size is slightly different from the total count in Table 5 because the cell counts have been rounded.

Discussion

Understanding who, where, why and when cellular agriculture derived foodstuffs will find willing consumers is crucial for the commercial viability of cellular agriculture and relatedly, its potential to make large-scale social impact. In this section, we discuss the overall consumer acceptance of PF made egg products and the factors that may encourage consumer acceptance. Our results offer insight into the market level enthusiasm for PF made food products, while also illuminating for whom and why PF products will attract interest and adoption. Notably, our paper examined differences between countries, and the different dietary identities and habits that correspond to varying levels of enthusiasm for PF made food products. Our research also offers an overview of the driving reasons consumers see for the adoption of a PF egg product.

Overall acceptance

The results show that there were significant differences between countries on some specific aspects of product acceptance, but not on overall product acceptance. Overall, a substantial proportion of consumers, i.e., between 51 to 61% of participants in all three countries surveyed, were at least willing to try out PF egg products. These figures are comparable to past findings on consumer acceptance of cultivated meat, another cellular agricultural product (see Bryant and Barnett, 2020 for a review). However, compared to a previous study on consumer acceptance of PF cheese, which had an acceptance rate of over 70%, consumer acceptance of PF egg is relatively low (Zollman Thomas and Bryant, 2021).

Given that cellular agricultural products offer common benefits such as food safety and lower environmental footprints, it would be interesting for future studies to explore why there is a difference in consumer acceptance of PF egg as compared to PF cheese. It is possible that consumers are more accepting of PF cheese simply because dairy is the most established PF product category and has already made market inroads in some geographies (Good Food Institute, 2023b). However, there may be other possible explanations such as perceived product novelty or naturalness. Conventional eggs and meat are unprocessed ingredients, whereas conventional cheese already comes across as a processed food product (Monteiro et al., 2018). The idea of cheese being industrially processed may be more familiar and palatable to consumers, as well as the product format,

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TABLE 4 Hierarchical regression model showing demographic and dietary variables predicting product acceptance of precision fermentation egg.

	Germany		USA		Singapore	
	B (SE)	*P*	B (SE)	*p*	B (SE)	*p*
Step 1						
Demographic variables						
Age	−0.01 (0.00)***	<0.001	−0.01 (0.00)***	<0.001	−0.00 (0.00)	0.083
Gender (ref = females)	−0.10 (0.07)	0.153	0.09 (0.07)	0.171	0.27 (0.05)***	<0.001
Income	0.18 (0.06)**	0.002	0.07 (0.05)	0.179	0.11 (0.04)**	0.006
Degree of urbanization	0.06 (0.04)	0.161	0.11 (0.04)*	0.011	–	–
Education	−0.04 (0.02)	0.116	0.08 (0.03)**	0.002	−0.01 (0.02)	0.780
R^2	0.045		0.056		0.036	
Adjusted R^2	0.041		0.051		0.032	
F	9.487***		11.837***		9.289***	
Step 2						
Demographic variables						
Age	−0.01 (0.00)***	<0.001	−0.01 (0.00)***	<0.001	−0.01 (0.00)**	0.002
Gender (ref = females)	0.03 (0.07)	0.641	0.10 (0.06)	0.109	0.27 (0.05)***	<0.001
Income	0.14 (0.06)*	0.012	0.08 (0.05)	0.109	0.14 (0.04)***	<0.001
Degree of urbanization	0.03 (0.04)	0.412	0.09 (0.04)*	0.031	–	–
Education	−0.05 (0.02)*	0.027	0.07 (0.02)**	0.002	−0.02 (0.02)	0.474
Current diet (ref = Omnivore)						
Flexitarian	0.78 (0.07)***	<0.001	0.52 (0.07)***	<0.001	0.33 (0.06)***	<0.001
Vegetarian	0.91 (0.13)***	<0.001	0.48 (0.19)*	0.014	0.33 (0.12)**	0.005
Vegan	1.15 (0.18)***	<0.001	0.79 (0.23)***	<0.001	0.47 (0.20)*	0.018
Others	−0.24 (0.27)	0.366	0.16 (0.15)	0.301	0.24 (0.18)	0.175
R^2	0.184		0.111		0.072	
Adjusted R^2	0.177		0.103		0.065	
ΔR^2	0.139		0.055		0.036	
F	24.858***		13.774***		9.663***	
ΔF	42.113***		15.343***		9.712***	
Step 3						
Demographic variables						
Age	−0.01 (0.00)***	<0.001	−0.00 (0.00)	0.053	−0.00 (0.00)	0.356
Gender (ref = females)	0.00 (0.07)	0.954	0.02 (0.06)	0.736	0.23 (0.05)***	<0.001
Income	0.12 (0.05)*	0.033	0.03 (0.05)	0.550	0.13 (0.04)***	<0.001
Degree of urbanization	0.04 (0.04)	0.373	0.08 (0.04)*	0.049	–	–
Education	−0.04 (0.02)*	0.032	0.06 (0.02)*	0.012	−0.03 (0.02)	0.137
Current diet (ref = Omnivore)						
Flexitarian	0.71 (0.07)***	<0.001	0.43 (0.07)***	<0.001	0.26 (0.06)***	<0.001
Vegetarian	0.83 (0.13)***	<0.001	0.14 (0.19)	0.480	0.23 (0.12)	0.054
Vegan	1.06 (0.19)***	<0.001	0.60 (0.23)**	0.007	0.29 (0.20)	0.143
Others	−0.24 (0.26)	0.357	0.07 (0.15)	0.628	0.26 (0.17)	0.135
Frequency of consumption						
Organic eggs	0.09 (0.03)***	<0.001	0.09 (0.03)**	0.003	0.07 (0.03)*	0.014
Free range eggs	0.01 (0.03)	0.735	0.03 (0.03)	0.200	0.05 (0.02)*	0.019
Normal eggs	0.02 (0.03)	0.499	0.05 (0.02)*	0.040	0.01 (0.02)	0.614

(Continued)

TABLE 4 (Continued)

	Germany		USA		Singapore	
	B (SE)	P	B (SE)	p	B (SE)	p
Plant-based egg alternative	0.13 (0.03)***	<0.001	0.17 (0.03)***	<0.001	0.13 (0.03)***	<0.001
R^2	0.216		0.186		0.136	
Adjusted R^2	0.206		0.175		0.125	
ΔR^2	0.032		0.075		0.064	
F	20.927***		17.327***		12.980***	
ΔF	10.041***		22.619***		18.272***	

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Degree of urbanization was not included in the regression analyses for Singapore.

TABLE 5 Reasons that will attract participant to buy precision fermentation egg product across countries for the weighted sample.

	Germany N=1,001 n (%)	USA N=1,001 n (%)	Singapore N=1,004 n (%)
Reasons that will attract one to buy product			
Less use of antibiotics	88 (8.8%)	29 (2.9%)	39 (3.9%)
Better animal welfare	221 (22.0%)	93 (9.3%)	77 (7.6%)
Great taste	57 (5.6%)	97 (9.7%)	74 (7.4%)
Less environmental impact	66 (6.6%)	81 (8.1%)	76 (7.6%)
Curiosity	182 (18.2%)	181 (18.1%)	136 (13.5%)
Fits with a vegan diet	47 (4.7%)	22 (2.2%)	40 (4.0%)
Use of new technology	16 (1.6%)	39 (3.9%)	32 (3.1%)
No cholesterol	75 (7.5%)	101 (10.1%)	111 (11.0%)
Protein content	28 (2.8%)	74 (7.4%)	57 (5.7%)
Price	62 (6.2%)	93 (9.3%)	171 (17.1%)
Health	69 (6.9%)	116 (11.6%)	149 (14.8%)
No egg allergens	16 (1.6%)	22 (2.2%)	13 (1.3%)
Others (e.g., Safety, etc)	4 (0.4%)	8 (0.8%)	11 (1.1%)
None/not interested	71 (7.1%)	45 (4.5%)	18 (1.8%)

unlike a PF egg product which would not be sold as individual, shelled eggs. In evaluating the naturalness of a food, consumers consider not only the content of the end-product, but also the processes it has undergone as well (Rozin, 2006). Food that has been processed by traditional (i.e., older) means may be perceived as more natural than food that has been processed by recently developed technologies (Etale and Siegrist, 2021). Compared to PF cheese, PF eggs may be perceived as a more novel or unnatural product that can potentially induce food neophobia. Finally, people may simply perceive the production of dairy to be more objectionable than that of eggs, finding more reasons to replace dairy products in their diets. Consumers' stated reasons for being attracted to a PF egg product and how these precipitate differences in acceptance compared to other cellular agriculture products will be examined in the following sections.

Demographics

A number of demographic characteristics have been hypothesized to predict attitudes and behaviors with regard to APs. However, extant findings on the impact of demographic variables on consumer acceptance of APs are mixed (Nguyen et al., 2022). While some studies show that demographics influence consumer acceptance of APs (e.g., Gómez-Luciano et al., 2019; Orkusz et al., 2020), others report that the impact of demographics is insignificant (e.g., de Boer et al., 2013; Birch et al., 2019; Barton et al., 2020).

In our results, older and higher educated participants predicted lower acceptance of the product in Germany while higher educated participants in the U.S. were more likely to accept the product. In both Germany and Singapore, higher income participants were more likely to accept the product. In Singapore, males were more likely to accept the product as compared to females. In the U.S., living in a more urbanized environment was associated with higher product acceptance.

In step 2 of our hierarchical regression, age was a statistically significant predictor of enthusiasm for the product across countries, but our largely diffuse results mirror wider findings around AP acceptance, with variable and/or weak connections seen between demographics such as gender or degree of urbanization and enthusiasm, while generally showing a prevailing trend that younger and more educated consumers are more likely to accept

APs (de Boer et al., 2013; Birch et al., 2019; Gómez-Luciano et al., 2019; Siegrist and Hartmann, 2019; Wilks et al., 2019).

In our results, only one demographic factor consistently predicted acceptance of PF egg products across regression steps and in at least two countries – income. That is, an increase in income was related to higher acceptance of PF egg products. This finding is supported by Tucker (2014), which showed that higher income positively affected consumers' perception of APs. One possible explanation for the income-acceptance relationship may be perceived affordability. That is, the higher one's income, the more affordable a novel food product such as PF egg is perceived to be. Price – and by extension, affordability – was found to play an important role in motivating AP consumption and in moderating consumer demand for APs (Slade, 2018). In a recent study, the Good Food Institute (2022) reported price to be a barrier to the consumption of APs: e.g., "consumers ranked price as the second-most important factor (behind taste) to encourage or discourage them from purchasing a plant-based product" (Good Food Institute, 2022, p. 5).

A second possible explanation for the relationship between higher income and higher acceptance of PF egg is that individuals with higher-income backgrounds have been associated with higher novelty-seeking scores (Lahti et al., 2006). As novelty seeking is one of the factors that can drive AP consumption (Tan et al., 2016; Apostolidis and McLeay, 2019; Mancini and Antonioli, 2019), we posit that higher-income individuals are more likely to engage in novelty-seeking behavior, which in turn drives their willingness to consume PF egg. This is an area that merits further research.

Dietary identities

As public awareness around the health, environmental and ethical consequences of unmoderated animal product consumption and production have risen, a greater share of consumers are changing their dietary identities to reflect this concern (Sanchez-Sabate and Sabaté, 2019).

Given that the development of cellular agriculture was born from a willingness to directly address concerns around unhealthy, unsustainable and degrading food practices (Mattick, 2018), examining how those pursuing alternative diets evaluate the appeal of cellular agriculture has been a natural focus for much of the research community when examining the societal adoption of novel foodstuffs (Stephens et al., 2018). Our research too examines the relationship between dietary identities and a willingness to consume a PF made egg product, with some findings corroborating existing research around the acceptance of cellular agriculture foods (Bryant et al., 2020; Zollman Thomas and Bryant, 2021), and some indicating notable differences in the dynamics toward attitudes surrounding a PF produced egg substitute. The predictive power of these dietary identities stands in contrast to our demographic data, which gently reiterated findings around age while also pointing to a moderate relationship between income and product enthusiasm.

Flexitarians

Our work provides insight into the volume of consumers electing to pursue alternative diets currently, as well as the relationship between these choices and an openness to embrace PF made foodstuffs. Our data replicates many existing surveys' findings regarding the level of vegans, vegetarians and flexitarians in Germany, Singapore, and the USA (Ho, 2020; Dagevos, 2021), with, once again particularly notable levels of flexitarians in Germany (forming a higher overall percentage of society than omnivores), as well as displaying the highest levels of veganism and vegetarianism among our sampled countries. While apparently closer to Europe in terms of culinary traditions and social traditions, Germany and Singapore were seen to be closer in level of animal product abstainers than the USA, showing a smaller percentage of its population to identify as flexitarians, vegetarians or vegans than Singapore or Germany.

In accordance with previous findings, our results showed strong predictive power to be associated with diet on the embrace of a novel food product (Szejda et al., 2021; Zollman Thomas and Bryant, 2021). Of all surveyed traits, diet had the strongest influence on acceptance, exhibiting both a stronger and more statistically significant relationship with PF egg acceptance than other examined demographic traits such as age, gender, income and education. Specifically, flexitarians were significantly more likely, in every country, to see themselves as future consumers of PF produced egg products than omnivores.

This finding, viewed in conjunction with the recorded burgeoning of the flexitarian movement (Dagevos, 2021), suggests that as more of society begins to acknowledge a need, and develops a readiness to reduce consumption of animal products, a wider pool of consumers are likely to be drawn to the fruits of cellular agriculture, in particular, PF made food. The noted capacity of new technologies to prompt wider societal norm changes (Verbeek, 2011) may well create a compounding effect, of awareness and necessary change arising from the introduction of PF made egg, particularly in an area such as egg production, which currently draws comparatively less public attention for its impact on the environment or the welfare of chickens than mammalian-based agriculture (Alonso et al., 2020).

Vegetarians and vegans

While an anticipated advantage of cultivated meat and PF food categories is its capacity to move beyond audiences typically served by plant-based animal substitute products (Silva and Semprebon, 2021), understanding the acceptance and enthusiasm of this consumer grouping, namely vegans and vegetarians, provides insight both into the foothold to be gained in this dedicated section of the market, and to understand whether and how new products will widen the range of consumers substituting away from animal products.

Existing research shows cultivated meat and, to a lesser extent, PF made dairy products, to be of strongest interest to those who currently consume meat and dairy respectively, with flexitarians having generally displayed the highest willingness to adopt products made through cellular agriculture (Bryant, 2020; Zollman Thomas and Bryant, 2021). Vegan consumers are notable in displaying more ambivalence toward products created through these new technologies than vegetarians or flexitarians (Baum et al., 2022), with potential reasons being the animal cell origins of cultivated meat, an aversion to unnaturalness or their success in having already removed meat and dairy from their diets (Faccio and Nai Fovino, 2019). Indeed, within groups reducing animal product consumption, enthusiasm for cultivated meat or PF is typically lowest among vegans, higher among vegetarians and again higher among flexitarians (Bryant et al., 2019).

Our findings regarding the acceptability of a PF produced egg products are highly notable given the reversal of this trend, with our regression showing vegans to display the highest levels of enthusiasm for such a product, with flexitarians showing relatively lower enthusiasm than vegans and vegetarians. These relationships hold in all of the observed countries. The reversal of this relationship suggests that consumers of PF made egg may share relatively more similarities with the consumers of plant-based APs, which are relatively more favored by vegans (Smart Protein Project, 2021), than the anticipated consumers of cultivated meat, or even PF made dairy products.

Numerous factors could explain this, related to the relationship of both flexitarians and vegans to eggs and existing egg alternatives, and their perceptions of other cellular agriculture technologies. Relationships that warrant further research is whether for some vegans, egg may be a more difficult product to find suitable replacements for, or similarly, the attitude of flexitarians toward eggs. As mentioned, eggs are products that are not viewed as carrying an equivalent moral burden to that of the beef or dairy industry (Alonso et al., 2020), with these attitudes potentially explaining why relatively more flexitarians are less interested by the use of novel technologies to replace eggs. Flexitarians often cite environmental reasons as the grounds for their dietary choices (Sanchez-Sabate et al., 2019), and just as these groups may hold a diminished association of chicken eggs to the most deplorable animal-welfare conditions, chicken eggs too are likely not associated with the dire environmental consequences that beef and dairy are (Hartmann et al., 2021). Finally, it may simply be that for many vegans who are relatively less enthusiastic about the use of cell culturing or the genetic engineering of microorganisms, applying PF technology in this setting is simply more acceptable than the animal biopsies necessary to begin the cell-culturing process, or the identical replication of dairy cow DNA in microorganism hosts, than the replication of egg proteins in microorganisms.

Consumption habits

While it is necessary to view the acceptance of PF egg through a social and political lens, considering the attitudes and heuristics driving acceptance of new food technology, the focuses of consumer scientists - consumer habits and purchasing patterns - are also highly relevant when seeking to understand the anticipated adoption and purchase decisions of consumers, and certainly more neglected when examining acceptance of novel foods, given scant primary data. While some studies have investigated the role of dietary behaviors in predicting attitudes to plant-based products (Kester, 2023) or cultivated meat (Malek and Umberger, 2021), little research thus far has examined consumers' granular dietary behavior, such as the choices and degree of consumption of conventional products and existing substitutes, with focus on how these relate to novel food products, especially PF made products. When noting that the impact of cellular agriculture depends not on the volume of consumers likely to adopt it, rather the volume of consumers' purchases of animal products that are eschewed given its introduction, this focus is overdue in the field.

In this way, our observations about the relative differences in egg consumption behavior between countries, and their observed relationship to PF acceptance, should be of especial note, not just for those seeking to understand the mechanics of a societal shift toward cellular agriculture, but also for those looking to understand how markets and product categories will be impacted by PF's emergence.

Our results show significant differences in the frequency of different categories of egg consumption across countries, with German respondents reporting a higher level of organic, and free-range egg consumption, while consuming lower levels of 'normal eggs' (i.e., eggs with no higher-welfare certifications). While Singaporeans reported the highest levels of 'normal' egg consumption, they reported the lowest levels of organic and free-range egg consumption, with Americans falling, respectively, between the other two countries in terms of consumption levels. These values demonstrate immediately the differences between German, American and Singaporean attitudes toward egg consumption, and the behavior already exhibited that reflect context, assessments and priorities within their "egg-buying" environments.

We found that the frequency of consuming plant-based eggs was positively related to the acceptance of PF produced egg products. Prior research has found high levels of consumer dissatisfaction with existing vegan substitutes for animal products (Rondoni et al., 2021). As such, it is likely that consumers who consume vegan egg substitutes remain on the lookout for better alternative proteins, which can more holistically satisfy their varied demands for nutrition, safety, and sensory appeal on top of being cruelty-free and planet-friendly, hence their observed enthusiasm for a PF egg product.

Similarly, a strong positive relationship between organic egg consumption and consumer acceptance of PF produced egg products suggests that those who do consume eggs, but exert relatively more effort to consume higher welfare standard eggs are more likely to embrace PF produced egg products. This fits with Heidemann's work into cultivated meat acceptance which linked the emergence of cultivated meat with support for organic animal rearing (Heidemann et al., 2020). Seeing the direction and strength of these relationships in all countries suggests that consumer behavior acts as a relatively robust means to anticipate the types of consumer segments and habits that PF egg consumption will correspond to, in juxtaposition to factors such as age, gender and education.

Reasons

Beyond an examination of the demographics and dietary trends cellular agriculture will likely synchronize with, an examination of the reasons why consumers are attracted to PF made food is a necessary undertaking. Existing research shows animal product reduction to be driven by both personal motives, such as health and taste, and prosocial concerns, including environment and animal welfare (Armstrong Soule and Sekhon, 2019), with the adoption of meat replacement products majorly derived from environmental factors, with lower impact derived from health messaging (Silva and Semprebon, 2021; Ye and Mattila, 2021). The examination of the reasons consumers find compelling poses additional insights into why people are likely to make the shift from traditional animal-based foods to PF products, offering help to identify the consumer priorities and opportunities that may facilitate a shifting of societal protein consumption.

The present study revealed that the driving forces behind the trial of PF egg products differed among the three countries. German participants were primarily motivated by their perception of the products' benefits for animal welfare and reduced use of antibiotics. By contrast, Americans were drawn to the health benefits, such as a lack of cholesterol of PF egg products. Singaporean participants were found to be influenced by a combination of health and price considerations.

Interestingly, across all three countries, participants indicated that they are open to consuming PF egg products because of their curiosity. It is reasonable to argue that as PF products are currently one of the most innovative alternatives to animal-made products, many potential consumers are attracted to this novel food as a result of their curiosity and novelty-seeking tendency. In fact, this is consistent with the prior finding that novelty seeking has been shown to be a key driver for promoting the consumption of APs (Apostolidis and McLeay, 2019; Mancini and Antonioli, 2019). It would be worthwhile for future research to examine the sustained interest or acceptance of PF products after first-time consumers have tried the products to satisfy their curiosity.

Another notable finding is that among the three countries, particularly within Singapore and the USA, that respondents listed health-related reasons (i.e., use of less antibiotics, no cholesterol) for explaining their openness to PF egg products, suggesting the potential for overlap both with personal and prosocial reasons for a PF egg product. This aligns with the evidence that health concerns or health consciousness act as drivers or barriers to accept plant-based meat alternatives (e.g., Siegrist and Hartmann, 2019) and cultivated meat (e.g., Verbeke et al., 2015; Adámek et al., 2018; Grasso et al., 2019). The current finding echoes prior research that health is a highly relevant factor that prospective consumers deliberate about when considering the acceptability of APs, including new PF products.

As previously mentioned, interest in a PF egg product was moderately lower than interest for a PF dairy product, of which consumers perceived significant environmental benefits over standard dairy products, with a comparable perception of health qualities (Zollman Thomas and Bryant, 2021). The absence of environmental reasons provided by consumers in our survey could explain some of the difference seen between PF product categories.

Conclusion

The findings of this research suggest that PF made egg products are likely to find a willing market, with a substantial proportion (51–61%) of consumers in the USA, Germany and Singapore at least willing to try out such a product. While there were significant differences between countries on some aspects of product acceptance, overall acceptance of the product was comparable across the countries. The strongest predictor of acceptance was dietary identity, with flexitarians being significantly more likely to accept the product than omnivores in all countries, with vegetarians and vegans displaying still higher enthusiasm, in contrast to major findings around cultivated meat and PF dairy acceptance. Other predictors of acceptance included degree of urbanization in the USA and age in Germany. Consumption habits also significantly predicted acceptance, with those consuming higher welfare standard eggs, and those consuming plant-based egg substitutes being more likely to accept the product. Reasons for acceptance included health, animal welfare, price and curiosity.

Future research is warranted to examine two major areas: deeper insight into the nature and mechanics of consumer acceptance of PF products, and, how this can be altered by experiences, framing and targeting. A more detailed examination of the differences between countries is warranted, as well as the impact of demographics, dietary identity and consumption habits on acceptance, especially when sampling the product, or being exposed to it in a supermarket, restaurant or food-service environment. Our research did not control the scepticism or trust that consumers placed in the product performance and product claims. Future research should examine the extent to which consumers believe product claims, how this shapes expectations of the product, and how these factors interact with willingness to try and buy. In this respect, the efficacy of different strategies to overcome scepticism, neophobia and reluctance would be of particular value for those looking to market PF based products to consumers, or, equally, discourage their uptake.

Further fruitful avenues of research may include direct examinations of the differences in consumer acceptance of PF egg compared to PF cheese or other products, and to examine the levels of sustained interest or acceptance of PF products after initial curiosity is satisfied, and when, if ever, consumers would consider completely substituting away from conventional eggs. Finally, research should investigate the reasons why vegans and vegetarians are comparatively more likely to accept PF egg than flexitarians, and how this could inform the marketing and positioning of different PF products.

The findings of this research and the outcomes of future research may well prove instrumental in normalizing the consumption of PF produced egg products, in turn altering how we eat. In parallel, the uptake of PF products may well "denormalise" the vast, industrialized processes behind contemporary animal product consumption, in turn altering how they live.

Data availability statement

The datasets presented in this article are not readily available because data is not readily available to outside researchers. Requests to access the datasets should be directed to oscar@formo.bio.

Ethics statement

The studies involving human participants were reviewed and approved by SMU Institutional Review Board. The participants provided their informed consent online to participate in this study.

Author contributions

OZT: conceptualization, translation, and data collection. OZT, MC, and AL: research design, survey instrument design, and analysis. TF, MC, SN, and OZT: data processing and data analysis. All authors contributed to the drafting and editing of the manuscript and approved the submitted version.

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OZT was employed by Formo Bio GmbH.

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Supplementary material

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Plant protein consumption is associated with body mass index among women of reproductive age in Indonesia

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Introduction: One of the known determinants of obesity in Southeast Asia countries, including Indonesia, is the nutritional transition, which is indicated by fast changes in food production, dietary habits, and physical activity. With rising incomes, plant protein from grains, tubers, and legumes is gradually being replaced by animal protein from poultry, eggs, dairy, and red meat. This change is identified as a protein transition. Different choices of protein sources in the diet have varying health effects. However, there is limited information on the Asian population on the role of protein consumption on the increasing obesity prevalence. Therefore, this study aimed to investigate the association of protein sources consumption with body mass index (BMI) among women of reproductive age in Indonesia.

Methods: This study used secondary data from the 2018 Indonesia Food Barometer (IFB) conducted using a quantitative cross-sectional survey. A total of 467 Indonesian reproductive-aged women (20–49 years) were included in this study. Dietary intake, including protein consumption, was obtained using 24-h dietary recall. Multiple linear regression was applied to find the association of protein consumption with BMI with a *p*-value <0.05 considered as a significant outcome variable.

Results: The Mean BMI was 25.02 kg/m2, median of animal and plant protein was 28.01 g/day and 25.37 g/day, respectively. Consumption of plant protein was significantly associated with BMI after adjusting for marital status and age (*p*-value = 0.043; R^2 = 0.080). The quality of plant protein should be considered to prevent obesity problems among women of reproductive age.

KEYWORDS

body mass index, Indonesia, obesity, plant protein, protein consumption, women of reproductive age

1. Introduction

The global health challenge recognizes obesity as an increasing health problem among women of reproductive age in low and middle-income countries (LMICs) (1). According to the World Health Organization (WHO) (2) in 2020, about 22% of the world's adult population (17% of men and 25% of women) were obese. Southeast Asia (SEA) countries exhibit varying prevalence of obesity, ranging from below 10% to nearly 30% (3–5). Indonesia, in particular, experienced a 6.4% increase in obesity between 2013 and 2018 (6, 7), with a higher prevalence among women (29.3%) as compared to men (14.5%). Specifically among women of reproductive age, the prevalence of obesity was 24.7% (6). Being obese is a significant risk factor for non-communicable diseases (8) and it also contributes to adverse maternal and fetal complications (9).

The nutritional transition, characterized by rapid changes in food production, eating habits, and physical activity, is a known predictor of obesity prevalence in SEA countries, including Indonesia (9, 10). As lower and middle-income countries experience rising incomes, there is a shift from plant to animal proteins in their diets, while the overall protein content remains constant (10–12). In contrast, high-income countries are promoting a transition towards plant-based proteins. Thus, protein intake appears to be determined not only by economic factors but also by geography, religion, ethnicity, modernization, and culture (12–14).

Evidence suggests that different types of protein have varying impacts on health. Higher protein consumption, animal-derived proteins, particularly from processed and red meat, has been linked to an increased risk of overweight and obesity (15). In contrast, plant protein has shown positive association with changes in waist circumference and reduced weight (16). Healthy plant-based dietary patterns emphasizing whole grains, fruits/vegetables, nuts/legumes, vegetable oils, and tea/coffee have been associated with lower weight gain, while less-healthy plant items such as fruit juices, potatoes, and refined grains have been consistently linked to increased weight gain (17).

Despite the significance of proteins in dietary guidelines, there is limited research on the association between protein consumption and the rising obesity prevalence in Asian populations. Therefore, this study aims to investigate the association of protein source consumption with body mass index (BMI) among women of reproductive age in Indonesia. By leveraging the 2018 Indonesia Food Barometer (IFB) dataset, this study aims to explore the relationship between protein source consumption and body mass index (BMI) among women of reproductive age in Indonesia.

2. Materials and methods

2.1. Study design and population

This study utilized available data from the 2018 Indonesia Food Barometer (IFB) study dataset. The IFB employed a quantitative cross-sectional study design and was conducted in six provinces across Indonesia, consist of West Sumatra, Jakarta, West Java, East Java, Bali, and South Sulawesi. Data collection for the IFB took place through in-person interviews conducted between March and July 2018. Subsequently, the present analysis was conducted between November 2022 to April 2023. The present analysis involved 467 women of reproductive age in the IFB dataset, which fulfilled the following criteria, i.e.: women aged 20–49 years and did not have special conditions, such as pregnant and lactating. Women experiencing changes in diet due to illness were excluded in the study. The sample was determined using total sampling, which means that all respondents who meet the inclusion and do not meet the exclusion criteria for this study was included. The amount of sample was already above the required minimum sample size needed, which was calculated to correlate protein consumption and obesity based on the previous study (18).

2.2. Socio-demographic characteristics

Socio-demographic variables consist of age, level of education, wealth index, type of residence, marital status, and occupational physical activity. Age was categorized as younger age (20–34 years old) and older age (35–49 years old). Level of education was captured as lower education (primary – lower secondary) and higher education (upper secondary – college). For wealth index, the scores were calculated based on principal component factors analysis with varimax rotation and were split into tertiles, with T1 as the lowest income, T2 is the middle income, and T3 is the highest income. Type of residence was separated into rural and urban area. Marital status was identified into two categories, which were unmarried and married. In addition, occupational physical activity (OPA) was defined as the type of activity that is associated with a job. OPA classification was determined by Steeves et al. (19).

2.3. Measurement of body mass index

This study used BMI score as an indicator of obesity. BMI is a straightforward index calculated by dividing an individual's weight in kilograms by the square of their height in meters (kg/m^2). This measurement is commonly used to classify overweight and obesity in adults. Data was collected using anthropometric measurement, following a standardized procedure (20). The measurements were taken using a stadiometer to determine the height and a scale to measure weight.

2.4. Assessment of dietary intake

Dietary intake data were collected by single 24-h food recall, using multiple sources method (MSM). MSM was used for evaluating nutrient data and calculating usual nutrient intakes from 24-h nutrient data using the cut point method (21). To assess inter-relationship between nutrient intakes of individuals to individual nutritional status, multiple recall should be applied. The MSM enabled to combine 24-h recall data with multiple 24-h recall data from the 20% subjects that was available. Initially, individuals were asked to write down everything they had eaten and drunk the day before the survey, from the time they woke up until they went to bed at night. For each food item, respondents estimated the quantities consumed using a food photograph bool of portion sizes (20). Protein consumption was calculated using a customized version of Nutri-Survey for Windows 2007. The software was manually updated with the latest available version of the Indonesian Food Composition Table from 2017. This table included 1,128 foods, including cooked dishes and menus, categorized into 13 food groups. It provided data on energy, water content (moisture), and 19 different nutrients. For this study, the nutrients of interest were energy (measured in kcal) and protein (measured in grams). For dishes or food not listed in the Indonesian FCTs, the recipe approach was used to calculate the nutrients.

The variable of protein sources consumption in this study consisted of four components, which were total protein intake, animal-based protein consumption, plant-based protein consumption, and the ratio of animal to plant-based protein. Total protein intake is defined as the sum of animal-based and plant-based protein intake based on a single 24-h recall, measured in grams per day (22). Animal-based protein sources consisted of poultry, eggs, milk and dairy products, fish and seafood, red meat, and pork. For plant-based protein, included grains and legumes. Furthermore, the ratio of animal to plant-based protein was calculated as the total gram of animal-based protein divided by the plant-based protein ratio (23). This ratio provides insight into the proportion of protein consumed from animal sources compared to plant sources. Additionally, other dietary intakes, including energy, carbohydrate, and fat, were also assessed using the same 24-h recall method, with measurements reported in grams per day (24). Dietary intake was adjusted by energy using energy residual method. To ensure the quality of the dietary data and minimize potential bias, energy under- and over-reporting was estimated using the predicted total energy expenditure (pTEE) technique (25). This technique applied cut-off to identify cases where participants may have provided inaccurate information about their energy intake. Specifically, individuals whose reported energy intake fell outside the range between 40–160% of the predicted total energy expenditure were flagged for further consideration and analysis.

2.5. Statistical analysis

The SPSS version 25 was used for data analysis. Statistically significant differences between groups were analyzed using an independent t-test for two groups and One-way ANOVA for more than two groups. Pearson correlation was conducted to analyze the correlation between protein and other dietary intakes with BMI score. Furthermore, Multiple Linear Regression analysis was applied to the association between protein consumption and other covariate variables with BMI score with a p-value <0.05 and 95% CI.

3. Results

The distribution of the socio-demographic characteristics and BMI among the subjects is presented in Table 1. The age distribution of the respondents was relatively balanced, with 50.3% categorized as younger age and 49.7% as older age. The majority of the subjects (60%) had attained higher education, resided in urban areas (55.2%), were married (83.3%), and had a low-intermediate level of occupational physical activity (80.9%). The wealth index was divided into tertiles, with 26.1% classified as having a low wealth index, 37.3% a middle wealth index, and 36.6% a high wealth index. The mean of subject's BMI score was 25.02 kg/m^2.

Table 2 shows that the subjects had a mean total protein intake of 55.98 g/d. In terms of protein sources, the subjects had a median intake of 28.01 g/day of animal-based protein and 25.37 g/day of plant-based protein. Additionally, the median ratio of animal to plant-based protein consumption was 1.5. The average energy, carbohydrate, and fat intakes were 1584.76 kcal/day, 205.10 g/day, and 59.70 g/day, respectively.

The association between protein consumption and BMI among women of reproductive age, after adjusting for covariate variables, is presented in Table 3. The analysis was conducted separately for total protein intake, animal-based and plant-based protein consumption, and the ratio of animal to plant-based protein ratio, to understand each association with BMI score. The initial covariate-adjusted in the analysis were carbohydrate intake, fat intake, age, level of education, marital status, and occupation physical activity (Supplementary Tables S1, S2). However, further analysis found that age and marital status significantly confounded the association between protein consumption and BMI.

The study found that total protein intake did not show a significant association with BMI score, after adjusting for confounding variables. However, there was a tendency indicating a positive correlation, suggesting that higher protein intake results in a higher BMI score. Similarly, the association between animal-based protein consumption with BMI was not significant, indicating no link between animal protein and BMI in this study. In contrast, the plant-based protein was found to be significantly associated with BMI score, suggesting higher plant protein consumption is linked with higher BMI. In terms of animal-to-plant-based ratio, there was no significant association with BMI score among women of reproductive age after adjusting with the covariate variables.

