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Challenges facing sustainable protein production: Opportunities for cereals

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ABSTRACT

Rising demands for protein worldwide are likely to drive increases in livestock production, as meat provides ~40% of dietary protein. This will come at a significant environmental cost, and a shift toward plant-based protein sources would therefore provide major benefits. While legumes provide substantial amounts of plant-based protein, cereals are the major constituents of global foods, with wheat alone accounting for 15–20% of the required dietary protein intake. Improvement of protein content in wheat is limited by phenotyping challenges, lack of genetic potential of modern germplasms, negative yield trade-offs, and environmental costs of nitrogen fertilizers. Presenting wheat as a case study, we discuss how increasing protein content in cereals through a revised breeding strategy combined with robust phenotyping could ensure a sustainable protein supply while minimizing the environmental impact of nitrogen fertilizer.

Key words:: sustainability, protein nutrition, grain protein content, innovative breeding

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THE ROLE OF ADEQUATE PROTEIN NUTRITION IN THE HUMAN DIET

Food security is central to the sustainable development goals, and plenty of research is focused on providing an adequate food supply for the ever-increasing world population while protecting the environment. However, ensuring food security does not simply involve increasing the overall food production, as maintaining a balanced, nutritious diet that includes various essential micro- and macronutrients is equally important. One essential macronutrient is nitrogen (N), which animals need to synthesize protein for growth and repair. Proteins provide 4 kcal energy per gram and are made of chains of amino acids that can be broadly defined as essential or non-essential, with essential amino acids being those that our bodies cannot produce and must obtain from our diet. Only 21 out of roughly 500 amino acids identified in nature make up the proteins in the human body, with methionine, histidine, valine, leucine, isoleucine, phenylalanine, threonine, lysine, and tryptophan considered essential (Hou et al., 2015). Of these essential amino acids, valine, leucine, and isoleucine play a vital role in protein synthesis, energy production, and metabolic signaling (Holeček, 2018). Some amino acids like cysteine, tyrosine, arginine, and proline are referred to as conditionally non-essential as their synthesis is limited under pathophysiological conditions (Lopez and Mohiuddin, 2021).

A deficiency in dietary protein can result in protein-energy undernutrition and can range from subclinical deficiencies in the form of stunted growth and low weight for age in children to hair loss, skin atrophy, or edema (Waterlow, 1973). Protein deficiency has repeatedly been referred to as a primary cause of child malnutrition, particularly in developing countries, and malnutrition causes 50% of child deaths under the age of 5 in the developing world (Black et al., 2003). The recommended daily protein intake ranges from 0.75 to 1.6 g per kg of bodyweight, depending on age and gender. The quality of ingested protein is also vital as poor-quality protein can accelerate muscle decline and aging (Levine et al., 2014). Therefore, adequate protein intake is essential to healthy living. Recent reviews have discussed future protein supply and demand challenges (Aiking, 2011; Henchion et al., 2017), supply of animal-derived proteins (Boland et al., 2013), supply of high-quality protein (Wu et al., 2014; Minocha et al., 2019), and alternative protein sources (de Souza-Vilela et al., 2019; Wang and Xiong, 2019; Ismail et al., 2020). Here, we will briefly discuss the major challenges currently facing protein nutrition.

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Is protein nutrition a challenge?

In 2017, the protein demand for the 7.3 billion world population was estimated at 202 million tonnes per annum (Henchion et al., 2017). A study based on FAO data between 1961 and 2011 presented three projected scenarios of protein demand for an estimated world population of 9.6 billion (current projection is 9.8 billion) by 2050. All three scenarios assumed that current per capita protein consumption rates for the then 7.3 billion population would be maintained but made different assumptions for the estimated 2.3 billion additional people. **Scenario 1** assumed that the protein demand of the additional population would match the rate consumed by developing countries, as the UN estimates that population growth will come from the developing world (UNDESA, 2019). **Scenario 2** assumed that protein would be consumed at the average global rate, and **Scenario 3** assumed that the rate would match that of developed countries, as developing countries have shown a significant increase in per capita consumption in recent decades (FAO, 2022). The three scenarios estimated increased demands of 32%, 33%, and 43%, respectively (Henchion et al., 2017). Other sources have estimated an increase of 57% in global protein demand by 2050 (Alexandratos and Bruinsma, 2012). To fulfill a 50% increase in protein demand, intensive livestock and arable farming is expected, as they contribute more than 99% of the global protein supply.

CHALLENGES ASSOCIATED WITH ANIMAL-BASED PROTEIN SOURCES

Historically, the major sources of protein nutrition have been animal-based (e.g., eggs, dairy products, meats, and fish) and plant-based (e.g., soybeans, chickpeas, lentils, broad beans, cereals) commodities, with alternative sources such as insects, algae, and aquaculture gaining attention more recently. Desirable nutritional attributes of animal-based proteins include the high ratio of essential to non-essential amino acids and the balanced profile of essential amino acids (WHO, 2007). By contrast, many plant-based protein sources have a low ratio of essential to non-essential amino acids and a poor profile of essential amino acids. For example, lysine content in cooked chicken breast can be up to 2.7%, whereas it is about 0.3% in wheat. Plant- and animal-based foods provide ~65% and ~35% of the protein in human diets, respectively. Various animal-based sources range in protein content from 14% to 32%, whereas plant-based sources range from 5% to 30% (Table 1). Consumption of animal-sourced protein is higher in developed countries, with plant- and animal-based foods providing 32% and 68% of protein to consumers in the United States (Wu et al., 2014). Moreover, the demand for animal-based sources is increasing with the trend of rising meat consumption in developing countries.

Since 1961, the average global per capita meat consumption has increased by 20 kg (Figure 1). On a worldwide basis, the average annual meat consumption, which was 40 kg per capita in 2013, is estimated to increase to 51.5 kg by 2050. Increased meat production will require a large-scale increase in animal farming. Moreover, one-third of global cereal production is used to feed livestock (FAO, 2017), and it is expected to stagnate over the next few decades (Herrero, 2013). Thus, increasing meat production on such a massive scale will be challenging and costly to the environment.

Climate risks associated with increased livestock production

FAO's Tackling Climate Change Through Livestock (2013) report estimated an annual emission of 7.1 billion tons CO_2-eq from livestock (Gerber et al., 2013), accounting for 20% of the 35.3 billion tons of global greenhouse gas (GHG) emissions (Ritchie and Roser, 2020). A comparison of GHG emissions from different food sources (Figure 2A) illustrates that emissions from animal-based sources are more than 10 times higher than those from plant-based sources. Similarly, land use per unit of protein production by animal sources is more than 100 times higher than that of cereals (Figure 2B). Apart from GHG emissions and habitat losses, an increased shift toward animal-based foods can also result in the introduction of pathogens into food systems (Figure 2C). Encroachment by humans into natural habitats through animal farming, hunting, and urbanization leads to habitat fragmentation that can cause the emergence of infectious diseases (Newbold et al., 2015). Examples include the epidemic causing coronavirus diseases, all of which originated from bats and market civets (Goldstein et al., 2018; Cui et al., 2019). The potential for spillover of these bat-borne coronaviruses to humans through animal foods has been evidenced in the literature (Cui et al., 2019). Ecosystem imbalance due to deforestation and biodiversity loss is another outcome of intensive animal farming, which intensifies human–wildlife interactions. The risk of biodiversity loss could be lowered by reducing livestock farming.

