SUSTAINING PROTEIN NUTRITION THROUGH PLANT-BASED FOODS: A PARADIGM SHIFT

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SUSTAINING PROTEIN NUTRITION THROUGH PLANT-BASED FOODS: A PARADIGM SHIFT

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Editorial: Sustaining protein nutrition through plant-based foods: A paradigm shift

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Editorial on the Research Topic
Sustaining protein nutrition through plant-based foods: A paradigm shift

Proteins are one of the vital building blocks of life. The entire protein complement of an organism and its building block amino acids are derived from food. Plants provide a good source of proteins for human and animal diets. The emerging global trend for a vegan diet has put the spotlight on the scientific dimensions of plants as a source of sustainable proteins. The Research Topic "*Sustaining Protein Nutrition through Plant-Based Foods: A Paradigm Shift*" provides a comprehensive Research Topic of original research and reviews covering the multifaceted dimensions of the role of plant proteins in human nutrition and health.

What are the different sources of plant proteins and how do they affect human health? The question is comprehensively explored by Langyan, Yadava, et al. who discuss functional properties and various health issues linked with plant-based proteins. Interestingly, plants could be source analogs of meat, milk, and egg. Cost-effective extraction and processing technologies, exploration of different food wastes as an alternative source of plant proteins, their environmental impact, and studying their effect on nutrition are important goals. Translational research for upscaling emerging technologies for improving plant proteins' bioavailability, digestibility, and organoleptic properties is expected to open new dimensions of the utilization of plant proteins.

Intake of a nutritious diet, including adequate intake of protein, is also necessary to fight pandemics, like COVID-19 (Mortaz et al.). How specific dietary profiles might help to augment public health strategies and reduce the rate and severity of COVID-19 is an open question. Supplementation of amino acid arginine significantly increases T cell function as well as enhances their numbers compared with control subjects.,

Similarly, a deficiency of amino acid methionine significantly decreases serum levels of IgG, IgA, and IgM antibodies and the relative percentage of $CD3^+$, $CD3^+/CD8^+$ and $CD3^+/CD4^+$T lymphocytes in the serum. Poor intake of sulfur-containing amino acids, like methionine and cysteine, significantly reduces the hydroxyl radical scavenging activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), making the host susceptible to viral infection. The production of interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) α is also dependent on the adequate availability and metabolism of sulfur-containing amino acids. These aspects of proteins, human nutrition, and disease progression need to be explored further, and plant-based foods and nutraceuticals as a source of these critical amino acids need to be researched and developed in the future.

Apart from their roles during the pandemic period, the geographical aspect of the protein story is also highlighted here. How locally available and adaptable plant species can transform the protein story of a particular population, was originally researched by Atuna et al.. They assessed the nutrient value and desirability of eight improved soybean varieties, for use in soymilk, tofu, and as an ingredient to enhance staple foods in the African nation of Ghana. The soybean varieties evaluated were generally desirable for soymilk and tofu production. For every kilogram of soybean used to produce either soymilk or tofu, 3.17–3.97 kg of fresh okara—a pulp byproduct of soymilk and tofu—was also produced. Recipe refinements using okara with cassava may help fill the protein gap among the vulnerable populations of the West African Sub-region, particularly in Ghana by improving the protein quality of ready-to-eat foods.

Like soybean, maize, especially quality protein maize (QPM) is considered a significant leap toward improvement in the nutritional status of rural masses in African and other developing countries. This maize contains a higher concentration of essential amino acids, particularly lysine and tryptophan, in its kernel endosperm. Kaur et al. have optimized protein quality assay in normal and QPM kernels effectively and helped in reducing the defatting time by 24 h and protein estimation by 3 h as compared to the already established protocol. This is expected to provide a further boost in the development and commercialization of QPM. Wheat is another cereal that is also a good source of protein and has received considerable attention, especially due to its bread and bakery-making quality (Goel et al.). In this line, Allai et al. have reported a novel process to make a pregelatinized cereal bar, containing whole wheat flour, whole barley flour, and whole corn flour, blended with Indian horse chestnut flour. Khalili et al. conducted a meta-analysis, cataloging studies aiming at the application of different types of nuts and their positive effects in improving glycemia, dyslipidemia, inflammation, and oxidative stress.

The importance of legumes as protein-rich food can never be underestimated. Apart from the leading legume crops, there is a need to rediscover the potential of multifaceted orphan legumes as a sustainable source possessing high nutritional values (Ramya et al.). Viana and English have demonstrated that dehulling and germination are potential processing methods that may be used to improve the physiochemical characteristics of salt-extracted protein concentrates from yellow eye beans (*Phaseolus vulgaris* L.), a rich source of dietary protein.

In the arena of valorization of agricultural waste for human protein, the use of leftover oilseed cakes could be a game changer (Singh et al.). Oilseed cakes exhibit higher angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity compared to other protein isolates and thus could be a source of health-promoting products. The Food and Agriculture Organization of the United Nations (FAO) cautions that protein could be a limiting macronutrient in the human diet in the future, therefore, there is a need to intensify efforts for developing different kinds of methods and processes to extract protein from oilseed cakes. The development of faster analysis methods for protein quantification in oilseed crops, including the one reported here (Langyan, Bhardwaj et al.), can further provide necessary tools for scientific research on oilseed feedstocks for edible proteins. Castor meal is a by-product of oil extraction from the castor plant and is generally considered toxic for humans. However, Amoah et al. show, for the first time, the positive effects of castor meal on the growth, feed utilization, immune response, digestive enzyme activities, and intestinal health of a fish known by the name of hybrid grouper, which has potential as a fishery medicine. The use of oilseed cake proteins in human food and health products is very limited at present, which requires further research on the utilization of these enormously rich protein sources in the diet.

The story of plant proteins remains incomplete, without addressing the basic agronomy of crops that are targeted as a source of proteins. Agronomic interventions, like intercropping and field management in specific agro-ecologies, are essential in making these crops high yielding, profitable, and economically sustainable for farmers on one hand and getting cost-competitive protein feedstock for the food technology industry on the other hand (Wang et al.).

In conclusion, this Research Topic on plant-based foods for sustaining protein nutrition incorporates a wide canvass of original research, reviews, and meta-analysis covering multiple, if not all, aspects of current progress in this area. It is hoped that researchers will appreciate the vast diversity of the nature of the problem and feel motivated to take up new dimensions of research that will make plants a central player in protein nutrition. It would be a great contribution to fighting protein malnutrition and meeting Sustainable Development Goals.

Author contributions

SL and PY contributed to the conceptualization and writing the primary draft. TB and TK edited the manuscript and supervision. SL, PY, TB, and TK approved the final draft.

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Conflict of interest

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Nutritional Impact and Its Potential Consequences on COVID-19 Severity

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Background: During late 2019 a viral disease due to a novel coronavirus was reported in Wuhan, China, which rapidly developed into an exploding pandemic and poses a severe threat to human health all over the world. Until now (May 2021), there are insufficient treatment options for the management of this global disease and shortage of vaccines. Important aspects that help to defeat coronavirus infection seems to be having a healthy, strong, and resilient immune system. Nutrition and metabolic disorders, such as obesity and diabetes play a crucial role on the community health situation in general and especially during this new pandemic. There seems to be an enormous impact of lifestyle, metabolic disorders, and immune status on coronavirus disease 2019 (COVID-19) severity and recovery. For this reason, it is important to consider the impact of lifestyle and the consumption of well-defined healthy diets during the pandemic.