TABLE 1 Socio demographic characteristic and BMI score of the subjects.

Variables (n = 467)	Mean ± SD	Frequency (n)	Percentage (%)
BMI score (kg/m^2)	25.02 ± 4.58		
Age			
Younger age (20–34 years old)		235	50.3
Older age (35–49 years old)		232	49.7
Level of education			
Lower education		187	40.0
Higher education		280	60.0
Wealth index[a]			
Wealth T1		122	26.1
Wealth T2		174	37.3
Wealth T3		171	36.6
Type of residence			
Rural		209	44.8
Urban		258	55.2
Marital status[b]			
Unmarried		78	16.7
Married		389	83.3
Occupational physical activity[c]			
High		65	13.9
Low-intermediate		402	86.1

[a]Wealth Index was divided into tertile, with T1 as the lowest income, T2 is the middle income, and T3 is the highest income.
[b]Marital status was categorized as unmarried (single/divorced) and married (living together or not).
[c]Occupational physical activities (OPA) defined as the type of activity that is associated with a job. OPA classification was determined by Steeves et al. (19).

TABLE 2 Protein and other dietary intake of the subjects.

Variables	Mean ± SD	Median (Min – Max)	E (%)
Total protein intake (g/d)	55.98 ± 10.75		14.9
Animal-based protein consumption (g/d)		28.01 (0.00–103.56)	7.76
Plant-based protein consumption (g/d)		25.37 (5.80–70.27)	6.59
Ratio of animal-based to plant-based protein		1.50 (0.00–7.43)	
Energy intake (kcal)	1584.76 ± 371.97		
Carbohydrate intake (g/d)	205.10 ± 34.69		54.44
Fat intake (g/d)	59.70 ± 12.21		35.76

All multivariate analyses in Table 3 show that BMI score was associated with age and marital status among women of reproductive age. Older age and married women were shown to be associated with higher BMI. All models explained only 8% of the variation of BMI among women of reproductive age, indicating that there were more factors other than those used in the study to explain BMI variation among women of reproductive age.

TABLE 3 Association of protein consumption with BMI among women reproductive age in Indonesia.

Variables	BMI scores			
	β	SE	95% CI	*p*-value*
Model 1[a]				
Constant	10.146	7.736	−5.057 – 25.349	0.190
Age	1.364	0.429	0.521–2.207	**0.002**
Marital Status	1.850	0.151	0.603–3.099	**0.004**
Total protein intake (g/d)	0.043	0.028	−0.011 – 0.098	0.108
Model 2[b]				
Constant	16.960	6.351	4.479–29.441	0.008
Age	1.349	0.429	0.507–2.192	**0.002**
Marital Status	1.832	0.635	0.584–3.080	**0.004**
Plant-based protein consumption (g/d)	0.052	0.026	0.002–0.102	**0.043**
Animal-based protein consumption (g/d)	0.009	0.018	−0.027 – 0.045	0.620
Model 3[c]				
Constant	22.830	5.265	12.483–33.177	<0.001
Age	1.364	0.429	0.522–2.207	**0.002**
Marital Status	1.830	0.636	0.581–3.079	**0.004**
Ratio animal to plant-based protein	−0.335	0.208	−0.745 – 0.074	0.108

[a]Total protein intake and BMI score adjusted with sociodemographic characteristic and other dietary intake.
[b]Animal protein, plan protein, and BMI score adjusted with sociodemographic characteristic and other dietary intake.
[c]Ratio of animal to plant-based protein and BMI score adjusted with sociodemographic characteristic and other dietary intake.
*Multiple linear regression with enter method (sig. *p*-value < 0.05), printed bold.

4. Discussion

The study population represented women of reproductive age in Indonesia, which characteristics were almost similar to the Indonesian population according to the 2017 Indonesia Demographic and Health Survey, 42% were categorized as younger age, 72% were married, 52% resided in the urban area, more than half women reproductive age had upper middle wealth index. However, a different characteristic was shown in the level of education, only 42% women of reproductive attend higher education (26). Prior study had shown that women have higher risk of obesity compared to man (27, 28). Obesity among women of reproductive age is rising despite maternal undernutrition still being prevalent, especially in LMICs. Furthermore, the consequences of obesity among obese women not only lead to several adverse maternal but also fetal complications during pregnancy, delivery, and postpartum (9).

The subjects' mean total protein intake was 55.98 g/d, which was consistent with earlier study (13, 29), but slightly below the recommended protein daily values for Indonesian women of 60 g/d (30). The average energy intake of the subjects was 1584.76 kcal/day, consistent with previous research, which found that women of reproductive age consume approximately 1,500 kcal per day (22, 31). Dietary intake of this study was adjusted by energy using energy residual method. Energy adjustment should be applied to separate the effect of energy intake from specific nutrient in relation with the diseases or nutritional status (32).

The average eating pattern in Indonesia is characterized by more dependence on a single staple. Non-starchy foods accounted for 30% of total dietary energy intake, substantially below the global average of 50% (33). However, the findings of this study have shown a slightly higher median intake of animal compared to plant protein with the ratio of animal to plant protein is 1.5. It indicated a shift in protein consumption among Indonesian. In 2014, the average consumption of protein was 53.81 grams, which rose by 8.17 grams in 2020 become 61.98 grams (34). In terms of the sources of protein, it also reported that there was an overall increase in fish and seafood, meat, and egg consumption. Contrary, there was a reduction in cereal consumption (34). This finding was supported by a longitudinal analysis of food expenditures, which found lower expenditure shares for staple foods and higher expenditure shares for meat, eggs, and milk products (35). Majority of animal sources come from poultry as well as fish and seafood. Similar to previous reports, Indonesia has one of the highest fish consumption rates in the world, as well as a diversity of high-protein soy products such as *tahu* (tofu) and tempeh. Meat and dairy consumption are modest by global standards but vary by cultural group and are increasing as income rise (36). Grains were most consumed food sources among plant-based protein compared to legumes. The prior study also shows a similar result on protein food group consumption (13).

This study found that total protein intake and animal-to-plant protein sources ratio had no association with BMI. Previous studies showed inconsistency in the associations between protein intake and obesity indices. Similar to the current result, some studies showed no associations between protein intake and obesity (16, 37). However, other research found association of protein with obesity (23, 38–40). Other studies even showed that higher-protein diets (1.0–1.5 g/kg body weight) were associated with lower BMI and waist circumference. The potential health effects of higher-protein diets appear to be more pronounced in overweight individuals than in normal-weight and obese individuals (39). The result explained that protein intake above the acceptable macronutrient distribution range can increase risk factors for high WC among women of childbearing age (38). Different findings of protein and obesity could be also due to the different proportions of animal protein and plant protein consumption (23). It has been suggested that a certain amount of protein is required for maintaining normal body weight and waist circumference, hence up to this threshold, only the quantity of proteins is beneficial.

This study further found no significant correlation between animal-based protein intake and BMI score among women of reproductive age in Indonesia. Similar to total protein intake, there are inconsistent results regarding animal protein intake and obesity. Some studies showed no association (16), some reported found a positive associations (41), and some showed a negative association between animal protein consumption with obesity (42, 43). Nonetheless, the rise in animal-source food products has both positive and adverse health effects. On the one hand, a few extra grams of animal-sourced foods can significantly improve the nutrition problems, such as undernutrition and micronutrient deficiency in developing countries. On the other hand, excessive consumption of animal-sourced foods (>18% of total energy) is associated with high saturated fat intake and higher mortality (24). Animal proteins are often accompanied by some quantities of saturated fats and cholesterol which might have deleterious effects on health (44).

In contrast to the result on animal protein, the study showed that plant-based protein consumption was significantly correlated with higher BMI among women of reproductive age in Indonesia after being adjusted by other covariate variables. In contrast, most of the previous studies consistently showed an inverse association between plant protein and obesity. Every 1-gram increase in plant protein was related to a 0.046 kg decrease in fat mass. Overall, a 19.2 g increase in plant protein in the vegan group was related to a 0.88 kg reduction in fat mass. The decrease in fat mass was also linked to a higher diet of plant protein, such as vegetables, grains, legumes, and fruits, as well as a lower diet of animal protein (18). Differences in weight gain distinguish for certain foods and beverages might be attributed to varied portion sizes, eating behaviors, satiety effects, or displacement of other foods. Consumption of various plant-based diet indices was linked to diverse degrees of weight gain (45).

The positive association between plant protein consumption and obesity in this study may be related to the quality of food sources of protein. In Indonesia, the main contributor of plant protein was grain (13, 34, 36). Previous research found that a 1-SD rise in an unhealthy form of a plant-based diet index (emphasizing refined grains, potato/fries, sweets, and sweetened drinks/juices) was linked to a 0.36 kg greater weight gain (45). Less-healthy plant foods, such as fruit juices, potatoes, and refined grains, have been consistently linked to an increased risk of weight gain in the unhealthful pro-vegetarian diet pattern (17).

Moreover, different protein sources also vary in amino acids in their ability to either prevent or induce obesity (46). Some research has reported that amino acids, including branched-chain amino acid (BCAA), tryptophan, and glutamate, can promote obesity (47–49). Amino acids such as glutamate are well known can increase obesity risk (47, 48). The positive correlation of glutamate with the obesity-related indices may be related to the regulation of food intake. Glutamate is abundant in wheat protein. Excess wheat consumption may have resulted in an increase in glutamate levels as well as an increase in BMI, visceral fat, and subcutaneous fat area in certain participants (47). Obesogenic amino acid tryptophan is also found in some plant sources, such as dried fruits, nuts, and oats. Furthermore, gut bacteria modulate the effect of plant protein on BMI by promoting appetite suppression. Gut bacteria metabolize choline and L-carnitine to create trimethylamine (TMA), which is then oxidized to produce trimethylamine-N-oxide (TMAO). TMAO has been linked to the development and risk of atherosclerosis (50). Plant-based foods such as avocado and legumes contain trace levels of L-carnitine. On the other hand, while red meat and eggs are high in choline, plant-based meals, including soybeans, potatoes, and most legumes, are also high in choline.

Protein is an essential component in the dietary guideline. Besides the quantity, the quality of protein sources should be considered. Even though some research shows a protective association between plant protein with BMI score, it is also possible that not all plant-based dietary patterns have beneficial effects on body weight. This finding provides specific information regarding the association between the consumption of different protein sources (i.e., plant vs. animal sources) with nutritional status among women of reproductive age, as similar studies are lacking in Indonesian settings. Understanding different outcomes on the nutritional status resulting from different protein sources is beneficial to design specific dietary recommendations. The present study did not separate the sources of protein based on the food groups, e.g., whole grains vs. refined grains, and processed vs. unprocessed meat as one of the limitations. Besides, information on the food preparation and cooking method is lacking in this study. Moreover, the clinical limitations of BMI should be considered. BMI is a surrogate measure of body fatness because it is a measure of excess weight rather than excess body fat. Further research on protein sources consumption with other nutritional status measures may be considered and focus on specific protein food groups to understand more the contribution of the food groups to the nutritional status.

5. Conclusion

In conclusion, high consumption of plant-based protein is associated with higher BMI scores. For future recommendations, the quality of plant protein should be considered besides the amount. Women of reproductive age should be encouraged to increase their intake of healthy plant foods while reducing their intake of less-healthy plant food (such as refined grains, potato/fries, sweets, and sweetened drinks/juices) for improved health outcomes. Furthermore, the government needs to improve health promotion and education activity regarding dietary intake, food choices, and obesity.

Data availability statement

The data used for this study was obtained from the 2018 Indonesia Food Barometer Study from SEAMEO RECFON. Data is available for public use by contacting the data owner. Requests to access these datasets should be directed to research@seameo-recfon.org.

Ethics statement

The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo Hospital with a letter numbered KET-162/UN2.F1/ETIK/PPM.00.02/2023 for secondary data analysis in which written informed consent for participation was not required. However, written informed consent was obtained from all participants in the primary data collected through the 2018 Indonesian Food Barometer (Ethical Clearance Number: 927/UN2.F1/ETIK/2017).

Author contributions

FS, HK, and JF conceived and designed the study. HK and JF involved in the primary data collection of IFB 2018, which was used for this study. FS analyzed the data. FS, HK, and JF interpreted the data. FS drafted the manuscript. 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/fnut.2023.1243635/full#supplementary-material

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Young consumers' perceptions of and preferences for alternative meats: an empirical study in Japan and China

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Introduction: Alternative meats have the potential to shape a sustainable food system. This study examined young consumers' perceptions of and preferences for plant-based and cultured meats. Since comparative studies on consumer preferences for alternative meats in different key Asian markets remain insufficient, this study was conducted in Japan and China, both of whom have promising alternative meat markets in Asia.

Methods: We conducted a discrete choice experiment and co-occurrence networks among 2006 ($n = 887$ in Japan and $n = 1{,}119$ in China) young consumers. This study adopted a treatment-control design where respondents in the treatment groups received health information on the use of antibiotics in meat production.

Results: Respondents in both countries perceived meat alternatives to be substitutes to conventional meat and associated them with plant-based proteins, processed products, and health benefits. In general, Japanese and Chinese respondents differed in their preferences for burger patties but had similar preferences for other attributes. Respondents in both countries were willing to pay a premium for "antibiotic-free," "traceable," and low carbon footprint labeling. This study reveals the heterogeneity of consumer preferences and the complexity of the impact of information interventions on consumer preferences.

Discussion: Plant-based meat is already available on the market in both countries, whereas cultured meat is still in the research and development stage. Hence, young consumers were more familiar with plant-based meat than cultured meat. It is worth noting that young Japanese consumers preferred cultured meat to conventional meat. This is attributed to the concerns about food security and food animal welfare. Furthermore, this study found that information intervention can induce and direct respondents' attention to an aspect of alternative meats that is negatively perceived. Based on the findings, this study has three implications for promoting alternative meat products: marketing messaging, food labeling, and product development.

KEYWORDS
alternative meat, co-occurrence network, cultured meat, discrete choice experiment, perception, plant-based meat, preference, young consumer

1. Introduction

Currently, the steady increase in global meat demand is shaping an unsustainable food system. From 2001 to 2021, global meat production increased from 237.0 million to 357.4 million tonnes (FAO, 2023). According to OECD/FAO (2022), global meat consumption is projected to increase by 15% before 2031 with the growth of the world's population. Climate change will be exacerbated by the expansion of the livestock sector (Grossi et al., 2019; Rehman et al., 2021), which emits approximately 14.5% of all human-derived greenhouse gas (GHG) emissions (Gerber et al., 2013). Further, livestock farming consumes extensive natural resources, such as water and land (Herrero et al., 2009; Thornton, 2010), and biodiversity is threatened by the loss of natural habitats (Batchelor et al., 2015; Machovina et al., 2015). In addition to environmental hazards, animal welfare (Gallo and Huertas, 2016; Sinclair et al., 2019), food security (Hibino et al., 2023), and the diseases caused by meat consumption (De Smet and Vossen, 2016; De Oliveira Mota et al., 2019; Espinosa et al., 2020) are rapidly becoming topics of grave concern.

Plant-based and cultured meat products are expected to meet the growing demand for meat while contributing to sustainability (Lee et al., 2020). Plant-based meat is manufactured by extracting proteins from protein-rich plants, such as soybeans, wheat, and peas (Wang Y. et al., 2023). Plant proteins have long been used as meat substitutes (Lee et al., 2020). In recent decades, vegetarians became interested in traditional plant-based meat products, such as veggie burgers (Broad, 2020). To better imitate the characteristics of real meat, novel plant-based meat products undergo improvements in nutritive value and sensory experiences, including taste and texture (Rubio et al., 2020). Cultured meat is another type of alternative meat that is produced by the extraction of stem cells from animals and use of *in vitro* cell culture and tissue engineering (Post, 2012). It enhances the flavor of meat and adjust fatty acid composition using technical methods such as controlling the medium's composition (Bhat and Hina, 2011). Producers can add desired nutrients or compound cells to the medium to enhance nutrition (Van Eelen, 2007).

The shift in consumption from conventional to alternative meats is viewed as a step toward sustainable development. From the environmental perspective, plant-based meat is more sustainable than animal meat in terms of natural resource consumption, carbon emission, and energy use (Hadi and Brightwell, 2021). As cultured meat is still not produced on a large scale, it is still unclear whether it is conducive to environmental sustainability, requiring a future life cycle assessment of its production system (Lynch and Pierrehumbert, 2019). Additionally, an increasing number of studies are focusing on animal welfare issues in the livestock industry (Gallo and Huertas, 2016; Sinclair et al., 2019). Although cultured meat production requires stem cells from animals, alternative meat production eliminates the need for livestock slaughter. Furthermore, with the rising demand for meat, plant-based and cultured meats can address sustainability challenges related to food security (Li, 2020; Hibino et al., 2023). In terms of health, plant-based and cultured meats can reduce the diseases associated with meat consumption. According to epidemiological studies, there is a positive association between red meat consumption and the occurrence of cardiovascular disease and colorectal cancer (Aykan, 2015; Zhong et al., 2020). Moreover, Intensive livestock production may contribute to the transmission of zoonotic diseases from animal hosts to human beings (Zinsstag et al., 2007).

Marketers and the media currently promote alternative meat products to realize the aforementioned benefits (Santo et al., 2020). Although plant proteins have a long consumption history, new plant-based meat products are being developed today using new technologies (Lee et al., 2020). Cultured meat is an emerging high-technology product with no history of consumption. As of June 2023, only Singapore and the United States allow the commercial sale of cultured meat (Food Frontier, 2023). To promote these products, researchers are focusing on tailoring novel plant-based products to consumer expectations and introducing consumers to the novel concept of cultured meat.

With a view to making alternative meats appealing to consumers, many studies examine consumer preferences regarding plant-based and cultured meats (Van Loo et al., 2020; Ortega et al., 2022; Washio et al., 2023). Factors affecting the acceptance of alternative meats include familiarity (Hoek et al., 2011; Mancini and Antonioli, 2019), health concerns (Food Frontier, 2023), taste and texture (Michel et al., 2021), unnaturalness (Weinrich et al., 2020), food security (Hibino et al., 2023), and animal welfare (Valente et al., 2019). Earlier studies investigated the role of food labeling, such as nutritional labels (Apostolidis and McLeay, 2016; Profeta et al., 2020; Wang et al., 2022), environmental labels (Apostolidis and McLeay, 2016; Profeta et al., 2020; Ortega et al., 2022), animal welfare labels (Ortega et al., 2022), origin labels (Apostolidis and McLeay, 2016; Profeta et al., 2020), and brand labels (Apostolidis and McLeay, 2016; Van Loo et al., 2020), in determining consumers' meat and alternative meat choices. In addition, researchers examined the effects of information interventions, including health information (Wang et al., 2022; Bazoche et al., 2023), environmental information (Van Loo et al., 2020; Wang et al., 2022; Bazoche et al., 2023), and technological information (Van Loo et al., 2020), on consumer preferences.

Although earlier studies provide valuable insights into consumer preferences for alternative meats, we identified two important research gaps in these studies. First, young consumers' perceptions of and preferences for alternative meats have not been sufficiently examined. Although some studies have reported that younger consumers are more likely to purchase alternative meats (Slade, 2018; Van Loo et al., 2020), only a few of them have delved into the underlying reasons driving these preferences or examined their specific perceptions of alternative meats. Young consumers, especially Generation Z, are often associated with sustainable consumption (e.g., Dabija et al., 2020; Dragolea et al., 2023). As they are poised to become a dominant force in the consumer market, exploring their perceptions and preferences is crucial for the development of the alternative meat market. Second, comparative studies on consumer preferences between different Asian countries are limited. A report on alternative proteins emphasized the importance of the Asian market and indicated variations in alternative meat markets in different Asian countries (Food Frontier, 2023). However, there is a lack of research comparing consumers' perceptions of and preferences for alternative meats in Asian countries. To overcome these research gaps, this study answers the following questions:

> RQ1. How do young consumers' perceptions of alternative meat products vary by country?

> RQ2. How do the preferences of young consumers for the different attributes of conventional and alternative meat products differ by country?
>
> RQ3. How can alternative meat products be promoted among young consumers in these countries?

This study was conducted in Japan and China, both of which have promising potential for alternative meat markets in Asia. In 2022, China had the largest meat substitutes market revenue in the world at 2.0 billion United States dollars, while Japan ranked second in Asia with 285 million United States dollars, following China (Statista Research Department, 2023). While plant-based meat is widely available in both the countries (Food Frontier, 2023), cultured meat is not yet allowed for sale in either country but its research and development efforts are in effect. Notably, China incorporated cultured meat in its 14th Five-Year Plan (Sheldon, 2022), and a Japanese cultured meat research consortium aims to demonstrate its manufacturing equipment at the Expo Osaka 2025 to promote public awareness of cultured meat (Anzo, 2023). There are some differences in the consumption of alternative meats between Japanese and Chinese consumers. For example, Chinese consumers (60.1%) have more experience consuming plant-based meat than Japanese consumers (23.9%) (Cross Marketing, 2021; Wang G. et al., 2023). In addition, the most important aspect considered by Japanese consumers when purchasing plant-based meat is flavor, whereas the aspect examined by Chinese consumers is health attributes (Food Frontier, 2023). Therefore, young Japanese and Chinese consumers are likely to have significant differences in their perceptions of and preferences for alternative meats.

The remainder of this paper is organized as follows: Section 2 explains the research methodology, whereas Section 3 presents the study's results. Further, Section 4 addresses the research questions based on our results, and Section 5 summarizes the findings and limitations of the study.

2. Materials and methods

To answer the research questions, online surveys were conducted among young Japanese and Chinese consumers. This study adopted a discrete choice experiment (DCE) and co-occurrence networks.

2.1. Experimental design materials and methods

DCEs are attribute-based experimental techniques that are applied in various fields to examine individuals' preferences for goods or services (Dinh et al., 2021; Phillips et al., 2021; Lizin et al., 2022). In particular, DCEs are widely used to explore consumers' preferences for meat products (Apostolidis and McLeay, 2016; Profeta et al., 2020; Van Loo et al., 2020; Ortega et al., 2022; Wang et al., 2022). DCEs form choice sets, and respondents select the most preferred option from two or more alternatives based on their evaluation of the attributes (Aizaki et al., 2014). In unlabeled DCEs, choice sets comprise multiple hypothetical profiles (i.e., alternatives) with fixed attributes and variable levels (Van Dijk et al., 2016). Compared to labeled DCE, unlabeled DCE is more appropriate for use in situations where consumers are unfamiliar with products, since it enables a better exploration of consumer trade-offs between different decision-making attributes (De Bekker-Grob et al., 2010).

Based on the DCE, we adopted a treatment-control design to test how information intervention affects consumer preferences (Grilli and Curtis, 2021). Information interventions were included in earlier studies on consumer preferences for alternative meat products (e.g., Van Loo et al., 2020; Wang et al., 2022; Bazoche et al., 2023), as well. In our study, respondents assigned to the treatment group received health information before answering DCE questions. To ensure that the respondents completely understood the information, they were asked to take a comprehension test. The respondents who answered incorrectly the first time were asked to repeat the reading, and those who answered incorrectly again were excluded from the study.

Prior to the formal survey, two focus groups (FGs) were conducted to gain a preliminary understanding of young consumers' perceptions of alternative meat products and examine which attributes and levels should be used in the DCE (Louviere et al., 2000). FGs typically consist of six to eight members (Finch and Lewis, 2003). We recruited six Japanese and eight Chinese participants who were 18 to 25 years of age. The Chinese and Japanese FGs were conducted on December 23, 2022, and January 6, 2023, respectively. Both the FGs were implemented online using Zoom, an online meeting software. The FGs were recorded using Zoom, and the informed consent of participants was obtained in advance.

2.1.1. Unlabeled DCE design

The first step in designing a choice experiment is identifying the product. To examine the meat preferences of young consumers in Japan and China, product selection criteria was two-fold: the product should (1) be popular among young consumers and (2) have minimal differences in terms of cooking style across the countries. Thus, burgers were considered the ideal product for this study. Although not indigenous to Japan or China, burgers are popular among young consumers in both countries (GlobalData Consumer, 2023; Mori, 2023). Additionally, we found that some FG participants exhibited greater familiarity with burger prices compared to raw meat prices.

The next step was to determine attributes and levels. Table 1 depicts the five finalized attributes and their corresponding levels, all of which were same for both countries, except the price levels. The selection of attributes prioritized the ones that are demand-related, measurable, and policy-relevant (Blamey et al., 2002). Attributes and levels were selected based on earlier studies and finalized based on the feedback provided in FGs; FG participants confirmed whether these attributes reflected their interest in selecting burgers and whether the levels were reasonable. Based on FG discussions, we made some adjustments to the attributes. For example, we excluded the calorie attribute because participants indicated that calorie information barely affected their purchase decisions regarding burger products.

Finally, five attributes and their corresponding levels were determined. First, burger patties were selected to examine young consumers' preferences for meat alternatives. We included plant-based and cultured meats, since plant-based patties are now widely available and cultured meat will likely be served in the coming years (Van Loo et al., 2020). The second attribute was price, which was considered the most important factor affecting consumers' choice of meat products (Merlino et al., 2018; Xu et al., 2019). All price levels were obtained by analyzing market prices, and their reasonableness was confirmed by FGs. The antibiotic claim was selected as the third attribute. Antibiotics are commonly used in the livestock industry for economic benefits; however, the abuse of antibiotics can pose a huge threat to public health (Ghimpețeanu et al., 2022). The "no claim" level indicates that the product does not specify antibiotic use. In an earlier study, the public in Germany, Italy, and the United States revealed a negative attitude toward the use of antibiotics in the livestock industry (Busch et al., 2020). The fourth attribute was the traceability of the burger patty. Traceability systems ensure food safety, and consumers are usually willing to pay a premium for "traceable" labeling (Ortega et al., 2011; Zhou et al., 2022). The last attribute was carbon footprint. According to the Parliamentary Office of Science and Technology (2006), the carbon footprint of a product refers to the total greenhouse gasses released throughout its production life cycle. Based on the data provided by earlier studies (Berners-Lee, 2011; Poore and Nemecek, 2018), we roughly estimated the carbon footprint of a burger and set the values to 1-, 4-, 7-, and 10-kg CO_2eq. FG participants understood that the difference between the lowest (1 kg) and highest (10 kg) values was significant.

2.1.2. Questionnaire design

The questionnaire for treatment groups comprised eight components: (1) sociodemographic characteristics, (2) dietary preferences, (3) consumption experience and intention to consume alternative meat products, (4) perceptions of meat alternatives, (5) knowledge tests on plant-based and cultured meat, (6) information intervention for the treatment groups, (7) comprehension test, (8) DCE choice sets, and (9) two psychological scales (i.e., green consumption value (GCV) and food neophobia scale (FNS)). The questionnaire for the control groups included all the eight components, except 6 and 7. A sample questionnaire is included in Supplementary material S1.

TABLE 1 Depiction of attributes and levels.

Attribute	Level		Information sources
	Japan	China	
Burger patty	Conventional meat Plant-based meat Cultured meat		Lee et al. (2020), Slade (2018), and Van Loo et al. (2020)
Price (JPY/CNY)	500 550 625 750	20 22 25 30	Analysis of available products FGs
Antibiotic claim	No claim Antibiotic-free		Busch et al. (2020) and Yang and Renwick (2019)
Traceability of the burger patty	Not traceable Traceable		Ortega et al. (2011) and Zhou et al. (2022)
Carbon footprint (CO_2eq)	1 kg 4 kg 7 kg 10 kg		Berners-Lee (2011) and Poore and Nemecek (2018) FGs

1 JPY = 0.007 USD (August 8, 2023); 1 CNY = 0.139 USD (August 8, 2023). CNY, Chinese Yuan; JPY, Japanese Yen; FG, focus group; USD, US dollar.

The components 1–5 and 9 were designed to obtain deep insights into consumer preferences. Alternative meat products are often considered environmentally friendly (Hadi and Brightwell, 2021); hence, we used the GCV created by Paço et al. (2019) to examine any correlation between GCVs and meat preferences. We also adopted the FNS designed by Pliner and Hobden (1992) to investigate whether food neophobia could be a predictor of young consumers' preference for alternative meat products. Both GCV and FNS used a 7-point Likert-type scale.

To clarify how young consumers perceive alternatives to meat, we designed an open-ended question asking respondents to create free associations about meat alternatives and input them in the form of single words or sentences (4). Free association is an effective technique to examine consumers' perceptions of things, since the associations made by people with cue words (i.e., meat alternatives) depend on their experience (Nelson et al., 2004).

To examine young consumers' knowledge of alternative meats, we designed True or False questions on the production of plant-based and cultured meats (5). Respondents were asked to read two statements and select their responses among "True," "False," and "I do not know." These statements were based on earlier studies (Van Loo et al., 2020; Wang Y. et al., 2023).

For DCE choice sets (8), we adopted an orthogonal main effect design to reduce the number of choice sets to 32 from 192 (= 3 × 4 × 2 × 2 × 4) potential choice sets (Lorenzen and Anderson, 1993). Since too many DCE questions can be psychologically stressful for respondents (Aizaki et al., 2014), the 32 choice sets were divided into two blocks, and participants were randomly assigned to one of the blocks. Before answering the DCE questions, respondents were instructed to imagine purchasing a burger at a fast-food restaurant. This was based on a market analysis of burger prices in fast-food restaurants, which was further confirmed by FGs. According to Ortega et al. (2022), consumption location does not affect consumer preferences for alternative meat products. To ensure that respondents could accurately understand the attributes, we explained the meaning of the antibiotic claim and provided the definitions of traceability and carbon footprint. Once they understood this information, respondents were asked to answer eight DCE questions. Figure 1 depicts a sample of the DCE questions used in the survey.

Prior to asking DCE questions, we provided health information on the use of antibiotics in meat production (Figure 2) to the treatment groups (6). We provided the following explanatory text along with Figure 2, as well:

Intensive livestock production can lead to the transmission of zoonotic diseases, such as the mad cow disease, from animal hosts to human beings (Zinsstag et al., 2007). Plant-based and cultured meats can reduce the risk of contracting the diseases associated with the consumption of conventional meat. Moreover, they can be produced without the use of hormones or antibiotics (Wang et al., 2022).

2.2. Data collection

In this study, we recruited 2,154 respondents aged 18–25 years who were registered with survey companies ($n = 1{,}000$ for Japan; $n = 1{,}154$ for China). The Japanese survey was conducted by Freeasy

Which of the following burgers do you prefer to buy?

	Burger 1	Burger 2
Burger patty	Conventional meat	Plant-based meat
Price	550 Yen	750 Yen
Antibiotic claim	No claim	Antibiotic-free
Traceability of the meat patty	Not traceable	Traceable
Carbon footprint (CO2eq)	10 kg	4 kg

- ○ Prefer to buy burger 1
- ○ Prefer to buy burger 2
- ○ Neither of them

FIGURE 1
Example of a discrete choice experiment question.

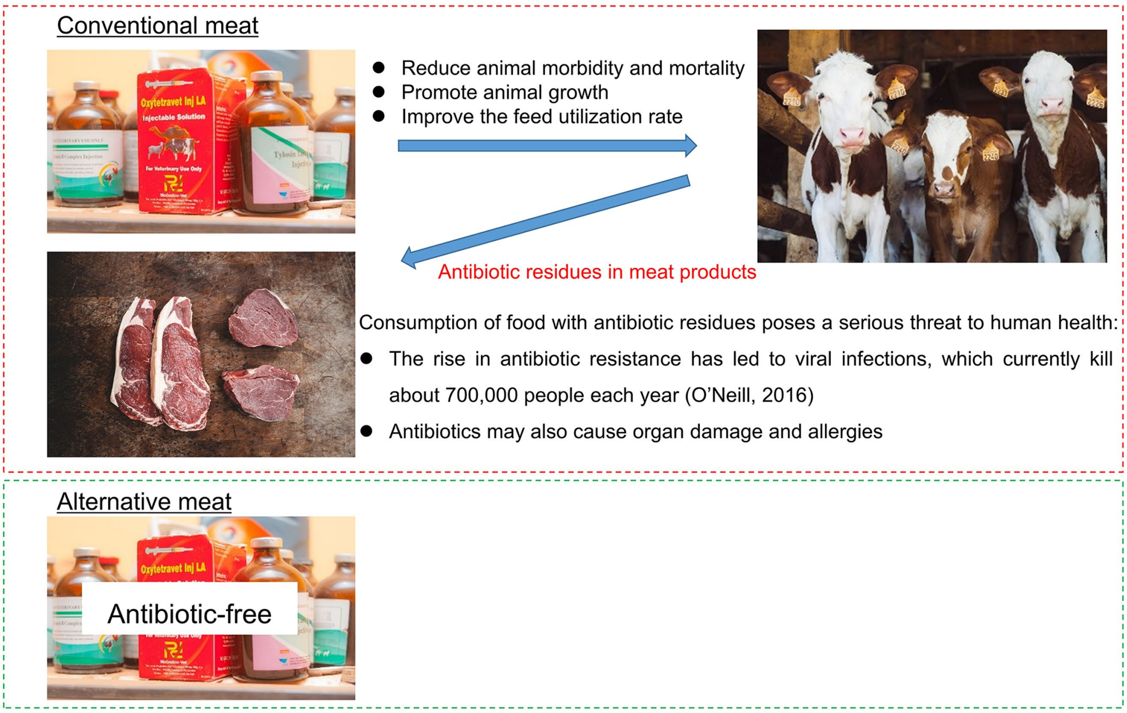

FIGURE 2
Health information for the treatment groups. (Adapted from Ghimpețeanu et al. (2022), Haiping et al. (2021), Hendrickson et al. (2020), and O'Neill, 2016. Photo courtesy: Unsplash).

(2023) from March 10 to March 11, 2023, and the Chinese survey was conducted by Wenjuanxing (2023) from April 8 to 12, 2023. These are professional online survey companies based in Japan and China. In each country, respondents were randomly assigned to one of four questionnaires (i.e., two blocks of choice design × control and treatment groups). After excluding the respondents who failed to pass

TABLE 2 Sociodemographic characteristics of the sample.

	Japan (n = 887)		China (n = 1,119)	
Gender	Male	49.9%	Male	45.2%
	Female	50.1%	Female	54.8%
Disposable income (JPY/CNY)	Below 20,000	43.0%	Below 1,000	11.6%
	20,000 to below 50,000	40.0%	1,000 to below 2,000	56.7%
	50,000 to below 100,000	13.9%	2,000 to below 5,000	26.1%
	Above 100,000	3.2%	Above 5,000	5.6%

CNY, Chinese Yuan; JPY, Japanese Yen.

the comprehension test, a valid sample of 2006 was collected ($n = 887$ in Japan and $n = 1,119$ in China).