Antibiotic resistance associated with livestock production

Another issue with overreliance on animal products is the extensive use of antibiotics in animal production. High-intensity livestock farming often comes with use of antibiotics to kill or reduce the growth of bacterial pathogens. Animal farming therefore encourages formation of resistant bacterial strains, which can present downstream problems in terms of health and costs for livestock, humans, or the environment (Van Boeckel et al., 2015). Although antibiotics are also used in plant agriculture to prevent bacterial diseases such as fire blight or bacterial spot in high-value crops (Haynes et al., 2020), this use appears limited. Available data suggest that the United States is the largest user of antibiotics in plant agriculture, although the magnitude is much lower compared with livestock agriculture (0.1% vs. 75% of antibiotic use in the United States) (FAO, 2019). Plant-derived protein sources may therefore reduce antimicrobial pollution, provided that antimicrobial use is regulated and monitored. These data encourage a reduction in livestock farming and the development of alternative protein sources for a sustainable future.

Progress and limitations on research for sustainable sources

Scientists are working on multiple fronts to tackle the issue of sustainable protein production, and considerable progress has been made in terms of low-emission livestock production in rangeland farming and alternative protein sources such as insects, algae, and aquaculture. However, concerns exist about the sustainability of these sources.

Sustainable practices in rangeland farming such as rotational grazing systems to reduce overgrazing and soil degradation can

Source	Animal based	Protein content (g/100 g)
Meat	chicken breast (grilled, without skin)	32
	pork chop (lean, grilled)	29–32
	beef steak (lean, grilled)	31
	lamb chop (lean, grilled)	29–31
Fish	tuna (canned in brine)	25
	salmon (grilled)	25
	cod (baked)	24
	mackerel (grilled)	20
Seafood	crab (canned in brine)	18
	mussels (cooked)	18
	prawns (cooked)	15
Eggs	chicken egg (whole, boiled)	14
Dairy	whole, semi-skimmed and skimmed milk	3–5
	cheddar cheese	25
	reduced-fat cheddar	28
	cottage cheese	9
	plain Greek-style yogurt	6
	plain low-fat yogurt	5
Source	**Plant based**	**Protein content (g/100 g)**
Pulses	red lentils (boiled)	22 (7)
	lentil flour (air-classified fraction)	50–57
	chickpeas (canned)	25 (7)
	lentils (boiled)	25 (9)
	chickpea hummus	11
Beans	soybeans (green, cooked)	22 (12)
	soybean (air-classified fraction)	55–60
	tofu (steamed)	8
	kidney beans (canned)	23 (7)
	baked beans	5
	lima beans	11
	kidney, black, navy, cannellini beans	12.5
Nuts	almonds	21.1
	walnuts	14.7
	hazelnuts	14.1
	peanut (butter)	25 (26)
	sunflower seeds	17
	almond milk	0.4
Grains	wheat, bread	10–15
	rice, long grain	7–8
	corn	9–10
	oat	15–17
	barley	9–12

Table 1. Protein content from different food sources.
*Sources: British Nutrition Foundation (BNF, 2021), Johns Hopkins Medicine (Medicine, 2019), and USDA (USDA, 2019).

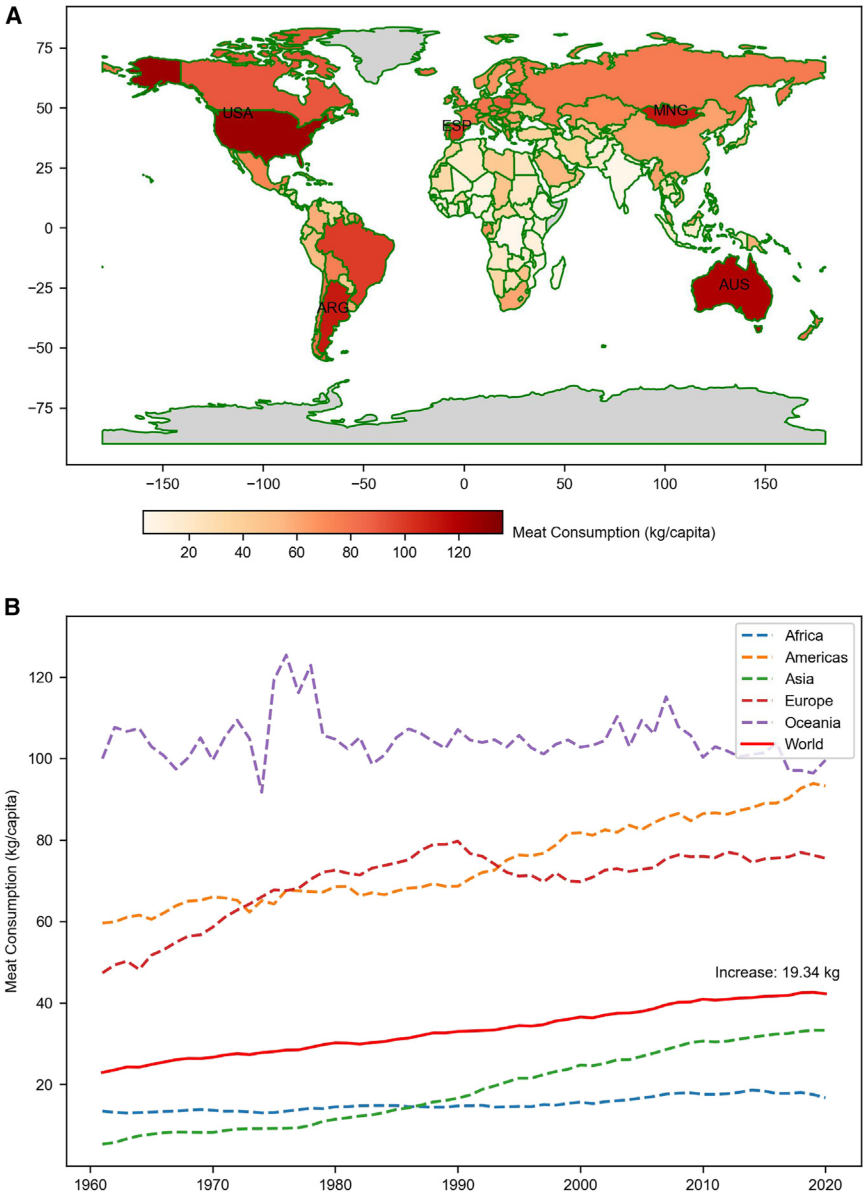

Figure 1. Rising trends in global meat consumption.

(A) Average meat consumption per capita in 2020. Labels are added for countries with over 100 kg per capita consumption.