Aims: In this review, we summarise recent findings on the effect of nutrition on COVID-19 susceptibility and disease severity and treatment. Understanding how specific dietary features might help to improve the public health strategies to reduce the rate and severity of COVID-19.

Keywords: COVID-19, SARS-CoV-2, probiotics, nutrition, proteins

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INTRODUCTION

The recent outbreak of coronavirus disease 2019 (COVID-19), caused by a new zoonotic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1), is a great threat to public health all over the world (2). As of May 20th 2021, variants of the coronavirus SARS-CoV-2 have infected more than 165 million people globally and resulted in 3.42 million deaths (3). Beyond prevalence and mortality, the restrictions and lockdown measures that are needed to control the COVID-19 pandemic evolved in a global economic and social crisis, severely affecting the people's well-being, mental health and social support (4). The direct consequences of COVID-19 on an individual represents a spectrum of clinical severity with some patients being asymptomatic or having only mild upper respiratory tract symptoms whilst some subjects have severe pneumonia characterised by fever, cough, dyspnoea, bilateral pulmonary infiltrates and acute respiratory injury requiring

ventilation (5–8). Approximately 20% of patients develop severe respiratory illness with an overall mortality of 2.3% (3). The impact of SARS-CoV-2 infection is not limited to the respiratory system, but it affects the kidney, gut, eyes, heart, and brain among other organs. Together, the effect on these target organs may have profound and prolonged consequences on COVID-19 severity, and on recovery (5–8). The body's mental and physical status and fitness are important factors in keeping one's immune system balanced and resilient and thereby able to mount a proper response against SARS-CoV-2 (9, 10). Obesity and type 2 diabetes are therefore examples of key risk factors for COVID-19 (11). Obesity is associated with dysfunctional adipose tissue, metabolic dysfunction, multi organ damage, endocrine disruption, impaired immune function, and low grade (sub) chronic inflammation (12). Moreover, obesity along with low physical activity and fitness, is the leading cause of type 2 diabetes or metabolic syndrome (T2DM), which is causally linked with elevated angiotensin-converting enzyme 2 (ACE2) expression (13).

The high prevalence of these risk factors, is for a significant part, associated with the pattern of nutrition such as increased consumption of high amounts of saturated fat (high fat diet, HFD), refined carbohydrates and low levels of fibre and antioxidants. Balanced nutrition has a potentially important role in the maintenance of immune homeostasis and resilience and for this reason resistance against disease including infections with viral and bacterial pathogens. Malnutrition has prolonged effects on physical and mental health by influencing gene expression, cell activation, and interfering with signalling molecules that shape and modulate the immune system (14). Thus, poor nutrition and an unhealthy diet might significantly weaken the immune system and increases susceptibility to infectious disease including SARS-CoV-2.

Disparities in nutrition or obesity are impacted by cultural background and closely correlated with severe COVID-19-related outcomes (15). The hospitalisation rates for COVID-19 positive subjects among Native and Latin Americans are higher than that of White Americans which could be attributed to malnutrition (15, 16). Another example of the impact of cultural background and socio-economic status on severity of COVID-19 is evidenced in Islamic countries with poor healthcare systems, lack of facilities particularly during the religious tradition of Ramadan fasting (17). During Ramadan, Muslims may have trouble in maintaining exercise, which negatively affects immune health. On the other hand investigations of health related effects of Ramadan Fasting also show beneficial effects of reduced meal frequency and caloric restrictions on insulin sensitivity, a reduction in oxidative stress and inflammation (17).

Indeed, nutrition and obesity play a crucial role in the fate of viral infectivity in general and the community health situation during this present pandemic. In this review, we summarise recent findings regarding the impact of nutrition on the variation in COVID-19 disease severity and also its potential impact on the control of the disease during the current pandemic. Understanding the dietary pattern that is deleterious to COVID-19 survival might help to improve public health strategies toward reducing the spread of COVID-19 and designing new approaches for control and maybe even treatment of this new disease.

PATHOGENESIS OF COVID-19 DISEASE

*S*ARS-CoV-2 virus primarily affects the respiratory system, although other organ systems are involved as well. Lower respiratory tract infection-related symptoms including fever, dry cough, and dyspnoea were reported in the initial case series from Wuhan, China (6). In addition, headache, dizziness, generalised weakness, vomiting and diarrhoea were observed (18). Although COVID-19 is mainly a respiratory disease, the gastrointestinal system can also act as a reservoir for SARS-CoV-2 (19). In addition; neurological manifestations are also reported in most hospitalised COVID-19 patients (20).

It is now widely recognised that the respiratory symptoms of COVID-19 are extremely heterogeneous, ranging from minimal symptoms to significant hypoxia with acute respiratory distress syndrome (ARDS) (8, 21). In the first reports from Wuhan, the time between the onset of symptoms and the development of ARDS was as short as 9 days, indicating that the respiratory symptoms could progress rapidly (6). ACE2is identified as a functional receptor for SARS-CoV-2 (22). Structural and functional analysis showed that the SARS-CoV-2 spike protein binds to the ACE2 receptor (23–25). ACE2 expression is high in the lung, heart, ileum, kidney and bladder (26). More specifically the ACE2 receptor is highly expressed on the apical side of lung epithelial cells in the alveolar space (27, 28). This correlates with the fact that early lung injury was often seen in the distal airways (29).

Genetic susceptibility can be a major factor in the host response to infectious diseases where inborn errors of the immune system are often critical (30). Differences in clinical outcomes of COVID-19 may also be determined by genetic susceptibility. Old age, gender and comorbidities including hypertension, diabetes, respiratory system disease and cardiovascular disease have all been identified as being closely associated with disease severity and mortality and represent significant risk factors (31).

COVID-19 morbidity and mortality rise dramatically with age and co-existing health conditions, including cancer and cardiovascular diseases. While most infected individuals recover, even very young, and otherwise healthy patients may unpredictably succumb to this disease (32). Questions still remain as to how susceptibility and outcome factors relate to SARS-CoV-2 infection.

In this line the greater severity of the disease was associated with maladapted immune responses and host ACE2. However, some other genetic parameters for SARS-CoV-2 receptor and entry gene expression and function have been described (33).

An intact immune system is essential for an effective defence against invading microorganisms. However, due to the immunological defects seen with COVD-19, there is reduced scope for a defence to be mounted against SARS-CoV-2 (34). The massive production of cytokines and chemokines observed during COVID-19 infection, the so-called "cytokine storm," leads

to broad and uncontrolled tissue damage and results in plasma leakage, enhanced vascular permeability and disseminated and vascular coagulation. This excessive proinflammatory host response is responsible for the pathological outcomes such as acute lung injury (ALI) and ARDS seen in severe SARS-CoV-2 patients, which typically leads to death.

Men are at a greater risk of severe symptoms and worse outcomes from COVID-19 than women. The precise reason for this discrepancy is not fully understood, but genetic factors, the effects of sex hormones such as oestrogen and testosterone as well as differences in immune cell function such as that of mast cells may be important factors (35).

Prostate cancer patients who were receiving androgen-deprivation therapy (ADT), a treatment that suppresses the production of androgens that fuels prostate cancer cell growth, had a significantly lower risk of SARS-CoV-2 infection (36). This suggests that blocking androgens in men is protective against SARS-CoV-2 infection. There is also evidence that males and females have different levels of receptors that recognise pathogens or that serve as an ingress point for SARS-CoV-2. Whilst there is currently no conclusive evidence for a role of ACE2 receptors and associated proteases being differentially expressed in males compared to females, it remains a potential contributing factor.