2.3. Data analysis

2.3.1. Discrete choice experiment analysis

We applied mixed logit models to the DCE analysis. The mixed logit model is a prominent discrete choice model because it can approximate any random utility model (McFadden and Train, 2000). Because of its high degree of flexibility, the model is widely used in various fields of research (Arteaga et al., 2022). The mixed logit model includes random parameters and enables researchers to identify heterogeneity in choice preferences (Greene and Hensher, 2007).

In the random utility framework, the utility function can be expressed as follows:

$$U_{nsj} = V_{nsj} + \varepsilon_{nsj}, \quad (1)$$

where U_{nsj} denotes the utility obtained by consumer n by selecting alternative j in choice situation s, which can be separated into an observed component (V_{nsj}) and a residual unobserved component (ε_{nsj}) (Hensher et al., 2015). In the mixed logit model, the observed component (V_{nsj}) can be specified as follows:

$$\begin{aligned} V_{nsj} = {} & ASC + \alpha Price + \beta_{1,n} Burger\ patty \\ & + \beta_{2,n} Antibiotic\ claim + \beta_{3,n} Traceability \\ & + \beta_{4,n} Carbon\ footprint, \end{aligned} \quad (2)$$

where ASC refers to the alternative-specific constant; α is the mean coefficient of price, which is fixed; and other coefficients (i.e., $\beta_{1,n}$ to $\beta_{4,n}$) represent random parameters that are assumed to be normally distributed. To capture the interaction effects of consumer characteristics and choice preferences, we added the following interaction terms to the model:

$$\begin{aligned} V_{nsj} = {} & ASC + \alpha Price + \beta_{1,n} Burger\ patty \\ & + \beta_{2,n} Antibiotic\ claim + \beta_{3,n} Traceability \\ & + \beta_{4,n} Carbon\ footprint + \beta_{5,n}\left(Burger\ patty \times GCV\right) \\ & + \beta_{6,n}\left(Burger\ patty \times FNS\right), \end{aligned} \quad (3)$$

where the interaction terms between *Burger patty* and *GCV* and between *Burger patty* and *FNS* are included. We adopted effects coding instead of dummy coding for nominal variables (i.e., *Burger patty*, *Antibiotic claim*, and *Traceability*) to avoid confusion among base-level variables and *ASC* (Hensher et al., 2015). The base levels were "conventional meat," "no claim," and "not traceable." To make the results more intuitive, we reversed the carbon footprint codes during data analysis, for example, the carbon footprint of 4 kg was coded as "-4" in the actual analysis.

Based on the mixed logit model, we computed consumers' willingness to pay (WTP) by dividing the estimated parameters of non-price attributes by the price parameter (Croissant, 2020). The original utility function (Equation 1) can be rewritten as follows:

$$U_{nsj} = ASC + \alpha Price + \beta'_{,n} Non\text{-}price\ attribute + \varepsilon_{nsj}, \quad (4)$$

where $\beta'_{,n}$ refers to the set of random parameters of non-price attributes. V_{nsj} was divided into two parts, price (i.e., $\alpha Price$) and non-price attributes (i.e., *ASC*, $\beta'_{,n}$ *Non-price attributes*). In this setting, a non-price attribute's WTP can be expressed as follows:

$$WTP_{Non\text{-}price\ attribute} = -\frac{\beta_{,n}}{\alpha}. \quad (5)$$

We adopted Krinsky and Robb's method to calculate 95% confidence intervals and test for significant differences in the distribution of WTP between control and treatment groups (Aizaki et al., 2014). The entire DCE analysis was conducted by statistical software R, version 4.2.2.[1]

2.3.2. Co-occurrence networks

Co-occurrence networks were used to analyze respondents' free associations with meat alternatives. The co-occurrence networks present the words that often appear together and reveals different themes by grouping them (Higuchi, 2016a). We used KH Coder 3. Beta.07b,[2] which is a free software that performs quantitative analyses of texts. We used a Japanese lexical analysis engine (ChaSen) and a Chinese lexical analysis engine (Stanford POS Tagger) to extract words from the original text. We cleaned the data before creating the co-occurrence network by removing meaningless words (Yano et al., 2018). All operations were performed according to the KH Coder 3 manual (Higuchi, 2016b).

3. Results

3.1. Respondents' characteristics

In this study, a total of 2006 ($n = 887$ in Japan and $n = 1,119$ China) valid samples were collected. Table 2 depicts respondents' sociodemographic characteristics. The gender ratio of men to women of 20–24 years is 1.05 in Japan (Ministry of Internal Affairs and

1 https://www.r-project.org/
2 https://khcoder.net/en/

Communications, 2023) and 1.13 in China (Office of the Leading Group of the State Council for the Seventh National Population Census, 2022). The sample differed slightly from the national gender ratio, since the percentage of female respondents exceeded that of male respondents (1.00 in Japan and 0.83 in China). Further, national statistical data on the distribution of disposable income among young people were unavailable.

Table 3 depicts the dietary preferences of the sample. Most of the respondents in both countries were omnivores. The respondents adopted a diet without meat or fish (i.e., a vegetarian or vegan diet) were limited in both countries but significantly higher in Japan than China (5.9% of the Japanese sample compared to 1.6% of the Chinese sample) [t (1282) = 4.8653, $p < 0.001$].

The Cronbach's alpha values for GCV and FNS were 0.86 and 0.76, respectively, for Japan and 0.81 and 0.74, respectively, for China. All values of Cronbach's α were greater than 0.70, which indicated the internal consistencies of the two scales used in this study (Taber, 2018). The mean GCV of Japanese respondents was 4.03, whereas that of the Chinese was 5.05 [t (1820.7) = −23.264, $p < 0.001$]. The mean FNS scores were 4.15 for Japan and 3.64 for China, which indicated that Japanese respondents were more resistant to unfamiliar foods than Chinese respondents [t (1801.5) = 14.594, $p < 0.001$].

3.2. Respondents' knowledge and perceptions of alternative meat products

Figure 3 depicts respondents' consumption experiences regarding alternative meat products. Among Japanese respondents, 32.24% had eaten alternative meat products; the corresponding proportion of Chinese respondents was relatively high, 64.97% [(1) + (2)] [t (2004) = −15.388, $p < 0.001$]. In terms of consumption intention, 53.89% of Japanese respondents compared to 79.09% of Chinese respondents were willing to try alternative meat products in future [(1) + (3)] [t (1693.7) = −12.175, $p < 0.001$].

TABLE 3 Dietary preferences of the sample.

	Japan (n = 887)	China (n = 1,119)
Omnivore	87.1%	88.2%
Flexitarian	4.5%	9.4%
Pescetarian	2.5%	0.8%
Vegetarian	4.1%	1.5%
Vegan	1.8%	0.1%

Table 4 depicts respondents' knowledge of plant-based and cultured meats. Among Japanese respondents, 56.71% knew the raw materials of plant-based meat, and 18.15% of them were aware of how cultured meat is produced. In contrast, 78.02 and 27.44% of Chinese respondents answered the knowledge tests correctly, respectively.

The co-occurrence networks (Figures 4, 5) illustrate words with high co-occurrence in the respondents' free associations with meat alternatives. Eleven and eight subgraphs were identified in the Japanese and Chinese samples, respectively. The words in each subgraph are more closely associated with each other than with the words in the remainder of the network (Higuchi, 2016b). The size of the circle reflects the frequency of a word. To better interpret the results, we labeled each subgraph using a theme.

Similarities between the two samples were identified. First, respondents from both countries described the characteristics of meat alternatives and recognized them as substitutes to conventional meat (Subgraphs 1, 4, and 8 in Figure 4; Subgraph 1 in Figure 5). Second, the respondents perceived meat alternatives as products containing plant proteins (Subgraphs 10 and 11 in Figure 4; Subgraphs 4 and 8 in Figure 5). Third, meat alternatives were often associated with processed foods (Subgraph 6 in Figure 4; Subgraph 5 in Figure 5). Fourth, respondents expressed their concerns regarding health (Subgraph 7 in Figure 4; Subgraph 2 in Figure 5). Fifth, both samples included product experience (Subgraph 3 in Figure 4; Subgraph 3 in Figure 5).

However, there were differences between the two samples, as well. For example, Subgraph 9 in Figure 4 reveals that Japanese respondents perceived insects as a meat alternative. Although Chinese respondents mentioned some high-protein foods (Subgraph 6 in Figure 5), the terms in the subgraph do not include insects. In addition, Subgraph 7 in Figure 5 presents Chinese respondents' perceptions of the benefits of meat alternatives; here, the terms "environmental protection" and "health" co-occurred, whereas the term "environment" in the Japanese sample appeared in the subgraph depicting the characteristics of meat alternatives (Subgraph 1 in Figure 4).

3.3. Discrete choice experiment model estimates

Table 5 depicts the results of the mixed logit model estimates for cases without (model type 1) and with (model type 2) interaction

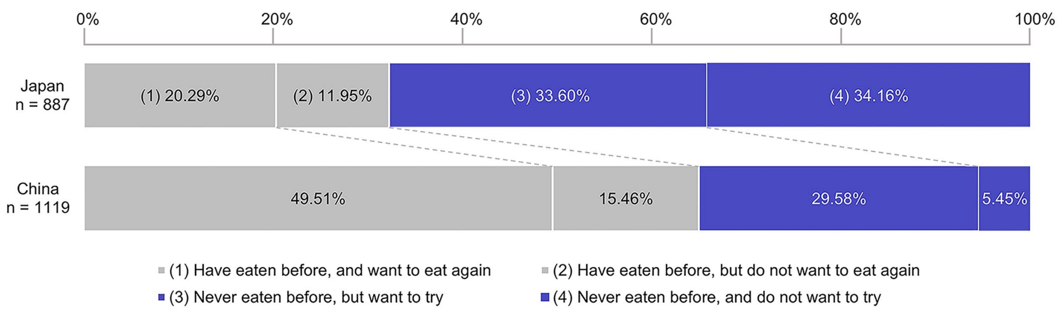

FIGURE 3
Alternative meat product consumption experience and consumption intention of respondents.

TABLE 4 Knowledge tests on plant-based and cultured meats.

Statement	Japan (n = 887)			China (n = 1,119)		
	True	False	I do not know	True	False	I do not know
1. Currently, the main raw materials of plant-based meat are soybeans, wheat, and peas.	**56.71%**	6.20%	37.09%	**78.02%**	3.84%	18.14%
2. Cultured meat, a type of alternative meat product, is produced by extracting stem cells from animals.	**18.15%**	11.39%	70.46%	**27.44%**	15.73%	56.84%

The correct answers are bolded.

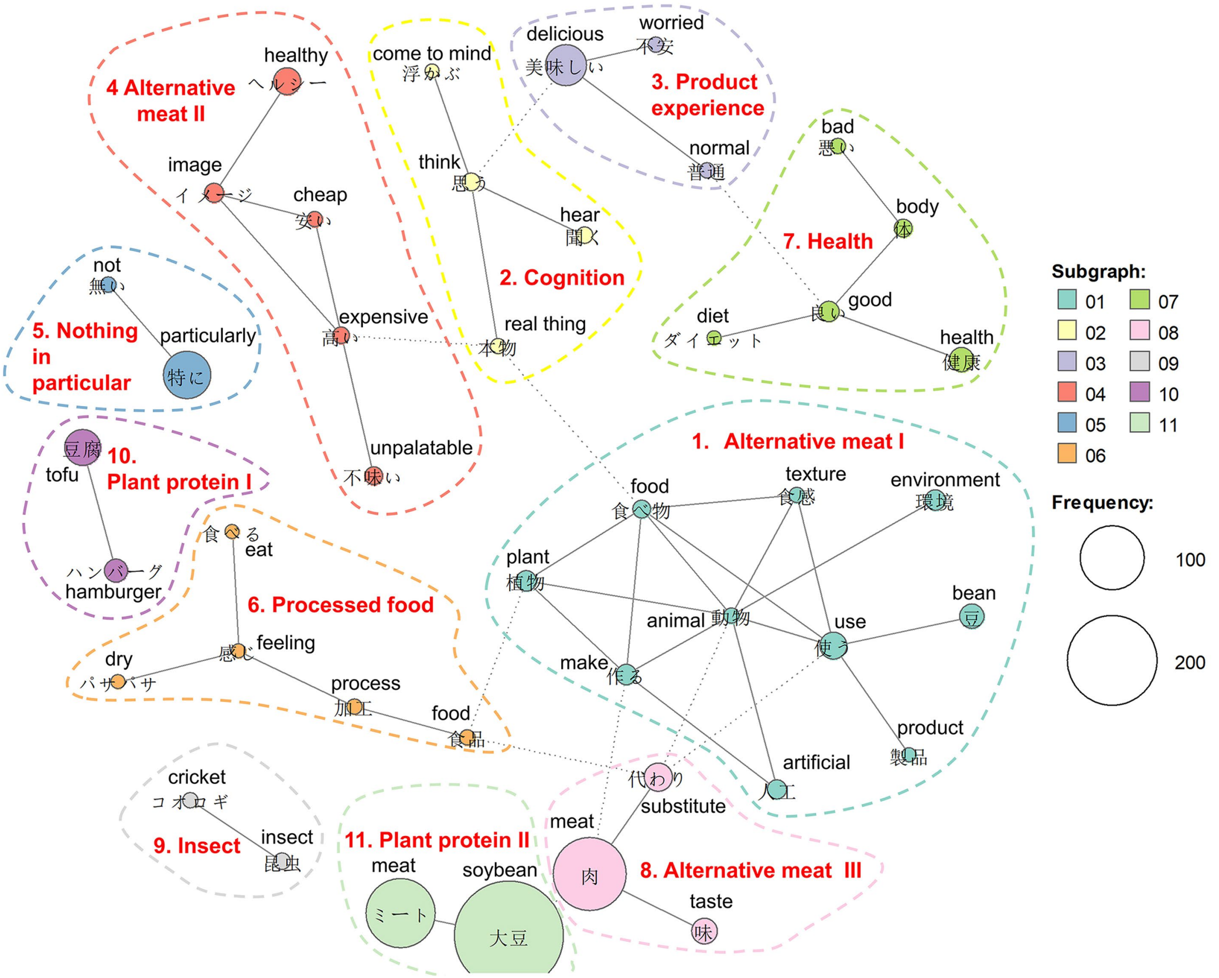

FIGURE 4
Co-occurrence network of Japanese consumers' free association with meat alternatives (n = 887).

terms. All the mean coefficients of the attributes in model type 1 were statistically significant. After including interaction terms, the signs and statistical significances of mean coefficients were identical to those in model type 1, except for "Plant-based meat" and "Cultured meat." When comparing models, the model with a lower Akaike information criterion (AIC) was considered better that the other (Mohammed et al., 2015), that is, model type 2 was the better estimate for all combinations in terms of AIC than model type 1. Therefore, hereafter, we focus on model type 2 alone. Our results found some interaction terms with statistical significance, such as the interaction between plant-based meat and GCV in the treatment groups of both countries. This indicates that respondents with a high GCV in the treatment group significantly preferred plant-based meat over conventional meat. The negative signs of the price coefficients were in line with the expectation that consumers' relative utility would decrease with the increase in price (Louviere et al., 2000). All standard deviations of random parameters, except "Traceability" in Japanese control and treatment groups, were significant in both models, which implies heterogeneity in consumer preferences.

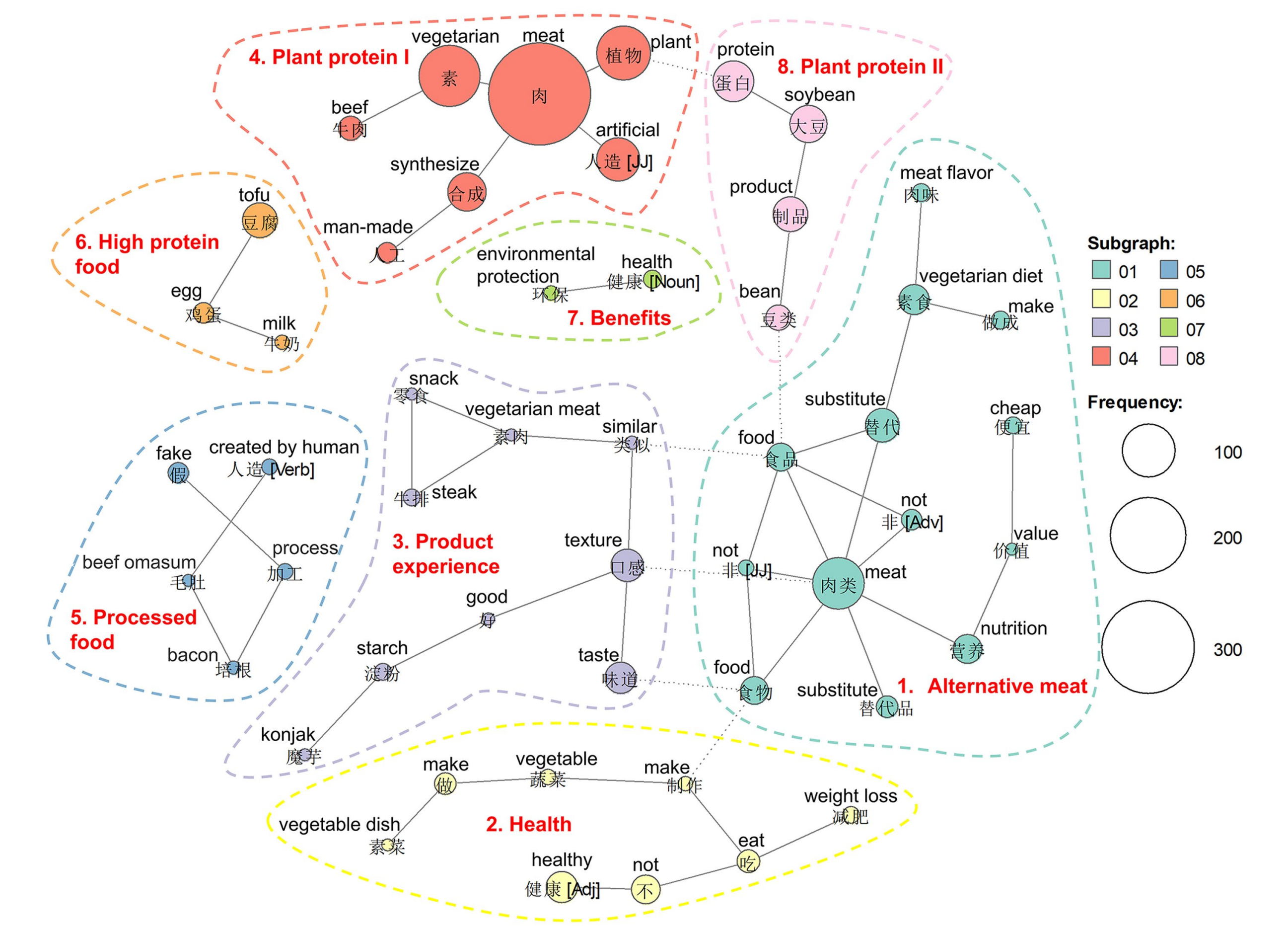

FIGURE 5
Co-occurrence network of Chinese consumers' free association with meat alternatives (n = 1,119).

Based on model type 2, the mean WTP was calculated for the items having statistical significance (Table 6). In general, Japanese and Chinese respondents differed in their preferences for burger patties but had similar preferences for other attributes. Without information intervention, Japanese respondents preferred cultured meat over conventional meat but had no significant difference in preference for conventional and plant-based meats, whereas Chinese respondents showed no significant preference for any type of meat. Moreover, respondents in the control and treatment groups from both countries preferred products with "antibiotic-free," "traceable," and low carbon footprint labeling.

Many dissimilarities and similarities were observed between control and treatment groups. The treatment groups of both countries preferred plant-based meat over conventional meat but had no clear preference between conventional and cultured meats. The significance levels indicated by asterisks in Table 6 demonstrate whether there was a significant difference in WTP between the groups. Compared with the control group, Chinese respondents in the treatment group had a significantly higher WTP for antibiotic claims; however, Japanese respondents' WTP for antibiotic claims did not differ significantly between the two groups. The WTP for low carbon footprint in the treatment group of Japanese respondents was significantly lower than that in the control group.

4. Discussion

4.1. Familiarity with and perceptions of meat alternatives [RQ1]

Our study revealed that Japanese respondents had less experience in consuming meat alternatives than Chinese respondents (see Figure 3). Among the Japanese respondents, 32.24% had consumed meat alternatives; this figure is higher compared to the survey finding that 23.9% of Japanese respondents between the ages of 20 and 69 had consumed meat alternatives (Cross Marketing, 2021). This may be because young consumers are more willing to consume meat alternatives (Van Loo et al., 2020). In comparison, 64.97% of Chinese respondents had consumed meat alternatives, which is consistent with the findings of Chung et al. (2023) that 60.1% of Chinese respondents had consumed plant-based meat. A study conducted in four major Chinese cities found that 85% of respondents had consumed plant-based meat (Wang G. et al., 2023), which may reflect the situation in first-tier cities, whereas our data align more closely with the national average. In addition, we found that the Japanese respondents had less positive consumption intentions than their Chinese counterparts (see Figure 3). This is explained by earlier findings, which indicate that individuals' familiarity with alternative

TABLE 5 Mixed logit model estimates.

	Japan $n = 887$				China $n = 1,119$			
	Control		Treatment		Control		Treatment	
	Model type 1	Model type 2	Model type 1	Model type 2	Model type 1	Model type 2	Model type 1	Model type 2
Mean coefficient								
ASC	3.494*** (0.235)	3.508*** (0.234)	4.271*** (0.231)	4.273*** (0.232)	4.691*** (0.217)	4.693*** (0.218)	4.343*** (0.216)	4.349*** (0.216)
Plant-based meat	−0.248*** (0.049)	0.249 (0.320)	−0.165*** (0.047)	−0.637* (0.319)	−0.113** (0.042)	−0.177 (0.315)	−0.129** (0.043)	−0.689* (0.319)
Cultured meat	−0.326*** (0.046)	0.701* (0.315)	−0.347*** (0.045)	−0.232 (0.303)	−0.570*** (0.043)	−0.349 (0.318)	−0.365*** (0.041)	−0.208 (0.297)
Antibiotic claim	0.259*** (0.036)	0.256*** (0.036)	0.326*** (0.035)	0.325*** (0.035)	0.502*** (0.034)	0.505*** (0.034)	0.652*** (0.038)	0.653*** (0.038)
Traceability	0.237*** (0.038)	0.238*** (0.038)	0.284*** (0.038)	0.283*** (0.039)	0.587*** (0.039)	0.590*** (0.039)	0.594*** (0.040)	0.595*** (0.040)
Carbon footprint	0.138*** (0.014)	0.142*** (0.014)	0.113*** (0.012)	0.114*** (0.012)	0.045*** (0.009)	0.045*** (0.009)	0.043*** (0.009)	0.044*** (0.009)
Price	−0.004*** (0.000)	−0.004*** (0.000)	−0.005*** (0.000)	−0.005*** (0.000)	−0.112*** (0.008)	−0.112*** (0.008)	−0.104*** (0.008)	−0.104*** (0.008)
Plant-based meat: GCV		0.051 (0.048)		0.142** (0.047)		0.063 (0.044)		0.109* (0.047)
Cultured meat: GCV		−0.058 (0.046)		−0.012 (0.044)		0.030 (0.043)		0.051 (0.045)
Plant-based meat: FNS		−0.171** (0.059)		−0.025 (0.057)		−0.068 (0.057)		0.003 (0.059)
Cultured meat: FNS		−0.193** (0.060)		−0.016 (0.055)		−0.103 (0.056)		−0.114* (0.054)
Standard deviations of the random parameters								
sd.Plant-based meat	0.675*** (0.073)	0.647*** (0.073)	0.580*** (0.069)	0.562*** (0.069)	0.525*** (0.065)	0.521*** (0.065)	0.584*** (0.068)	0.575*** (0.068)
sd.Cultured meat	0.555*** (0.082)	0.540*** (0.082)	−0.439*** (0.085)	−0.441*** (0.085)	0.660*** (0.065)	0.652*** (0.065)	0.519*** (0.069)	0.510*** (0.070)
sd.Antibiotic	−0.54*** (0.064)	−0.547*** (0.064)	−0.546*** (0.064)	−0.549*** (0.064)	0.447*** (0.062)	0.448*** (0.061)	0.549*** (0.060)	0.548*** (0.060)
sd.Traceability	0.057 (0.142)	0.013 (0.149)	0.028 (0.177)	0.038 (0.170)	0.249** (0.093)	0.252** (0.093)	0.336*** (0.081)	0.339*** (0.081)
sd.Carbon footprint	0.443*** (0.024)	0.450*** (0.024)	0.342*** (0.019)	0.344*** (0.020)	0.168*** (0.012)	0.168*** (0.012)	0.177*** (0.012)	0.178*** (0.012)
AIC	6343.85	6326.38	6195.80	6194.52	7269.59	7268.04	7107.07	7101.14
Log-likelihood	−3159.9	−3147.2	−3085.9	−3081.3	−3622.8	−3618.0	−3541.5	−3534.6
Number.obs	10,800	10,800	10,488	10,488	13,800	13,800	13,056	13,056

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Standard errors are depicted within parentheses. ASC, alternative specific constant; GCV, green consumption value; FNS, food neophobia scale; AIC, Akaike information criterion.

meat products affects their acceptance (Hoek et al., 2011; Mancini and Antonioli, 2019).

The knowledge tests (Table 4) corroborated the findings of earlier studies that consumers are more familiar with plant-based meat than cultured meat (Wang, 2022; Takeda et al., 2023). Plant-based meat is already on the market in both countries, whereas cultured meat is still in the research and development stage and remains unavailable for sale (Food Frontier, 2023). In Japan, food chains, such as Mos Burger and Freshness Burger, have already introduced plant-based burgers with soy patties (Anzo, 2021). Marukome Co., Ltd., a top miso company in Japan, sells a range of alternative meat products made from soybeans (Marukome, 2023). Plant-based meat products, such as vegetarian chickens, have a long history in China, as well. In recent years, food brands such as Starbucks and KFC launched plant-based

TABLE 6 Comparison of WTP estimates for control and treatment groups by country.

	Japan (JPY)			China (CNY)		
	Control	Treatment	Significance level	Control	Treatment	Significance level
Burger patty						
Plant-based meat (compared to conventional meat)		−128.31 [−265.41, −7.60]			−6.63 [−12.77, −0.64]	
Cultured meat (compared to conventional meat)	159.27 [17.05, 301.84]					
Antibiotic claim						
Antibiotic-free (compared to "no claim")	58.10 [41.97, 75.78]	65.44 [50.99, 81.42]		4.51 [3.83, 5.33]	6.28 [5.38, 7.41]	**
Traceability						
Traceable (compared to "not traceable")	54.04 [35.35, 74.82]	57.04 [40.02, 75.04]		5.27 [4.43, 6.31]	5.73 [4.69, 6.94]	
Carbon footprint						
	32.28 [25.96, 40.32]	22.89 [17.89, 28.54]	*	0.40 [0.25, 0.57]	0.42 [0.26, 0.62]	

WTP was computed only for statistically significant variables in model type 2. The figures in brackets represent 95% confidence intervals. Asterisks indicate the statistically significant difference between control and treatment groups (calculated according to the method by Krinsky and Robb). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

meat products in China, and internationally renowned plant-based meat brands, such as Beyond Meat, entered the Chinese market (Ye, 2023). These alternative meat products that are already on the market have increased consumers' awareness of plant-based meat. We also found Chinese respondents to be more knowledgeable of alternative meats than Japanese respondents (see Table 4). This is expected since the Chinese have had more consumption experience (see Figure 3).

Co-occurrence networks (Figures 4, 5) revealed young consumers' perceptions of meat alternatives in detail. Five similarities were identified between the two samples. First, young consumers perceived meat alternatives as substitutions to conventional meat with specific characteristics, rather than supplements. Second, they associated meat alternatives with plant protein, which is consistent with the findings of Michel et al. (2021). This can be attributed to the plant-based products that are already available in the market. A Chinese study noted that "vegetarian chicken" and "Buddha duck" were made from plant-based ingredients; this has caused consumers to associate plant protein with meat (Wang, 2022). Third, meat alternatives are considered processed products. This is not surprising, because alternative meat products undergo several processing procedures to mimic the taste, texture, and appearance of real meat. Fourth, the health theme appeared in both samples. An earlier study found food safety to be an important factor influencing the food purchasing behavior of Japanese consumers (Sasaki et al., 2022). For example, after the first case of Bovine Spongiform Encephalopathy infection was reported in Japan in 2001, Japanese consumers' demand for beef decreased significantly (Godo, 2015). Similarly, Chinese consumers became highly concerned about the safety of meat products after the reporting of several food safety scandals (Wang, 2022). The final similarity between the two samples is that the respondents mentioned product experience, which probably influenced their consumption preferences.

We observed two differences in the co-occurrence networks, as well. First, Japanese respondents associated meat alternatives with insects, whereas Chinese respondents did not. Since edible insect products are widely available in both countries, we attributed this difference to young Chinese consumers' tendency to perceive insects as protein supplements, rather than meat substitutes. The second difference is that environmental benefits appeared in different themes in the two samples. The co-occurrence of "environmental protection" and "health" in the Chinese sample implies that respondents perceived a strong link between these two benefits. However, in the Japanese sample, "environment" did not co-occur with "health" to form a separate subgraph; rather, they appeared in the subgraph depicting the characteristics of alternative meat. This implies that Japanese respondents perceived environmental benefits as a characteristic of alternative meat but did not associate them with health. This may cause young Japanese consumers to make a trade-off between environmental benefits and health in their preferences, which is explained in Section 4.2.

4.2. Consumer preferences for conventional and alternative meat products [RQ2]

Model types 1 and 2 presented different consumer preferences for burger patties. The results of model type 1 were straightforward, with all signs of alternative meats being negative and statistically significant. These results were consistent with the findings of earlier studies on meat alternatives (Van Loo et al., 2020; Washio et al., 2023). However, by including GCV and FNS, model type 2 demonstrates the complexity of consumer preferences. Since model type 2 had a better AIC for all cases, the model without interaction terms (model type 1) might be a misleading aspect in this study. Therefore, we focused on the results obtained using model type 2.

4.2.1. Plant-based meat

Our results revealed similar preferences for plant-based meat between young Japanese and Chinese consumers. The control groups did not show significant preferences between plant-based and conventional meats; however, the treatment groups showed a negative WTP for plant-based meat compared to conventional meat (Table 6). The negative effect of food health information on consumer preferences for plant-based meat was not recorded by earlier studies (e.g., Ortega et al., 2022; Wang et al., 2022). A possible explanation is that our health information was not sufficiently convincing, and consumer attention was directed toward the health benefits of alternative meat. In FGs, we found negative perceptions of the health benefits of plant-based meat. One Japanese participant believed that plant-based meat could not provide the same amount of protein as conventional meat. Further, Chinese FG participants stated that "plant-based meat may contain a large number of food additives" and that "plant-based dishes may be cooked with high levels of oil and salt." In co-occurrence networks, respondents associated meat alternatives with processed foods (Figures 4, 5). It is noted that ultra-processed plant-based meat products have harmful health consequences (Flint et al., 2023). A high sodium content is another concern for consumers (Bohrer, 2019). Therefore, treatment groups may have been influenced by such negative health perceptions due to which they showed a preference for conventional meat over plant-based meat.

While the treatment groups showed a negative preference for plant-based meat, in general, respondents with a high GCV in the treatment groups preferred plant-based meat to conventional meat (Table 5). There are two possible explanations for this observation. First, consumers with a high GCV were aware of the negative effects of antibiotic use on the environment. For example, antibiotics can cause water and soil pollution and alter environmental microbiota (Martinez, 2009). Second, consumers with a high GCV were highly concerned about their health and were easily convinced by the provided health information. Although no studies have directly proved this causal relationship, consumers' environmental attitudes are significantly influenced by their health attitudes (Ritter et al., 2015). However, this preference was not observed in the control groups. This can be attributed to a lack of awareness of the negative environmental impact of conventional meat production (Hartmann and Siegrist, 2017).

Japanese respondents with a high FNS in the control group preferred conventional meat to plant-based meat, whereas Chinese respondents with a high FNS showed no preference (Table 5). Food neophobia is often considered a barrier to the acceptance of alternative meats (e.g., Hoek et al., 2011; Siegrist and Hartmann, 2020). The difference in preferences between the two countries is probably because the Japanese respondents were less familiar with plant-based meat than their Chinese counterparts (see Figure 3; Table 4). In the treatment group, Japanese respondents with a high FNS showed no preference between conventional and plant-based meats. For consumers with a high FNS, familiarity is a prominent consideration in making food choices; they consider familiarity more important than health concerns (Karaağaç and Bellikci-Koyu, 2022). While the information intervention directed respondents' attention to health, Japanese respondents with a high FNS cared more about the knowledge provided by the information intervention, which increased their familiarity with plant-based meat.

4.2.2. Cultured meat

Our results revealed that the Japanese control group preferred cultured meat to conventional meat, whereas the Chinese control group had no preference. This contradicts the findings of earlier studies indicating that cultured meat is less preferred than conventional meat (e.g., Van Loo et al., 2020; Ortega et al., 2022). There are two possible explanations for the positive preference toward cultured meat in the Japanese control group. First, the role of cultured meat in ameliorating world hunger significantly increases its consumer acceptance (Hibino et al., 2023). According to a Japanese survey, 55% of respondents agreed that cultured meat is a possible solution to global famine (Nissin Foods Group, and Hirosaki University, 2019). Moreover, Japanese domestic news and online articles often associate cultured meat with food security (Ishikawa, 2021; JBpress, 2021; NHK, 2023), which probably reinforces young consumers' awareness. Second, animal welfare can be one of the main reasons why consumers prefer cultured meat to conventional meat (Valente et al., 2019; Specht et al., 2020; Weinrich et al., 2020). Although the current production of cultured meat relies on real animals to obtain stem cells, it ensures a reduction in the number of animals slaughtered and reduces intensive animal husbandry (Rubio et al., 2020). A Brazilian study found that more than 80% of the respondents had limited knowledge of cultured meat; however, 63.6% said they would eat cultured meat, mostly out of concern for animal welfare (Valente et al., 2019). It is also considered one of the strongest positive drivers of the acceptance of cultured meat in Germany (Weinrich et al., 2020). A Japanese study found that 59.4% of respondents advocated the reduction of livestock suffering (Iwamoto and Kubota, 2022). Another study revealed that most Japanese respondents have a positive WTP for animal welfare (Sonoda et al., 2018).