(B) Changes in meat consumption trends from 1961 to 2020 across different continents and globally. Average global consumption has increased by nearly 20 kg. Figure created with data from Our World in Data (Ritchie et al., 2017).

help to reduce environmental impact, including GHG emissions (Baronti et al., 2022). Other strategies include improving feed efficiency, reducing waste and emissions from manure, and using renewable energy for farm operations. Recently, some farmers have started experimenting with adding seaweed to feed, as a small amount of dietary seaweed can significantly reduce methane emissions from livestock (Vijn et al., 2020). However, many challenges remain to be addressed to make these strategies sustainable for farmers. For example, **1)** implementation of new infrastructure can be expensive, particularly for small farms, **2)** challenges to dissemination of knowledge and technical expertise can limit farmers' ability to adopt these practices, **3)** regulatory frameworks may not support adoption of sustainable practices owing to a lack of government support or friendly policies, particularly in developing countries, and **4)** there is still limited research on best practices to reduce GHG emissions from livestock. Therefore, a collaborative effort between farmers, scientists, and policymakers will be required to make these practices sustainable and affordable for farmers.

Insects are already used in regional meals, including mopane caterpillars, palm weevil larvae, adult crickets, honeybees, and meal worms, and qualify as a nutritional alternative to meat. The protein content of insects is similar to that of meat but shows greater variation between species (10%–35%) (Payne et al., 2016). However, despite the potential of insects as sources of protein nutrition, certain issues remain. The largest variable is the reluctance to eat insects in many cultures, although people are willing to try insect products in unrecognizable forms like powders or supplements in pasta or crisps (Wilkinson et al., 2018). More pioneering work is needed to assess the capacity of insects to rival or replace livestock protein.

Algae encompass different, unrelated multi- and single-celled photoautotrophic organisms and are considered to be a potential alternate food source (Figure 3). The microalgae *Spirulina* sp. and *Chlorella vulgaris* have relatively high dry-weight protein contents of up to 58% and 63% (Tokuşoglu and Üunal, 2003; Becker, 2007). Spirulina also has 180% more calcium than milk and 670% more protein than tofu (Capelli and Cysewski, 2010). Besides being a rich protein source, algae are also used as production platforms for food supplements such as vitamins, pigments of visual or antioxidant value, fatty acids, and polysaccharides (García et al., 2017). Algae can accumulate significant biomass relatively quickly, function as a CO_2 sink, require no farming space for cultivation, and can grow wherever water and nutrients are available, including in seawater. Given the many benefits of algae, why are they not more broadly established as a major food source? A major reason is their taste, smell, and texture. Many microalgae have distinctive aromas, including earthy, seafood/seashore, cucumber, or smelly summer pond (Francezon et al., 2021). The digestibility of microalgae can also vary greatly depending on the presence and composition of cell walls, with cyanobacteria generally showing higher scores (Niccolai et al., 2019). Although digestibility can be improved, it may require costly procedures such as heating, drying, enzyme treatments, or fermentation. Many microalgae can be genetically

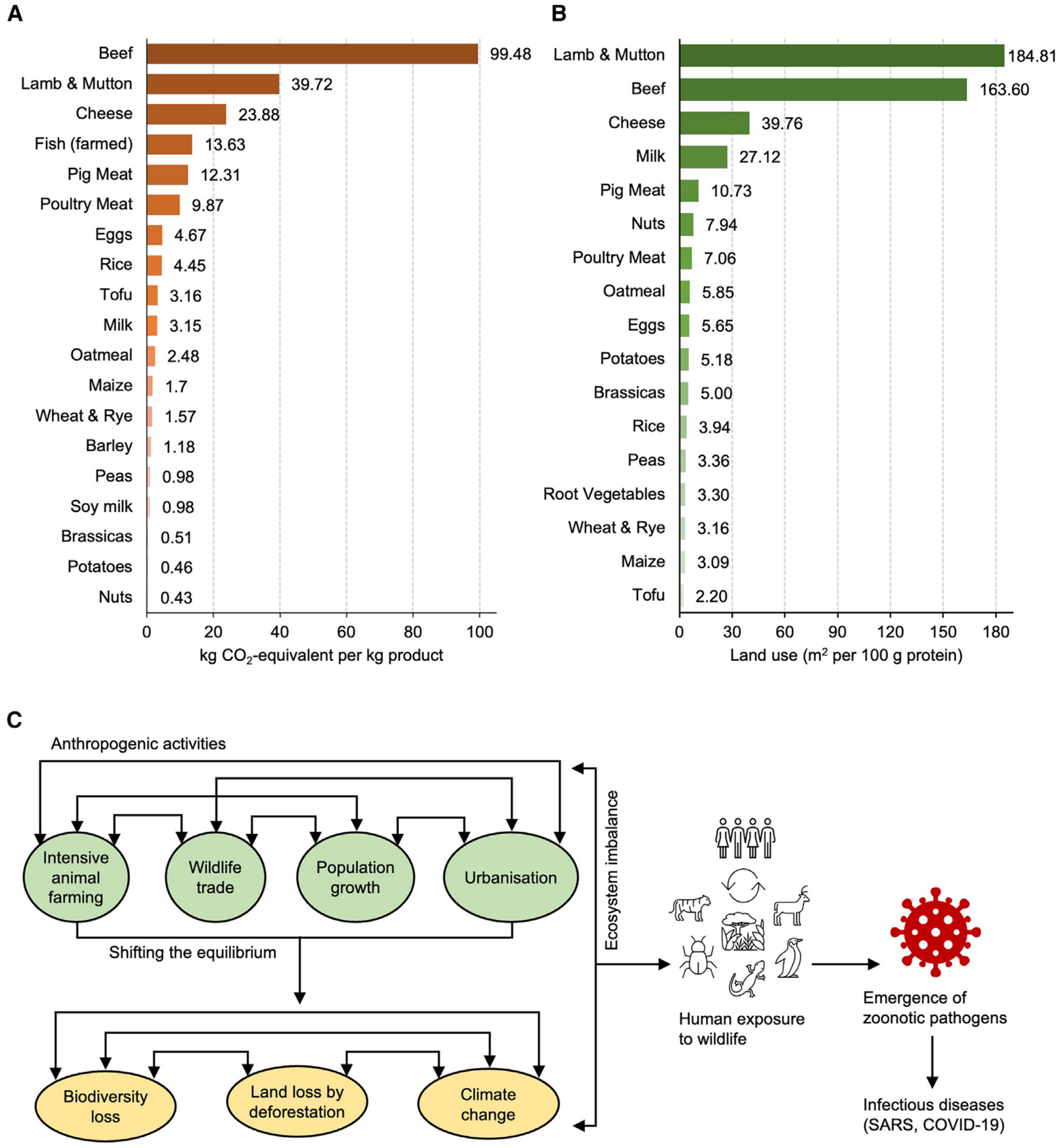

Figure 2. Climate and pathogenicity risks associated with increased animal farming.

(A) Greenhouse gas emissions from meat and dairy are highest, whereas emissions from plant-based foods are more than 10 times lower.

(B) Meat products, especially beef and mutton, use at least 100 times more land area per 100 g of protein than cereals. These data further emphasize how livestock production contributes to global warming and land losses. The graphs were produced using data from Poore and Nemecek (Poore and Nemecek, 2018) and Our World in Data (Ritchie and Roser, 2020).