PHYSICAL INACTIVITY, MALNUTRITION, AND COVID-19

Balanced nutrition is an important determinant in immune function against infectious disease in general (14). Poor nutrition and an unhealthy diet significantly weakens the immune system and increases susceptibility to infectious disease (37). A reduction in physical activity and a higher energy intake have been observed as a consequence of pandemic isolation measures which is especially worrisome since they both enhance the risk of a more severe outcome of COVID-19 (38). This is particularly true in middle-aged and elderly people where physical inactivity negatively impacts cardio-vascular functional capacity, body weight, metabolic function, muscle strength, haemostatic factors and immune functions (39). Moderate, but not vigorous exercise, enhances immune processes resulting in lower incidence of upper respiratory tract infections (39). **Figure 1** summarises how levels of exercise and diet affect immune functions. A suboptimal diet may significantly affect the susceptibility to COVID-19 infection as well as the downstream consequences including severity, recovery and the potential for re-infection in different patient populations (40). Diets with a high consumption of saturated fatty acids (SFA), sugars, refined carbohydrates, and low levels of fibre and antioxidants modulate the balance between the adaptive and innate immune responses leading to an impaired host defence against viruses (41). In addition, these diets are associated with a higher prevalence of COVID-19 risk factors and the long term recovery from COVID-19 infection (42).

SFA-rich diets induce chronic activation of the innate immune system while inhibiting adaptive immunity. In fact, high SFA diets induce a lipotoxic state which could activate toll-like receptor (TLR) 4 on the surface of macrophages and neutrophils and lead to chronic activation of the innate immune system. This, in turn, may trigger other inflammatory signalling pathways and the production of proinflammatory mediators (41, 43). The expression levels of TLR9 and levels of endogenous triggers for TLR9 activation are also influenced by diet which has been proposed to contribute to a severe outcome of COVID-19 in vulnerable patients (44).

A high fat diet (HFD) and obesity increases TLR9 expression in visceral adipose tissue in mice and human (45). HFD induces excess production of nucleic acids and related protein antigens worsening metabolic inflammation through activation of macrophages and expansion of plasmacytoid dendritic cells (pDCs) in the liver (46). In animal models, HFD also increases macrophage infiltration into the lung tissue and alveoli. A similar process may underlie the high rate of inflammation in lung epithelial cells and the alveolar damage seen in obese COVID-19 patients or those with evidence of metabolic syndrome (47). Furthermore, carbohydrates, sugars and a HFD increase oxidative stress and thereby impair the proliferation and maturation of both B and T cells and induce apoptosis which together results in suppression of the adaptive immune response to viral infection (48).

In animal models of influenza infection, a HFD enhanced lung damage and delayed the onset of the adaptive immune response. This was associated with impaired memory T cell function and a reduced capacity to respond to antigen presentation and clearance of the influenza virus (48). The mechanism(s) causing the increased lung damage are unclear but may involve programmed cell death (49–52).

As a result, the elderly, patients with comorbidities, and those with risk factors for COVID-19 should be cautious with the consumption of unhealthy diets that could pose an increased risk to COVID-19 severity. A healthy, balanced diet should contain the necessary macro- and micronutrients, vitamins, minerals, and maybe even unique microbes such as probiotics that can restore and maintain immune function (53).

Proteins, vitamins and minerals have, for a long time, been considered important factors in health and resistance against infection due to their impact on immune homeostasis (54). The immune-effect of natural herbal medicines such as Shuang-Huang-Lian oral liquid during upper respiratory tract infections may be explained, at least in part, by specific proteins, and other active ingredients (2, 55). A recent comprehensive meta-analysis regarding the effect of nutrition status on the immune response to respiratory viral infection reported that vitamins and minerals play a determinant role in the ability to mount an immune defence against respiratory viral infection and are associated with the severity of infection outcome (56).

In the current COVID-19 pandemic, there are reports of vitamins and minerals affecting the severity of infection and mortality. For example, low prealbumin levels is associated with increased severity of ARDS in patients with SARS-CoV-2 (57). Vitamins A, B complex, C, D and E, and trace elements have an important role in the prolonged and effective stimulation of the immune system (58, 59). Thus, deficiencies in vitamin and trace element levels could result in a more detrimental fate in response

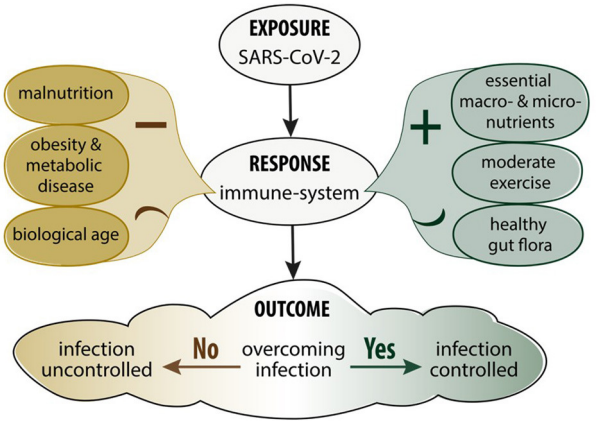

FIGURE 1 | Impact of nutrition, metabolic disease and exercise on the immune response and SARS-CoV-2 infection.

to viral infections including SARS-Cov2 (60). Some studies also suggest beneficial effects of natural compounds.

In summary, the nutritional status of an individual has a significant impact on not only the susceptibility to, but also the severity of, COVID-19 infection. The next section provides additional details concerning the impact of proteins, vitamins and minerals in viral respiratory infections that might help finding new strategies for the prevention and control of SARS-CoV-2 infection (**Table 1**).

Proteins

Proteins are critical factors in immune-nutrition and essential for the production of, for example, immunoglobulins, and cytokines. Dietary proteins are digested to their constituent amino acids and dietary protein deficiency reduces plasma concentrations of most amino acids. Amino acids, such as arginine are the precursor of polyamines that play a significant role in the regulation of DNA replication and cell division. In addition, optimal antibody production requires a sufficient plasma arginine level. Supplementation with arginine significantly increases T cell function as well as enhancing their numbers compared with control subjects (61). Furthermore, arginine is essential for the generation of nitric oxide by macrophages, an essential component of the innate immune response. In contrast, methionine has an important role in the growth, development and histological structure of immune organs and enhances macrophage phagocytic activity (62). Methionine deficiency also decreases lymphocyte activities and inhibits the proliferation and differentiation of B and T cells (63). Methionine also plays a role in both humeral and cellular immunity since methionine deficiency significantly affects antibody titre and decreases serum levels of IgG, IgA, and IgM. Furthermore, methionine deficiency decreases the relative percentage of $CD3^+$, $CD3^+/CD8^+$, and $CD3^+/CD4^+$T lymphocytes (64). Given the importance of T cell immunity in the defence against COVID-19, this aspect of methionine deficiency is essential in the prevention of, and reduction in the severity of infection.

Reduction of sulphur-containing amino acids in the serum significantly reduces the hydroxyl radical scavenging activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) which helps to protect the host against viral infection (3, 4). Thus, methionine deficiency can result in oxidative damage and lipid peroxidation, which will lead to a failure in cellular immunity.

Amino acids are also important components for cytokine production. The production of interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF) α is strongly dependent on the metabolism of sulphur-containing amino acids including methionine and cysteine (65).