The negative impact of information intervention was reflected in Japanese respondents' preference for cultured meat, as well. In contrast to the control group, the Japanese treatment group no longer preferred cultured meat over conventional meat but tended to treat them equally. Their attention can be directed toward the health benefits of alternative meats by providing health information, and there was a negative perception of the health benefits of cultured meat (e.g., Tucker, 2014; Hocquette et al., 2015). This likely undermines Japanese respondents' preference for cultured meat to a certain extent.

Interestingly, we found different effects of the information intervention on the preference for cultured meat of respondents with a high FNS in the two countries. Our health information positively influenced the Japanese respondents' preferences for cultured meat. Japanese respondents with a high FNS in the control group preferred conventional meat to cultured meat, which was not surprising because the respondents were not familiar with cultured meat (see Table 4). In the treatment group, Japanese respondents with a high FNS showed no preference between conventional and cultured meat, which implies that our health information could increase the trust in cultured meat of Japanese respondents with a high FNS. However, the information intervention had a negative impact on Chinese respondents' preference for cultured meat. One possible explanation is that our health information made Chinese respondents with a high FNS realize that they lacked knowledge of the health benefits of cultured meat. Most of them did not know how cultured meat was produced (see Table 4).

Respondents with a high GCV in the treatment groups of both countries preferred plant-based meat to conventional meat; however, they did not show any preference between cultured and conventional meats. This is attributed to the perceived unnaturalness of cultured meat (e.g., Tucker, 2014; Weinrich et al., 2020). An earlier study found that the consumers who were ready to pay a premium for environmentally friendly products were among those who were the most concerned about the naturalness of food (Lockie et al., 2004). Therefore, the perceived unnaturalness of cultured meat can be a barrier to its acceptance by consumers with a high GCV.

4.2.3. Antibiotic claim

Respondents in both countries preferred "antibiotic-free" over "no claim" labeling, which indicates that young consumers had a negative attitude toward antibiotic use in meat production. A meta-analysis by Yang and Renwick (2019) found a similar result that consumers were willing to pay a high premium for hormone- or antibiotic-free livestock products. The abuse of antibiotics may lead to the human consumption of food contaminated with antibiotic residues, and antibiotic resistance poses a serious threat to human health (Ghimpețeanu et al., 2022). Due to the perceived health benefits of such food products, young consumers were willing to pay a premium for "antibiotic-free" labeling. However, this may also indicate that young consumers are unaware of the benefits of antibiotic use in the livestock industry. Proper antibiotic use can improve animal welfare and enhance food safety, which are often overlooked by consumers (Busch et al., 2020).

With information intervention, Chinese respondents showed significantly stronger preference for "antibiotic-free" labeling, whereas Japanese respondents did not. This could be because the information intervention reminded the Chinese respondents of the food safety scandals associated with antibiotic residues that came to light. For example, in 2012, when China's KFC chicken supplier used antibiotics and hormones to accelerate the growth of poultry, excessive levels of antibiotics were detected in the chickens (Hornby Lucy, 2013). Another possible reason is that the information intervention stimulated Chinese respondents' awareness of the experiment's implementation. Given that the information intervention centered on antibiotics, Chinese respondents in the treatment group may have felt that they were expected to favor "antibiotic-free" labeling. However, in a real-life consumption scenario, consumers may not place a higher premium on "antibiotic-free" labeling when presented with marketing messages about antibiotics.

4.2.4. Traceability

Our study found that respondents in both countries were willing to pay a premium for "traceable" labeling; this result aligns with the findings of several earlier studies (e.g., Ortega et al., 2011; Wu et al., 2015; Zhou et al., 2022). This indicates the positive attitude of young consumers in both countries toward the establishment of traceability systems for alternative meats. The information intervention did not have a significant impact on the preference for "traceable" labeling, which is reasonable since our health information did not include traceability.

Japanese respondents showed homogeneity in their preferences, whereas Chinese respondents showed heterogeneity. In the Japanese sample, the standard deviations of the random parameter (sd. Traceability) were not statistically significant; this indicates the homogeneity of Japanese respondents' preferences. Currently, Japan has mandatory traceability systems for beef and rice and encourages food business operators to establish traceability systems for other food products (Jin and Zhou, 2014). The homogeneous preferences of Japanese respondents regarding traceable products may be the result of their awareness of the benefits of traceability systems. In comparison, Chinese respondents showed heterogeneity in their preferences for traceability. An earlier study using an extended theory of planned behavior model found that factors such as face consciousness, trust, and policy support affected Chinese consumers' purchase intentions for traceable products (Ding et al., 2022). Household income and education level were also identified as two factors contributing to the heterogeneity in Chinese consumers' preferences for traceability (Wu et al., 2015).

4.2.5. Carbon footprint

The respondents in both countries preferred low carbon footprint labeling, which is consistent with the findings of earlier studies (e.g., Apostolidis and McLeay, 2016; Carlsson et al., 2022). Notably, we observed a significantly lower WTP for low carbon footprint labeling in the Japanese treatment group than the control group. In other words, young Japanese consumers may make trade-offs between health and environmental benefits. Yang et al. (2021) found a similar substitution effect, in which the consumer premium decreased with the simultaneous appearance of health-related and low-carbon attributes.

4.3. Implications for promoting alternative meat products [RQ3]

4.3.1. Marketing messaging

Marketing messages can increase consumers' familiarity with, and positive perceptions of, alternative meats (Apostolidis and McLeay, 2016; Tosun et al., 2021). Since young consumers in both countries currently have limited knowledge of alternative meats, particularly cultured meat, marketing messages have the potential to significantly influence their preferences. For example, nutritional information significantly increased the WTP for plant-based meat among consumers in Beijing (Wang et al., 2022). Our results indicated heterogeneity in consumer preferences in both Japan and China (Table 5), which implied the diversity of consumer segments. This study identified the following two consumer segments by estimating the interaction terms in both countries: respondents with a high GCV and those with a high FNS. They differed in their preferences for burger patties, and the information intervention had different effects on their preferences (Table 5). Hence, marketing messages should be customized to suit different consumer segments in both countries (Tosun et al., 2021).

We found that providing certain information alone could unexpectedly reduce consumers' preferences for alternative meat in both countries. Such messages can increase consumers' knowledge but can induce and direct consumers' attention to an aspect that is negatively perceived. An American study revealed that when respondents received only technical information on cultured meat production, the perceived unnaturalness discouraged them from consuming it (Bryant and Dillard, 2019). To promote alternative meat efficiently, marketers in Japan and China should disseminate designed messages about the product to consumers in a multidimensional manner to overcome the diversity of consumer segments and avoid triggering negative consumer perceptions.

4.3.2. Food labeling

Food labeling can be an effective method to promote alternative meat (Apostolidis and McLeay, 2016; Profeta et al., 2020; Ortega et al., 2022). Our study revealed that young consumers in both countries were willing to pay a premium for "antibiotic-free," "traceable," and low carbon footprint labeling. The premium for "antibiotic-free" and "traceable" labeling reflects respondents' health concerns. Globally, the largest use of antibiotics is in agriculture and, today, the consumer demand for antibiotic-free food is increasing steadily (Larsen, 2018). The "antibiotic-free" labeling enables young consumers in both countries to positively assess the health benefits of alternative meat. Since the information intervention significantly enhanced Chinese respondents' preference for "antibiotic-free" labeling, providing education or sending marketing messages about the health risks of antibiotic residues would make "antibiotic-free" labeling very effective in China.

Traceability is another credence attribute of food products. Food companies use different levels of traceability labeling to differentiate their products from the products of their competitors (Liu et al., 2019). Japan has introduced a traceability system for beef products and enacted the Beef Traceability Act (Godo, 2015). Consumers can trace beef information online by entering the product's ID number (MAFF, 2023). In comparison, China's food traceability system remains inadequate to this day. The lack of food supply chain databases, insufficiency of relevant laws and regulations, and use of outdated traceability technologies are challenges to establishing a sound food traceability system in China (Tang et al., 2015). To increase consumers' confidence in alternative meat products, both countries must incorporate the traceability of alternative meat products into the construction of food traceability systems.

Since alternative meat is often marketed as an environmentally friendly product, carbon footprint labeling has the potential to encourage alternative meat consumption (Apostolidis and McLeay, 2016). Both Japan and China have started implementing carbon footprint labeling for various products (Fu, 2023; SuMPO, 2023). The carbon footprint labeling of alternative meat products is expected to be beneficial in attracting environmentally concerned consumers in both countries. However, in FGs, we found that participants from both countries had a poor understanding of the concept of the carbon footprint, with one participant confusing it with carbohydrates. Therefore, policymakers and marketers must enhance young consumers' awareness of carbon footprint labeling in both countries. In this study, we adopted specific carbon footprint values (i.e., 1-, 4-, 7-, and 10-kg CO_2eq) that may not be easily comparable by consumers in real consumption situations. However, as carbon footprint labeling becomes more popular and public awareness of environmental protection increases, consumers may become more sensitive to specific carbon footprint values. Notably, compared with the control group, the Japanese treatment group showed a significantly less WTP for low carbon footprint labeling. Therefore, in Japan, the combination of carbon footprint labeling and marketing messages on health benefits should be applied with caution.

4.3.3. Product development

Alternative meat products should mimic real meat products to attract meat consumers in both countries. As respondents in both countries were predominantly omnivores, with only a small percentage being vegetarians and vegans (Table 3), the effect of dietary preference on consumers' perceptions of and preferences for alternative meats cannot be inferred. However, targeting meat consumers appears to be the most strategic and profitable approach for alternative meat producers in both countries, aligning well with the goal of promoting sustainable food consumption. In addition, we found that respondents in both Japan and China perceived meat alternatives as substitutes to conventional meat and mentioned product experience in free associations about meat alternatives (Figures 4, 5). Therefore, in future, alternative meat products should be similar to conventional meat in terms of product experience, such as taste and texture, in both countries. Some food retailers have already adopted this marketing strategy. For example, Burger King and Impossible Foods co-created the Impossible Whopper, which emphasizes the similarities between new plant-based meat products and real meat products (Schwab, 2019).

5. Conclusion

A dietary shift from conventional to alternative meats is often considered beneficial in shaping a sustainable food system. To promote alternative meat consumption, many earlier studies examined consumer preferences for alternative meats (e.g., Apostolidis and McLeay, 2016; Profeta et al., 2020; Wang et al., 2022). However, few studies have investigated young consumers' perceptions and preferences for alternative meats, and comparative studies on consumer preferences for alternative meats among key markets in Asia remain limited. Our study applied DCE and co-occurrence networks to examine the perceptions and preferences of young Japanese and Chinese consumers regarding plant-based and cultured meats.

Our study has several important findings. First, Japanese respondents were less familiar with alternative meats than Chinese respondents; however, they had some similar perceptions of meat alternatives. For example, respondents in both countries perceived meat alternatives to be substitutes to conventional meat and associated them with plant-based proteins, processed products, and health benefits. Second, our results revealed young consumers' preferences for plant-based and cultured meats. Notably, Japanese respondents preferred cultured meat to conventional meat. Third, respondents from both countries showed heterogeneity in their preferences for plant-based and cultured meat. Further, we examined the preferences of two consumer segments: respondents with a high GCV and those with a high FNS. The estimates of various consumer segments' preferences for alternative meats facilitate the development of effective marketing messages (Tosun et al., 2021). Fourth, our results revealed the complexity of the impact of information interventions on consumer preferences. The information intervention can have a positive impact on consumer preferences for alternative meats, such as the preference for plant-based meat among respondents with a high GCV in both countries. Interestingly, the health information on antibiotics can also have an unanticipated negative impact on consumer preferences for alternative meats. This may be because the information intervention directed consumers' attention to an aspect that was negatively perceived (e.g., the health benefits of plant-based meat). Fifth, the respondents in both countries had a positive WTP for "antibiotic-free," "traceable," and low-carbon footprint labeling. Hence, the adoption of health and environmental labeling can make alternative meat appealing to young consumers in both countries.

Our study has some limitations: First, this study adopted a stated preference survey, whose results might not be consistent with real consumption behavior (Nguyen et al., 2015; Wang et al., 2022). It would be interesting to corroborate the results by conducting revealed preference studies to avoid hypothetical bias. Second, burgers were the only products considered in our study. Young consumers may have different perceptions of alternative meats for different foods. An earlier study found that consumers in the United Kingdom had a lower WTP for cultured beef burgers than that for conventional beef burgers but a similar WTP for cultured and conventional chicken nuggets (Vural et al., 2023). Hence, future research should compare consumers' perceptions of alternative meat applications for different types of foods. Third, edible insects were not included as a promising meat alternative in this study. Insect proteins are superior to plant proteins in terms of total protein levels, essential amino acid content, and bioavailability (Lee et al., 2020). Moreover, insect farming is less expensive and more environmentally friendly than livestock farming (Gravel and Doyen, 2020). Therefore, edible insects form an important part of the alternative meat market; accordingly, future research should examine young consumers' perceptions of and preferences for edible insects. Fourth, future research should examine Japanese and Chinese consumers' attitudes toward the use of "meat" labels on alternative meat products. A United States study found that more than 70% of respondents were opposed to the use of "beef" labeling on plant-based and cultured meat products (Van Loo et al., 2020). Consumers may be confused or misled when these alternative products are labeled as meat. In the Japanese FG, a participant stated that she considered plant-based meat to be a mixture of plant ingredients and animal meat, rather than purely plant-based. Such misconceptions can lead to undesirable dietary shifts; for example, consumers may not realize that the protein content in purely plant-based meat is not equivalent to that in animal meat. Finally, our information intervention solely centered on the antibiotic use in conventional meat production, emphasizing the positive aspect of alternative meats. In a real marketing environment, consumers are exposed to various types of information, such as environmental and nutritional information, some of which may also be negative. Therefore, future research should test the effects of different information interventions on consumers' preferences. The continued exploration of effective marketing strategies is crucial, since products are continually updated and the consumer perceptions of alternative meats vary continuously.

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

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. TU: Data curation, Funding acquisition, Investigation, Project administration, Software, Supervision, Writing – review & 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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1290131/full#supplementary-material

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Amino acid and fatty acid profiles of perennial Baki™ bean

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To realize the potential of sainfoins to contribute to sustainable agriculture and expand on demonstrated uses and benefits, *de novo* domestication is occurring to develop perennial Baki™ bean, the trade name used by The Land Institute for pulses (i.e., grain legumes) derived from sainfoins. The objective of this study was to characterize amino acid and fatty acid profiles of depodded seeds from commercial sainfoin (*Onobrychis viciifolia*) seed lots, and compare these results with data published in the Global Food Composition Database for Pulses. The fatty acid profile consisted primarily of polyunsaturated fatty acids (56.8%), compared to monounsaturated (29.0%) and saturated fatty acids (14.2%), and *n*-3 fatty acids (39.5%), compared to *n*-9 (28.4%) and *n*-6 (17.6%) fatty acids. The essential fatty acid linolenic acid (18,3 *n*-3) was the most abundant fatty acid (39.2%), followed by oleic acid (18,1 cis-9) (27.8%), and the essential fatty acid linoleic acid (18,2 *n*-6) (17.3%). The amino acid profile consisted primarily of the nonessential amino acids glutamic acid (18.3%), arginine (11.6%), and aspartic acid (10.8%), followed by the essential amino acids leucine (6.8%), and lysine (5.8%). Essential amino acid content met adult daily requirements for each amino acid. This indicates that sainfoin seeds may be a complete plant protein source. However, further research is necessary to better understand protein quality, defined by protein digestibility in addition to the amino acid profile. By demonstrating favorable fatty acid and amino acid profiles to human health, these results contribute to a growing body of evidence supporting the potential benefits of perennial Baki™ bean, a novel, perennial pulse derived from sainfoins.

KEYWORDS

perennial grain crops, fatty acid profile, amino acid profile, Onobrychis, sainfoin, perennial Baki™ bean

1 Introduction

The Food and Agriculture Organization (FAO) of the United Nations defines pulses as grain legumes. These crops have a long history as foundational components of global agricultural and food systems (1). By converting atmospheric nitrogen into ammonia via symbiosis with rhizobia soil bacteria (2), legumes are ultimately linked to providing almost the entire amount of nitrogen that livestock and humans must obtain from diets (3, 4). This process allows pulses to accumulate twice the amount of protein of cereal grains (5). Pulses are uniquely positioned as a globally important staple food due to their ability to deliver a complete nutritional package. In addition to providing a sustainable and affordable source of protein, they are a vital source of dietary fiber, slowly digested carbohydrates, vitamins, minerals, and polyphenolics (6, 7). A well-established body of evidence links pulse consumption to a reduced risk of mortality from all causes (8–10). Yet, despite the numerous benefits of pulses to agricultural and food systems, and their potential to address myriad challenges facing agriculture and human health, pulses suffer from low adoption and are underutilized (11, 12).

In addition to these major pulse crops, there are many legume species of minor global economic importance that hold major agricultural importance as food and fodder for humans and animals as part of regional crop and food systems. However, most of these legume species are not well known outside of their primary production regions, where genetic diversity is maintained and improved through farmer-maintained landraces (13, 14). In the context of legumes, this leads to neglected and underutilized, or orphan, status, despite the ability to adapt to specific, often challenging, agroecological conditions and provide nutritional security (15–18).One such example of neglected and underutilized legumes are species in the *Onobrychis* genus (hereafter sainfoins). Sainfoins are temperate perennial legumes originating from central Asia with great potential for sustainable agriculture (19, 20). Sainfoins are undergoing *de novo* domestication at The Land Institute (Salina, KS, US) to develop Perennial Baki™ bean, the trade name used by The Land Institute for pulses derived from *Onobrychis* spp., as a perennial grain legume crop to expand on the demonstrated benefits and uses of sainfoins (21). Unlike all other pulses, which typically include annual species, sainfoins do not require replanting each year. Therefore, sainfoins, like other perennial grain candidates, provide continuous living cover and nitrogen fixation to improve soil health and reduce soil erosion (22–25). Throughout this paper, sainfoin is used to refer to the crop plant, while perennial Baki™ bean is used to refer to the grain legumes (i.e., pulses) derived from sainfoins.

This study is part of an ongoing effort to investigate the quality and safety of Baki™ bean for human consumption. Previously, we showed that Baki™ bean had protein content similar to soybean and lupin, fat content similar to chickpea, high dietary fiber and phytic acid content, and iron and zinc content comparable to most pulses (26). Several studies have also investigated seed composition within the genus *Onobrychis*. Notably, Tarasenko et al. (27), Ditterline (28), and Baldinger et al. (29) quantified amino acids, while Bagci et al. (30), Bakoglu et al. (31), Kaplan et al. (32), and Karataş et al. (33) quantified fatty acids. Complimenting these studies are monogastric animal feeding trials, which demonstrate the potential value of sainfoin seeds in diets of weenling pigs (29) and rats (34). To advance our previous work, the aim of this study was to characterize amino acid and fatty acid profiles in the context of data published in the Global Food Composition Database for Pulses (35).

2 Materials and methods

2.1 Seed material

Commercial seed companies and/or seed producers from Montana, US provided samples of named sainfoin (*Onobrychis viciifolia*) varieties for analysis (Table 1). Plants were harvested in either 2018 or 2020, in July or August based on individual company and producer schedules. While specific harvest times may vary, seeds are generally harvested when most seeds have reached physiological maturity. This ensure that the greatest quantity of high-quality seed is available for sale into the forage industry to establish new fields. Seed company and producer identities are not disclosed for privacy purposes. The seed samples ($N=9$) include the sainfoin varieties AAC Mountainview (36), Delaney, Eski (37), Shoshone (38), Renumex (39), and Rocky Mountain Remont. Rocky Mountain Remont is a selection from Remont.[1] See USDA NRCS Plant Materials Technical Note No. MT-91[2] for additional information on selected variety releases.

2.2 Sample preparation

Before analysis, Baki™ beans were removed from pods (i.e., depodded) using a Halstrop bench top dehuller. Following dehulling, a 3.571 mm sieve was used to separate the pods (i.e., hulls) and seeds. Then, a 2.778 mm sieve was used to separate the seeds into two fractions. The fraction that remained on the sieve was reserved for analysis of the whole seed (i.e., cotyledons and seed coat intact). Approximately 500 g of seed were haphazardly sampled from the total amount of seed available for analysis of each seed sample. All analyses were performed by Great Plains Analytical Laboratories (GPAL) (Kansas City, MO, US) unless otherwise noted. The GPAL quality assurance system is in accordance with International Electrotechnical Commission (ISO/IEC) 17,025:2018 and the Association of Official Agricultural Chemists (AOAC) Requirements for Food and Pharmaceutical Testing Laboratories.

2.3 Determination of fatty acid profiles

The Baki™ bean fatty acid profile was determined according to AOAC 996.06 with a detection limit of 0.003% (40). Briefly, the procedure consists of hydrolytic extraction, followed by methylation and analysis of the resulting fatty acid methyl esters (FAMEs) via capillary gas chromatography coupled with flame ionization detection.

2.4 Determination of amino acid profiles

The Baki bean™ amino acid profile was determined as described in Schuster (41), with a detection limit of 10 mg/100 g sample. Briefly, two different reagents were used to derivatize primary and secondary amino acids, before separation on a reverse phase column and detection using a diode array detector. Amino acid content was adjusted to mg/g protein by dividing by crude protein content, which has been previously reported for each sample by Craine et al. (26).

2.5 External datasets

To compare the Baki bean™ amino acid and fatty acid profiles analyzed using the methods described above to other pulse crop species, data for pulse crop species were downloaded from the Food and Agriculture Organization/International Network of

1 https://www.montanaseeds.com/about-us

2 https://www.nrcs.usda.gov/plantmaterials/mtpmctn12043.pdf

TABLE 1 Baki™ bean sample ID, grower code, variety code, variety, and year ($N = 9$).

ID	Grower Code	Variety Code	Variety	Year
R-S-18	R	S	Shoshone	2018
R-D-18	R	D	Delaney	2018
R-R-18	R	R	Rocky Mountain Remont	2018
W-D	W	D	Delaney	2020
W-R	W	R	Rocky Mountain Remont	2020
W-M	W	M	AAC Mountanview	2020
W-Rx	W	Rx	Renumex	2020
CS-E	CS	E	Eski	2020
CS-S	CS	S	Shoshone	2020

Food Data Systems (FAO/INFOODS) Global Food Composition Database for Pulses (Version 1.0 - uPulses1.0–2017) (35). Data for raw seeds were reported on an edible portion, dry matter content basis. For comparisons, data for the following pulses were selected *Cicer arietinum* (L.) (chickpea), *Lens culinaris* (Medik) (lentil), *Phaseolus vulgaris* (L.) (common bean), *Pisum sativum* (L.) (pea), *Vicia faba* (L.) (broad bean or fava bean) *Vigna radiata* (L.) R Wilczek (mung bean), and *Vigna unguiculata* (L.) Walp (cowpea).

Data for *Glycine max* (L.) (soybean) was downloaded from by the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) FoodData Central (42).

2.6 Statistical analyses

When not already present in this form, data were adjusted to a dry matter basis using moisture content. All values, unless otherwise noted, are reported on an edible portion dry matter basis (EPDM). All statistical analyses, unless otherwise noted, were performed using the R statistical software (43). The summarise function (44) or functions in base R were used to generate summary statistics (e.g., count, mean, standard deviation). The standard error of the mean was calculated and reported along with mean values. To test the null hypothesis that the crop species did not differ significantly with respect to the content of each individual, a Kruskal Wallis test was performed for each analyte. *Post hoc* analysis consisted of pairwise comparisons between crop species to determine whether mean values significantly differed, which was performed using Fisher's least significant difference test with Bonferroni corrected p values. The Kruskal Wallis tests and Fisher's least significant difference tests were performed using the agricolae package in R (45).

3 Results

3.1 Fatty acid profile

Fatty acid profiles for the sainfoin varieties are provided in Table 2. The content of 45 individual fatty acids was determined, representing the various fatty acids groups. These include saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3 (i.e., n-3) fatty acids, and omega-6 (i.e., n-6) fatty acids. Other cis and trans isomers of certain amino acids are also reported.

Of the 45 fatty acids, 25 were present below the detection limit (0.003) and the content of each is therefore reported as <0.003 in Table 2. These include butyric acid (4:0), caproic acid (6:0), heptanoic acid (7:0) caprylic acid (8:0), capric acid (9:0), lauric acid (12:0), tridecanoic acid (13:0), myristoleic acid (14:1), 10-pentadecenoic acid (15:1), elaidic acid (18:1 trans-9), other trans isomers of 18:1, other cis and trans isomers of 18:2, nonadecanoic acid (19:0), eicosadienoic acid (20:2 n-6), eicosatrienoic acid (20:3 n-3), homo-gamma-linolenic acid (20:3 n-6), eicosapentaenoic acid (20:5 n-3), heneicosanoic acid (21:0), erucic acid (22:1 n-9), docosadienoic acid (22:2 n-6), docosapentaenoic Acid (22,5 n-3), docosahexaenoic acid (22,6 n-3), tricosanoic acid (23,0), and nervonic acid (24,1 n-9).

The remaining 20 fatty acids were present at levels above the detection limit (i.e > 0.003). Notable fatty acids, found to occur in the highest amounts, include, from highest to lowest amount, alpha-linolenic acid (18:3 n-3), oleic acid (18:1 cis-9) and linoleic acid (18:2 n-6). Of the n-3-6-9 fatty acids, n-3 had the highest content, followed by n-9 and n-6. The sainfoin varieties had a narrow range (0.01 g/100 g sample) in values for the ratio of n-6 to n-3 fatty acids (i.e., n-6/n-3).

A comparison of the content of various fatty acids groups, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), between Baki™ bean, other pulse crops, and soybean is provided in Table 3 as g/100 g sample and in Figure 1 as a percent of total fatty acids. The fatty acid profile of sainfoin seeds was comprised primarily of MUFA, followed by PUFA, and is most comparable to broad bean and pea (Figure 1). The crops differed significantly with regards to SFA ($\chi^2_{8,52} = 45.33$; $p < 0.001$), MUFA ($\chi^2_{8,52} = 46.66$; $p < 0.001$), PUFA ($\chi^2_{8,52} = 41.58$; $p < 0.001$), and FA ($\chi^2_{8,52} = 41.03$; $p < 0.001$).

3.2 Amino acid profiles

Essential amino acid (EAA) profiles are provided in Table 4. The crop species differed significantly with regard to content of histidine ($\chi^2_{8,52} = 35.55$, $p < 0.001$), isoleucine ($\chi^2_{8,52} = 35.04$, $p < 0.001$), leucine ($\chi^2_{8,52} = 35.97$, $p < 0.001$), lysine ($\chi^2_{8,52} = 35.04$, $p < 0.001$), methionine ($\chi^2_{8,52} = 35.37$, $p < 0.001$), threonine ($\chi^2_{8,52} = 36.77$, $p < 0.001$), tryptophan ($\chi^2_{8,52} = 34.99$, $p < 0.001$), and valine ($\chi^2_{8,52} = 35.89$, $p < 0.001$). No difference was found for phenylalanine content ($\chi^2_{8,52} = 15.54$, $p < 0.0494$).

TABLE 2 Baki™ bean fatty acid profiles, representing samples ($N = 9$) from named varieties.

Fatty Acid	Minimum	Maximum	Mean ± SD	Mean ± SD (% total FA)
n-3 Fatty Acids	2.81	3.70	3.25 ± 0.30	39.54 ± 1.08
Alpha-Linolenic Acid (18:3 n-3)	2.79	3.66	3.22 ± 0.29	39.20 ± 1.11
n-9 Fatty Acids	1.94	2.75	2.34 ± 0.24	28.37 ± 0.97
Oleic Acid (18:1 cis-9)	1.90	2.68	2.29 ± 0.23	27.76 ± 0.95
n-6 Fatty Acids	1.28	1.64	1.45 ± 0.16	17.64 ± 1.01
Linoleic Acid (18:2 n-6)	1.26	1.59	1.43 ± 0.15	17.32 ± 1.04
Palmitic Acid (16:0)	0.67	0.93	0.78 ± 0.10	9.42 ± 0.39
n-6/n-3 ratio	0.40	0.50	0.45 ± 0.03	5.47 ± 0.63
Stearic Acid (18:0)	0.19	0.29	0.24 ± 0.03	2.96 ± 0.13
Vaccenic Acid (18:1 cis)	0.06	0.08	0.07 ± 0.01	0.79 ± 0.05
Behenic Acid (22:0)	0.05	0.07	0.06 ± 0.01	0.71 ± 0.05
Arachidic Acid (20:0)	0.03	0.04	0.04 ± 0.00	0.45 ± 0.02
Eicosenoic Acid (20:1 n-9)	0.03	0.04	0.03 ± 0.003	0.36 ± 0.01
Lignoceric Acid (24:0)	0.02	0.02	0.02 ± 0.003	0.23 ± 0.02
Conjugated Linoleic Acid (18:2)	0.01	0.03	0.01 ± 0.008	0.15 ± 0.08
Myristic Acid (14:0)	-	0.01	0.01 ± 0.004	0.13 ± 0.05
Margaric Acid (17:0)	0.01	0.01	0.01 ± 0.002	0.13 ± 0.01
Arachidonic Acid (20:4 n-6)	0.01	0.01	0.01 ± 0.002	0.12 ± 0.01
Pentadecanoic Acid (15:0)	–	0.01	0.01 ± 0.004	0.09 ± 0.05
Gamma Linolenic Acid (18:3 n-6)	–	0.01	0.00 ± 0.00	0.05 ± 0.07
Other Cis Isomers (18:1)	–	0.01	0.00 ± 0.00	0.04 ± 0.06
Margaroleic Acid (17:1)	–	0.01	0.00 ± 0.00	0.04 ± 0.05
Palmitoleic Acid (16:1)	–	0.01	0.00 ± 0.00	0.02 ± 0.04
Nonanoic Acid (9:0)	–	0.01	0.00 ± 0.00	0.01 ± 0.04

FA, fatty acids; –, Fatty acids with values below detection limit (<0.003 g) are omitted; fatty acids presented in descending order by mean value. Units reported as g/100 g sample, unless otherwise noted, on an edible portion dry matter (EPDM) basis.

TABLE 3 Total content of each fatty acid group for each crop.

Crop	N	SFA	MUFA	PUFA	FA
Baki bean™	**9**	**$1.17^{a} \pm 0.15$**	**$2.39^{a} \pm 0.24$**	**$4.68^{a} \pm 0.42$**	**$8.23^{a} \pm 0.79$**
Broad Bean[1]	2	$0.29^{cd} \pm 0.08$	$0.38^{cd} \pm 0.08$	$0.82^{cd} \pm 0.31$	$1.50^{bc} \pm 0.45$
Chickpea[1]	6	$0.65^{a} \pm 0.10$	$1.22^{a} \pm 0.32$	$2.68^{ab} \pm 0.16$	$4.55^{a} \pm 0.37$
Common Bean[1]	10	$0.37^{bc} \pm 0.07$	$0.15^{d} \pm 0.03$	$0.92^{c} \pm 0.15$	$1.43^{bc} \pm 0.22$
Cowpea[1]	4	$0.56^{a} \pm 0.06$	$0.31^{bc} \pm 0.14$	$0.92^{bc} \pm 0.10$	$1.80^{ab} \pm 0.27$
Lentil[1]	7	$0.24^{d} \pm 0.05$	$0.26^{c} \pm 0.07$	$0.55^{d} \pm 0.10$	$1.04^{c} \pm 0.20$
Mung Bean[1]	3	$0.55^{ab} \pm 0.12$	$0.08^{d} \pm 0.01$	$0.71^{cd} \pm 0.21$	$1.34^{bc} \pm 0.34$
Pea[1]	10	$0.31^{cd} \pm 0.04$	$0.40^{b} \pm 0.10$	$0.90^{c} \pm 0.22$	$1.60^{b} \pm 0.34$
Soybean[2]	1	3.15^{a}	4.82^{a}	12.31^{a}	27.77^{a}

FA, fatty acids; S, saturated; MU, monounsaturated; PU, polyunsaturated. Units reported as g/100 g sample, edible portion dry matter (EPDM) basis. Within each column, values that share a letter are not statistically different (alpha = 0.05).
[1]FAO (35).
[2]USDA ARS Food Data Central.

Nonessential amino acid (NEAA) profiles are provided in Table 5. The crops differed significantly with regards to alanine ($\chi^2_{8,52} = 37.26$, $p < 0.001$), arginine ($\chi^2_{8,52} = 43.75$, $p < 0.001$), aspartic acid ($\chi^2_{8,52} = 35.59$, $p < 0.001$), cystine ($\chi^2_{8,52} = 40.47$, $p < 0.001$), glycine ($\chi^2_{8,52} = 40.94$, $p < 0.001$), glutamic acid ($\chi^2_{8,52} = 36$, $p < 0.001$), proline ($\chi^2_{8,52} = 32.24$, $p < 0.001$), serine ($\chi^2_{8,52} = 38.06$, $p < 0.001$), tyrosine ($\chi^2_{8,52} = 31$, $p < 0.001$).

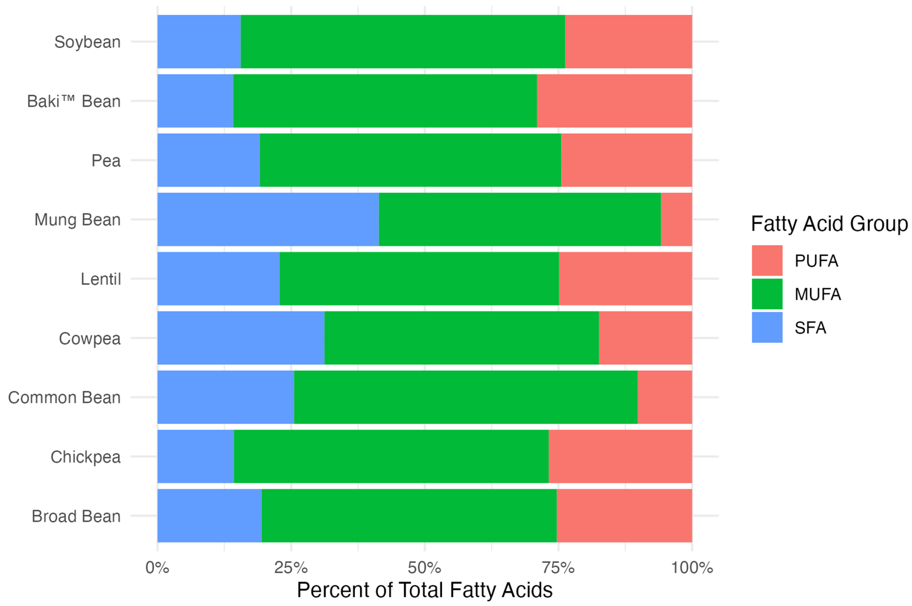

FIGURE 1
Fatty acid groups, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), shown for each crop species as the percentage of total fatty acids.