(C) Anthropogenic activities such as intensive animal farming, wildlife trade, population growth, and urbanization lead to imbalances in natural habitats because of biodiversity loss, deforestation, and climate change. These factors are interconnected and often overlap. As a result, humans are exposed to wildlife, which can cause the spillover of zoonotic pathogens into human populations and can lead to infectious diseases.

altered relatively easily (with the curious exception of Spirulina) (Jester et al., 2021); however, the growth of genetically modified algae in economically viable open ponds is currently debated because of ethical concerns (Henley et al., 2013). Another limitation to farming freshwater algae is an adequate supply of water, as in Australia, Northern Africa, the Middle East, and Western China. In summary, despite the many benefits of algae, significant obstacles remain to be overcome before they are considered as viable alternatives to meat.

In recent years, aquaculture—farming fish in controlled environments such as water ponds or cages—has gained attention as a potential alternative source of protein. Aquaculture appears to be more sustainable than traditional animal farming because it uses less land and water and has lower GHG emissions (Subasinghe et al., 2009). Also, fish are excellent sources of protein, omega-3 fatty acids, and other essential nutrients. Aquaculture can be extended to any area and can easily provide a supply of fish and protein to areas where other sources are scarce.

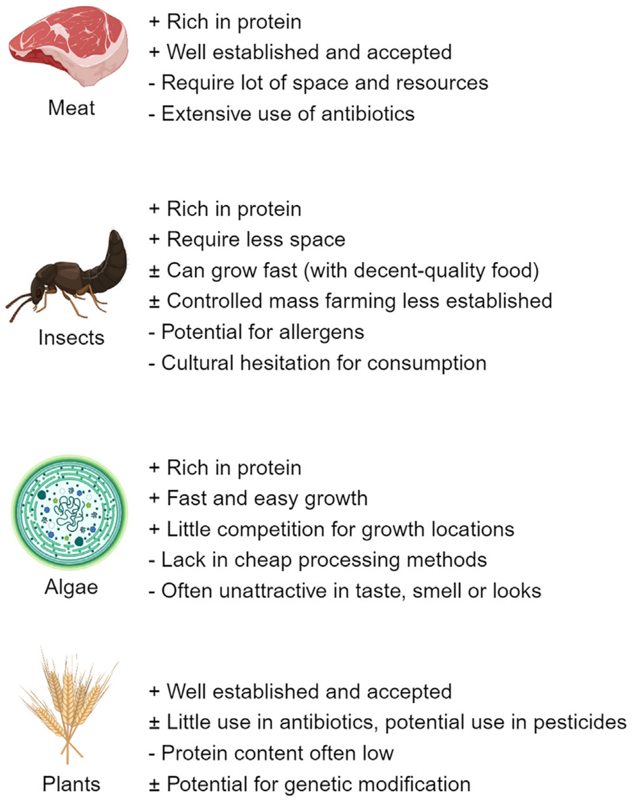

Figure 3. Potential of insects and algae to provide protein in comparison with meat and plants.

The diagram shows advantages and disadvantages of each of these sources in the context of potential protein supply. + refers to advantage, – refers to disadvantage, and ± refers to intermediate effect. *Figure created with **BioRender.com**.

However, there are also challenges associated with aquaculture. These include the large amounts of feed required, which comes from wild fish populations in the form of fishmeal or fish oils (Cao et al., 2015). This raises a question about the sustainability of feed used in aquaculture, particularly if it contains non-renewable ingredients. Other issues such as disease management and environmental effects of nutrient pollution and escapees from farms are concerning. In the next sections, we will discuss how plants can contribute toward global sustainable protein production.

ENHANCING PLANT-BASED PROTEIN SOURCES

Given the limited promise of alternative sources such as insects and algae, producing more plant-based protein may be a viable and sustainable solution to meet the growing demand for protein and reduce the use of animal-based foods for protein supply. Most plant-based foods come from cereal grains, and the protein supply per kg of different grains is 3–10 times lower than that of meat. For example, chicken breasts (grilled) can provide 32 g protein per 100 g, whereas wheat, as the highest protein cereal, provides between 10–15 g per 100 g dry weight, depending on the variety (BNF, 2021). Furthermore, plant-based protein often has less than the recommended amounts of essential amino acids, like lysine (Gorissen et al., 2018). Historically, it has been difficult to achieve high protein content in grain because traditional breeding programs have focused on producing high yielding varieties based on phenotypes such as plant height, heading date, grain number, grain weight, and pathogen resistance (Bedő and Láng, 2015). Once the selection process is completed and a variety has been produced from an initial screen of 1000s of lines, only then are complex traits such as protein content analyzed. However, by this stage, substantial genetic diversity has already been lost. This may have led to the notion that increased grain protein is often associated with decreased grain carbohydrate and yield (Tabbita et al., 2017). An example of reduced genetic diversity is a major allele that controls protein content in wheat (*Gpc-B1*, discussed later in detail), which was found in a wild emmer and is not present in modern cultivars (Avivi, 1978). One obstacle faced during traditional breeding is that the window between harvest and sowing is narrow in many regions, and time is required to assess lines in a large breeding program. Nonetheless, emerging high-throughput phenotyping technologies provide new opportunities to screen complex traits early in the breeding program. For example, **hyperspectral imaging** (glossary) can rapidly and non-destructively phenotype grain protein content (GPC) in large-scale breeding trials (Caporaso et al., 2018). To complement these phenotyping approaches, improved genetic resources, including sequenced TILLING populations or diverse germplasm collections and gene editing technology like CRISPR-Cas9, provide opportunities to discover novel genetic factors that can enhance our understanding of the biology underpinning complex traits like protein content.

Other plant sources, such as pulses or duckweed, have considerably higher protein content than cereals; however, they do not contribute significantly to the overall human diet in developed countries. For example, average global pulse consumption (21 g/day) contributes only 3% of total dietary energy, and this number has remained stagnant since 1985 (Rawal and Navarro, 2019). Therefore, a significant increase in overall cereal production or cereal protein levels would be needed to ensure a sustainable protein supply. However, intensive farming also puts pressure on required resources, mainly nitrogen (N) and water. High crop yields and protein levels are only possible when N uptake is high, and commercial crops therefore remove substantial amounts of soil N (Ewel et al., 2019), typically coming from fertilizers. However, increased N fertilizer application is expensive for farmers, contaminates the soil, and raises human health concerns. Higher amounts of nitrates found in agricultural lands under N fertilization can lead to ground water contamination and cause serious health issues. Some examples include the production of methemoglobin by the activity of oral/stomach bacteria that convert nitrates into nitrites, which leads to inhibition of hemoglobin activity (blood disorders), or the endogenous formation of N-nitroso compounds (carcinogenic) when nitrites react with stomach acids (Ward, 2009). Moreover, N fertilizers contribute to GHG emissions, through either burning of fossil fuels during manufacture or denitrification of nitrates into N_2O by soil bacteria. Human-induced N_2O emissions, mainly driven by the addition of N fertilizers to croplands, have increased by 30% since the 1980s (Tian et al., 2020). Another major issue

Commodity	Price (US$)/kg[a]	Protein (g)/kg[b]	Protein price (US$)/kg[c]
Maize	0.27	104	2.56
Rice	0.34	81	4.24
Wheat	0.31	145	2.16
Oat	0.16	169	0.95
Soybean	0.59	406	1.44

Table 2. Price (US$) per kilo of protein from leading food crops of the world.