The effect of dietary proteins in improving immune function has been reported in cancer patients. In a clinical trial, whey protein isolate (WPI) enriched with Zn and Se improved cell-mediated immunity and antioxidant capacity in cancer patients undergoing chemotherapy. WPI is an alternative oral nutrition supplement (ONS) that contains high quality protein and amino acid profiles. WPI increases GSH function because of its cysteine-enriched supplementation, reduces oxidative free radical formation and prevents infection (5). This suggests that WPI supplementation may improve GSH levels and thereby enhance immunity in subjects at risk of COVID-19 as well as reducing the severity of the disease in patients already infected with SARS-CoV-2.

Vitamins

A healthy immune system may aid the prevention and treatment of patients with COVID-19 (62). Vitamins play an important role in normal immune function and their dietary levels tightly regulate immune reactions (66) (**Table 1**). For example, vitamins A and D increased humeral immunity following influenza vaccination in children (63, 67). Fasted individuals are encouraged to have sufficient and timely intake of healthy and functional foods including vitamins in order to maintain exercise performance and immune function (17).

Vitamin A is an important player in the regulation of both the cellular and humoral arms of the immune system and significantly increased the antibody response after anti-viral vaccination (56). Vitamin A acts *via* the nuclear retinoid acid receptor (68, 69) and regulates the proliferation and differentiation of immune cells and modulates the expression of proinflammatory cytokines including TNFα and IL 6 (70, 71).

A protective role of vitamin A has been indicated against in a variety of lung infections, HIV, and malaria (72, 73). In animal models of corona virus infection, the levels of plasma retinol and retinol-binding protein is significantly reduced and mortality from respiratory infections decreases in those with adequate vitamin A within their diets (74, 75). As a result, we postulate that vitamin A supplementation may make a useful contribution in combating the risk of susceptibility to COVID-19 infection and reducing the severity of the disease in patients.

B group vitamins are key players in metabolic pathways particularly those of organic molecules. Furthermore, the important role of B group vitamins including folic acid, B12, and B6 in immune function is well known. For example, the active form of vitamin B6, pyridoxal phosphate, is a cofactor

for many metabolic processes particularly transamination or breakdown of amino acids and the metabolism of important immunomodulatory mediators (76, 77). These metabolic pathways are also important in viral infection suggesting that a balance intake of these vitamins is necessary in the regulation of the viral immune response. In particular, they regulate the function of natural killer cells and cytotoxic $CD8^+$ lymphocytes and thereby contribute to effective viral clearance (78).

Vitamin D is fat soluble and known as a multifunctional agent in a broad range of bodily functions including immune reactions (79). Vitamin D receptors (VDRs) are expressed in a broad range of respiratory epithelial and immune cells and vitamin D activation is induced by cytokines and TLRs within the respiratory tract (79, 80). Epidemiological studies indicated the importance of vitamin D in the immune defence against influenza A and B, parainfluenza and respiratory syncytial virus (RSV) (81, 82). Interestingly; low levels of serum vitamin D enhanced the risk of both upper and lower respiratory tract infections (83). It has been reported that serum vitamin D levels of $\geq$95 nmol/L significantly reduced the rate of acute viral respiratory tract infections two-fold (60).

On the other hand, low levels of vitamin D are associated with enhanced levels of inflammatory cytokines and an increase in the incidence of many diseases. Importantly, vitamin D deficiency is associated with increased thrombotic episodes, obesity, and diabetes which are frequently observed in severe COVID-19 patients (84). An inhibitory and antiviral activity of vitamin D in human nasal epithelial cells infected with SARS-CoV-2S has been reported (85).

Vitamin D deficiency has shown an important role in reducing the risk of severe disease and mortality in COVID-19 patients. In Chicago, more than half of COVID-19 related deaths occurred in African-American individuals known to have vitamin D deficiency (86). Indeed, regions with the highest rates of COVID-19 mortality are those with a high prevalence of vitamin D deficiency (66). Indeed, a meta-analysis indicates that low serum levels of vitamin D is significantly associated with the risk, seriousness and mortality of COVID-19 (87). Although the area is controversial, the limited current data suggests that higher serum vitamin D levels favour a decreased risk of COVID-19 infection and mortality (88). It is reasonable, therefore, to suggest that regular vitamin D supplementation would be of benefit to individuals at greater risk of infection or of developing severe disease (89).

Vitamin E is a potent regulator of host immune functions due to its antioxidant capacity. This enables vitamin E to modulate multiple immune and inflammatory responses including T-cell proliferation, granulocyte phagocytosis, and cytotoxicity through effects on gene transcription (90–93). This explains why vitamin E deficiency is accompanied by impairment of both humoral and cellular immunity (94). Although vitamin E supplementation increased the risk of pneumonia in smokers (95), vitamin E had a therapeutic benefit in chronic hepatitis B (HB) patients in a small pilot randomised clinical trial (RCT) (96). In another RCT, vitamin E treatment led to higher anti-HBe seroconversion in children (97). A computational analysis to assess the ability of FDA-approved drugs to block coronavirus binding to ACE2 or transmembrane protease, serine 2 (TMPRSS2) and downstream transcriptomic profiles indicated that vitamin E, ruxolitinib and glutamine were likely to significantly attenuate infection by SARS-CoV-2 (98). This needs to be confirmed in human studies.

Vitamin C boosts many aspects of the immune system including cell signalling, phagocytosis, antibody production, immune cells proliferation and leukocyte migration to the site of infection (99). Furthermore, vitamin C mediates many physiological events, such as hormone production and immune homeostasis and acts as an essential antioxidant and enzymatic co-factor in many cellular functions (58).

Animal studies highlight its role in improving the production of interferons (IFN)α and β in response to influenza A virus and this may explain its ability to protect against coronavirus infection (100). Indeed, higher serum levels of vitamin C is associated with a reduced incidence of pneumonia and lower respiratory tract infections (101, 102). In addition, vitamin C reduces the duration and severity of the common cold (58), and of upper respiratory tract infections (101).

Vitamin C also promotes the repair of the damaged tissues (58) and high-dose intravenous vitamin C has a beneficial effect in patients with virus-induced ARDS which results from severe lung damage (103). Since ARDS is evident in many subjects with severe COVID-19 it supports the concept that vitamin C may be useful in the treatment of COVID-19 (104). Further studies are required to demonstrate a link between COVID-19 incidence and severity with systemic vitamin C levels.

Interestingly, apart from individuals with impaired glucose 6-phosphate activity and renal failure, no adverse effects of large doses of intravenously or orally administrated vitamin C have been detected (105, 106).

Minerals

In addition to vitamins, several minerals have a beneficial and supportive role in enhancing antiviral immune responses and thus could be beneficial in controlling COVID-19 (**Table 1**). Zinc plays a pivotal role in the immune system particularly in antiviral and antibacterial immunity (107). Zinc deficiency is associated with an increased susceptibility to infectious and viral diseases and studies have shown that the zinc status is a critical factor that can influence immunity against viral infections (108). In patients infected with torque tenovirus (TTV), injection of a high dose of zinc enhances the immune response (107). On the other hand, low-dose supplementation of zinc together with selenium improved the humoral immune response to influenza vaccine and increased antibody titres (109).