A comparison of the content of various groups of amino acids (e.g., EAA, NEAA) between sainfoin, pulse crops, and soybean is provided in Table 6. The crops differed significantly with regards to content of total amino acids ($\chi^2_{8,52} = 36.19$, $p < 0.001$), EAA ($\chi^2_{8,52} = 34.77$, $p < 0.001$), branched-chain amino acids (BCAA) ($\chi^2_{8,52} = 36.4$, $p < 0.001$), sulfur amino acids (SAA) ($\chi^2_{8,52} = 36.9$, $p < 0.001$), aromatic amino acids (AAA) ($\chi^2_{8,52} = 23.68$, $p < 0.01$), NEAA ($\chi^2_{8,52} = 37.12$, $p < 0.001$), and the ratio of essential to nonessential amino acids (EAA/NEAA) ($\chi^2_{8,52} = 40.66$, $p < 0.001$).

3.3 Amino acid daily requirements

A comparison of EAA content to adult (>18 years old) daily requirements is provided in Figure 2. Mean values for all crops met the daily requirements for histidine, leucine, lysine, AAA, threonine, tryptophan, and valine. The mean value for mung bean failed to meet isoleucine and SAA daily requirements. Additionally, lentil, broad bean, mung bean, and common bean mean values failed to meet SAA daily requirements. While the pea mean value (22.4 mg/g protein, dry matter) met the SAA requirement, within one standard error of the mean (2.6 mg/g protein, dry matter) pea fails to meet this requirement.

Since the values are reported as mg/g protein in Figure 2, compared to g/100 g sample as in Table 4, the maximum and minimum values vary. For example, soybean had the maximum value for lysine content (g/100 g sample) and mung bean had the minimum value (Table 4), compared to soybean having the maximum value for lysine content (mg/g protein) and Baki™ bean having the minimum value (Figure 2). Furthermore, Baki™ bean had the minimum value for leucine, lysine, and AAA, followed by mung bean and broad bean in each instance, and valine, followed by chickpea and mung bean. Lentil had the minimum value for histidine, followed by pea and broad bean; mung bean had the minimum value for isoleucine, followed by Baki™ bean and broad bean; common bean had the minimum value for SAA, followed by mung bean and broad bean; lentil had the minimum value for threonine, followed by Baki™ bean and broad bean; and broad bean had the minimum value for tryptophan, followed by lentil and pea. Soybean had the maximum value for each EAA, except for histidine in which Baki™ bean, followed by cowpea and soybean, had the maximum value and SAA, in which chickpea, followed by Baki™ bean and soybean, had the maximum values.

4 Discussion

4.1 Favorable fatty acid profile

A limited number of studies provide empirical data for fatty acid profiles of seed samples from sainfoins, representing various species with the *Onobrychis* genus. For instance, Tarasenko et al. (27) analyzed seed samples of *O. arenaria*, Bagci et al. (30) analyzed *O. major*, *O. altissima*, *O. hypargyrea*, and *O. huetiana*, Bakoglu et al. (31) analyzed *O. fallax*, Wijekoon et al. (46) analyzed *O. viciifolia* (cv. Melrose), and Kaplan et al. (32) analyzed 20 different genotypes of *O. viciifolia*. In general, fatty acid profile of sainfoin seeds is primarily composed of alpha-linolenic acid, oleic acid (18:1 n-9), linoleic acid (18:2 n-6). However, among these species, the fatty acid profiles vary with regard to the predominant fatty acids. Bagci et al. (30) found higher values for linoleic acid (31.5–51.8%) and Bakoglu et al. (31) found higher values for oleic acid (52.56%), with each representing the most abundant fatty acid. This is compared to our results, where we found linolenic acid to be the most abundant fatty acid (39.12%), which agrees with Tarasenko et al. (27) (41.41%) and Kaplan et al. (32)

TABLE 4 Essential amino acid content of each crop.

Crop	N	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
Baki bean™	**9**	**1411^{a} ± 77**	**1258^{a} ± 81**	**2322^{a} ± 136**	**1982^{a} ± 112**	**568^{a} ± 42**	**1324.73^{a} ± 78.06**	**1288^{a} ± 63**	**392^{a} ± 13**	**1544^{a} ± 93**
Broad Bean[1]	2	716^{ab} ± 23	1115^{ab} ± 35	1995^{ab} ± 64	1720^{abc} ± 57	182^{b} ± 5.8	1170^{a} ± 42	954^{ab} ± 31	238^{bc} ± 7.6	1230^{abc} ± 42
Chickpea[1]	6	632^{b} ± 74	933^{bc} ± 49	1652^{b} ± 100	1478^{c} ± 114	292^{ab} ± 53	1293^{a} ± 74	825^{b} ± 71	224^{c} ± 20	946^{c} ± 45
Common Bean[1]	10	687^{b} ± 47	1000^{abc} ± 137	1893^{ab} ± 200	1573^{bc} ± 161	263^{ab} ± 59	1285^{a} ± 147	1064^{a} ± 66	276^{ab} ± 36	1273^{ab} ± 210
Cowpea[1]	4	823^{ab} ± 31	1073^{ab} ± 107	1803^{ab} ± 168	1635^{abc} ± 76	397^{a} ± 43	1353^{a} ± 104	943^{ab} ± 41	278^{ab} ± 28	1189^{abc} ± 196
Lentil[1]	7	681^{b} ± 103	1163^{a} ± 78	2106^{a} ± 107	1974^{a} ± 108	235^{b} ± 13	1320^{a} ± 94	937^{b} ± 80	261^{abc} ± 29	1401^{a} ± 74
Mung Bean[1]	3	662^{b} ± 59	656^{c} ± 141	1687^{b} ± 224	1383^{c} ± 121	239^{ab} ± 59	1150^{a} ± 157	910^{b} ± 87	244^{bc} ± 24	1069^{bc} ± 139
Pea[1]	10	605^{b} ± 68	1002^{abc} ± 110	1784^{b} ± 198	1756^{ab} ± 192	243^{ab} ± 26	1175^{a} ± 136	924^{b} ± 112	237^{bc} ± 34	1175^{abc} ± 132
Soybean[2]	1	1210^{ab}	2174^{a}	365^{a}	2985^{a}	603^{a}	2341^{a}	1948^{a}	652^{a}	2238^{a}

Values reported as mean value ± standard deviation. Units reported as mg/100 g sample, edible portion dry matter (EPDM) basis. Within each column, values that share a letter are not statistically different (alpha = 0.05).
[1]FAO (35).
[2]USDA ARS Food Data Central.

(33.15–41.22%). Moreover, we also found oleic acid as the second most abundant, which agrees with the results of Tarasenko et al. (27), Bagci et al. (30), and Kaplan et al. (32). Wijekoon et al. (46) report comparable amounts of linolenic (25.7%) and oleic acid (25.2%), followed by linoleic acid (20.0%). Interestingly, we detected myristic acid in our samples (0.000–0.133%), as did Bagci et al. (30) (0.2–0.9%), Wijekoon et al. (46) (0.30%), and Kaplan et al. (32) (0.00–0.36%), while Tarasenko et al. (27) and Bakoglu et al. (31) found this fatty acid to be absent in *O. arenaria* and *O. fallax*, respectively. Additionally, it appears that the presence or absence of erucic acid (22:1 n-9) varies across species. We found erucic acid content to be below the detection limit. Erucic acid content was also found to be absent in *O. fallax* (31), *O. major*, *O. altissima*, and *O. hypargyrea* (30), but was detected in *O. arenaria* (0.24%) (27) and *O. huetiana* (1.6%) (30). A lack of erucic acid is favorable, because this fatty acid is regulated in Europe, the U.S., Australia, and New Zealand to maintain content in oils below 5% (Europe) and 2% by weight, respectively (47, 48). Sainfoin fatty acids appear to be predominantly unsaturated, based on our results (85.8%), and those reported by Wijekoon et al. (46) (68.6%) and Kaplan et al. (32) (85.72–89.50%). While we did not test for this specifically, Kaplan et al. (32) showed that genotype had a significant effect on both *O. viciifolia* fat and fatty acid content. This indicates that genetic diversity may exist and could be used during the breeding process to influence fatty acid content and composition. In lupin (*Lupinus albus*), genotype and genotype-by-environment interaction have been shown to significantly impact total FA, MUFA, PUFA, and n-6/n-3 ratio, while genotype had a significant impact on oil content (49). Studies investigating how genotype, environment, management, and their interactions impact the fatty acid profiles of sainfoin seeds should be conducted. This information will be valuable for producers and breeders interested in identifying sources of variation and the extent of variation in fatty acid content and composition.

Linoleic (18:2 n-6) and alpha-linolenic acid (18:3 n-3) cannot be synthesized by the body and must be acquired through the diet. Therefore, these fatty acids are defined as essential fatty acids (50). We found the essential fatty acids alpha-linolenic acid and linoleic acid to be the most abundant and third most abundant fatty acids in Baki™ bean Because humans lack the enzymes to convert between n-6 and n-3 fatty acids, the proportion of n-6 to n-3 fatty acids (i.e., n-6/n-3 ratio) is of particular concern for nutritionist and dietitians who advocate for an appropriate balance to optimize health, growth, and development (50). While a ratio of 1/1 to 4/1 is recommended, most Western diets are considerably imbalanced with a ratio 15/1–16.7/1 (51). With a mean value of 0.447 (2/5 ratio), Baki™ bean appears to have a lower n-6/n-3 ratio compared to other pulses and is most similar to the *Phaseolus* group, including navy bean (0.91), kidney bean (0.81), and black bean (0.90). This group is contrasted by much higher ratios for chickpea (19.67) and broad bean (14.59) (52). Excessive intake of n-6 fatty acids and insufficient intake of n-3 can lead to several chronic diseases, such as cardiovascular disease, diabetes, and several cancers, which are prevalent in Western societies, and increasingly prevalent in developing countries where diets are being transformed by the influence of Western consumption patterns and the availability of cheap, energy dense foods (51, 53–56). These foods include meat and dairy products from corn and soy fed animals, high n-6 vegatable oils (e.g., corn, soy, sunflower, cottonseed), and processed foods comprised primarily of corn and soy (57). Even though Baki™ bean and other pulse crops generally

TABLE 5 Nonessential amino acid content of each crop.

Crop	*N*	Alanine	Arginine	Aspartic Acid	Cystine	Glutamic Acid	Glycine	Proline	Serine	Tyrosine
Baki bean™	**9**	**1360^{a} ± 73**	**3938^{a} ± 398**	**3677^{a} ± 240**	**492^{a} ± 23**	**6222^{a} ± 478**	**1703^{a} ± 73**	**1624^{a} ± 108**	**1769^{a} ± 118**	**1098^{a} ± 60**
Broad Bean[1]	2	1105^{bc} ± 35	2615^{ab} ± 78	2980^{ab} ± 99	333^{ab} ± 11	4655^{ab} ± 148	1150^{abc} ± 42	1105^{ab} ± 35	1300^{ab} ± 42	881^{ab} ± 28
Chickpea[1]	6	969^{c} ± 51	2097^{bc} ± 256	2487^{b} ± 133	412^{ab} ± 198	4007^{b} ± 329	862^{d} ± 45	1,036^{ab} ± 148	1188^{b} ± 101	663^{b} ± 52
Common Bean[1]	10	1107^{bc} ± 64	1475^{d} ± 91	2951^{ab} ± 167	137^{c} ± 48	3918^{b} ± 225	1050^{c} ± 104	1101^{ab} ± 280	1411^{a} ± 97	732^{ab} ± 169
Cowpea[1]	4	1075^{bc} ± 75	1763^{cd} ± 270	2736^{b} ± 317	161^{c} ± 57	4258^{ab} ± 300	987^{cd} ± 140	1128^{ab} ± 85	1152^{b} ± 182	747^{ab} ± 106
Lentil[1]	7	1410^{a} ± 148	2140^{bc} ± 123	3363^{a} ± 258	306^{ab} ± 56	5063^{a} ± 530	1197^{ab} ± 64	1339^{a} ± 103	1321^{ab} ± 94	773^{ab} ± 67
Mung Bean[1]	3	1197^{ab} ± 115	1620^{cd} ± 165	2850^{ab} ± 363	187^{bc} ± 20	4247^{ab} ± 591	1733^{a} ± 145	1147^{ab} ± 116	1653^{a} ± 140	635^{b} ± 89
Pea[1]	10	1102^{bc} ± 125	2179^{b} ± 237	2885^{ab} ± 318	308^{ab} ± 51	4269^{ab} ± 481	1103^{bc} ± 122	1029^{b} ± 115	1172^{b} ± 153	785^{ab} ± 107
Soybean[2]	1	2112^{a}	3478^{ab}	5638^{a}	722^{a}	8685^{a}	2074^{a}	2624^{a}	2600^{a}	1698^{b}

Values reported as mean value ± standard deviation. Units reported as mg/100 g sample, edible portion dry matter (EPDM) basis. Within each column, values that share a letter are not statistically different (alpha = 0.05).
[1]FAO (35).
[2]USDA ARS Food Data Central.

TABLE 6 Total content of various amino acids groups by each crop.

Crop	*N*	AA	EAA	BCAA	SAA	AAA	NEAA	EAA/NEAA
Baki bean™	**9**	**33976^{a}± 2160**	**12090^{a}± 628**	**5124^{a}± 301**	**1060^{a}± 63**	**2423^{a}± 137**	**21885^{a}± 1535**	**0.55^{c}± 0.01**
Broad Bean[1]	2	25444^{ab} ± 827	9321^{ab} ± 308	4340^{ab} ± 141	515^{abc} ± 17	2051^{ab} ± 71	16123^{ab} ± 520	0.58^{bc} ± 0
Chickpea[1]	6	21996^{b} ± 1484	8276^{b} ± 446	3531^{b} ± 187	704^{a} ± 215	1957^{ab} ± 118	13720^{b} ± 1057	0.60^{b} ± 0.02
Common Bean[1]	10	23195^{b} ± 1317	9313^{ab} ± 902	4165^{ab} ± 521	401^{c} ± 87	2017^{ab} ± 280	13882^{b} ± 724	0.67^{a} ± 0.07
Cowpea[1]	4	23495^{ab} ± 2004	9492^{ab} ± 604	4065^{ab} ± 396	558^{ab} ± 44	2100^{ab} ± 201	14002^{b} ± 1441	0.68^{a} ± 0.03
Lentil[1]	7	26991^{a} ± 1015	10079^{a} ± 607	4670^{a} ± 236	541^{ab} ± 66	2094^{ab} ± 138	16912^{a} ± 782	0.60^{bc} ± 0.05
Mung Bean[1]	3	23267^{ab} ± 2648	7999^{b} ± 973	3411.66^{b} ± 500	426^{bc} ± 76	1785^{ab} ± 246	15268^{ab} ± 1678	0.52^{c} ± 0.01
Pea[1]	10	23732^{ab} ± 2648	8900^{ab} ± 987	3961^{b} ± 439	551^{ab} ± 71	1960^{ab} ± 241	14831^{ab} ± 1662	0.60^{bc} ± 0.00
Soybean[2]	1	47431^{a}	17800^{a}	8062^{a}	1326^{a}	4038^{a}	29631^{a}	0.60^{bc}

AA, amino acids; EAA, essential AA; BCAA, branched-chain AA (leucine, isoleucine, valine); SAA, sulfur AA (methionine, cystine); AAA, aromatic AA (phenylalanine, tyrosine); NEAA, nonessential AA; EAA/NEAA, ratio of essential to nonessential AA. Values reported as mean value ± standard deviation. Units reported as mg/100 g sample, edible portion dry matter (EPDM) basis. Within each column, values that share a letter are not statistically different (alpha = 0.05).
[1]FAO (35).
[2]USDA ARS Food Data Central.

have lower lipid and fatty acid content compared to oil seeds and oil legumes (e.g., soybean and peanut), they can still serve as an important source of fatty acids in human diets. In the context of increasing the intake of pulses in diets, fatty acid content and composition becomes increasingly important. For example, in a review of fatty acid profiles of selected pulses, data presented by Hall et al. (58) (% out of total fat) shows that linoleic acid is the primary fatty acid for chickpea (57%) and lentil and pea (48%). Linolenic acid content is highest for kidney (46%), great northern (43%), pinto (43%), navy (40%), mung (36%), and black (36%) beans. Conversely, linolenic acid content is lowest for lentil (12%), pea (10%), lupin (9%), and chickpea (2%). Finally, oleic acid is the most abundant fatty acid for lupin, and the second most abundant for chickpea, lentil, mung bean, and pea. Therefore, pulses can differ in their fatty acid profile, especially with regard to fatty acids essential to human health. For Baki™ bean, the composition of the fatty acid profile indicates that it can provide beneficial fatty acids for human nutrition, due to the high proportion of polyunsaturated to monounsaturated and saturated fatty acids, the relatively high content of the essential fatty acid alpha-linolenic acid (18:3 n-3), and high proportion of n-3 fatty acids compared to n-6 fatty acids, especially compared to other pulse crops. Specifically, Baki™ bean had higher SFA content than the values reported for broad bean, common bean, lentil, and pea, higher MUFA content than content than broad bean, common bean, cowpea, lentil, mung bean, and pea, higher PUFA content than broad bean, common bean, cowpea, lentil, mung bean, and higher FA content than broad bean, common bean, lentil, mung bean, and pea (Table 3). Enhancing these components could be a target of biofortification, or the breeding of crops to increase nutritional value (59, 60), as has been proposed for chickpea (61).

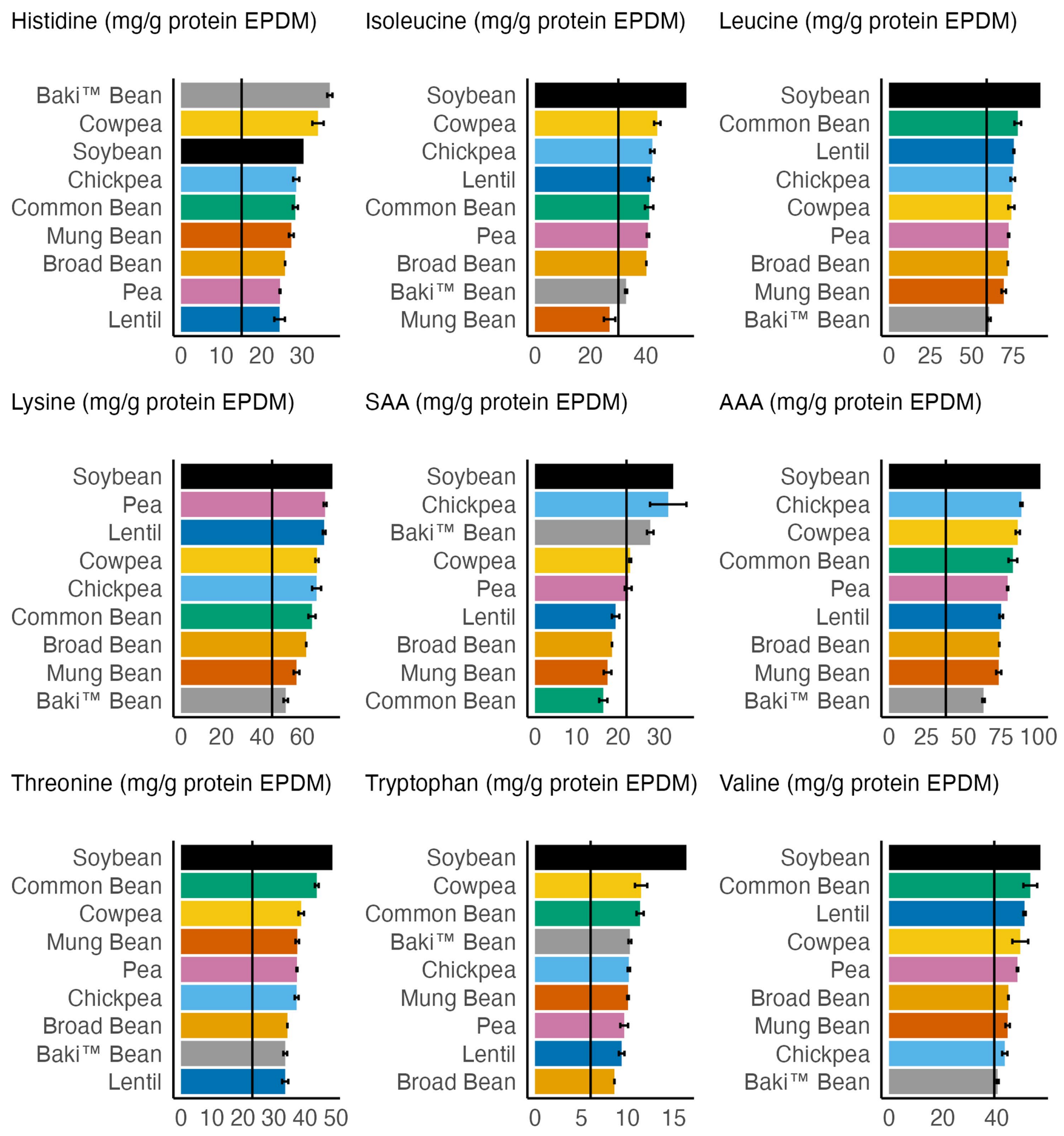

FIGURE 2
Comparison of essential amino acid content of each crop to adult daily requirements (solid, black vertical line) (FAO/WHO/UNU, 2007). Error bars represent one standard error of the mean.

4.2 Amino acid profiles of sainfoin seeds

Tarasenko et al. (27) also report data for *O. arenaria* amino acid content. It is worth noting that the values they report are not on a dry matter basis, and that the seeds analyzed had a reported moisture content of 8.5%. Adjusting the values they report to a dry matter basis (i.e., 0% moisture) and unit to mg/100 g sample allows for a more direct comparison. We found higher total AA content (33,975.65 versus 29,442.62). When comparing the content of EAAs, we found higher content for histidine (1,411.24 versus 1,016.39), the sum of leucine and isoleucine (3,580.03 versus 3,245.90), lysine (1,982.03 versus 1,737.70), the sum of phenylalanine and tyrosine (2,423.05 versus 2,163.93), proline (1,624.00 versus 1,497.27), tryptophan (392.25 versus 142.08), valine (1,544.10 versus 1,398.91), and slightly higher content for threonine (1,287.62 versus 1,267.76). We found lower content for the sum of methionine and cystine (1,060.39 versus 1,191.26). Several factors could have contributed to these differences, such as the differing species and varying production methods.

Few additional studies provide insights into *Onobrychis* spp. amino acid content. Ditterline (28) found amino acid composition of sainfoin seeds to be comparable to soybean meal, and our amino acid profile results are comparable to those reported in Table 28 in their study. Baldinger et al. (29) analyzed the content of a limited number of amino acids, including lysine, tryptophan, methionine and cysteine content. They report a slightly lower amount of lysine. Futhermore, we found a slightly higher amount of tryptophan, as well as methionine and cysteine, compared to their results. Interestingly, Baldinger et al. (29) found a ratio for Lysine:Met+Cys:Threonine:Tryptophan in sainfoin of 100:56:60:17, which they claim to be close to the ideal ratio of 100:60:65:18 recommended for piglets with 5–20 kg body weight (62). They also report that this ratio was higher than the ratio of reported for peas of 100:33:53:13 (63). Ultimately, their findings indicate that sainfoin seeds could be a viable option for inclusion in weanling pig diets 10–16% compared to peas or soybean cake.

In this study, Baki™ bean had higher content of each of the nine essential amino acids, except for methionine and phenylalanine, than chickpea and mung bean. Additionally, Baki bean™ had higher methionine content than broad bean and lentil, higher histidine content than common bean, lentil and pea, higher leucine content than pea, higher lysine content than common bean, higher threonine content than lentil and pea, and higher tryptophan content than broad bean and pea (Table 4). Considering nonessential amino acid content Baki bean™ had higher alanine content than broad bean, chickpea, common bean, cowpea, and pea, higher arginine content than, chickpea, common bean, cowpea, lentil, mung bean, and pea, and higher aspartic acid content than chickpea and cowpea. Additionally, Baki™ bean had higher cystine content than common bean, cowpea, and mung bean, higher glutamic acid content than common bean and chickpea, higher glycine content than common bean, chickpea, cowpea, and pea, higher proline content than pea, higher serine content than chickpea, cowpea, and pea, higher tyrosine content than chickpea and mung bean (Table 5). Finally, Baki™ bean had higher total amino acid content than common bean and chickpea, higher essential amino acid content than chickpea and mung bean, higher branched chain amino acid content than chickpea, mung bean, and pea, higher sulfur amino acid content than common bean and mung bean, and higher nonessential amino acid content than chickpea, common bean, and cowpea. Sainfoin bean had a lower ratio of essential to nonessential acids than chickpea, common bean, and cowpea (Table 6).

4.3 Potential complete protein

In addition to their possible uses and value in animal diets, pulses are regarded as an important source of protein in human diets. Traditional human diets have relied on complimentary combinations of cereals and pulses as a solution to satisfying protein and amino acid requirements. Typically, the low-lysine content of cereals is supplemented by the content in pulses and the low-SAA content of pulses are supplemented by the content in cereals (5, 64, 65). Therefore, this strategy helps to mitigate the risk of limiting amino acid content in the diet. We define limiting amino acid content as insufficient content of a single essential amino acid, or multiple amino acids, when compared to the respective adult daily requirements established by the World Health Organization (WHO) and FAO (66). The crops analyzed in this study had a narrow range in values for the ratio of essential amino acids to nonessential amino acids, indicating that this level of analysis is not as informative as considering the content of individual amino acids. Our analysis of the FAO pulses data set shows that mung bean, lentil, broad bean, and common bean can have limiting essential amino acid content, especially for the sulfur amino acids (SAA) methionine and cysteine. Comparatively, we did not identify any limiting essential amino acids in the Baki™ bean samples analyzed. This indicates that seeds from sainfoins may provide a complete protein source with respect to satisfying essential amino acid requirements. Future studies are necessary to not only corroborate the results we provide regarding the amino acid profiles, but to also investigate how different combinations of genotypes, environments, management practices and processing techniques influence amino acid content and composition. Even though the ability of legumes to fix nitrogen through symbiotic associations with specific species of *Rhizobium* bacteria is believed to enhance the stability of seed protein content across environments (67), significant effects of environment and genotype-by-environment on seed protein content have been shown for *Vigna stipulacea* (68), *Lens culinaris* (69), *Cicer arietinum* (70). Moreover, genotype can also impact seed protein content. For example, Baptista et al. (71) found that certain bean and cowpea genotypes had amino acid scores close to meeting requirements. Moreover, in a study of cooked pulses, Nosworthy et al. (72) report amino acid scores (content/reference requirement) for the sulfur amino acids (methionine + cysteine) ranging from a limiting value of 0.59 for split red lentils and split green peas to a value of 1.08 for chickpeas that exceeds requirements. Conversely, chickpeas had the lowest score for tryptophan (0.61), compared to the highest score found for black beans (0.95). Scores for lysine, the amino acid typically limiting cereals, ranged from 1.16 for red kidney beans to 1.40 for whole green lentils. Sulfur fertilization and later harvest time can increase cysteine and methioine content, as has been shown for lentils (73). As with fatty acid content and composition, this information will be valuable for producers and breeders focused on improving sainfoin protein quality.

5 Conclusion

This study builds on evidence supporting the potential of sainfoin as a novel pulse crop. We quantified the amino acid and fatty acid profiles of Baki™ bean, representing seeds from named sainfoin varieties grow in the western US by commercial seed producers, and made comparisons to pulse crops using data reported by the FAO. Baki™ bean amino acid and fatty acid content was found to be higher than certain pulse crops. Baki™ bean fatty acids were primarily polyunsaturated, compared to monounsaturated and saturated fatty acids. The fatty acid profile was primarily composed of n-3 fatty acids, followed by n-9 fatty acids and then n-6 fatty acids. We found the essential fatty acid linolenic acid (18:3 n-3) to be the most abundant fatty acid, followed oleic acid (18:1 cis-9), and the essential fatty acid linoleic acid (18:2 n-6). When comparing essential amino acid content to adult daily requirements, Baki™ bean met the requirements for each amino acid. Moreover, we found that Baki™ bean, in addition to chickpea, soybean, cowpea, and pea, met sulfur amino acid requirements, which are typically limiting for pulses, as evidenced by lentil, broad bean, mung bean, and common bean failing to meet requirements. Future studies are required to further investigating the promising amino acid and fatty acid profiles found in this study for Baki™ bean.

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

EC: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SB: Conceptualization, Methodology, Project administration, Writing – review & editing. MŞ: Conceptualization, Writing – review & editing. TP: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. BS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1292628/full#supplementary-material

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Development of a regionalized dynamic weighting method for the environmental impact of alternative protein sources

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Accurate environmental analysis is central to addressing food product impacts, yet uncertainty shrouds the effectiveness of life cycle assessment (LCA) weighting methods, particularly for alternative protein foods and different countries. Our approach characterizes environmental impact weighting based on total or specific production impacts at the country level, facilitating relevance assessment. We have developed an innovated methodology to calculate weights for alternative foods such as crickets, mealworms, black soldier flies, cultured meat, *Chlorella*, and *Spirulina*. This method integrates their country-level eco-potential linked to environmental impacts, and addresses challenges in existing methodologies-policy changes, contextual adaptation, method specificity, intangible values. Relative impact weights, normalized by arable land and population, cover greenhouse gas emissions, land use, water use and energy use. Eco-potential points for alternative protein sources are derived by dividing their impact values by the relative country-level weights. In addition, eco-potential points for conventional protein sources are calculated for comparison, highlighting disparities. The results show a dynamic eco-potential influenced by evolving country-level *per capita* impacts that influence food product impacts. Comparison of literature based LCAs with our weighted country-level impacts highlights an alignment between absolute emissions and relative impact weightings in certain cases. Moreover, we have developed a parallel methodology to calculate eco-potential points for selected alternative food proteins based on protein supply in countries. This calculation is based on 17 years of data and multiplies the protein supply by the average environmental impact of selected sources (GHGE, water, land and energy use). This results in country-level weighted impacts (CWI), or eco-potential points that are aligned with protein supply. Combining the CWI from the four indicators gives the combined eco-potential values for selected alternative proteins.

The comparison of the product's CWIs for GWP, WU, EU and LU showed that certain impact categories with higher CWI or eco-potential points can contribute to the higher combined eco-potential point. The eco-potential points of different impact categories also varied between countries.

KEYWORDS

life cycle assessment, eco-potential points, relative impact weights, alternative protein sources, country weighted impact

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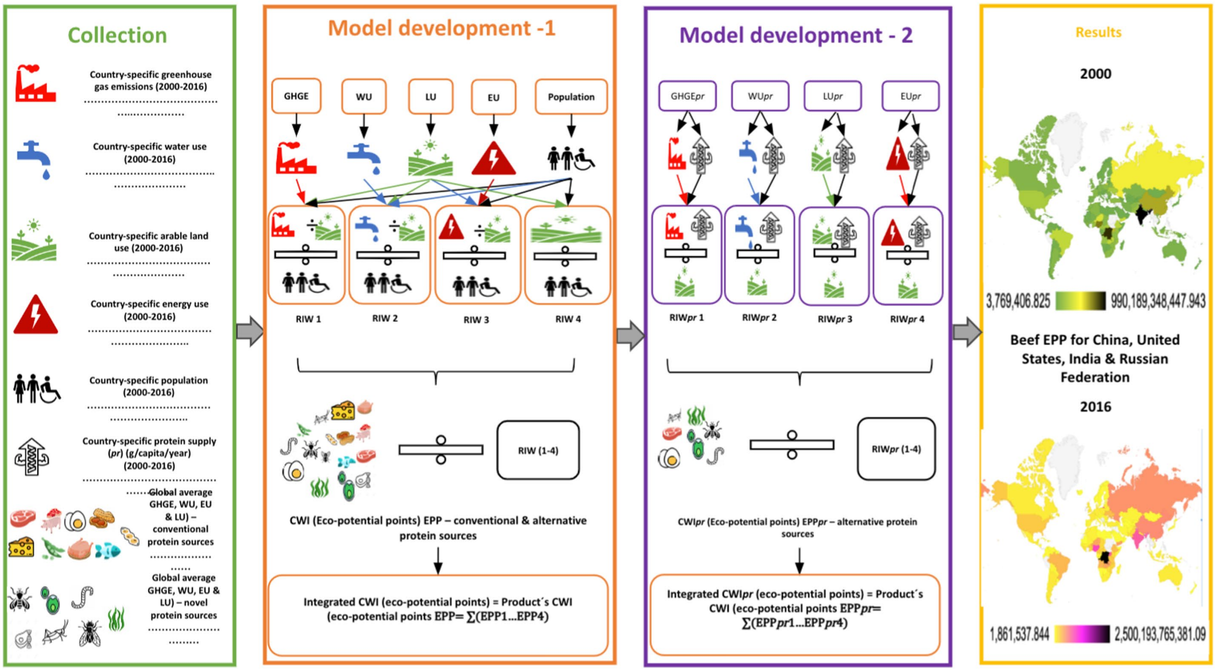

GRAPHICAL ABSTRACT

Pr, protein; GHGE, greenhouse gas emissions; WU, water use; EU, energy use; LU, land use; RIW, relative impact weight; CWI, country weighted impact; EPP, eco potential point; GHGEpr, greenhouse gas emissions per protein supply; WU*pr*, water use per protein supply; LUpr, land use per protein supply; EU*pr*, energy use per protein supply; CWI*pr*, country weighted impacts per protein supply.

Highlights

- Country relative impact weights (RIWs) were shown to be dynamic over time with changes in factors such as total GHG, water use, arable land use, energy use and population.
- Product's impact, weighted against the relative impact weights (RIWs) in selected impact categories was influenced by the dynamic changes in the RIWs over time.
- Product's country weighted impacts (CWIs), when combined for the selected impact categories, reflected the combined potential for the increased impact in the selected categories and for the country of interest.
- Product's absolute emissions or resources use could be higher, but its CWI or eco-potential could be lower in the same country, or they could be the same.

1 Introduction

Effective management and policy interventions in the field of climate change mitigation and environmental impact reduction require a reliable assessment system capable of reflecting the current state of the environment in a dynamic and regional perspective (Smetana et al., 2015; Beaussier et al., 2019). Assessments of food systems sustainability are crucial to address global challenges like climate change and environmental degradation. Studies like Vermeulen et al. (2012) highlights inefficiencies in the food supply chain. These assessments provide a comprehensive understanding of social, economic, and environmental factors, guiding evidence-based interventions for long-term sustainability (Willett et al., 2019).