[a] Price of commodity from https://markets.businessinsider.com/commodities (accessed: Sep. 6, 2022).

[b] Protein data were collected at the USDA nutrient data laboratory (USDA, 2019).

[c] Calculated using the equation: (commodity price per kg/protein grams per kg) × 1000 = protein cost per kg (Dolson, 2021).

with increased crop production in commercial agriculture is the risk of genetic uniformity within a crop species, which can drive pathogen susceptibility. For example, the 1970–71 epidemic of Southern Corn Leaf Blight (SCLB) in several Corn Belt states in the United States caused more than 50% yield loss in some regions and 20%–30% yield loss on average (Ullstrup, 1972). Almost 85% of US corn fields had been planting Texas cytoplasmic male sterile (Tcms) corn in 1970, and SCLB only affected Tcms corn. Increasing crop production through modern agriculture thus remains a challenge.

The question then remains: how can we ensure a sustainable supply of protein for the ever-increasing world population? Here, we propose new approaches for increasing major plant-based protein sources.

Major plant-based protein sources

Major plant-based protein sources, after legumes, are the cereal grains, particularly maize, rice, and wheat, which together provide 42% of the total protein in developing countries (Kropff and Morell, 2019). Legumes are a healthy source of protein, have a lower GHG footprint, and improve soil fertility through nitrogen fixation. Some disadvantages include unstable annual yields and low consumption in some regions, e.g., Caucasus and Central Asia. Fortunately, a recent review is available on the role of legumes in addressing protein nutrition challenges (Semba et al., 2021). Cereals are a major component of our diet and provide more than 50% of global calorie intake; wheat alone accounts for 20% (FAO, 2022). Therefore, increasing protein levels in cereals can have a significant impact on the overall global protein supply. Among maize, rice, and wheat, wheat has a higher protein content, and the protein produced comes at a cheaper price for the consumer (Table 2). In this review, we will focus on wheat as a case study and discuss challenges to productivity and increasing protein content. The main increase in protein production must come from increased production per unit land area, as there is limited scope to increase cropping area.

Wheat productivity challenges and the impact of global climate change

Wheat is the third largest cultivated cereal crop and the most widely grown cereal; it has a broad impact on more countries than either maize or rice (Hanson, 2021). On average, ∼677 million tonnes of wheat have been produced every year from an average area of ∼217 million ha across the world since 2000 (FAO, 2022). Global wheat demand is estimated to increase by 60% by 2050. To help overcome challenges related to the economic and environmental costs of N fertilizers, recent research has focused on improving N-use efficiency (NUE) of crops, including wheat.

As NUE is a complex trait with many contributing processes, identifying the correct trait for improvement is not trivial, as key physiological and metabolic processes that influence N uptake and utilization are closely related to yield. These include the role of the root system, nitrate assimilation and its relationship to photosynthesis and post-anthesis remobilization and N partitioning, trade-offs between yield and quality (e.g., grain N content), and interactions with the capture and utilization of other nutrients. NUE is defined as a product of N uptake and N utilization efficiencies. Traits that influence N uptake efficiency in wheat are root size and morphology, root N transporters, and root interactions with microorganisms (Foulkes et al., 2009). The genetics of nitrate transport from soil to plant is not well understood in wheat. Two gene families of nitrate transporters, *NRT1* and *NRT2*, have been identified in *Arabidopsis* (Wang et al., 2012), and 6 and 10 ammonium transporter genes (*AMTs*) have been identified in *Arabidopsis* and rice, respectively, with *AMTs* associated with N uptake (Li et al., 2009; von Wirén et al., 2000). Some members of both gene families are expressed in root hairs (Gu et al., 2013; Duan et al., 2016) and are therefore likely to influence N uptake. Previous studies have suggested that nitrate and ammonium capture from soil might be improved by manipulating these N transporters in wheat (Liu et al., 2015); however, no success has been achieved to date. Key traits that influence N utilization efficiency include glutamine synthetase activity, which regulates nitrate assimilation (Bernard and Habash, 2009); manipulation of the photochemistry of Rubisco, which constitutes 30% of the total N in wheat leaves (Lawlor, 2002); post-anthesis **N remobilization** (glossary), which is crucial during the grain filling period when soil N falls short; and the stay-green trait, that features delayed **senescence** (glossary) to enhance the photosynthetic capacity of a plant (Thomas and Ougham, 2014). An inverse relationship between grain yield and protein content makes simultaneous genetic improvement of yield and grain protein a difficult task. Growers and breeders must therefore manage the contradictory objectives of increased yields and higher protein content. The grain protein deviation (GPD; deviation from the yield–protein linear regression) (Bogard et al., 2010) allows breeders to select for high protein content without an associated grain-yield penalty. Crops that are both high yielding and high in protein content absorb large quantities of N (Bogard et al., 2010). Because the majority of grain N originates from post-anthesis remobilization Gaju et al., 2014), rather than post-anthesis uptake, mechanisms to enhance reserve N accumulation in the canopy and **N remobilization** (glossary) efficiency should also be addressed in the genetic improvement of GPD.

Challenges for phenotyping GPC and NUE in wheat

To achieve the maximum genetic potential of a crop or engineer its genetics to improve a certain trait, it is critical to establish a relationship between genotype and phenotype. Therefore, high-throughput phenotyping of GPC or N-related traits is as important as genotyping, and all downstream experiments required to build a relationship between genes and GPC ultimately rely on accurate phenotyping.

Commonly used techniques for N and protein estimation in wheat are the Kjeldahl (Mariotti et al., 2008) and Dumas methods (Sáez-Plaza et al., 2013). Both methods are labor intensive and destructive. Some disadvantages are that the Kjeldahl method can only measure N bound to free amino acids, nucleic acids, proteins, or ammonium and not in other forms like nitrates and nitrites, whereas for the Dumas method, the high initial cost or small sample size required make it difficult to obtain representative samples, and incomplete combustion leads to inaccurate measurements (Mihaljev et al., 2015). Some of the other destructive methods for protein estimation in cereals are chromatographic and mass spectrometric methods; however, their application is more focused toward studying the type and quality of protein.

Commonly used and emerging non-destructive techniques for phenotyping proteins involve nuclear magnetic resonance (NMR), near-infrared spectroscopy (NIR), and **hyperspectral imaging (HSI)** (glossary). NMR is used to study 3D protein structures and identify novel proteins (Williamson et al., 1995), whereas NIR and HSI are used to determine protein content. HSI has developed significantly in recent years and is now used to study protein quality features in grains (Hu et al., 2021; Ma et al., 2022). In grain crops, the application of HSI has been extended to single-grain protein analysis. The potential to investigate single grains provides an advantage for HSI, because most techniques for protein estimation in wheat rely on extracting data for batches and ignoring variability among individual grains. However, previous studies have reported that batches typically have a bimodal protein content, and the mean value for the batch does not reflect the true value. For example, at least two independent studies of protein content in different classes of US wheat have reported variability across (Delwiche, 1995) and within batches (Delwiche, 1998) on a single-grain basis. Variability among single grains has also been reported for other traits in wheat, such as falling number (Caporaso et al., 2017), water uptake, and protein content of soft and hard wheats (Manley et al., 2011). These studies show evidence of single-grain heterogeneity in wheat and demonstrate that random selection of all low- or all high-quality samples can lead to misrepresentation of the batch. Therefore, the application of HSI to whole single grains has gained substantial interest from the food industry. Because HSI can phenotype protein content robustly and non-destructively, its use in breeding could help reduce the time and labor required to phenotype large populations and fast track genetic analysis to investigate underlying genes. There are, however, certain limitations to the use of HSI, such as the development of a good calibration model, especially when investigating genetically diverse germplasms. For instance, in the case of grain protein, if a population ranges from 5% to 20% GPC, the calibration model must include samples with these values so that future samples can be predicted. Therefore, one standard calibration model based on hexaploid wheat may not be effective for all kinds of wheat samples. Another challenge with outdoor HSI calibrations is noise introduced by inconsistent light. A recent review has discussed advances and challenges in the use of HSI for plant phenotyping (Sarić et al., 2022). Addressing these challenges would improve the use of technology for industrial application.