In *in vitro* experiments Zn inhibits the SARS-CoV-2 RNA polymerase (110). Interestingly, chloroquine that has some protective efficiency against coronaviruses acts as a zinc ionophore (111). In addition, zinc may supress ACE2 activity and regulate the production of IFNα to improve antiviral activity (108) and zinc also has an anti-inflammatory role by inhibiting NF-κB signalling (112) and modulating regulatory T-cell functions. This combination of actions may be important in sequencing the cytokine storm present in subjects with COVID-19 (112).

TABLE 1 | Overall role and impact of nutrition on immune function.

		Role and impact on immune responses
Protein		1. Production of cytokines and antibodies. 2. Regulation of both humeral and cellular immunity specially Tell immunity. 3. Regulation of DNA replication and cell division. 4. Generation of nitric oxide, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) as well as scavenging activity by immune cells.
Vitamins	A group vitamins	1. Antiviral immunity. 2. Regulation of the proliferation and differentiation of immune cells *via* nuclear retinoid acid receptor.
	B group vitamins	1. Immune metabolic pathways as co-factor. 2. Viral clearance *via* regulation of natural killer cells and cytotoxic $CD8^+$ lymphocyte functions.
	C group vitamins	1. Act as enzymatic co-factor and an essential antioxidant in boosting immune functions including phagocytosis, cell signalling, antibody production leucocyte migration, and hormone production.
	D group vitamins	1. Controlling inflammation in the lungs. 2. Proliferation and activation of viral specific immune cells *via* its receptor. 3. Upregulation of cytokines and their recruitment to the infected sites.
	E group vitamins	1. Antioxidant activity. 2. Gene transcription of proteins involved in T-cell proliferation, phagocytosis and cytotoxicity, regulate the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and modulate signal transduction.
Minerals	Zinc	1. Antiviral and antibacterial immunity, inhibition of viral RNA polymerase and ACE2 activity. 2. Involved in modulation of inflammatory cytokines. 3. Upregulation of Th1cytokine responses, activation of immune metabolic pathways.
	Selenium	1. Antioxidant and anti-inflammatory properties. 2. Increase in T-cell proliferation. 3. Upregulation of IL-10.
	Copper	1. Inhibition of viral replication and release. 2. Inhibition of viral-induced cell apoptosis. 3. Activity of ceruloplasmin, benzylamine oxidase and superoxide dismutase and improvement of the cell antioxidant status.
	Magnesium	1. Activator role in many of enzymatic reactions. 2. Regulation of nuclear factor-κB, Il-6, c-reactive protein, and other related signalling pathways.
Probiotics		1. Influencing immune reactions by up or down regulation of immune responses

Zinc-deficient populations are at an increased risk of infection by several viruses including human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (113). In a RCT, Zn increased Th1cytokine responses including the production of IL-2 and of INFγ in response to influenza vaccine (107). In another RCT, oral supplementation of high-dose zinc after stem cell transplantation demonstrated that zinc enhanced thymic function and the production of CD4 naïve T cells, helping to prevent the reactivation of TTV (107). However, in an elderly population, enhancing zinc plasma concentrations had no effect on the antibody response or on the number of lymphocytes present following influenza vaccination (114).

Selenium is another trace element with a broad range of effects from antioxidant to anti-inflammatory properties (115). Selenium supplementation resulted in both beneficial and detrimental effects on cellular immunity to influenza. Selenium supplemented subjects had a more rapid clearance of the poliovirus after vaccination for influenza. In this study, selenium induced a dose-dependent increase in T-cell proliferation and the production of IL-8 and IL-10. However, mucosal influenza-specific antibody responses were unaffected by selenium supplementation (116).

Copper has a crucial role in the development and differentiation of immune cells and mediates several antiviral responses (117). Chelates of thujaplicin and copper inhibited influenza virus-induced apoptosis *in vitro* supressed viral replication and release from the infected cells (118). In addition, intracellular copper interferes with the influenza virus life cycle (119). Appropriate copper intake optimises the antioxidant status and improves the serum level and activity of ceruloplasmin, benzylamine oxidase and superoxide dismutase (118, 120).

Magnesium also regulates immune function by controlling various aspects of immunity such as immunoglobulin synthesis and antibody-dependent cytolysis (121). Magnesium is an activator of many enzymatic reactions and is essential for a broad range of physiological functions (122). Magnesium intake supports different aspects of immune functions including regulation of NF-κB, IL-6, c-reactive protein, and other related signalling pathways (123). The major role of magnesium is in viral immunity which has been reported in many *in-vitro* and *in-vivo* studies (121, 124). A recent study reported that magnesium in combination with vitamin D and vitamin $B_{12.}$ Significantly reduce the proportion of severe COVID-19 patients needing intensive care (125).

Probiotics

SARS-CoV-2 infection of the gastrointestinal system affects gut inflammation both directly and indirectly following infection of intestinal epithelial cells through the ACE2 and transmembrane protease serine 2 (TMPRSS2) viral entry system. This results in

pronounced pro-inflammatory chemokine and cytokine release (126, 127). In addition, cellular and animal studies indicate that SARS-CoV-2 instigates an acute intestinal inflammatory response including elevated levels of faecal calprotectin and serum IL-6 and linked to clinical evidence of diarrhoea (127). To date, the rationale for using microbiome modulators such as pre and probiotics in COVID-19 is indirect. Two randomised controlled trials showed that critically ill patients on mechanical ventilation who were given probiotics (*Lactobacillus rhamnosus* GG, live *Bacillus subtilis*, and *Enterococcus faecalis*) developed substantially less ventilator-associated pneumonia compared with placebo (128, 129). Due to the similarities between severe COVID-19- and pneumonia-induced ARDS there is potential for this therapeutic approach being useful in COVID-19.

Patients with COVID-19 appear to have an altered gut microbiome with depletion of beneficial commensals (*Eubacterium ventriosum, Faecalibacterium prausnitzii, Roseburia, and Lachnospiraceae taxa*) and enrichment of opportunistic pathogens (*Clostridium hathewayi, Actinomyces viscosus, Bacteroides nordii*) (130). It is uncertain whether this difference is causal or downstream of other changes but again indicates that probiotics or microbiome manipulation may be useful in severe COVID-19 subjects. Disturbances in gut microbiota and their metabolites influence immune responses, inflammation and diseases of the lungs by mediating both over-active and under-active immune responses (131). Favourable implications of gut microbiota modulation in COVID-19 is speculated upon because a general imbalance of gut microbiota is commonly seen in elderly and immune-compromised patients and patients with other co-morbidities like type-2 diabetes, and cardiovascular disorders (132). To date however, the rationale for using probiotics in COVID-19 is derived from indirect evidence and more research is needed before any specific recommendations on probiotic use can be made (133).

CONCLUSION

The COVID-19 pandemic poses a significant threat to humans. Until the widespread availability of effective, long-term, vaccines, and effective treatment and prevention measures. An important therapeutic and preventive strategy, may be to reduce the incidence or severity of infection. This will involve having a healthy and resilient immune system. An individual's nutritional status has a significant impact on the susceptibility to COVID-19, response to therapy, and on the long-lasting consequences of infection. As such, it is critical to consider the impact of lifestyle and the consumption of healthy diets during the pandemic.

A good healthy balanced nutrition is vital in the recovery process for all patients with COVID-19, particularly those who have suffered cardiac distress, pulmonary distress, or those who have been critically ill due to the weight loss, frailty or sarcopenia associated with these conditions (134). These patients require individually tailored nutrition support, started early in their journey, that is sufficient and timed to enable optimal metabolic utilisation to aid recovery (134). Nutritional rehabilitation needs to be central to the community management of these patients' post-hospital discharge to ensure efficient and effective recovery and to reduce the risk of hospital re-admissions or the duration of long-COVID-19.