Regionalization in life cycle assessment (LCA) is not new, thus approaches based on regionalized characterization factors (Frischknecht et al., 2018), assessment methods and databases combined with geoinformatics systems (Mutel et al., 2011), downscaling of input-output analysis to regional level (Smetana et al., 2015) have already established a solid basis. However, such approaches are not sensitive to year-to-year changes in economy, society or emissions. In addition, Life Cycle Impact Assessment (LCIA) methodologies are subject to uncertainty. For example, LC-IMPACT methodology has circumstantial uncertainty such as which time horizon to take into account and what is the degree of certainty of the consequences (Frischknecht et al., 2018). While dealing with uncertainty in how we measure environmental impacts, it's important to also look at the effects of new products on the market. This shows that we need to use different methods when checking how these emerging products affect the environment.

Determining the environmental impacts of alternative and emerging products on the market is a complex and challenging task. Even ex-ante approaches in LCA (Cucurachi et al., 2018; Moni et al., 2019; Steubing and De Koning, 2021) do not assess the relevance of the environmental impacts of emerging products and technologies at the scale of producing regions and countries. Furthermore, alternative protein foods are characterized by limited data availability and potential variations in production practices and scales (Ververis et al., 2020). By definition alternative proteins are proteins which are derived from plants, animal cells, or fermentation, replicate or exceed the taste of traditional animal products with comparable or lower costs, requiring fewer resources and generating fewer negative environmental impacts. While plant-based and fermentation-derived options are available, cultivated meats are still in development (Good Food Institute, 2023).

Due to regional differences, the LCA at the production and the consumption stages vary considerably. The products consumed today have intercontinental environmental impacts as they are often produced in one country and delivered through global supply chains (Yang et al., 2019). Thus, LCA weighting results should represent the "price" of production in terms of country's capacity. Such a weighting system is essential for policy and decision makers who want to interpret LCA results in terms of a country's or region's environmental awareness (Castellani et al., 2016). Regional weighting is an important issue in the LCA of the extended supply chains, as data on environmental impacts of regions and of regional production are not available (Itsubo et al., 2015). Examples of regionalized weighting methodologies with their characteristics and limitations are presented in Table 1.

Our work builds on existing regionalized and weighting methodologies such as Eco-indicator 99, EDIP, Eco-scarcity 2006, Japanese LCIA, TRACI, and extends them by developing a model to incorporate reference to population and environmental impacts and conditions into the weighting methodology of LCA. The incorporation of country based weighting factors, based on real environmental impact data from the countries, into the LCA should provide a solid basis for dynamic analysis of alternative or emerging products and technologies. In this study, we develop a methodological approach based on the eco-potential of conventional and alternative protein sources which discloses the additional environmental impacts in different countries. We have also delved into the crucial concept of the rebound effect, as outlined by Matraeva et al. (2022), which denotes an increase in overall resource consumption despite efforts to reduce individual usage, including the integration of 'green' technologies.

The term "eco-potential" is basically developed for this research work and is related to the original term "Ecopoints" or ecological scarcity LCIA method using midpoint approach (Sharaai, 2012). Eco-potential points are different from the already used LCIA eco-points in that they give us the high and low scores for the country-level environmental impacts of the product, with high scores representing the higher potential for lower impacts and low scores representing the higher probability of higher environmental impacts. The reason behind choosing the specific conventional protein sources was because of the data availability for its country-based impact from the study done by Poore and Nemecek (2018). The regionalized (country-based) environmental impacts from the study is a good base for comparing the results obtained from our methodology. Since we wanted to analyze the regional environmental impacts of future foods, the data for the future foods especially the (alternative protein sources) was gathered which was available from the study done by Parodi et al. (2018). Finally, we demonstrate the advantages and limitations of our approach to determining the relevance of the country-level and dynamic environmental impacts of alternative foods by analyzing the results and comparing them with other studies. In this study, we present a useful and promising weighting system for policy and decision makers to interpret LCA results related to protein sources in the context of a country's environmental awareness. Our approach offers valuable insights that can enhance the applicability of LCA findings for informed decision-making."

TABLE 1 Existing weighting methods and their associated principles/characteristics and their limitations.

Existing regionalized weighting methods	Weighting principles/characteristics	Limitations
Eco-indicator 99	In this procedure weighting is done at the endpoint level (damage category level in ISO) (PRé Sustainability, 2020)	Use of panel approach supports value choices depicting the importance of people, experts, organizations, regions, political agendas or costs (Pizzol et al., 2016). It is a limitation because the use of panel approach can be biased and not very fair. People in the panel might have different opinions, and important things could be left out. This makes the results not very reliable or fair
EDIP	Impersonates political reduction targets based on the urge to reduce the impacts and preparedness to make necessary changes (Stranddorf et al., 2005)	Method not effectual for estimating the improvements of products that reduce impacts which either has a favorable current condition or has not yet been turned into a precise policy objective (Pizzol et al., 2016)
Eco-scarcity 2006	Based on governmental policies in the corresponding countries (Muhl et al., 2020)	Policy based targets are difficult to precisely translate into weights because the targets does not cover all the elementary flows and impact categories (Pizzol et al., 2016)
Japanese LCIA	Based on endpoint modeling (named LIME) uses a survey-based approach (Inaba and Itsubo, 2018)	Only limited to G20 countries. Survey sheet is not fully understood in the developing countries and thus weighting factors are needed to be developed which includes the developing countries (Publications Office of the European Union, 2011)
TRACI	Employing the willingness to pay as a base reference, valuing impacts and damages in terms of costs, and middle impacts weighting (Kalbar et al., 2016). Compared to weights obtained using other methods, monetary units may be more known and simpler to relate to for most audiences	The methodology only covers use value and is too case-particular for endpoint usage. Also, apposition from the audience to assign monetary values on biodiversity or human life also limits its applicability (Pizzol et al., 2016)

EDIP, environmental design of industrial products; LCIA, life cycle impact assessment; LIME, life cycle impact assessment method based on endpoint modeling; TRACI, tool for reduction and assessment of chemical and other environmental impacts.

The study relied on a stepwise approach to calculate normalized values and impact weights according to the impact indicators relevant for the selected country (Table 1). Food production accounts for a significant share of environmental impacts in the developed countries (Nemecek et al., 2016). Indicators such as GHGEs, WU, ALU, and EU were chosen because they are directly related to agri-food systems and reflect the critical environmental parameters for food production. In fact, more than a quarter (26%) of the world's GHGE come from food and agriculture (Poore and Nemecek, 2018). In particular, the consumption of proteins from animal sources leads to significant environmental impacts. The consumption of animal proteins is a well-known contributor to greenhouse gas emissions, water consumption, biodiversity loss, and impacts on nutrient cycling due to agricultural production (Ernstoff et al., 2019). Some environmental and demographic factors (country arable land, country population) were considered as normalization factors. The size of the human population and the rate of its growth are now contributing significantly to the extinction of biodiversity (Crist et al., 2017).

2 Methods

2.1 Model development

The first step was to calculate the relative impact weights (RIWs) by collecting the data for the country's total GHGE, WU, EU, LU and population for a time scale of 17 years. The collected data is for calculating the relative impact weight (RIW) results for the environmental impact categories as mentioned above. In the second step, the CWIs or eco-potential points of the product were calculated by dividing the environmental impacts of the product (GHGE, WU, ALU, and EU) by the RIWs calculated in the first step. In the third and fourth step the same methodology for calculating the RIW, CWI and eco-points was applied to the environmental impacts of the protein supplies in the selected countries only. We then carry out an analysis of the eco-potential of nine conventional protein sources [i.e., peas, soybeans (tofu), groundnut, beef, cheese, pork, eggs, fish (farmed) and poultry meat] using the developed weighting methods according to geographical aspects (148 countries), resource (land, climate, water and energy uses), time scale (17 years) and population aspects. Next, we assess the applicability of the eco-potential weighting method to identify the additional potential environmental impact of alternative protein sources (i.e., crickets, mealworms, black soldier fly, housefly, cultured meat, *Chlorella*, and *Spirulina*) in relation to the aforementioned aspects and the impact of the current protein supply.

The relative environmental impacts and eco-potentials of the target products were calculated in four application steps as explained in the Table 2. In the first step (I), impact categories (country level, annual values) of arable land use, greenhouse gas emissions, water use, and energy use were selected. In the second step (II), normalized values of relative impact weights (RIW) are calculated in 4 sub-steps for the selected impact categories. In step (II), the four impact indicators (ALU, GHGE, WU, and EU) are normalized by the ALU and population in the selected countries (RIW1–4). Similarly, RIWpr1–4 (protein related) values are also calculated. For the calculation of the protein related RIW, in the first step (Ia), data on the dietary protein supply (g/capita/day) per country and the environmental impacts (GWP, WU, EU, and ALU) of the food protein sources were collected. An average of the selected protein sources was again calculated for the environmental impact indicators (GWP, WU, EU, and ALU). The average values of these impact indicators were then multiplied by the converted amount of protein supply (g/capita/year). In step (IIa), values for RIWpr1–4 are calculated by normalizing the protein-related impact indicators (ALUpr, GHGEpr, WUpr and EUpr) by ALU and population. In step (III), the eco-potential points (EPP1–4) for the target food products in the different countries are calculated by dividing the overall average environmental impacts of the product by the country's total impact weights (RIW1–4) and similarly with the protein-related Relative impact weights (RIWpr1–4) in step (IIIa). Finally, in steps (IV and IVa) the product-specific eco-potential points (EPP1–4) were integrated by summing them to obtain the final result.

2.2 Data collection

First, data were collected for total GHGE, WU, EU, ALU and population for 148 countries (Supplementary Table S1). Data for resource use and GHGE were only available for 148 of the 195 countries. The weighting method is intended to be dynamic and should therefore reflect the variations in the annual environmental impacts and indicators used as weights. To determine the sensitivity of the proposed weights to annual variations, data were collected for from 2000 to 2016 (more recent data were not available). Data on the environmental impacts of conventional and alternative protein sources were also collected (Supplementary Table S1).

Data on environmental impact indicators of global warming, water use and land use for conventional protein sources were collected from Poore and Nemecek (2018), while data on the environmental impacts of alternative protein sources were collected from different sources (Supplementary Table S1). In addition, the original environmental impact data, expressed as total average yields for each product presented in the data source, expressed in terms of its 100 g nutritional units (NU), were converted to 1,000 g or 1 kg of protein per product. The calculations behind these conversions are presented in Supplementary Table S1. The data were converted to show the environmental impact of the global average yield of 1 kg of protein per product. The environmental impact values for conventional and alternative protein sources are presented below in Tables 2, 3.

3 Results

3.1 Analysis of relative impact weights

It's clear from the results for the country specific RIWs, that they are dynamic over time. Many factors that change over time (e.g., GHGE, WU, ALU and EU) contribute to the temporal changes in the results. Only the factors GHGE, WU, EU, and ALU were selected because water-land-energy emissions are an important basis to reduce the GHGE from agricultural activities (Deng et al., 2021). For example, RIW2 for China reflects the dynamics associated with increasing GHG emissions and decreasing arable land (Figure 1). Similarly, the values of the other RIWs (1–4) depend on the initial impacts considered (arable land, GHGE, WU, and EU) and the values used for the normalization of the impacts (population and arable

TABLE 2 Methodological framework for estimating the relative environmental impact and eco-potential of protein source production.

Type of indicator	Application steps	Characteristics (examples)	Application steps	Characteristics (examples)
	Food products (production system, service)		Protein sources (supply)	
Weighting categories (indicators)	I. Selection of impact categories (country level, annual values)	1 ALU 2 GHGE 3 WU 4 EU	Ia. Selection of impact categories relevant to protein supply (country level, annual values)	Protein supply: 1 ALUpr 2 GHGEpr 3 WUpr 4 EUpr
Relative impact weights (RIW)	II. Calculation of RIW (normalized values)	$RIW1 = ALU \div N$ $RIW2 = GHGE \div ALU \div N$ $RIW3 = WU \div ALU \div N$ $RIW4 = EU \div ALU \div N$	IIa. Calculation of protein related RIWpr	$RIWpr1 = ALUpr \div ALU \div N$ $RIWpr2 = GHGEpr \div ALU \div N$ $RIWpr3 = WUpr \div ALU \div N$ $RIWpr4 = EUpr \div ALU \div N$
Country-level weighted impacts (CWI), expressed in eco-potential points	III. CWI calculation for target product	$EPP1 = (Food\ ALU \div RIW1)$ $EPP2 = (Food\ GHGE \div RIW2)$ $EPP3 = (Food\ WU \div RIW3)$ $EPP4 = (Food\ EU \div RIW4)$	IIIa. CWI calculation for target protein sources (supply)	$EPP1pr = (Protein\ source\ ALU \div RIWpr1)$ $EPP2pr = (Protein\ source\ GHGE \div RIWpr2)$ $EPP3pr = (Protein\ source\ WU \div RIWpr3)$ $EPP4pr = (Protein\ source\ EU \div RIWpr4)$
Integrated eco-potential	IV. Integrated eco-potential calculation for specific product	$EPP = \sum(EPP1 \ldots EPP4)$	IVa. Integrated eco-potential calculation for specific protein source	Protein specific integrated $EPP = \sum(EPPpr1 \ldots EPPpr4)$
Meaning of the country weighted impacts	Potential of increased environmental impact caused by production (of food) in relation to the relevant country's resources. Expressed in eco-potential points		Potential of increased environmental impact caused by protein source production (or supply) in relation to the impact caused by the current conventional protein supply. Expressed in eco-potential points (protein supply)	

ALU (ALUpr), arable land use (arable land use of protein supply); GHGE (GHGEpr), greenhouse gas emissions (greenhouse gas emissions of protein supply); WU (WUpr), water use (water use of protein supply); EU (EUpr), energy use (energy use of protein supply); RIW1–4 (RIW1–4pr), relative impact weights, normalized impact values (relative impact weights for protein supply); CWI, country weighted impacts (impacts of the products weighted against normalized values in a specific category), EPP1-4 (EPP1-4pr), eco-potential (eco-potential of protein products); *N*, population of a country.

TABLE 3 Environmental impacts from conventional protein sources (Sources can be found in Supplementary Table S1).

Conventional protein sources	Land use (m^2/NU)	GHGE ($kgCO_2eq$/NU)	Stress-weighted water use (L/NU)	Energy use (MJ/NU)
Beef	1,640	500	174,190	132.2
Cheese	400	110	819,070	250.2
Peas	34	4	125,780	348
Pig meat	110	76	413,270	123.33
Eggs	57	42	162,060	224.5
Poultry meat	71	57	81,860	92.22
Groundnuts	35	12	236,050	18.2
Fish	37	60	182,290	221.07
Soybeans (tofu)	22	20	31,960	19

m^2, meter square; NU, nutritional unit; GHGE, greenhouse gas emissions; $kgCO_2eq$, kilograms of carbon dioxide equivalent; L, liter; MJ, megajoule.

land). The dynamics of the indicated factors would also determine the dynamics of RIWs. RIWs calculated for 148 countries are included in Supplementary Table S1.

3.2 Country-level weighted impact analysis for targeted products

Country weighted impacts represent the impact of a product in a selected impact category, weighted by the relevant relative impact weight (RIW). Temporal dynamic changes in the country weighted impacts (country-level eco-potential) are caused by the dynamic weights as discussed above (Figure 1), but also by changes in the impacts of the product in the same category (if the changes are known). By definition "temporal dynamic changes" means the changes in the relative impact weights (country's eco-potential) triggered by the change in the country's total environmental impacts throughout the year. The country weighted impact (CWI) from GHGE, based on 1 kg of beef production, is shown in Figure 2. The analysis shows that the eco-potential is highest in

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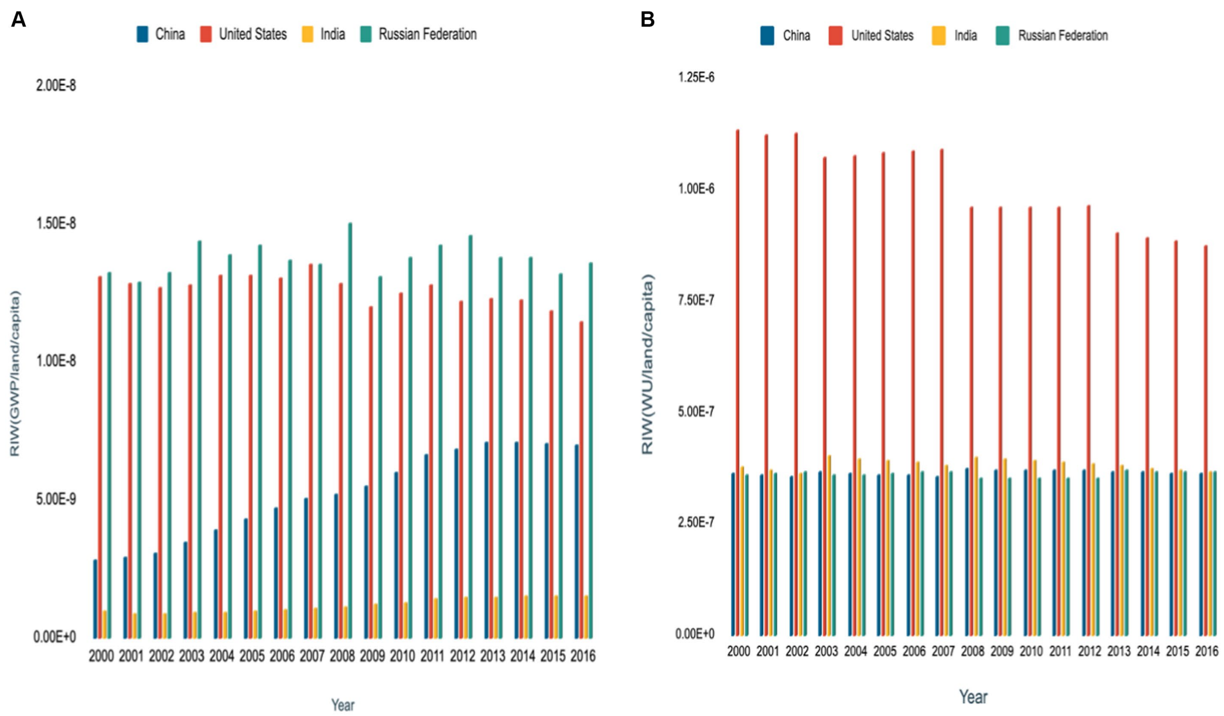

FIGURE 1
RIW2 and RIW3 relative impact weight based on **(A)** GHGE and **(B)** water use normalized by arable land and population for China, United States, India and Russian Federation (graphs has a factor of 10^{-9}). RIW, relative impact weight; GHGE, greenhouse gas emissions.

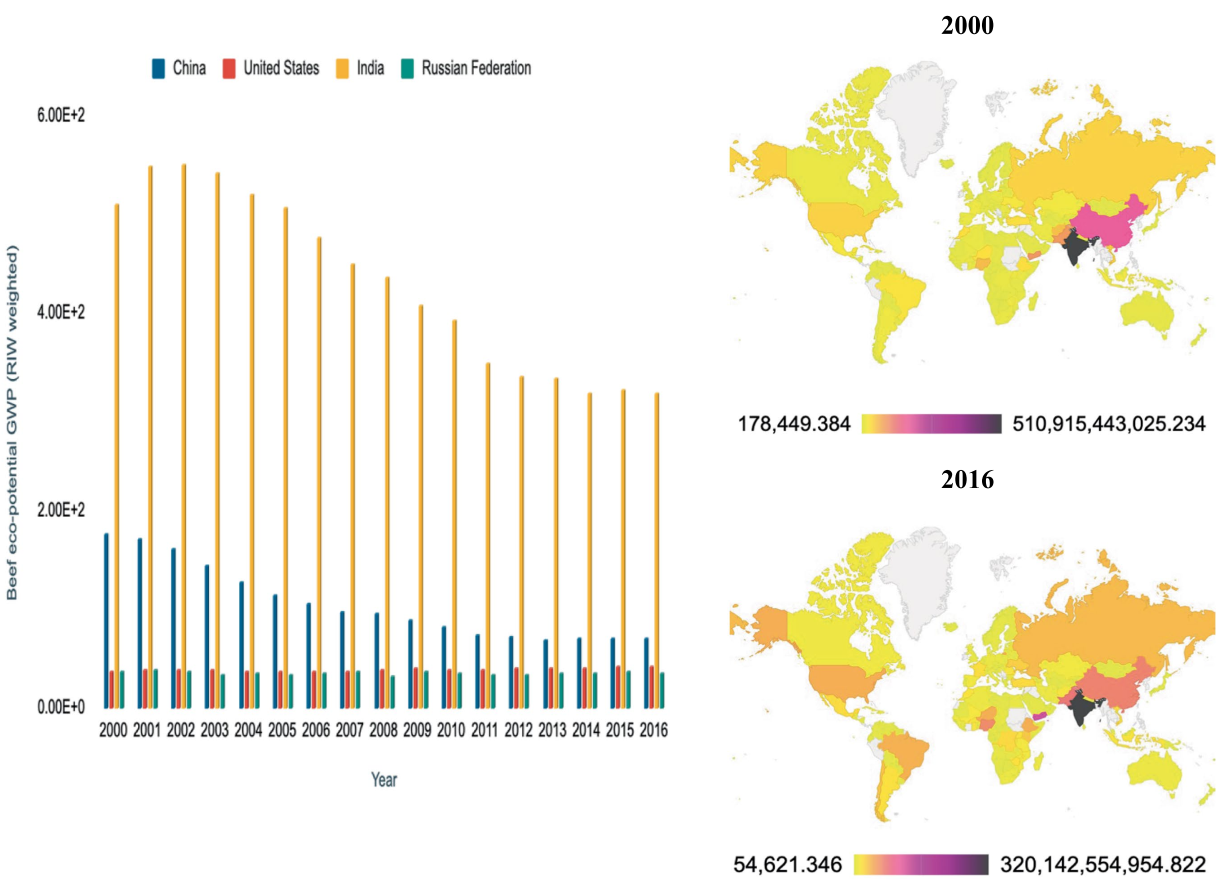

FIGURE 2
CWI weighted impact (GHGE based) for the production of 1 kg of beef (graph has a factor of 10^9). CWI, country-level weighted impact; GHGE, greenhouse gas emissions.

India and lowest in the United States and the Russian Federation. The values of the eco-potential for beef production is highest in India as the country's relative impact weight (RIW) for GHGE is lowest (Figure 1) which means there is lesser burden on the environment when the country's total GHGE were normalized by its arable land use and population. So, producing beef in the country would relatively have a lower additional impact when compared with the other chosen countries (China, United States, and Russia). Calculated CWIs for 148 countries are provided in Supplementary Table S1.

3.3 Integrated eco-potential analysis for conventional and alternative protein sources

The same normalization and weighting base defined for relative impact weights (RIW) and country weighted impacts (according to the methodological framework in Table 1) allows to obtain results in points that can be summarized to reflect the combined potential for the increased impact in the selected categories and for the country of interest. These combined eco-potential points obtained for food products or protein sources show the relative potential environmental impact of the production of the food products with respect to the country-specific situation, taking into account several impact categories (see Table 4).

The eco-potential of beef production (Figure 3) showed that, among comparable countries, India could be a representative country for beef production with the lowest incremental environmental impact. The incremental impact of beef production is much lower relative to the amount of resources normalized to population and available arable land (Figure 3). In contrast, the United States is the worst performing country, where the additional environmental impacts of one unit of beef production is associated with a higher additional environmental burden due to the country's existing environmental impacts. Similar trends are observed for alternative protein products when the same weighting system is applied (Figure 4). Therefore, the application of higher integrated relative impact weights results in less beneficial eco-potential (potential for environmentally beneficial production of new products). The graphs for the other products can be found in Supplementary Table S1.

3.4 Relative impact weight analysis based on protein supply

the same methodology was used to calculate the RIWs. The main difference here is that it focuses only on the impacts of the level of protein supplies in the different countries. The results for the country specific RIWs show that they are dynamic over time. The average environmental impacts values of the selected alternative protein sources were calculated and multiplied by the amount of protein supplies in the countries. The average environmental impacts for the four environmental impact indicators of the protein supplies (g/capita/year) in the countries were divided by the arable land use per country. The results for the RIW of global warming according to the protein supplies from alternative protein sources in Figure 5, show that China and Russia have the highest weights for total GHGE according to protein supplies from alternative protein sources. Overall, the results show that the RIW for GHGE or resource use will be higher in those countries where the relative emissions or resource use is comparatively higher than their arable land use and population and vice versa.

3.5 Country-level weighted impact analysis of protein supply for target products

As calculated above, the country weighted impacts represent the impact of a product in a selected impact category, weighted by the relative impact weight (RIW). Dynamic changes over time in the country weighted impacts (country-level eco-potential) are caused by the dynamic weights as discussed above (Figure 5). In addition, changes in protein supply in countries over time also affect the eco-potential of the target product. The target product used as an example shown in Figure 6 is *Tenebrio molitor* (mealworm). The results show that the production of 1 kg of protein from mealworm has the highest GHG eco-potential in India compared to the other countries. A higher eco-potential indicates the highest potential for lower additional GHGE weighted by arable land *per capita*. Country-level weighted impacts (CWI) of a product indicates its eco-potential in a particular environmental impact category. The higher bars in Figure 6. Indicate that there is a higher probability of lower additional environmental impacts when compared with other countries.

TABLE 4 Environmental impacts from alternative protein sources (Sources can be found in Supplementary Table S1).

Alternative protein sources	Land use (m^2/NU)	GHGE (kg CO_2eq/NU)	Stress-weighted Water use (L/NU)	Energy use (MJ/NU)
Crickets (*Acheta domesticus*)	155	2.35	21,132	96
Mealworms (*Tenebrio molitor*)	72.63	12.24	23,000	1842.69
Black soldier fly (*Hermetia illucens*)	37.34	14.79	2549.39	103.24
House fly (*Musca domestica*)	0.06	2.66	194.5	40.57
Cultured meat	14.69	30.90	1707.69	437.48
Chlorella	7.75	128.10	7,391	2306.48
Spirulina (*Arthrospira platensis*)	4.76	112.89	1900	1842.69

m^2, meter square; NU, nutritional unit; GHGE, greenhouse gas emissions; kgCO_2eq, kilograms of carbon dioxide equivalent; L, liter; MJ, megajoule.

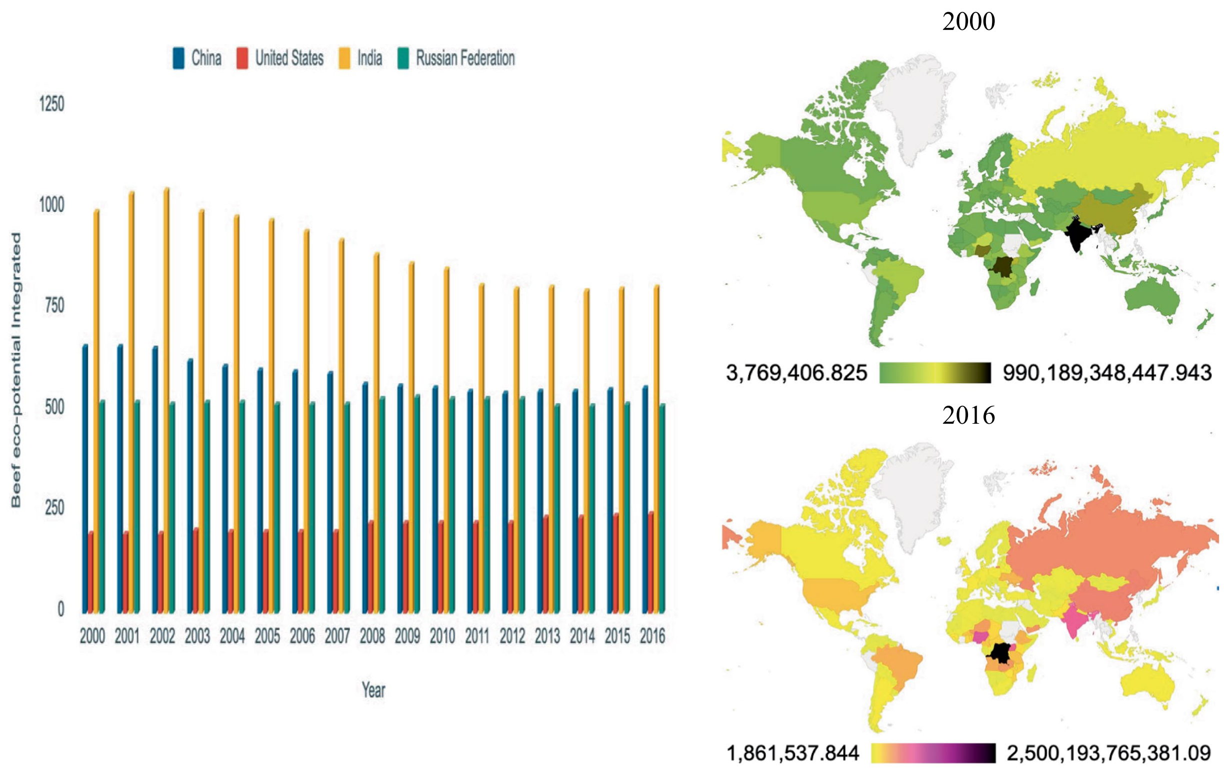

FIGURE 3
Eco-potential of beef production, values expressed in eco-potential points, values for impact of beef production are from Nemecek et al. (2016) and University of Warwick (2013) (graph has a factor of 10^9).

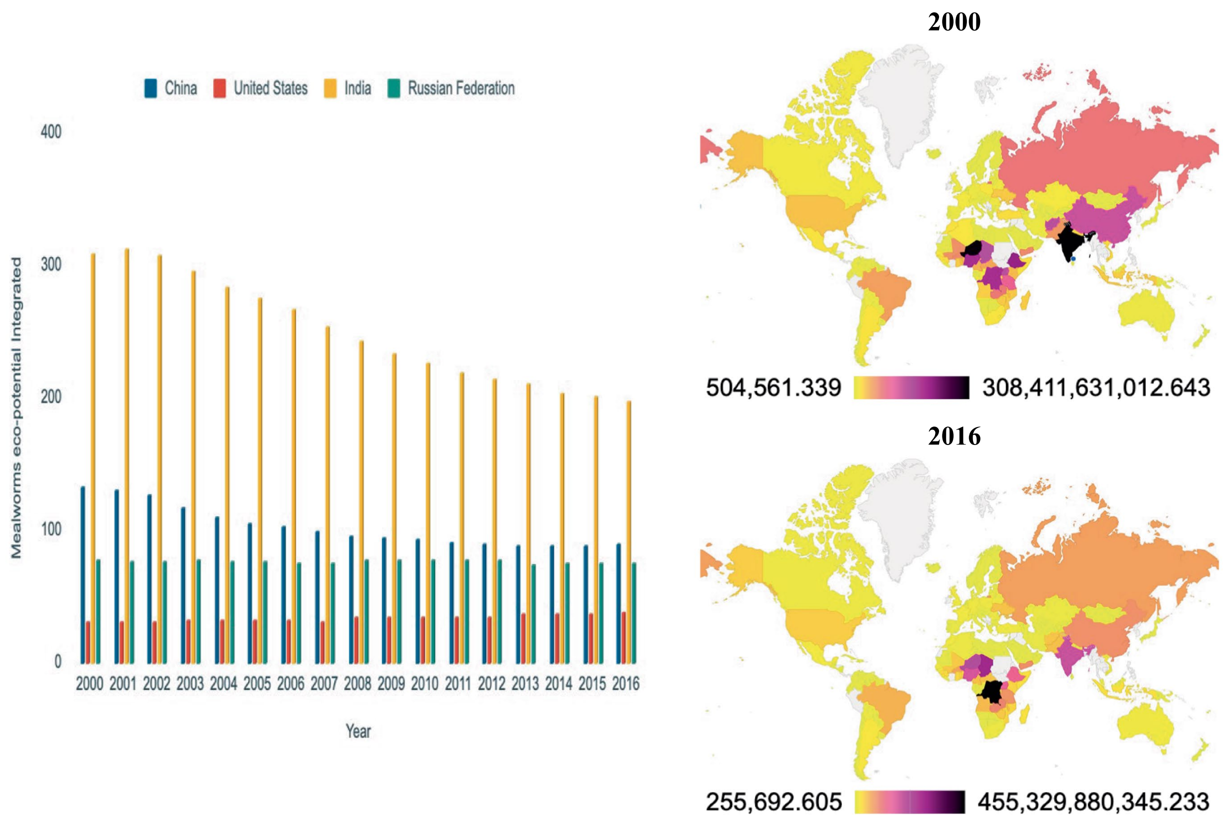

FIGURE 4
Eco-potential of *Tenebrio molitor* (mealworm) production, values expressed in eco-potential points, values for impact of mealworm production are from Parodi et al. (2018) and Miglietta et al. (2015) (graph has a factor of 10^9).

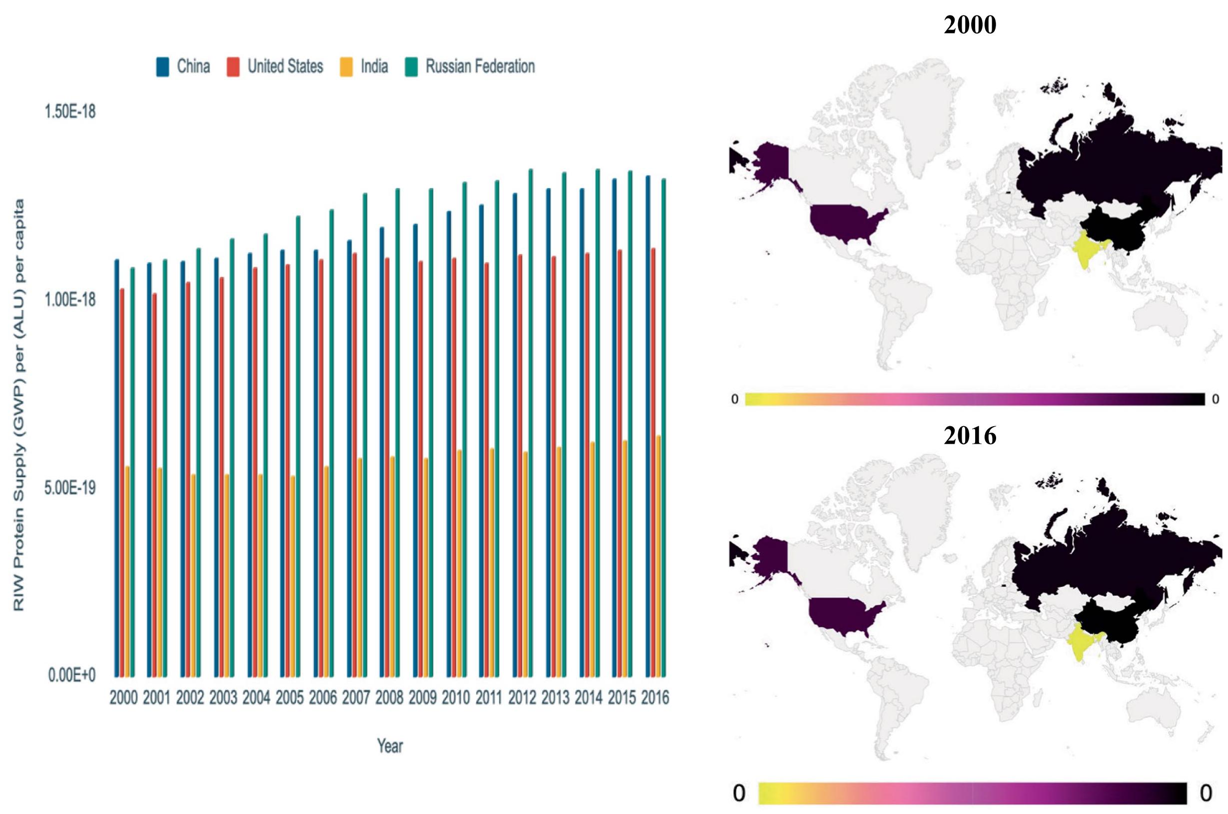

FIGURE 5
Relative impact weight (RIW)—protein supply (alternative protein sources) based on GHGE normalized to arable land and population (maps and graph has a factor of 10^{-9}). RIW, relative impact weight; GHGE, greenhouse gas emissions.