Promising remote-sensing technologies for field-based phenotyping of NUE traits include chlorophyll fluorescence imaging to measure photosynthesis, infrared thermometry as a proxy for canopy photosynthesis, and spectral reflectance vegetation indices, which can provide accurate estimates of crop biomass and N content. A full review of these phenomics technologies is beyond the scope of this article. Fortunately, recent reviews of such phenomics methodologies are available (Araus et al., 2018; Yang et al., 2020). Regarding phenotyping for root system architecture traits, development of reliable and reproducible phenotyping technologies will again be critical for plant breeding. Promising techniques range from small-plant approaches (from filter paper and agar to rhizotrons) to field-based phenotyping (direct approaches, e.g., "shovelomics" or the soil-core break method and indirect remote-sensing approaches). Again, recent comprehensive reviews are available (Bekkering et al., 2020; Tracy et al., 2020).

Limitations to wheat protein quality

The quality of wheat proteins is widely debated owing to their weak nutritional profile and potential health concerns. For example, wheat contains less lysine (0.3%) than animal-based protein (i.e., up to 2.7%), and the high contents of proline and glutamine reduce its digestibility (Biesiekierski, 2017). Moreover, wheat gluten proteins can cause allergies, celiac diseases, and non-celiac gluten sensitivity in vulnerable individuals. The prevalence of gluten-related disorders has reportedly increased in recent years, reportedly affecting ~1% of the population. However, Shewry and Hey concluded that it was difficult to establish the extent to which these disorders had risen, because greater reporting could be due to improved diagnosis and awareness. On the other hand, wheat is not only a major source of food in many developing countries of Africa and Asia (~70% of total food intake) but also provides many essential nutrients, including dietary fibers, minerals, and B vitamins. Therefore, discouraging the use of wheat proteins could have a negative health effect overall (Shewry and Hey, 2016).

It is essential to note that breeding efforts have been directed toward improving the quality of cereal proteins, particularly by elevating lysine content. High-lysine lines and hybrids have been developed in maize using the naturally occurring *opaque2* and *floury2* mutants, which show an inverse correlation between lysine and prolamins (reviewed elsewhere; Yu and Tian, 2018). A similar strategy could be used in wheat to screen TILLING mutants of prolamin biosynthetic genes to improve lysine content. A recent study using genomic prediction recommended donor genotypes with a high lysine content from over 7000 winter wheat accessions (Berkner et al., 2023) and could further accelerate the breeding of high-lysine wheat. Moreover, gluten variants have been genetically engineered to reduce antigenicity (Mitea et al., 2010). These ongoing breeding efforts highlight the potential to mitigate the limitations of cereal proteins, including wheat, and the importance of a multifaceted strategy to ensure sustainable protein production.

GENETIC PROGRESS ON GPC REGULATION IN WHEAT

Early inroads into GPC improvement in wheat were made by investigating a collection of global wheat cultivars with trait variability in a Soviet Union wheat breeding program from a collection of the Vavilov Institute of Plant Genetic Resources (VIR) (Mitrofanova and Khakimova, 2017). Earlier studies considered climatic conditions and soil N richness as major external factors influencing variation in wheat GPC. VIR collections were grown in many parts of the Soviet Union with different soil fertility levels, leading to the understanding that soil fertility (N nutrition) was a major factor involved in GPC variation. The highest GPC varied between 17% and 19% in lines grown in southeastern regions where the soil N levels were higher (Ivanov, 1947). While the earlier studies indicated environment to be the major factor influencing GPC, later research found that genetic variation made a significant contribution to protein levels in wheat grain (Avivi, 1978), suggesting that the trait could be improved through breeding. However, progress has been limited by the phenotyping, genetic, and environmental factors described earlier in this review. Newly developed technologies can accelerate efforts to improve GPC levels in wheat by adoption of a revised breeding strategy, e.g., introduction of HSI early in the screening process to select for the best yield–protein trade-off, genetic screens of wild germplasm, or development of TILLING populations to identify new genetic variation (Figure 4, Key Figure).

Research into high-GPC wheat cultivars accelerated between the 1960s and 1980s, and a core collection of global wheat genetic resources with high GPC was created. The collection comprised wild, weakly domesticated, and cultivated wheat species (Mitrofanova and Khakimova, 2017). Between the 1970s and 1980s, intensive work on GPC improvement in wheat by intraspecific hybridization was carried out in the United States and Canada, and the average GPC in these regions rose by 0.5–3% (Shewry, 2007). However, over the years, GPC has been revealed as a highly polygenic trait that is strongly influenced by growing conditions and soil composition. Until the end of the 20th century, the gene pool available for study of wheat GPC variation was not fully explored. In the last two decades, with advances in molecular genetics and sequencing technology, the focus has shifted toward identification of loci and alleles that contribute to high or low GPC in wheat. The first indication of GPC loci was obtained from a 1978 study that reported some accessions of large grain wild emmer (*Triticum turgidum* var. *dicoccoides*) had GPC genes (Avivi, 1978). From these samples, the accession FA-15–3, which originated from Israel, was hybridized with the durum variety Langdon to investigate chromosomal effects on GPC (Joppa and Cantrell, 1990). The line carrying a pair of FA-15-3 chromosome 6B markers was highest in GPC. The locus on chromosome 6B was mapped and named *QGpc.ndsu-6Bb* (*Gpc-B1*) (Joppa et al., 1997). *Gpc-B1* was later introgressed into bread wheat (Mesfin et al., 1999) and physically mapped to within a 245-kb region (Distelfeld et al., 2006). In addition to this major locus on chromosome group 6, many other loci have been reported on different wheat chromosomes by independent quantitative trait loci mapping and genome-wide association studies; however, their roles in the biology of GPC have not yet been investigated or they have proven to be phenotypically unstable.