In this respect, access to healthy foods should be a priority for individuals and governments to reduce the susceptibility and prolonged effects of COVID19. Given the over-representation of minorities with the disease and those who also have poor nutrition, we should aim to increase the access to healthy fresh food as well as provide nutritional education to these at-risk subjects.

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EM and SA designed draft and wrote first version of manuscript draught. GB, GF, SM, JG, and IA revised and comments to the manuscript. All authors has seen and approved final version of manuscript.

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REVIEW
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Wheat Proteins: A Valuable Resources to Improve Nutritional Value of Bread

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Triticum aestivum, commonly known as bread wheat, is one of the most cultivated crops globally. Due to its increasing demand, wheat is the source of many nutritious products including bread, pasta, and noodles containing different types of seed storage proteins. Wheat seed storage proteins largely control the type and quality of any wheat product. Among various unique wheat products, bread is the most consumed product around the world due to its fast availability as compared to other traditional food commodities. The production of highly nutritious and superior quality bread is always a matter of concern because of its increasing industrial demand. Therefore, new and more advanced technologies are currently being applied to improve and enrich the bread, having increased fortified nutrients, gluten-free, highly stable with enhanced shelf-life, and long-lasting. This review focused on bread proteins with improving wheat qualities and nutritional properties using modern technologies. We also describe the recent innovations in processing technologies to improve various quality traits of wheat bread. We also highlight some modern forms of bread that are utilized in different industries for various purposes and future directions.

Keywords: wheat, seed storage proteins, baking, gluten, nutrition

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INTRODUCTION

Cereals have achieved their well-deserved importance all around the globe owing to their good nutritional profile. People misinterpret cereals as starch-rich foods even though they have proteins, vitamins, antioxidants, and some essential fatty acids, too (McKevith, 2004). Wheat, as bread, contributes maximum nutrients to the global population than any other single food source. The end-product quality of wheat is mainly dependent on wheat proteins and their processing techniques involving harvesting of the grain to the production of flour, which further decrease the bioavailability of some of the nutrients (Rustgi et al., 2019). The product quality is determined by the balanced composition of biochemical components in a seed such as seed storage proteins, starch, minerals, fibers, and phenolic compounds (Žilić et al., 2011). In addition, besides interactions between wheat and companion, additives can also have effects on nutritional quality of end-products. There is a continuous increase in demand for improved wheat products by consumers and baking industries (Dewettinck et al., 2008). Wheat proteins are the responsible agents governing the production of bread and other related end-products. Biotechnological tools

are also gaining importance in harnessing cereal proteins for better end-products (Verni et al., 2019; Shewry and Jones, 2020). Breeding *via* cross-hybridization in wheat crop have proved successful for the production of new superior end-use quality products (Kiszonas and Morris, 2018).

Wheat storage proteins are major determinants of wheat flour composed of gluten and non-gluten fractions out of which wheat end-product quality mainly depends on the gluten proteins. Gluten protein mainly provides the elasticity and extensibility of dough, which is unique for wheat, leading to diverse end-products. Gluten protein cysteine residues form disulfide bonds, which are basically the chemical bases for the physical properties of dough (Islam et al., 2019). Gluten was found to be degraded during germination (Michalcová et al., 2021). Worldwide studies are going on to assess various wheat varieties for producing enhanced quality bread (Lama et al., 2018). Various seed storage proteins alleles of wheat have also been explored to dissect their impact on end-product quality of wheat (Goel et al., 2018a,b; Altenbach et al., 2019). Additional work on enhancement of shelf-life was done to enhance its susceptibility to spoilage (Nionelli et al., 2020). Considering the wide acceptability of wheat bread and other related products, in the present review, we shall be discussing about wheat proteins impact on bread making, also other techniques that are revolutionizing the quality of today's bread, and the effect of interactions with other food components on the nutritional enhancement of bread.

BREAD MAKING

Bread is the product of baking of wheat flour mixed with water, salt, yeast, and flavor ingredients. The characteristic of wheat bread as physical attributes of texture, color, and volume are among the most important parameters taken into account by the consumers (Tebben et al., 2018). The mechanical properties of bread are often associated with the perception of freshness and elasticity that influence the consumption decision (Fagundes et al., 2018). The protein that is responsible for dough elasticity and formation of good bread is gluten produced by mixing gliadin and glutenin, which gives dough its elastic character (Peña, 2002). The gases produced during the rising of the dough and the ability of the dough to hold these gases makes a substantial difference in bread quality as illustrated in **Figure 1** (Janssen et al., 2021). The journey of bread making started during Neolithic times; history proves that the mixing of bread with other sources is not a recent tale. In the Second World War, it was called "National Loaf" in which the addition of calcium carbonate was done during that period to counter the expected deficiencies due to shortage of milk and cheese (Hayden et al., 2016).

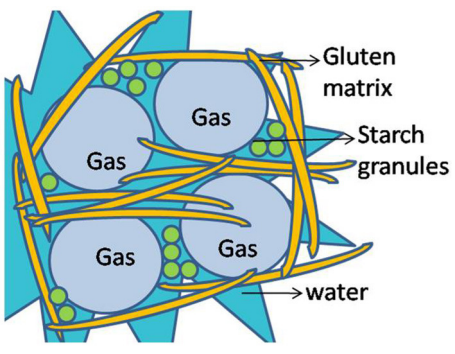

FIGURE 1 | Illustration of gas molecules entrapped in the gluten matrix of wheat dough.

The basic steps involved in bread making, including mixing, rising, kneading, baking, and cooling, have more or less remained constant since long. Mixing is simply a process causing the uniform distribution of ingredients and allows the formation of a gluten protein mesh-like network to give the product of bread (Guerrini et al., 2019). There is an optimum mixing time, which changes, depending on the flour and mixing method used, because too much mixing produces dough with reduced elastic properties. This results in the development of small unrisen and unmixed patches in the bread, giving the loaf a poor appearance from inside (Létang et al., 1999). Next to mixing, fermentation is done, during which dough slowly moves from a rough non-extensible dense mass into a dough with good gas holding and good extensibility properties. Besides this, breakdown of carbohydrates leads to the formation of alcohol and carbon dioxide that gives the bread its natural flavor and causes rising of the dough (Rosell, 2011). Kneading/molding is done to remove gas from a large hole formed during rising of the dough. The dough is then allowed to rise again and is kneaded if required by the particular production process being used. During the final rising (proving), the dough again fills with more bubbles of gas, and once this has proceeded far enough, the dough is transferred to the oven for baking (Cauvain, 2012).

The baking process transforms initial dough into a flavorful product, which is light and readily digestible. The penetration of intense heat increases the volume and size of the tiny gas cells (Ishwarya et al., 2018). At about 60°C, stabilization of the crumb begins, making the starch granules swell; as they get released in the presence of water, the outer wall of the starch granule cell bursts, making the inside starch form a thick gel-like paste that helps to generate the structure of the dough (Kumar and Sharma, 2018). As baking continues, the internal loaf temperature reaches ∼98–100°C. As the moisture is driven off, the crust heats up and eventually reaches the same temperature as the oven. During baking, crust temperature is over 200°C, and the internal temperature of crumb is about 98°C. The loaf is full of saturated steam, which must be evaporated. The whole loaf is cooled to about 35°C before slicing and wrapping can occur without damaging the loaf. In a bakery, special cooling areas are required to ensure efficient cooling before slicing and wrapping of the bread. This completes the process of bread making, which is then consumed by people all over the world (Pateras, 2007).