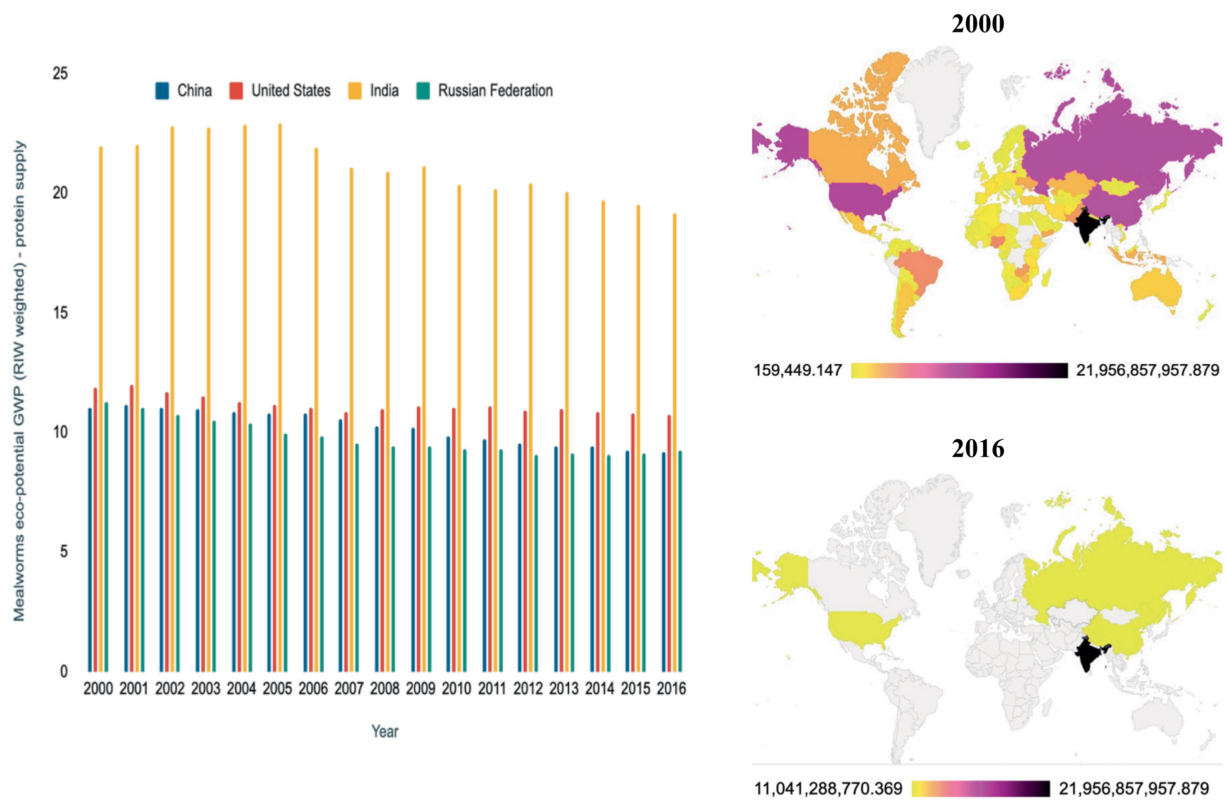

FIGURE 6
Country-level Weighted Impact CWI (GHGE based) by protein supplies to produce 1 kg of *Tenebrio molitor* (mealworm), (graph has a factor of 10^{9}). CWI, country weighted impact; GHGE, greenhouse gas emissions.

3.6 Analysis of integrated eco-potential of alternative protein sources according to country-specific protein supplies

The same methodology (according to the methodological framework in Table 1) as discussed above is applied here to obtain the combined eco-potential of the target product shown in Figure 7. The results show that the country weighted impacts (country-level eco-potential) (GHGE, EU, ALU and WU) from the total protein production of the alternative protein sources for mealworm alone, when integrated together, give India the highest eco-potential points when compared to other countries. The eco-potential of the mealworm alternative protein source showed that India could again be a representative country for the mealworm production, which would result in the lowest additional environmental impact. The additional impact of mealworm production will be much lower relative to the amount of resources normalized to population and available arable land (Figure 7). In contrast, Russia is the worst performing country, where the additional environmental impacts of one unit of mealworm production is associated with a higher additional environmental burden due to the existing environmental impacts from protein supply in the country. Graphs for the other products are presented in Supplementary Table S2.

3.7 Sensitivity analysis

CWIs for GWP, WU, EU and ALU are also compared with each other in order to show the dynamics between them. For example, below in Figure 8, CWIs (1–4) for 1 kg of beef are compared with each other. Analysis of the compared results shows differences in the range of eco-potential points between the selected environmental impact categories. The CWI (GWP) for 1 kg of beef is highest in India while the CWI (WU) is the second highest. CWI (EU and LU) shows the lowest eco-potential points. This shows that CWI (GWP) has a higher contribution to the cumulated eco-potential point when aggregated together with CWI (WU, EU, and LU). This means that the GWP of 1 kg of beef, normalized for arable land use and population, has the lowest environmental impact contribution when compared with water use, energy use and land use. While the CWI (WU) for 1 kg of beef is the highest in China, the United States and the Russian Federation and the CWI (GWP) is the second highest, this shows that the CWI (WU) has the highest contribution to the cumulated eco-potential point when aggregated with the CWI (GWP, EU, and ALU). This means that WU normalized by arable land use and population from 1 kg of beef has the lowest environmental impact contribution in China, the United States and the Russian Federation while CWI (GWP) has the second lowest environmental impact contribution. CWI (EU and ALU) had the lowest points in all countries, meaning that they have the most negative environmental impacts. When all the points are added up to cumulative eco-potential point, India shows the highest due to the higher contribution of CWI (GWP and WU) compared to other countries (Figure 9).

4 Discussions

4.1 Limitations and assumptions

The results of this study for the Country Weighted Impacts (CWI) of conventional protein sources for the environmental impact

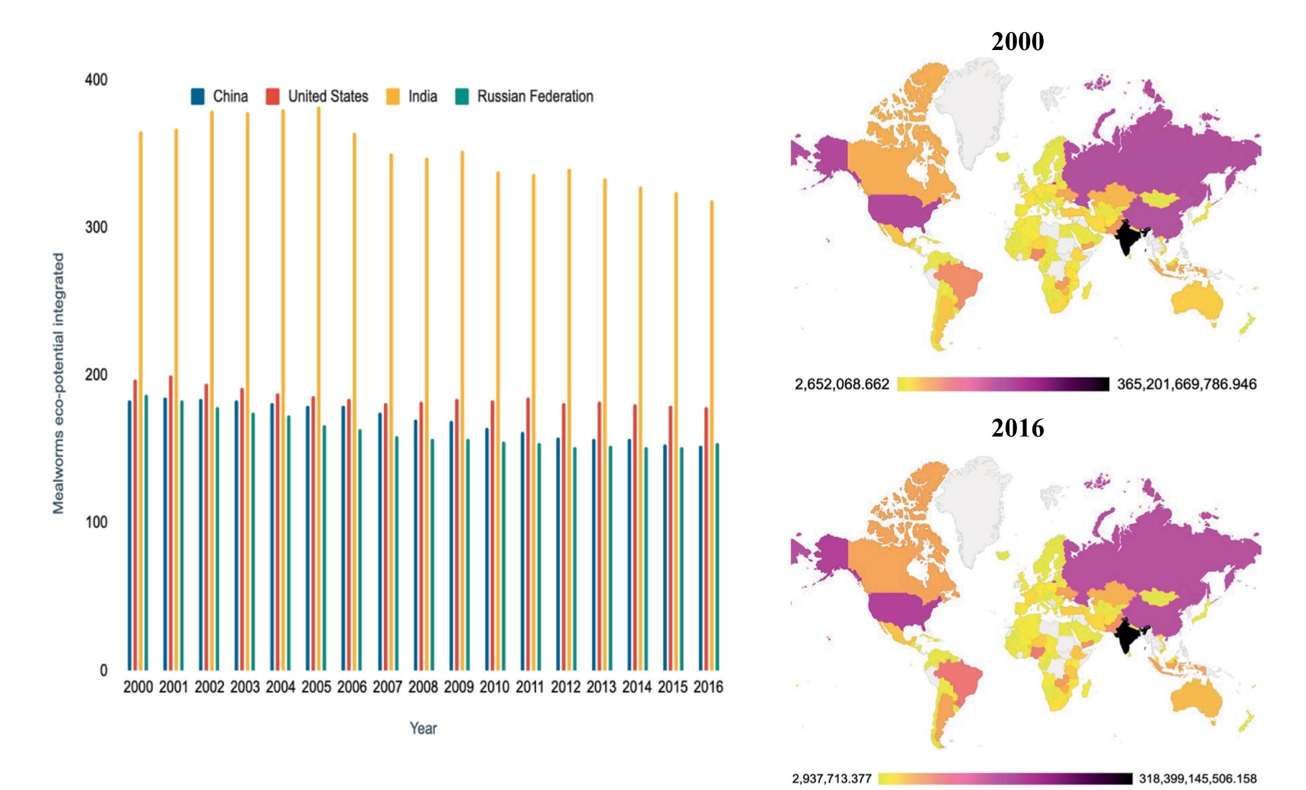

FIGURE 7
Eco-potential of *T. molitor* (mealworm) production according to protein supplies, values expressed in eco-potential points, values for the impact of *T. molitor* production are from Parodi et al. (2018) and Miglietta et al. (2015) (graph has a factor of 10^9).

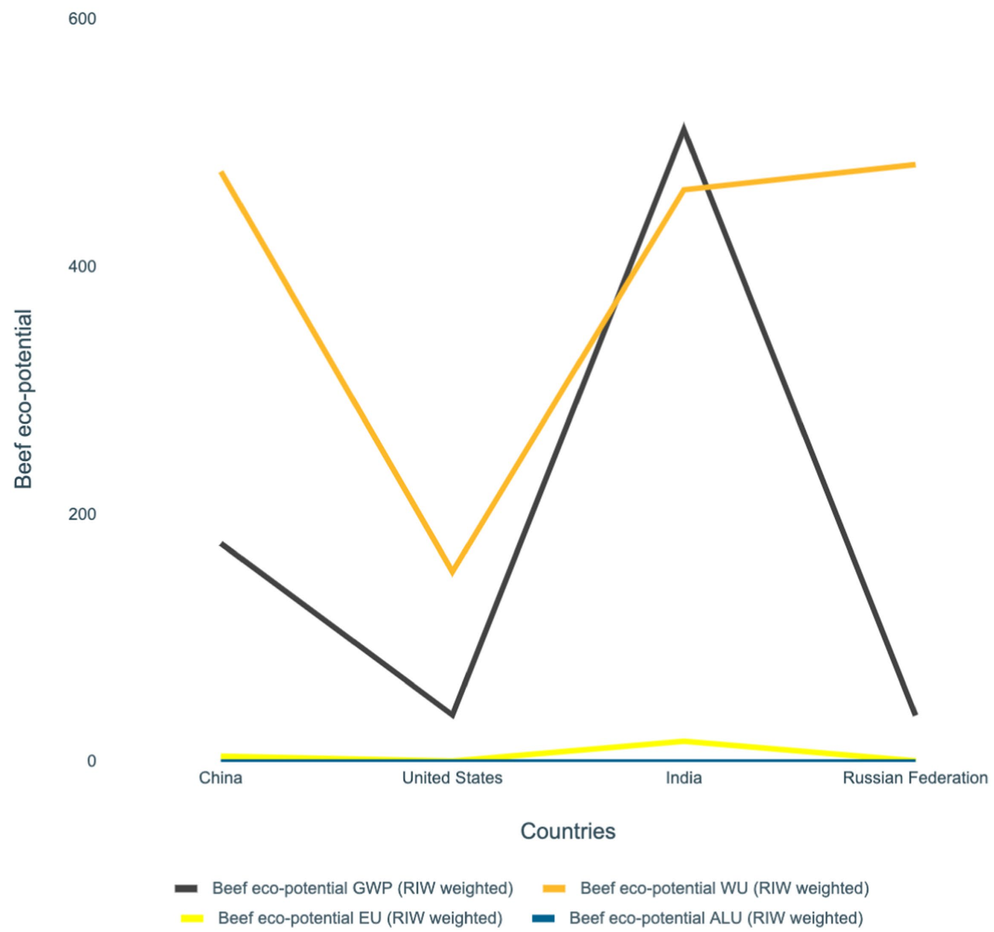

FIGURE 8
CWI (1–4) relative impact weights comparison for beef with considered parameters of GWP, WU, EU, and ALU (graph has a factor of 10^9). RIW, relative impact weight; GWP, global warming potential; WU, water use; EU, energy use; ALU, arable land use.

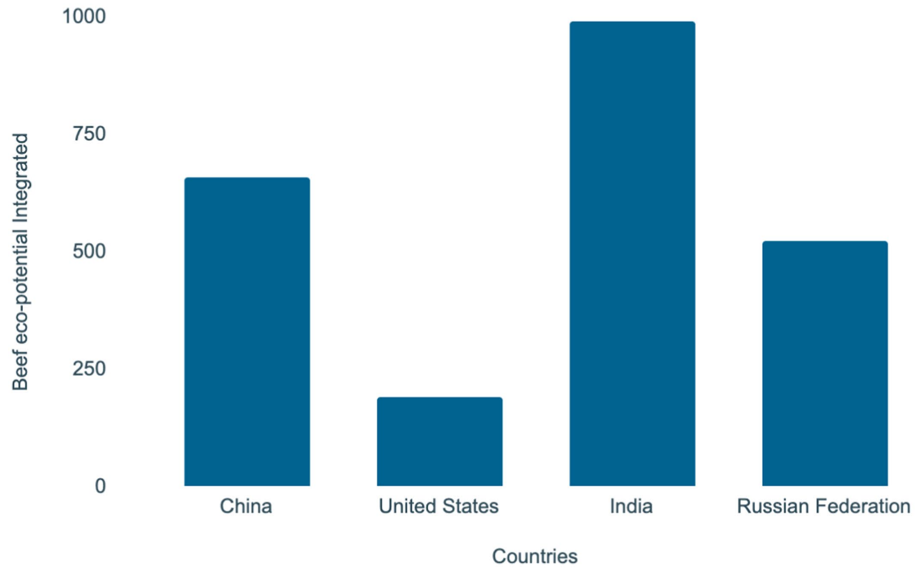

FIGURE 9
Cumulated beef eco potential points for the year 2000 (graph has a factor of 10^9).

categories of GWP, WU and ALU were compared with unweighted environmental impact values from the scientific literature (Poore and Nemecek, 2018). Comparisons were possible for the three environmental impact categories of GWP, WU and ALU. However, comparisons were not possible for all countries and energy use impact category due to a lack of data in the literature.

CWI values are dynamic and change annually. For the analysis of comparisons between CWI derived from conventional protein sources and their country-level absolute environmental impacts, an average CWI value from 2000 to 2016 was taken for each country, and the average value for the country-level absolute environmental impacts were taken from scientific literature.

It is important to note that the CWI indicates the eco-potential for the additional production of a unit in a particular impact category. Therefore, higher CWI values represent the better option for the production with a lower relative impact than the option with a lower CWI. The analysis shows that while the absolute emissions or resource use of the product may be higher in a given country, the CWI may be lower in the same country.

The analysis of the absolute impact values for the production of different protein sources and their weighted values showed that they vary. For example, based on the LCA results for GHGE (kgCO_2eq) from soybean, India has the highest GHGE while the GHGE-related CWI is the highest in India compared to other countries. Therefore, the values are not contradictory, but rather complementary, the absolute values indicate the high absolute negative impact of production while the proposed CWI indicates a high relative eco-potential of production compared to existing resources and emissions (probability of lower relative impacts).

If we consider soybean production in Canada (which has the lowest GHGE per FU among compared countries), then the absolute impact of tofu production is low (could be related to the geographical suitability and advances in production technologies). At the same time, Canada's CWI is low, indicating a low GHG related eco-potential. This implies that an additional unit of production would result in a relatively higher additional environmental burden in Canada. However, not all the impacts and their CWI are related in the same way. This shows that normalizing the impact values to the available resources and using the normalized values as weights it is possible to define the country-level eco-potential of a product that is not related to the absolute impact value of production.

4.2 Recommendations for enhanced application of LCA results

The proposed method for estimating the eco-potential of new products could be related to the eco-efficiency methods. While the concept of eco-efficiency reflects the current value of a product in relation to its function, it does not have a long-term strategy that can deal with rebound effects. Therefore, an increase in eco-efficiency may lead to an increase of consumption (rebound effect) and therefore a decrease in overall sustainability (Bjørn and Hauschild, 2012). Eco-efficiency focuses only on the resource consumption and waste emissions related to the current state of production, and rarely on the state required for long-term sustainability (Bjørn and Hauschild, 2012). Production may be considered eco-efficient in terms of the functional unit, but its relative impact in terms of the overall impact on the environment and available resources may be quite "costly."

The proposed eco-potential weighting system offers the possibility of an additional indicator that defines the relationship between environmental impacts and the available capacity of the environment at the country level in a dynamic approach. It is not intended to replace the environmental impacts of midpoint impact categories, nor is it intended to act as a substitute for eco-efficiency indicators.

In our methodology, we are not mentioning that there is an additional burden due to the country's existing environmental impacts. We normalize our results available arable land use *per capita*. So, the impact weights for country's depend on various factors, not just the total emissions. When a country's environmental impacts are higher than its current arable land use *per capita*, the burden shifts towards the impacts from food production/consumption. Even if a new product has a small impact, it adds to the burden per person's available land. This means more land will be needed with additional production. This burden shift can happen within a country, not to other countries.

Our approach aims to provide complementary measurements that include the relationship with the country's environment. For example, the production of a protein source is not beneficial to the ecosystem components, as all human activities are inherently harmful to the environment (considering only absolute impact values). On the other hand, country weighted eco-potential indicators can help to reduce environmental burden by shifting the perspective from product-based sustainability to ecological sustainability, thus contributing to a reduction in impacts relative to population, area and country-level impacts in a relevant category. In addition, countries with higher emissions and resource use defined *per capita* and arable land use can significantly reduce their environmental footprint by adopting alternative production systems (e.g., alternative proteins).

This study proposes a new methodology for calculating the eco-potential of alternative protein sources taking into account the environmental pillar of sustainability. Eco-potential is defined in the study as an additional environmental impact expressed relative to the existing environmental impact and the country's environmental capacity. This methodology helps to identify the relative environmental impacts of alternative protein sources in different countries, according to the country's total emissions and resource use (GHGE, WU, EU and ALU) *per capita* and on an annual basis.

The eco-potential scores for alternative protein sources varied over time and space. As the values for the relative impact weight (RIWs) based on GHGE normalized by population and arable land increased over time (2000–2016), so did the additional environmental impact burden of the alternative protein sources food products (expressed as CWI for 1 kg of beef) in Figure 2. This indicates the potential for the application of an eco-potential assessment methodology based on regional normalization and weighting factors. Comparison of the results with the scientific literature revealed inconsistencies in the representation of the results, which can be explained by the different assessment approaches.

Future studies should consider implementing the proposed weighting system for policy and decision makers, building on the concept of relative environmental sustainability (Bjørn and Hauschild, 2012). The eco-potential points obtained for the chosen protein sources could also further be used by the policy and decision makers when considering its production in a specific country based on its relative environmental impacts.

5 Conclusion

Overall, the proposed relative impact weights (RIW—specific category impact values normalized to population and available arable land area) could be used as indicators of the current state of environmental impact of a country in a specific impact category, eco-potential therefore represents a regionally relevant impact value of an individual product (whole production system) weighted by the existing level of impact and the regional carrying capacity.

Thus, depending on their relative sustainability in different countries, the developed eco-potential scores for food products can be linked to an overall goal of a state of absolute sustainability by maximizing benefits to ecological systems rather than focusing solely on the product-based eco-efficiency of reducing the damage. Producers should consider the relative environmental impacts of the available capacity of the productive land (arable land use) when considering the production of food products in a country. Considering both the absolute (food product's individual environmental impacts in relation to their production/consumption) and relative environmental sustainability (impacts in relation to its regional environmental conditions) or eco-potentiality can contribute to a more sustainable food system.

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

AF: Data curation, Investigation, Methodology, Writing – original draft. SG: Methodology, Supervision, Writing – review & editing. SS: Conceptualization, Methodology, Supervision, Writing – review & 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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Supplementary material

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Glossary

CF	Characterization factors
CO_2	Carbon dioxide
CO_2eq	Carbon dioxide equivalent
EDIP	Environmental development of industrial products
ESM	Ecological scarcity method
FU	Functional unit
GHG	Greenhouse gas
GHGE	Greenhouse gas emissions
WU	Water use
GWP	Global warming potential
CWI	Country weighted impact
L	Liter
LCA	Life cycle assessment
LCIA	Life cycle impact assessment
LIME	Life cycle impact assessment method based on endpoint modeling
MJ	Megajoule
NU	Nutritional unit
RIW	Relative impact weights
ALU	Arable land use
EU	Energy use
LU	Land use

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Enhanced viability and stability of the *Lactobacillus reuteri* DSM 17938 probiotic strain following microencapsulation in pea and rice protein-inulin conjugates

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Probiotics, which offer various health benefits can face challenges in terms of stability during food processing, storage, and gastrointestinal digestion. Therefore, this study aimed to improve the stability and survival of probiotics during various processing conditions and storage. To address this issue, the study was designed to microencapsulate *Lactobacillus reuteri* DSM 17938 within plant proteins (specifically rice protein (RP) and pea protein (PeP)) and their Maillard reaction conjugated with inulin by spray-drying. The encapsulation efficiency (EE%), stability during storage and temperature, and the viability after simulated gastrointestinal digestion of the microcapsules were examined. The results demonstrate that individual proteins exhibited lower EE%; however, the Maillard conjugates showed increased EE%, with RC (rice protein conjugates) displaying a higher EE% (96.99%) than PC (pea protein conjugates) (92.87%) ($p < 0.05$). Fourier Transform Infrared Spectroscopy verified the interaction between different functional groups of the proteins and Maillard conjugated and indicated the successful encapsulation of *Lactobacillus reuteri* DSM 17938 cells. The results also suggested that RC-encapsulated probiotic cells exhibited maximum survival upon gastrointestinal transit, with a decline of only 1.24 and 1.52 log CFU/g after gastric and complete simulated gastrointestinal digestion, respectively. The viability of probiotics encapsulated with RC and PeC showed improvement compared to those encapsulated with RP and PeP, particularly during refrigerated and room temperature storage, thermal challenge, and simulated gastrointestinal transit. Overall, these findings suggest that plant proteins and prebiotic inulin conjugates could serve as promising new encapsulation matrices for the encapsulation of probiotics in food applications.

KEYWORDS
probiotics, protein-prebiotic conjugate, viability, encapsulation, simulated gastrointestinal digestion

1 Introduction

Probiotics are a group of microorganisms that exert positive biological effects on the host when consumed in appropriate quantities (Hill et al., 2014). According to the previous reports, probiotic strains can treat constipation, decrease cholesterol levels, regulate the immune system, and maintain human gut health by preventing the growth of harmful bacteria (Kaur et al., 2021). A minimum of 10^8 colony forming units (CFU) per mL or g of probiotic food is required to exert a positive effect (Hill et al., 2014). However, several factors affect the survivability of probiotic strains during processing, storage, and gastrointestinal transit, ultimately limiting their beneficial effects at the targeted area (Arslan et al., 2015; Markowiak and Śliżewska, 2017). Therefore, maintaining the viability of probiotics during all stages of production until consumption is of paramount importance to food producers and scientists worldwide.

Microencapsulation is a promising technique for bacterial cell protection. Several studies have investigated the protective role of microencapsulation against the adverse conditions faced by probiotics (Ashwar et al., 2018; Fu et al., 2021; Devarajan et al., 2022; Hadidi et al., 2022; Yeung et al., 2023). The successful application of microencapsulation plays a major role in improving the viability and availability of cells throughout the stages of preparation and extends through the human gastrointestinal tract (Kailasapathy, 2002; Sarao and Arora, 2017). The viability of probiotics in food processing and formulations can be affected by the type of encapsulation material used (Loyeau et al., 2018; Ahmad et al., 2019; de Araújo Etchepare et al., 2020; Devarajan et al., 2022). Different food materials, such as milk proteins, plant proteins, and polysaccharides, have been used as wall materials for the encapsulation of probiotics (Afzaal et al., 2021; Rajam and Subramanian, 2022; Xu et al., 2022). Complex polysaccharides with prebiotic properties and various dietary proteins are the most frequently used wall materials for the microencapsulation of probiotic microorganisms (Fernanda et al., 2016). The food industry has been using spray-drying microencapsulation successfully for many years. The process generally involves the dispersion or dissolution of a core material in a solution of wall material to form a fluid mixture. This mixture is then sprayed into a heated chamber. As the solvent of the wall material evaporates, the small droplets transform into solid particles with the core material entrapped within the wall material matrix (Arslan et al., 2015; Burger et al., 2022). Spray drying is one of the oldest, most common, and economical techniques for producing large quantities of viable cells. Spray drying is frequently used as the optimal method for microencapsulation (Vaniski et al., 2021).

Protein-based delivery systems, in addition to their disadvantages of precipitation and aggregation (pH and ionic strength effects), exhibit poor stability against digestive enzymes in the gastrointestinal tract and are thus easily degraded, ultimately leading to a burst release of encapsulated bioactive compounds in the gastric environment instead of in the intestine (Marson et al., 2020; Nooshkam and Varidi, 2020). Although crosslinking proteins with other molecules could address the above issue, owing to health concerns, the application of chemicals as crosslinkers is limited (Li and Huang, 2015). Similarly, prebiotic oligosaccharides are known to have weak physical interactions with probiotics; therefore, microcapsules exert limited improvement in encapsulation efficiency (EE), and insufficient protective effects are delivered to encapsulated probiotic cells (Zhong et al., 2021). Therefore, the conjugation of proteins with carbohydrates via the Maillard reaction could improve protein stability and prebiotic interactions with proteins, improving the overall EE and microcapsule stability (Zhong et al., 2021). Various dietary proteins and polysaccharides and their conjugates have been used as wall materials for microencapsulation (Ahmad et al., 2019; Devarajan et al., 2022). However, researchers are now focusing on new sources of the wall matrix, specifically from plant proteins, owing to their sustainability and low cost of production compared to animal proteins (Hadidi et al., 2023; Islam et al., 2023). Plant protein isolates can be used to increase the nutritional value and functional properties of food (Lam et al., 2018). Moreover, combining plant proteins with prebiotics can improve the stability of the encapsulated probiotics (Gharibzahedi and Smith, 2021).

Pea and rice are good protein sources owing to their high nutritional value and bioavailability (Pietrysiak et al., 2018; Kiran et al., 2023). Pea and rice proteins are suitable for encapsulation due to their high solubility, ability to absorb water and fat, capacity for emulsion stabilization, and capability to form gels (Burger et al., 2022). The globulins of pea and rice proteins possess all the functional properties necessary for their successful incorporation into microencapsulation systems as wall materials (Hadidi et al., 2023; Islam et al., 2023). Recent studies have reported the utilization of pea and rice proteins for probiotic encapsulation (Varankovich et al., 2017; Vaniski et al., 2021). Nevertheless, to the best of our knowledge, the application of rice and pea proteins and prebiotic conjugates as wall materials for probiotics and their comparison with native proteins have not been reported. Therefore, this study aimed to investigate the utility of rice protein (RP), pea protein (PeP), their conjugates (RC and PeC), and prebiotics as wall materials for encapsulating the probiotic strain, *Lactobacillus reuteri* DSM 17938. *L. reuteri* DSM 17938 was chosen because it has been extensively researched, shows promising health benefits for gastrointestinal and immune health, has a well-known survival profile in the gut, and has a proven safety record in clinical trials. The physicochemical characteristics, morphology, and impact of RP, PeP, RC, and PeC on probiotic survival during gastrointestinal digestion were evaluated. Furthermore, the viability and stability of the probiotic organisms during storage at different processing temperatures and during transit via simulated gastrointestinal digestion (SGID) were investigated.

2 Materials and methods

2.1 Chemicals and reagents

RP (Protein content = 91.2%) and PeP (Protein content = 85.93%) were procured from ET Proteins (Public Republic of China). The prebiotic, inulin, was procured from the NOW Foods Company (Bloomingdale, IL, United States). DeMan, Rogosa, and Sharpe (MRS) medium were obtained from HiMedia Laboratories (Mumbai, India). The enzymes used to simulate gastrointestinal digestion, such as pepsin, trypsin, pancreatin, and bile salts, and other analytical chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade. *Lactobacillus reuteri* DSM 17938 https://www.sciencedirect.com/topics/food-science/lactobacillus was obtained from the Department of Food Science, UAEU.

2.2 Preparation of the rice and pea protein conjugates via the Maillard reaction

The rice and pea protein conjugates (RC and PeC) were produced via the Maillard reaction according to a previously optimized protocol in our laboratory (data not shown) following the protocols as described by Guo et al. (2022). Briefly, RP and PeP were mixed with inulin in a ratio of 1:1.25 (w/w) in deionized water. The pH was then adjusted to 9.0 with 1 M NaOH and the mixtures were stored at 4°C overnight for complete hydration. After this hydrated solutions were freeze dried overnight, and were heated at 80°C for 16 h (as per previously optimized conditions) under a controlled relative humidity of 79%. The resulting RC and PeC were stored under refrigerated conditions until further use.

2.3 Encapsulation of *Lactobacillus reuteri* DSM 17938 using RP, PeP, and their conjugates (RC and PeC)

Encapsulation of the probiotic bacterial culture, *L. reuteri* DSM 17938, was performed according to the methodology described by Algaithi et al. (2022). Briefly, the *L. reuteri* DSM 17938 strain was inoculated in sterile MRS broth and incubated for 18 h at 37°C. The cells were harvested via centrifugation (Digicen 21 R, Ortoalresa, Madrid, Spain) at 5000 × *g* for 5 min at room temperature and then washed twice with sterile peptone water to remove any lingering traces of spent broth. Thereafter, the solution was resuspended in sterile saline (0.9%) to obtain a bacterial cell suspension of 10^{11}–10^{12} CFU/mL. RP, PeP, and their conjugates (RC and PeC) were rehydrated in sterile deionized water at 5% (*w/v*) and *L. reuteri* DSM 17938 probiotic cells were added to obtain a final cell count of approximately 10^{8-9} CFU/mL. To facilitate the microencapsulation process, the mixture was stirred under sterile conditions for 2 h at 200 rpm and then spray dried using a pilot-scale spray dryer (Mini Spray Dryer B-290; BUCHI, Switzerland). The following parameters were used: an inlet air temperature of 180°C, an outlet temperature of 91°C, and a feed flow rate of 8.0 mL/min. The resulting powders were kept in airtight containers and stored under two storage conditions, refrigerated storage (4–8°C) and room temperature storage (25°C), for further analysis.

2.4 Encapsulation efficiency (EE)

The EE was determined according to the methodology described by Mudgil et al. (2022). Briefly, the viability of the *L. reuteri* DSM 17938 probiotic strain upon encapsulation using RP, PeP, RC, and PeC as wall materials was determined before and after the spray–drying process based on dry weight. Appropriate serial dilutions were prepared and pour plating was performed using sterile MRS agar. The colony forming units (CFU) for the free and encapsulated probiotic strains were determined after 48 h of incubation at 37°C. The EE was determined using the following equation (Eq. 1):

$$\text{Encapsulation Efficiency}\,(\%) = \frac{\log \text{CFU / g after spray drying}}{\log \text{CFU / g before spray drying}} \times 100 \qquad (1)$$

2.5 Structural characterization

2.5.1 Scanning electron microscopy

The microstructures of *L. reuteri* DSM 17938-loaded RP, PeP, RC, and PeC were analyzed after spray drying using scanning electron microscopy (SEM) (JSM-6010PLUS/LA scanning electron microscope, JEOL). Briefly, the samples were placed on an aluminum stub using a double-sided carbon tape and coated with a layer of gold through sputtering (108 Auto Sputter Coater, TED PELLA, INC). Micrographs were recorded under high vacuum to obtain digital images at the desired magnification.

2.5.2 Fourier transform infrared spectroscopy analysis

Structural changes in the *L. reuteri* DSM 17938-loaded RP, PeP, RC, and PeC samples after spray drying were further analyzed via Fourier transform infrared spectroscopy (FTIR) (Spectrum Two UATR, PerkinElmer, Waltham, MA, United States) over the range 450–4,000 cm^{-1}. For each spectrum, 32 scans were recorded. The spectral resolution was set at 4 cm^{-1}.

2.6 Viability of *Lactobacillus reuteri* DSM 17938 bacterial cells during storage, simulated gastrointestinal digestion conditions, and thermal treatment

2.6.1 Stability of probiotic cells during storage

As mentioned in Section 2.3, the spray-dried bacterial cells were stored under refrigerated (4°C) and room temperature (25°C) conditions for 28 days (4 weeks). Viability was assessed weekly throughout the 28 days of storage via serial dilution and plating onto MRS agar, as described above. Probiotic viability (%) was calculated using Eq. (2).

$$\textit{Cell viability}\,(\%) = \frac{\log \frac{\text{CFU}}{\text{g}} \text{ on storage day}}{\log \frac{\text{CFU}}{\text{g}} \text{ on day 0}} \times 100 \qquad (2)$$

2.6.2 Probiotic viability based on simulated gastrointestinal digestion

The probiotic viability of free and encapsulated cells under SGID was determined according to the methods by Ahmad et al. (2019) and Devarajan et al. (2022). Gastric fluid was made to pH 2.0 using 1 M HCl and contained the following chemicals; sodium chloride, potassium chloride and pepsin in concentrations of 94 mM, 13 mM and 2000 IU/mL of the fluid. Similarly, SIF (pH; 8.0) contained calcium chloride, potassium chloride, sodium bicarbonate, sodium chloride, bile salt, along with 4,000 mg pancreatin and 261 units of pancreatic lipase in a concentration of 3 mM, 10 mM. 85 mM 164 mM and 3.1 mM, respectively.