Biological regulation of GPC in wheat

After the *Gpc-B1* locus was physically mapped on the wheat genome, efforts were expedited to understand the GPC mechanism in wheat, and a gene encoding an NAC domain protein was identified (Uauy et al., 2006b). The gene was named *NAM-B1* owing to its phylogenetic similarity to the *Arabidopsis* No Apical Meristem (NAM) transcription factor protein. NAC proteins belong to a set of transcription factors that are one of the largest plant gene families and participate in the regulation of plant development, defense responses, and **senescence** (glossary) (Puranik et al., 2012). They have a conserved N-terminal region (the NAC domain) with five subdomains, A–E, and a C-terminal domain that is highly variable and contains the transcription activator site (Uauy et al., 2006b). Nucleotide comparison of *Gpc-B1* in FA-15–3 and Langdon wheats revealed that Langdon had a 1-bp insertion at position 933 that caused a frameshift mutation and resulted in a 327-amino-acid inactivated polypeptide (Uauy et al., 2006b).

A comparative study of near isogenic recombinant inbred lines of *Gpc-B1* active alleles and Langdon wheat revealed that *Gpc-B1* functional alleles increased GPC via post-anthesis **N remobilization** (glossary) from leaves to spikes. The study also reported a high level of free amino acids in the flag leaf at anthesis in lines carrying *Gpc-B1* active alleles, which suggested that *Gpc-B1* affected translocation of N from leaf to grain (source to sink) and was active well before grain formation (Kade et al., 2005). Another study investigated recombinant substitution lines segregating for *Gpc-B1* functional alleles. The lines carrying *Gpc-B1* active alleles showed rapid flag leaf chlorophyll degradation after 20–30 days from anthesis, faster yellowing of main spike peduncles, and reduced moisture content in grain and straw. Consequently, the grain filling period was reduced, and the lines senesced 4–5 days earlier and produced 10–15% higher GPC compared with wild-type lines that had non-functional *Gpc-B1* alleles (Uauy et al., 2006a).

In bread wheat, the *NAM* gene has four copies at the *Gpc-A1*, *Gpc-D1*, *Gpc-B2*, and *Gpc-D2* loci. *Gpc-B2* and *Gpc-D2* are paralogous copies of *Gpc-B1* on chromosome group 2 that have 91% DNA similarity and 98–100% protein similarity. When the transcript levels of all *NAM* genes were reduced by RNA interference in the bread wheat cultivar Bobwhite, a transgenic line with 40–60% reduced transcript levels (between the fourth and ninth day of anthesis) reached 50% flag leaf chlorophyll degradation 24 days later and main spike peduncle yellowness 30 days later than non-transgenic lines. Mature grains of the transgenic plants showed reductions of 30% in GPC, 36% in Zn, and 38% in Fe; however, no difference in grain size was detected relative to non-transgenic lines, which confirmed that the reduced GPC was a result of low N translocation from the leaf and not a dilution effect due to larger grains (Uauy et al., 2006b). A more

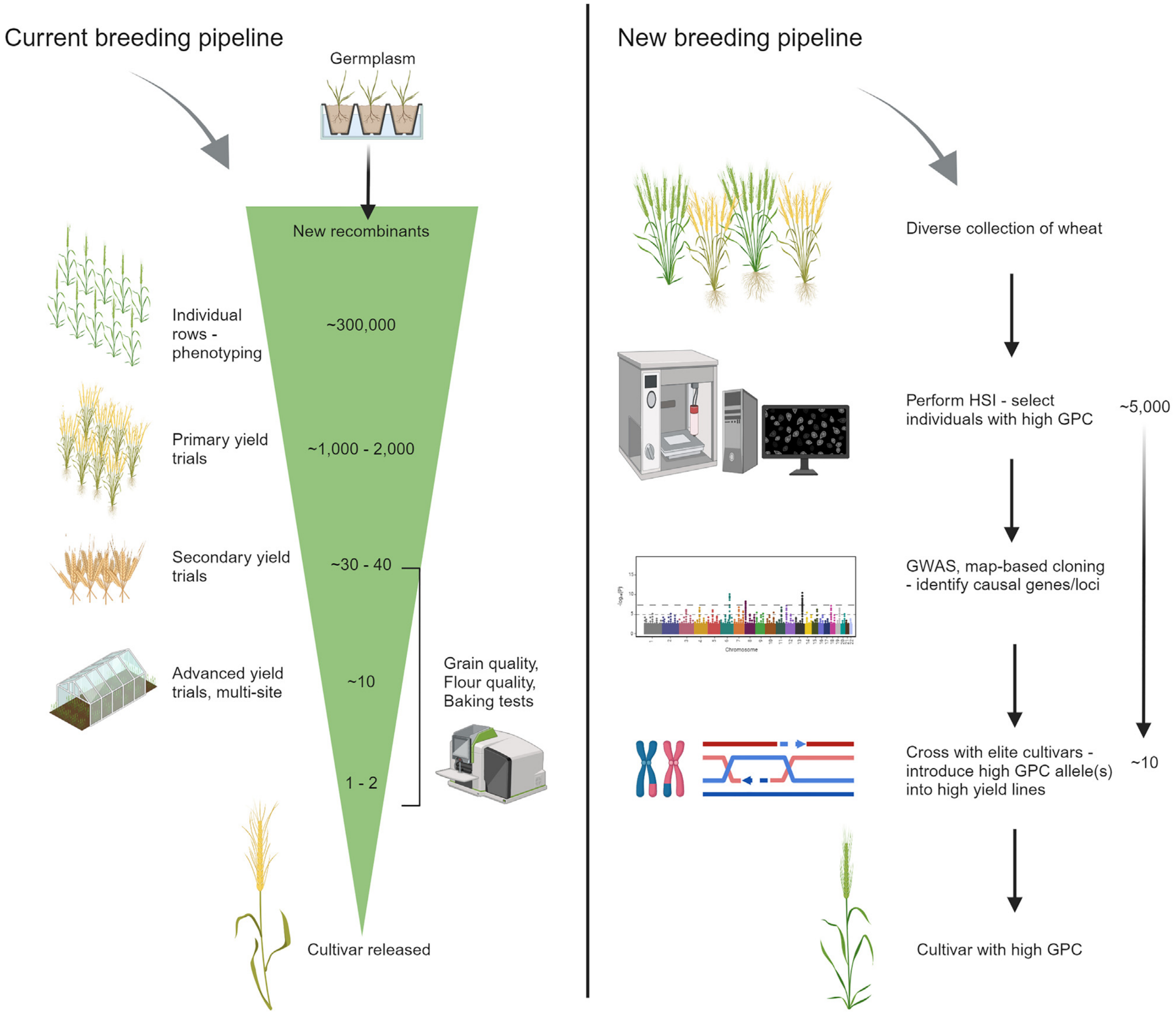

Figure 4. Comparison of current breeding pipeline and a proposed new breeding pipeline.
The current breeding pipeline follows the conventional breeding method in which thousands of rows are phenotypically screened to select for yield components like grain yield, height, heading, etc. In the later stages, a few selected lines are screened for grain and flour quality characteristics such as GPC; however much of the genetic diversity has been lost by this stage. The new (proposed) breeding pipeline can benefit from high-speed testing technology like hyperspectral imaging to rapidly and non-destructively test GPC in large populations in real time. In addition, advanced genetic tools can rapidly screen large populations for new variations on the basis of associations between phenotypes and genotypes. In this way, quality characteristics like GPC can be targeted much earlier in the breeding process when the germplasm has maximum genetic potential. *Figure created with **BioRender.com**.

recent study used next-generation sequencing to study the transcriptome profile of transgenic lines at the 12th day of anthesis. The findings indicated that the expression levels of several hundred genes involved in photosynthesis and metabolic processes were changed in the transgenic lines, leading to delayed **senescence** (glossary) and reduced grain protein, with gene activity changing markedly before visual signs of **senescence** (glossary) (Cantu et al., 2011).