The rising demand in the food industry emphasized the intervention of research to enhance aspects of improved bread for large production and longer shelf-life. Food additives such as emulsifiers, which belong to a general class of compounds known as surfactants, are used to raise dough strength and as crumb softener in bread quality.

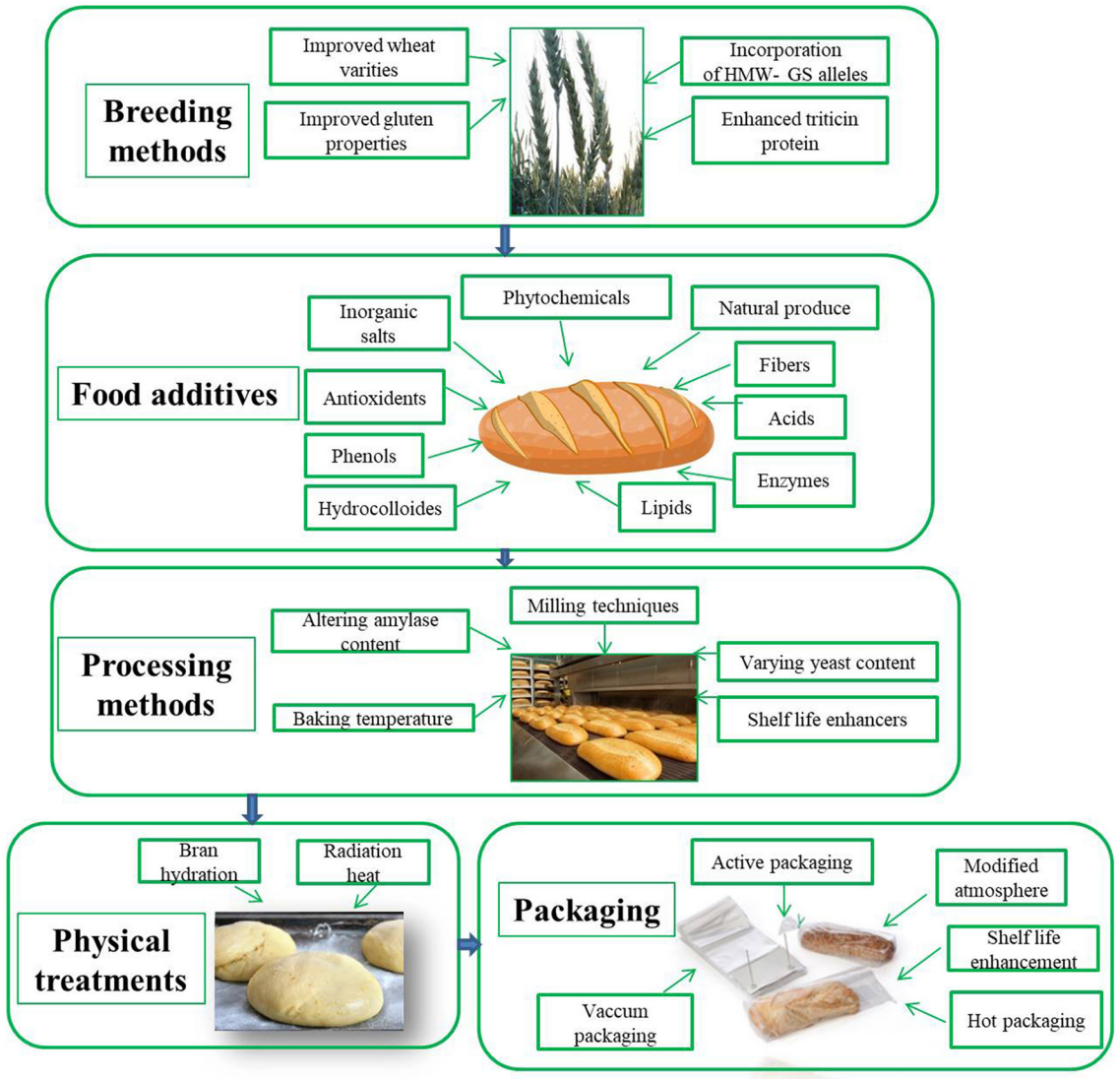

FIGURE 2 | Advancements in present day improving bread quality from breeding methods to processing techniques.

ROLE OF WHEAT PROTEINS IN BREAD MAKING

Bread making could be possible due to the viscoelastic properties of wheat doughs. These properties are a result of the structures and interactions of prolamins (a group of seed storage proteins) as observed in previous studies (Shewry et al., 1999). Seed storage proteins of wheat are comprised of the gluten proteins, comprising two prolamin groups, gliadin and glutenin, considered as the main creator of bread. Glutenin is comprised of polymers with subunits linked by disulfide bonds, which is significantly important for bread making (Shewry and Miflin, 1955). Qualitative or quantitative differences in the composition of seed storage proteins account for much of the variation in bread-making quality as observed in diverse wheat cultivars (Huebner and Wall, 1976; Payne, 1987; Goel et al., 2018a). A range of studies has been explored for the probable impact of wheat seed storage proteins and their role in bread making taking different allelic combinations (Gupta and Shepherd, 1989; Goel et al., 2015). A minute wheat seed storage protein, Triticin, was also thought to improve quality of bread product (Goel et al., 2015, 2018b). Extensive studies are also available on quantitative trait locus (QTL) analysis, depicting the role of the genetic loci on end-product bread quality and nutritional enhancement (Charmet et al., 2001; Li et al., 2009; Goel et al., 2019; Suliman et al., 2021). Owing to the huge genome size of the wheat, researchers focused on the synteny area of related cereal crops to study the responsible factors of wheat proteins further governing end-product quality (Quraishi et al., 2017). Furthermore, the biotechnological tools have been harnessed to dissect wheat

proteins variable actions in improving bread quality (Goel et al., 2017, 2020).

RECENT ADVANCES TO IMPROVE BREAD QUALITY

Production of Gluten-Free Bread

Improvement in the nutritional quality of wheat bread has always been on the priority list of bakers and wheat breeders because of its huge popularity throughout the world. When we look for the additives, one of the best option are legumes, as they are known as rich source of proteins, minerals, and bioactive health-promoting compounds, which may provide texture, structure, and baking quality to the end-products (**Figure 2**). They also have a low glycemic index, and therefore, their inclusion in bread has enhanced the food menu for people allergic to gluten. Furthermore, various laboratory experiments have proved that along with nutritional value, the viscoelastic properties of gluten-free bread can be improved with the addition of legumes like chickpea, soybean, and lupin (Melini et al., 2017). Industries have started adding barley to the wheat dough to enhance the fiber content without disturbing the glycemic index of traditional wheat bread and without negatively affecting its sensory characteristics (Cavallero et al., 2002). Major emphasis has been given these days toward the reformulation of bread and bakery products by altering the gluten content with the addition of functional compounds such as non-cereal flours, prebiotics, and additives (Elleuch et al., 2011). To improve sensory properties, shelf-life, and quality of gluten-free bread, flour from chestnut seeds, amaranth seeds, and psyllium seeds are added to the dough mix. It has been observed that the addition of prebiotics in dough prevents microbial growth and increases the shelf-life of bread (Rahaie et al., 2014). Production of gluten-free bread is an initiative over rising issues of celiac disorders; sorghum and potato starch were considered as potent options earlier for making bread gluten free. Further addition of hydroxypropyl methylcellulose and whey protein concentrate acts as a technological improver in bread dough, and it was stipulated from the observations that both can be efficiently used to obtain gluten-free protein-rich bread (Rustagi et al., 2018).