Briefly, free cells and encapsulated *L. reuteri* DSM 17938-loaded RP, PeP, RC, and PeC samples (approximately 8.0 log CFU/mL) were incubated with simulated gastric fluid (SGF) for 2 h followed by simulated intestinal fluid (SIF) for 3 h at 37°C. An aliquot of the sample was collected at each digestion stage, and cell viability was determined after pour plating with MRS agar, as described in Section 2.6.1. Viable cells were counted via plating onto MRS agar after 48 h of incubation at 37°C. The effects of simulated gastric digestion (SGD) and gastrointestinal digestion (SGID) processes on the viability of *L. reuteri* DSM 17938 in the free form and when encapsulated in the RP, PeP, RC, and PeC matrices were determined by calculating the Log

CFU/g of free *L. reuteri* DSM 17938 and the encapsulated cells using Eq. (3):

$$\text{Cell viability}\,(\%) = \frac{\log \frac{CFU}{g} \text{ after SGID}}{\log \frac{CFU}{g} \text{ before SGID}} \times 100 \quad (3)$$

2.6.3 Thermal stability

Thermal stability tests were performed according to Guo et al. (2022). Briefly, powdered encapsulated materials were suspended in sterile peptone water at approximately 8.0 Log CFU/g. The mixture was then heated at 50°C and 80°C for 5 and 10 min, respectively. Cell viability before and after thermal treatment was calculated as described in Section 2.6.1 and 2.6.2, by plating appropriate dilutions of cells onto MRS agar. Cell viability was calculated as previously described.

2.7 Statistical analysis

Microencapsulation of the probiotic was performed in three batches using the protein and prebiotic conjugates. Data analysis was carried out using SPSS 28.0 (IBM, Chicago, IL, United States), with one-way analysis of variance (ANOVA) and Tukey's multiple range test for separating the means between samples ($p < 0.05$).

3 Results and discussion

3.1 Encapsulation efficiency

The effectiveness of microencapsulating *L. reuteri* DSM 17938 probiotic cells using plant-based proteins (i.e., RP and PeP) and their inulin-based conjugates (i.e., RC and PeC) was assessed according to their cell viability; the results are presented in Figure 1. No significant differences were found between the EEs of the native rice and pea proteins, with EE values of 80.16 and 83.80%, respectively. However, the Maillard conjugates (RP + inulin and PeP + inulin) exhibited increased EE, with RC displaying a better EE% (96.99%) than PC (92.87%) ($p < 0.05$). Therefore, the encapsulation of probiotics in protein microparticles was less effective than encapsulation in conjugated protein microparticles. Protein/polysaccharide complexes or conjugates have been reported to be useful for the microencapsulation of probiotics due to their good flexibility while entrapping various types of probiotic cells, ultimately improving their survivability (Praepanitchai et al., 2019; Sun et al., 2022). For instance, whey proteins and isomalto-oligosaccharide-based Maillard reaction conjugates displayed higher EE for *Lactobacillus rhamnosus* than their native mixture, which aligns with the results of the current study (Liu et al., 2016). Similar results have been reported by other researchers, where Maillard reaction product (MRP) conjugates served as better wall materials than native proteins for the encapsulation of probiotics (Fu et al., 2021; Yeung et al., 2023). As reported previously, MRP conjugation leads to the creation of glycoproteins with balanced hydrophobic and hydrophilic interactions, and is better at lowering interfacial tension than native proteins, resulting in high encapsulation yields (Liu et al., 2016).

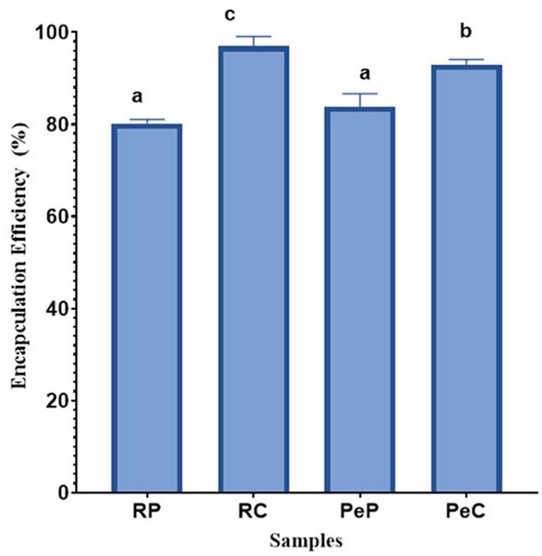

FIGURE 1
Encapsulation efficiency (%) of *L. reuteri* DSM 17938 into RP, PeP, and RC, PeC conjugate microparticles, after spray drying. Keynotes: RP: Rice protein; PeP: Pea proteins; RC: Rice conjugate; PeC: Pea conjugate. Different letters over the bars indicate statistically significant differences among samples ($p < 0.05$) ($n = 3$).

3.2 Morphological changes

SEM was used to examine the surface morphology of *L. reuteri* DSM 17938 free cells and cells encapsulated in RP, RC, PeP, and PeC. As shown in Figure 2A, free-cell micrographs revealed the typical morphological features of *L. reuteri* DSM 17938 cells: rod shaped, colonized in groups, and attached to each other. In contrast, native inulin particles had an irregular spherical morphology with a slightly smooth surface (Figure 2B). Figures 2C,F show the native rice and pea proteins, respectively. RP had round globular structures with rough surfaces and some cavities, while PeP had smaller globular structures with smooth margins. Figures 2D,E show the probiotics entrapped in RP and RC, respectively; *L. reuteri* DSM 17938 could not be seen even at higher magnifications (data not shown), indicating that the probiotic cells were successfully entrapped within the wall materials. Interestingly, upon encapsulation, the RP and RC particles exhibited smooth surfaces, contrary to those of the native rice proteins, RP-pro (Figure 2D) and RC-pro (Figure 2E); this could be attributed to the spray drying procedure, which permitted the rapid evaporation of water. The *L. reuteri* DSM 17938-loaded microparticles appeared to be stable in the interior cavities of the protein-prebiotic conjugate matrix. Moreover, prebiotic conjugation with RP and PeP decreased the porosity of the wall materials by filling the pores of the matrix, ultimately providing better coverage for the probiotics. The use of prebiotic-based wall materials in the encapsulation process can result in the formation of a protective layer around the probiotic bacteria, which can affect the overall morphology of the encapsulated particles. Similar entrapment results were obtained when *Lactobacillus plantarum* and *Lactobacillus casei* were loaded into soybean protein microparticles via spray drying (González-Ferrero et al., 2018). Furthermore, Mao et al. (2018) reported that the encapsulation of *Bifidobacterium longum* in Maillard reaction conjugates of soy protein isolates and carrageenan resulted in spherical cavity-like structures upon spray drying, providing better protection to probiotic cells.

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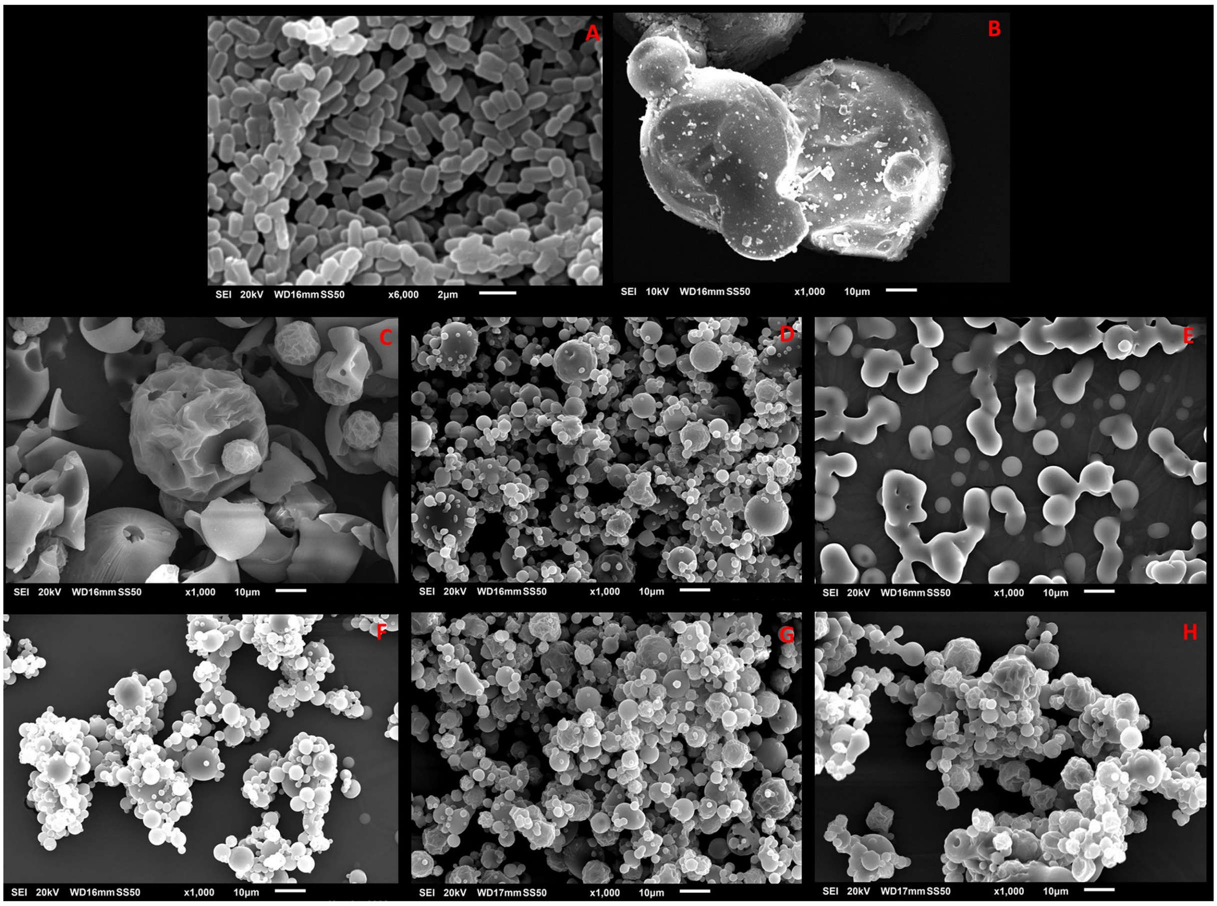

FIGURE 2
SEM images of *L. reuteri* DSM 17938 free cells **(A)**, Inulin **(B)**, native RP **(C)** RP-pro **(D)**, and RC-pro **(E)**, native PeP **(F)** PeP-pro **(G)**, and PeC-pro **(H)** microparticles after spray drying. Keynotes: RP: Rice protein; RP- pro: Rice protein with probiotics; RC-pro: Rice conjugate with probiotics; PeP: Pea protein; PeP- pro: Pea protein with probiotics; PeC-pro: Pea conjugate with probiotics.

3.3 Fourier transform infrared spectroscopy

To interpret the interaction between the probiotics and wall materials, Fourier transform infrared spectra were recorded in the range 450–4,000 cm^{-1}. The infrared spectra of the wall materials before and after probiotic encapsulation are presented in Figure 3A for RP and RC, and Figure 3B for PeP and PeC. Inulin sample displayed a characteristic peak between 3,267 and 3,340 cm^{-1}, which was attributed to stretching in the OH region (Olech et al., 2023). Proteins display unique spectral characteristics, with the amide I and II bands being the most prominent and occurring in the approximate wavenumber ranges of 1,600–1,500 cm^{-1} and 1700–1,600 cm^{-1}, respectively. In both the PeP and RP samples, the distinct bands at 1645 cm^{-1}, 1,652 cm^{-1}, 1,542 cm^{-1}, and 1,529 cm^{-1} originated from the stretching of C=O bonds in amide I and the vibrations of N-H bonds in amide II, respectively (Devarajan et al., 2022). In addition, the Maillard reaction consumed some carbonyl and amino groups, resulting in the creation of Schiff bases (C=N), pyrazines (C–N), and Amadori compounds (C=O). These changes led to variations in the intensity and positioning of peaks related to amide A, amide I, and amide II indicating conjugation of proteins with carbohydrates (Li et al., 2023). The intense absorption peak around 2,925 cm^{-1} for free cells could be mainly attributed to antisymmetric stretching vibration and bending vibrations of the C–H groups, and bending and vibrations of lipid molecules in the cell membrane (Chen et al., 2023). After conjugation, the intensity of the peak at 1034 cm^{-1} for RP and RC and 1,029 cm^{-1} for PeP and PeC increased, indicating the occurrence of structural and conformational changes upon conjugation. As the peak at 1301–1034 cm − 1 was attributed to the stretching vibrations of C– O and C– C bonds, together with the bending of C– H bonds, and identified absorption peaks serve for accurate indications for carbohydrates. Which are organic compounds composed of carbon, hydrogen, and oxygen atoms and the presence of C– O and C– C bonds in the absorption peaks suggests the presence of carbohydrates in the protein sample or complex protein further confirming conjugation of proteins with carbohydrates. Moreover, as observed in both spectra upon encapsulation, the characteristic peak of free cells at 2925 cm^{-1} decreased, suggesting their entrapment in the wall materials. Such findings suggest no interaction occurred between the wall materials and probiotics during microencapsulation; hence, the encapsulated probiotics could be easily released during gastrointestinal transit. Overall, upon encapsulation, no noticeable change was observed, except a slight peak shift.

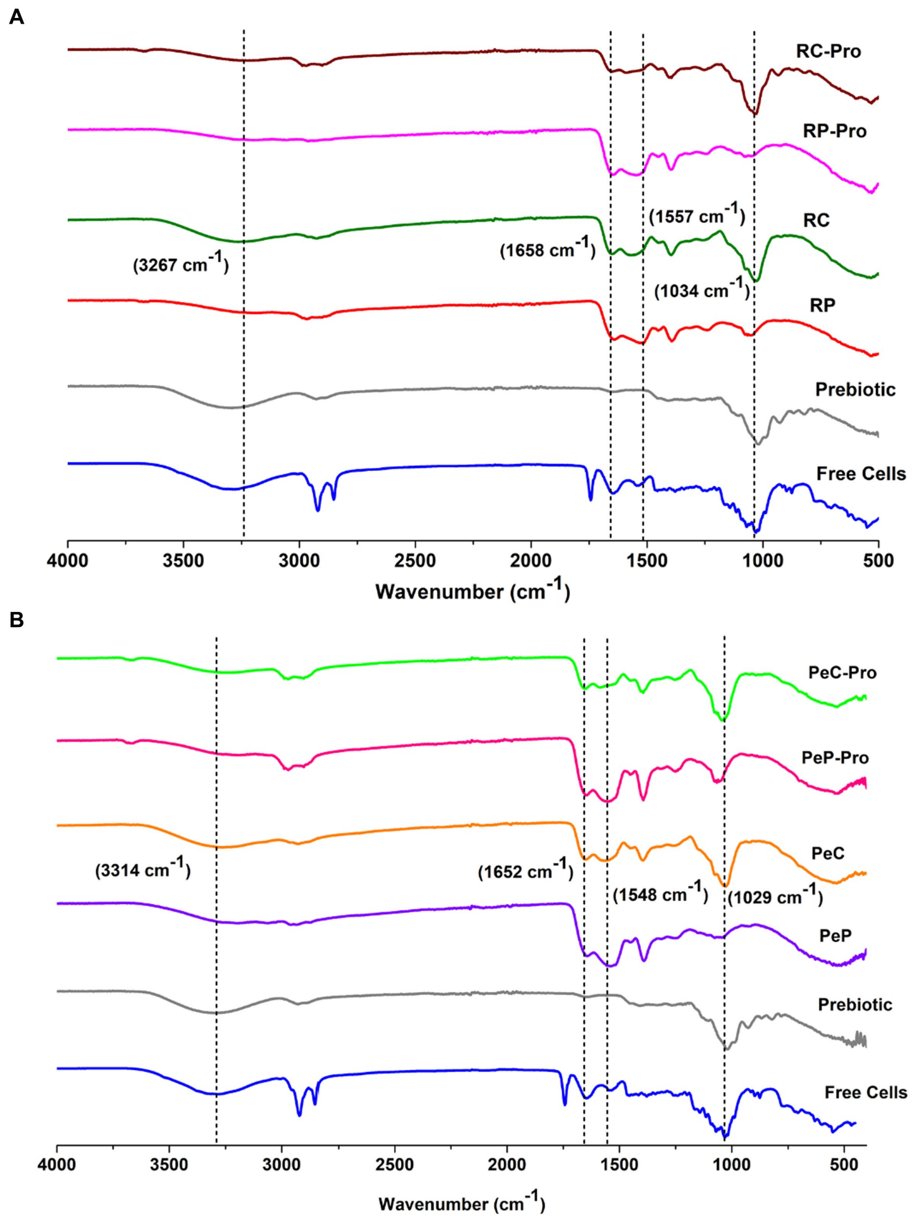

FIGURE 3
FTIR spectra of Prebiotic inulin, native RP **(A)**, native RC **(B)**, Prebiotic, *L. reuteri* DSM 17938 free cells, and the different *L. reuteri* DSM 17938 -loaded RP-pro, and RC-pro conjugate microparticles, after spray drying. For keynotes, please see legend for Figure 2.

3.4 Viability of encapsulated *Lactobacillus reuteri* DSM 17938 bacterial cells under simulated gastrointestinal digestion conditions, storage, and thermal challenge

3.4.1 Viability of encapsulated *Lactobacillus reuteri* DSM 17938 bacterial cells under simulated gastrointestinal digestion conditions

The harsh condition of the gastrointestinal tract serves as the main challenge in the successful delivery and survival of probiotics. Therefore, the survivability of free and encapsulated *L. reuteri* DSM 17938 probiotic cells in the RP, PeP, RC, and PeC microparticles after the gastric and complete-SGID phases was analyzed according to cell viability (%), and the obtained data are presented in Figure 4. The viability of free *L. reuteri* DSM 17938 displayed a significant decline of 4.52 log cells from an initial log count of 8.3 to 3.78 log CFU/g. However, the pea protein and conjugate did not result in any significant differences in probiotic cell survivability. Overall, cells encapsulated in PeP showed a 2.1 log reduction after gastric transit and 3.98 log reduction after complete SGID. Similarly, PeC-encapsulated probiotic cells showed a decline of 1.67 log CFU/g and 3.84 log reduction upon completion of SGID. Of note, PeC-encapsulated probiotic cells survived better under gastric conditions than PeP-encapsulated probiotic cells (Figure 4). Furthermore, probiotic cells encapsulated in the RP and RC matrices showed better survival than those encapsulated in PeP and its conjugate. Overall, RP-encapsulated probiotic cells displayed 1.57 and

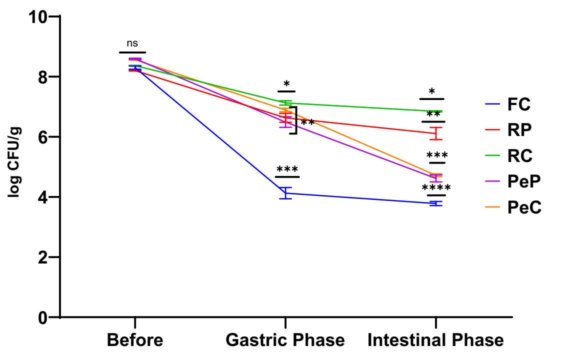

FIGURE 4
Viability of *L. reuteri* DSM 17938 free and encapsulated cells into RP, PeP, and RC, PeC conjugate microparticles, upon undergoing *in vitro* simulated gastrointestinal digestion. Pea conjugate. The level of different * at different phases indicate statistical difference among samples (p < 0.05) (n = 3). For keynotes, please see legend for Figure 2.

2.09 log reductions after gastric and complete SGID, respectively. These results suggest that RC-encapsulated probiotic cells exhibited maximum survival upon gastrointestinal transit, with a decline of only 1.24 and 1.52 log CFU/g, respectively after gastric and complete SGID. Overall, encapsulation in protein-prebiotic conjugates, such as in RC, can protect *L. reuteri* DSM 17938 under both gastric and complete-SGID conditions for their establishment in the intestine as well as their proliferation. The improved survival of probiotic cells encapsulated in the RC matrix could be ascribed to the fact that RC-microencapsulated probiotic cells were released in a controlled manner and maintained their viability and functionality during gastrointestinal transit. The results of the current study are consistent with those of a previous study that confirmed the enhanced viability of *L. plantarum* cells when encapsulated in whey protein-dextran conjugates were obtained via the Maillard reaction (Guo et al., 2022). Similarly, Loyeau et al. (2018) reported the enhanced viability of *Bifidobacterium animalis* subsp. *lactis* INL1 using whey proteins and dextran conjugates. Another study reported similar SGID findings when the Maillard reaction between soy protein isolate (SPI) and I-carrageenan (IC) was used to encapsulate *Bifidobacterium longum* (Mao et al., 2018); probiotic viability decreased by 2.38 log under simulated gastric conditions. Gunzburg et al. (2020) reported 8 logs stomach acid protection of *L. casei* that had been encapsulated by cellulose sulphate. These authors confirm that this improved survival artificial gastric juice also translates into the in vivo situation in mice that have been gavaged with cellulose sulphate encapsulated bacteria showing better survival and colonization. Overall, the conjugation of rice protein with the prebiotic, inulin, resulted in a conjugate with enhanced EE and enhanced protection under harsh SGID conditions.

3.4.2 Viability of encapsulated *Lactobacillus reuteri* DSM 17938 bacterial cells during storage

The success of probiotic supplements or products is entirely related to the viable counts maintained during the storage of food products. Therefore, a high number of live cells must be maintained throughout the shelf-life of probiotic food products. Microencapsulation has been reported to effectively increase probiotic survival during storage (Feng et al., 2020). To comparatively evaluate the effects of native proteins and their conjugates on probiotic survival, studies at room temperature (25°C) and refrigeration temperature (4°C) were performed weekly for up to 28 days (4 weeks) (Figure 5). Storage at 25°C for 28 days resulted in a decline in viable count among the encapsulated samples. The maximum decline was observed in samples encapsulated in the PeP wall matrix, with a reduction of 2.68 log CFU/g after 28 days of storage. However, the reduction in the PeC sample was less than that in the native protein-encapsulated sample, with the viable cell count reduced by 1.78 log CFU/g after 28 days of storage. Probiotic cells encapsulated in native RP were better protected than those encapsulated in native PeP based on a total reduction of only 0.9 log CFU/g, which is markedly better than that obtained with PeP conjugates and PeC. Overall, RC-encapsulated probiotic cells displayed the maximum survival rate throughout storage, and only a decline of 0.73 log CFU/g after 8 days of storage. The RP and RC microparticles showed significantly higher protective effects based on the slower decline in *L. reuteri* DSM 17938 with increasing storage time. Such findings suggest that encapsulation in these matrices is a beneficial strategy to increase cell viability during storage. The results obtained in this study were comparable to those obtained in another study, where *L. plantarum 21,805* was encapsulated in whey protein–dextran conjugates. Notably, a decline of approximately 4.86 log CFU/mL was found after 30 days of storage at room temperature (Guo et al., 2022). Similar results were reported for *L. reuteri* DSM 17938 encapsulated in camel milk proteins, where a better survival was observed after continuous storage for up to 90 days (Algaithi et al., 2022). Similarly, a study on the encapsulation of *B. animalis* in whey protein-dextran conjugates revealed substantial protection of the encapsulated cells even after 12 months of storage at room temperature (Loyeau et al., 2018). According to a study on the encapsulation of *B. animalis* in soy protein isolate and carrageenan conjugates, better protection was achieved with the conjugate than the native soy protein-carrageenan mixture at 20°C (Mao et al., 2018). Allahdad et al. (2022) reported that a mixture of pea and rice protein maintained a high concentration of viable probiotics during a 143 days storage period. Overall, these results indicate that plant proteins, upon conjugation with inulin, provide a better encapsulating matrix for the efficient protection and delivery of probiotic bacteria.

The results regarding the stability of encapsulated probiotic cells under refrigerated storage (4°C) for 28 days suggested better protection than storage at room temperature based on the higher cell viability (Figure 5B). During storage at room temperature, a maximum decline of 2.09 log CFU/g was observed in PeP-encapsulated samples, followed by RP-, PeC-, and RC-encapsulated samples, with declines of 1.23, 1.22, and 1.18 log CFU/g, respectively. In general, conjugated proteins induced significantly (p < 0.05) better protection of probiotic cells than the native proteins. Furthermore, higher viability of samples was observed at 4°C compared to room temperature. These findings align with those of other studies that suggested better survival of encapsulated cells at refrigeration temperatures than at room temperature (Loyeau et al., 2018; Mao et al., 2018; Guo et al., 2022; Yeung et al., 2023).

The continuous decline in the number of cells over prolonged storage at room temperature is largely attributed to injuries induced by the high temperature of spray drying, intrinsic temperature and desiccation resistance of the probiotic strain used, the characteristics of the encapsulated material, moisture content of the particles produced, and packaging conditions (Vaniski et al., 2021). The

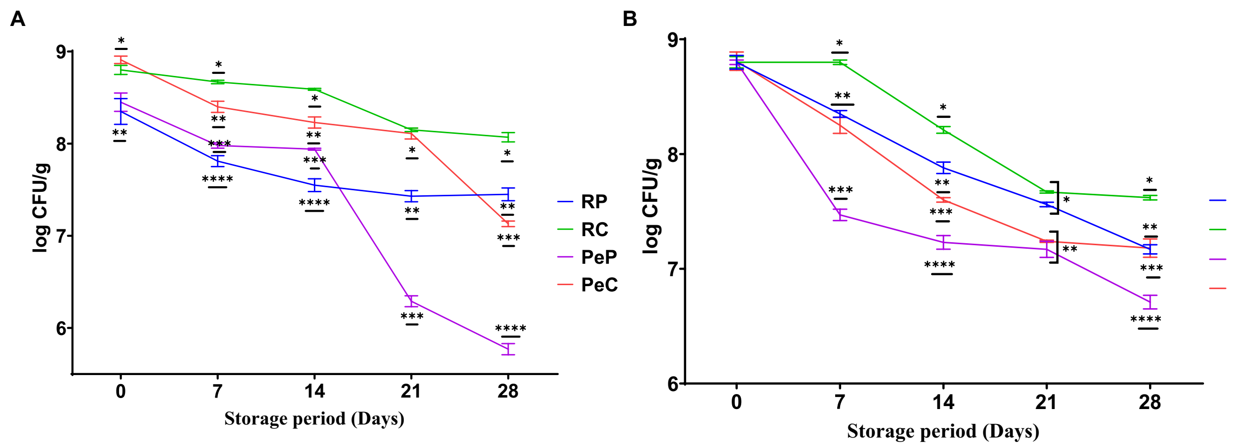

FIGURE 5
Storage stability of *L. reuteri* DSM 17938 into RP, PeP, and RC, PeC conjugate microparticles, during 28 days of storage at **(A)** room temperature 25°C; **(B)** refrigerator 4°C. The level of different * at different phases indicate statistical difference among samples ($p < 0.05$) ($n = 3$). For keynotes, please see legend for Figure 2.

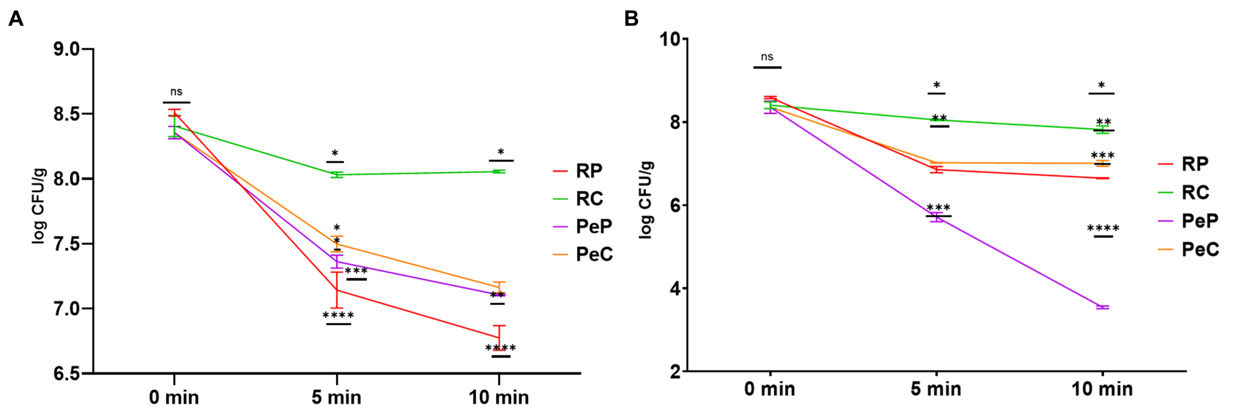

FIGURE 6
Thermal stability of *L. reuteri* DSM 17938 into RP, PeP, and RC, PeC conjugate microparticles, during 10 min of heat treatment at **(A)** 50°C; **(B)** 80°C. Different symbols on the lined at particular time period means statistically significant differences as analyzed via ANOVA ($p < 0.05$) ($n = 3$). For keynotes, please see legend for Figure 2.

higher protection by conjugated proteins could be due to their better moisture retention capacity imparted by prebiotics, which prevented excessive drying and improved cell viability during storage (González-Ferrero et al., 2018). Overall, the results of this study suggest that conjugated proteins can be used for probiotic microencapsulation to increase the viability of probiotic bacterial cells.

3.4.3 Viability of encapsulated *Lactobacillus reuteri* DSM 17938 bacterial cells during thermal challenge

Microencapsulation of probiotic bacteria is not only used to increase the long-term survival of cells during storage, but also to overcome the thermal conditions applied during food processing (González-Ferrero et al., 2018). The microencapsulation technology has proven to be a highly preferred option for protecting probiotics, boosting a decrease in thermal stress and improving the thermal tolerance of probiotic bacteria (Devarajan et al., 2022). The effect of thermal heating on the viability of probiotic-free and encapsulated *L. reuteri* DSM 17938 was assessed after heating for 10 min at 50 and 80°C (Figure 6). The microencapsulation of *L. reuteri* DSM 17938 in the RP, PeP, RC, and PeC groups were found to have significantly different effects on bacterial viability. Further, *L. reuteri* DSM 17938 -loaded PeP, PC, and RP microparticles could not withstand extreme heat treatments, with RP showing a decline of 1.36 and 1.73 log CFU/g after 5 and 10 min of heating at 50°C (Figure 6A). Interestingly, the ability of probiotic cells to tolerate heat was markedly improved after encapsulation in RC based on a loss of only 0.37 and 0.35 log CFU/g after 5 and 10 min, respectively (Figure 6A). Further, probiotic cells encapsulated in PeP showed a decline of 0.99 and 1.25 log CFU/g after 5 and 10 min of heating, respectively, whereas PeC-encapsulated cells showed a reduction of 0.85 and 1.19, respectively, after 5 and 10 min of heating, indicating better protection conferred by the conjugated proteins compared to native proteins. Overall, RC encapsulation of *L. reuteri* DSM 17938 provided greater protection than RP, PeP, or PeC alone, effectively reducing heat transfer from the surrounding environment to the cell interior (Ahmad et al., 2019; Algaithi et al., 2022).

Figure 6B displays the effect of high temperature treatment (80°C) on the survivability of encapsulated probiotics. PeP-encapsulated probiotics could not withstand high heat treatments, with a reduction of 2.64 and 4.81 log CFU/g after 5 and 10 min of heat treatment at 80°C. Similarly, substantial alterations in the viability of the probiotic were also observed in RP- and PeC-encapsulated cells based on a decline of 1.94 and 1.34 log CFU/g after 10 min, respectively. However, probiotic cells could tolerate high heat treatment (80°C) after encapsulation in the RC matrix, with only a decline of 0.58 log CFU/g after 10 min. These results align with those obtained by Ahmad et al. (2019), who found that encapsulation of probiotics in camel whey protein matrices led to enhanced protection of probiotics from thermal stress induced by 10 min of treatment. Similar results were obtained in a prior study as dextran conjugated whey microparticle led to enhanced thermal protection of *L. plantarum* probiotic cells compared to free cells (Guo et al., 2022). Loyeau et al. (2018) reported similar results for the viability of *Bifidobacterium* when encapsulated in whey protein dextran conjugates produced via the Maillard reaction. Based on these results, microencapsulation is an alternative for ensuring good stability of probiotics, as MRP-based protein carbohydrate conjugates are known to possess higher thermal stability than native proteins and can offer better protection to probiotics during processing and in the digestion process (Zhong et al., 2021; Guo et al., 2022).

4 Conclusion

This research explored the effect of native proteins (pea and rice) and their Maillard conjugates with inulin, formed by wet heating, on encapsulation of *L. reuteri* DSM 17938 cells by spray drying. All encapsulated *L. reuteri* DSM 17938 cells exhibited superior viability during simulated gastrointestinal transit. All encapsulated *L. reuteri* DSM 17938 cells exhibited superior viability upon SGID in these *in-vitro* studies. RC-encapsulated probiotic cells showed maximum survival with a decline of only 1.24 and 1.52 log CFU/g, respectively after gastric and complete SGID phase. Probiotics loaded in RC exhibited higher levels of storage survivability, which could be attributed to the advantageous microstructure of RC, ultimately offering higher protection. Furthermore, the thermal treatment and storage stability results indicated that RC-encapsulated probiotics tolerated harsh environments and provided superior protection to probiotic cells. Hence, microencapsulation of probiotic bacteria using rice protein-prebiotic (inulin) conjugates may be a useful and effective approach for the encapsulation of probiotic bacteria for further biofortification applications. Overall, this study presents a new source of matrices for probiotic carriers with high stability and protective effects.

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

PM: Methodology, Validation, Formal analysis, Investigation, Writing – original draft. FA: Formal analysis, Investigation, Methodology, Writing – original draft. HK: Data curation, Software, Validation, Writing – review & editing. MJ: Software, Writing – review & editing. AH: Writing – review & editing, Data curation, Formal Analysis. FH: Data curation, Formal analysis, Investigation, Writing – review & editing. SM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & 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.

The author(s) 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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