Limitations of current knowledge and emerging information on the biology of GPC in wheat

Many of the findings from different studies, as discussed above, indicate that the processes of **senescence** (glossary) and post-anthesis **N remobilization** (glossary) are associated with protein content in grain. The *Gpc-B1* locus affects post-anthesis translocation of N from leaf to grain and, as a result, has pleiotropic effects on **senescence** (glossary) kinetics, grain size and weight, and GPC. Since the identification of *Gpc-B1* more than 15 years ago, its effects on grain nutritional components, yield components, physiology, and bread-making quality have been investigated in several field trials worldwide (reviewed elsewhere; Tabbita et al., 2017). Studies have reported varying effects of *Gpc-B1* on yield components, with some reporting increases in yield and others reporting no difference. Despite the lack of clear evidence for a negative effect of *Gpc-B1* on yield, at least three independent studies have reported a negative correlation between these traits

(Chee et al., 2001; Brevis and Dubcovsky, 2010; Kuhn et al., 2016). Moreover, several studies have reported a negative effect of *Gpc-B1* functional alleles on grain weight and have attributed this to rapid **senescence** (glossary) and a short grain-filling period (Joppa et al., 1997; Uauy et al., 2006a; Brevis and Dubcovsky, 2010). Therefore, a consensus on the effect of *Gpc-B1* on yield components has not yet emerged, limiting its introgression into modern wheat cultivars.

Recently, new information has emerged on a novel mechanism that may promote higher protein accumulation in wheat grains without reducing key yield component traits (Dixon et al., 2022). In this recent study, Dixon et al. reported on the role of a *homeodomain/leucine zipper transcription factor Homeobox domain-2 (HB-2)* in protein accumulation in wheat grains through changes in **plant vasculature** (glossary). A single-nucleotide mutation in *HB-2* found in TILLING mutants resulted in more transcripts of this gene, which is expressed in cells surrounding xylem and phloem of stems. The mutants produce more vascular bundles than their wild-type siblings, which enhances their hydraulic conductance. Consequently, more N-based assimilates are translocated to the grain, and GPC increases significantly, with mutants producing grain with ~25% more protein than their wild-type siblings. Moreover, unlike results for *Gpc-B1*, the higher protein content of grain from heterozygous lines was not associated with reduced yield component traits such as grain size, grain weight, and grain number per inflorescence, suggesting that moderately higher *HB-2* expression could help to increase GPC without reducing yield (Dixon et al., 2022). This raises a new research question on the role of **plant vasculature** (glossary) in **N remobilization** (glossary) and regulation of grain protein accumulation (see outstanding questions). Emerging research on other crops, such as discovery of the *Teosinte high protein 9* (*THP9*) locus from the wild ancestor of maize that increases maize seed protein without affecting yield (Huang et al., 2022) and comparisons of storage protein synthesis between monocots and dicots (Yang et al., 2023), offers approaches for improving GPC in wheat and vice versa.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This article provides a broad overview of the future of plant-based protein, the importance of increasing protein production to feed the growing population, the effect of increased demand on the global climate through enhanced livestock production, and mitigation strategies to shift the focus toward sustainable, plant-based protein diets. Although considerable progress has been made in sustainable rangeland farming, many recent studies have highlighted the challenges associated with overreliance on meat production for protein supply. When we look at plant-based protein sources, pulses and legumes are considered because of their high protein content, but overall global consumption of pulses has not increased since 1965 (Oecd, 2022). On the other hand, cereals could provide major benefits owing to their overall impact as a major part of the global diet, and any increase in wheat protein content could thus have a massive effect on overall supply. We therefore propose that increasing the protein content of cereal grains through a revised breeding strategy in which innovative phenotyping and genetic information are incorporated early during trait selection could be a solution.

Using genetic and genomic approaches to understand the molecular control of protein accumulation in grain and exploiting this knowledge to engineer large quantities of high-quality protein could bring major advances in nutrition. Emerging high-speed testing approaches, such as **hyperspectral imaging** (glossary), offer robust and non-destructive phenotyping methods for protein content to help take advantage of improved genetic resources and knowledge in wheat. Abundant information on the genetics of protein accumulation is available, and new information on the role of **plant vasculature** (glossary) in the distribution of N-based assimilates to inflorescences and grain has recently emerged. Such information could be exploited in a multi-disciplinary approach that combines molecular genetics and high-speed testing to accelerate breeding for protein-rich crops, ultimately providing a solution for sustainable protein supply.

Outstanding questions

Future research could benefit from addressing the following questions.

1. **Does increasing the protein levels of cereals affect the quality of the producible protein, and if so, is this trade-off beneficial?** This review discusses an alternative and more sustainable approach to meet the rising protein challenge through boosting protein levels in cereals, as they are major contributors to the global food supply. A potential area to explore is the quality of protein from cereals as an alternative to protein from animal- or other plant-based sources.
2. **Is there potential to improve high-speed testing methods like hyperspectral imaging in order to screen protein fractions for protein quality determination?** With the emergence of imaging techniques such as hyperspectral imaging into plant breeding, future research on the suitability of these methods for non-destructive investigation of protein fractions in grain would benefit grain and protein quality estimation.
3. **Do wheat mutants with altered vasculature that show potential for increased grain protein behave like wild types that may have been treated with low nitrogen?** Nitrogen fertilizers, which contribute to environmental and soil pollution, are positively correlated with GPC. Given the recently discovered role of plant vasculature in enhancing GPC without affecting yield, investigation of how plants with altered vasculature behave in low-nitrogen soils could benefit research on reduced fertilizer use in cropping systems, ultimately benefiting the environment.

GLOSSARY AND NOTE

Plant vasculature, also known as the plant vascular system, is a set of conducting tissues that transport water and nutrients throughout the plant body. Plants that contain vasculature are called vascular plants or tracheophytes, which comes from the Greek word *trachea* (meaning windpipe) and refers to water-conducting tissues.

Senescence and nitrogen remobilization: Senescence, also known as biological aging, is directly linked to grain protein accumulation through the remobilization of nitrogen from vegetative tissues to grain after fertilization. Rapid senescence increases GPC but can result in reduced grain carbohydrates and yield.

Hyperspectral imaging is an emerging high-throughput technique for phenotyping grain quality traits like protein content. It combines a broad-spectrum camera with near-infrared spectroscopy to extract spectral and spatial information from an object in three-dimensional space, which can then be used to predict the protein content of a single grain through statistical modeling. Use of hyperspectral imaging early in plant breeding can help to minimize phenotyping time and capture the maximum genetic potential of germplasms.

This review includes **secondary analysis of existing data** that are publicly available from FAO at https://www.fao.org/faostat/en/?#data/QCL, https://www.fao.org/faostat/en/#data/FBS, and Our World in Data at https://ourworldindata.org/meat-production. Further information about the terms of use and licenses are available at https://www.fao.org/contact-us/terms/db-terms-of-use/en/.

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