Improving Texture and Fiber Content

There are different classes of wheat flour that have been gown in different climatic zones around the globe. For example, five classes of wheat are grown in the United States, having their own properties in bread making, like soft white wheat is a special kind that has low moisture content and gives white product such as Asian-style noodles, pastries, and exquisite cakes. Another class is soft red winter wheat, which provides excellent baking and milling properties for making flat breads, cookies, and pretzels (Nebraska Wheat Board, 2020). In certain parts of North America, bread making is practiced by using white wheat flour in which other fibers, germ fractions, phytochemicals, and other important nutrients are generally concentrated. As compared to whole grain bread, white flour products have minimal dietary fibers and non-nutrient phenolics (Xu et al., 2019). On the other hand, hard white wheat is similar to red wheats in its characteristics but has sweet taste and is used in yeast breads, tortillas, and ramen noodles. Hard red winter wheat is mostly used for making pan bread, all-purpose flour, flat breads, and hard rolls, while hard red spring wheat, also called aristocrat of wheat, is used for making pizza crust, bagels, rolls, and hearth breads (Nebraska Wheat Board, 2020).

Therefore, in the direction of quality and texture improvements, several additions have been tried in different proportions such as the addition of course grains, dietary fibers, pectin extracts, and natural coloring and flavoring substances. These fortifications not only improved the nutritional properties of bread but also enhanced its texture and storage. In this light, Angelino et al. investigated the effect of dietary fibers and phenolic compounds on the properties of bread dough and finished bread (Angelino et al., 2017). The phenolic compounds in the form of apple pectin and fruit phenolic extracts showed enhanced antioxidant activity and storability of final bread as compared to the untreated bread (Sivam et al., 2011), although these changes in antioxidant properties entirely depend on the choice of pectin extract (kiwi, apple, and other fruits) (Rupasinghe et al., 2008). Research confirmed that the addition of pectin into bread dough enhances polymeric cross-linking with bread particles of high molecular weight (Sivam et al., 2012).

Increasing Nutritional Value

Providing healthy and safe fresh bakery products and fulfilling expectations of consumers are big challenges before organic farmers, millers, and bakers. The quality of raw organic produce depends upon various factors, *viz*., genotype, crop management, soil fertility, and crop rotation practices, which can be modulated as per market requirements, whereas the nutritional quality, taste, and flavor of bakery products varies with changes in the milling and baking process. Among crop management practices, nitrogen application plays an important role in achieving acceptable yield levels of good bread-making qualities. Canadian researchers have established the fact that the quality of bread can be affected by the cultivation practices of wheat to be used in bread making (Mason et al., 2007). The researchers found that organically cultivated wheat produces more nutritive bread (high in protein value indicating excellent grain quality for yeast leavened bread), whereas conventionally grown wheat results in stronger textured bread (Annett et al., 2007). Wheat bran proteins (WBPC) inclusion was observed in bread formulations and studied to determine the impact on nutritional properties without deleterious effects on quality (Alzuwaid et al., 2021). Manganese application through seed treatments (seed priming) is a cost-effective method for improving the productivity of bread wheat particularly in alkaline calcareous soil (Ullah et al., 2018). Additionally, the novel wheat varieties with pigmented grains (black, purple, and blue) with higher amounts of anthocyanins and other phenolics than the traditional wheat varieties can be effectively utilized to bake bread of some medicinal values as well, which may have preventive properties against cancer and chronic diseases (Sharma et al., 2018). The antimicrobial property was reported to improve with the addition of phenolic compounds in many baking products,

improving health benefits and extending the shelf-life of bread (Xu et al., 2019).

INNOVATIONS IN BREAD PROCESSING APPROACHES

Bread Milling

The damage to flour starch, amylase activity, particle size, and ash content of dough largely affects the baking performance of bread. These qualities are modified with milling techniques that in return modifies baking performance and nutritional properties of bread. The mineral bioavailability in bread can be increased using sourdough techniques (acidification process) in the baking method. The responses of bread quality parameters to milling and baking techniques have allowed identifying positive and negative characters of wheat bread, as baking has a multidisciplinary approach (Abecassis et al., 2008).

Addition of Different Yeast Concentrations

Birch et al. (2014) stipulated that the level and strain of yeast along with temperature and duration of fermentation have a significant effect on the aroma of bread crumb. The strain and amount of yeast added to the flour mix control fermentation activities, and modifications in temperature along with time during the fermentation process may alter oxidation of lipids present in flours. The fortification for improving the quality and protein content of homemade bread had also been tried using nutritional yeast. Such fortified bread boosts the nutritional status of poor people and reduces the incidence of protein deficiency diseases. Variable concentrations of yeast (1–15%) is known to increase protein percent in homemade bread; however, fortification with only lower concentrations (1–3.5%) of yeast is acceptable in the market, as higher quantities of yeast alter the taste of bread to an unacceptable level by its consumers (Harusekwi et al., 2014). The impact of addition of organic acids in improving bread quality was studied in China. They analyzed the yeast activity, proteolysis, and amylolysis by adding acetic acid, malic acid, fumaric acid, lactic acid, and citric acid to bread dough ingredients. The organic acids increased specific volume of the bread, whereas they lowered moisture content, pH value, and hardness of bread, which resulted from high yeast activity (Su et al., 2019). Since organic acids improve bread quality, the effects of addition of lactic acid bacteria in dough mix on bread texture and quality have been studied by food technologists. Out of many strains of *Lactobacillus plantarum*, LAB strains (LB-1, F-3, and F-50) exhibited antifungal activity and found useful for making bread to extend shelf-life. In fact, among these three strains, LB-1 significantly improved water holding capacity, viscosity, elasticity, and extensibility of sourdough (Sun et al., 2020).

The baking interventions include sourdough, which is prepared through natural fermentation using lactobacilli and yeast. The lactic acid produced by the action of lactobacilli adds taste and good keeping qualities to sourdough. It reproduces nutritionally superior, fiber-rich, gluten-free bread with improved mineral bioavailability, making it unsuitable for celiac persons. Poutanen et al. (2009) stated that the inclusion of natural yeast and bacteria in bread dough results in the solubilization of proteins and polysaccharides present in cell wall, which change the texture of baked bread and absorption of nutritional and non-nutritional compounds. The process of natural fermentation may also lead to the synthesis of novel bioactive compounds and metabolites such as prebiotic oligosaccharides.

Loaf Volume and Sensory Qualities

The researchers working on the qualities of loaf stated that the consumers are attracted to higher loaf volume and weight, which is a positive economic character for retail marketing. The buyers often believe that bread with higher loaf volumes offers more substance for similar prices. Shittu et al. (2007) explained that the varying rates of gaseous output and starch gelatinization capacity are responsible for variable loaf volumes, which result from differences in time and temperature of baking. Industries keep these factors in mind to attract the consumers by making little changes in the bread-making process. The crumb moisture content and loaf volume of bread are significantly affected by baking temperatures (Shittu et al., 2007), while dried crumb hardness, bread loaf weight, and density levels can be altered with differential baking durations at variable temperatures. Ghorbani et al. (2019) observed that bread baked at 320°C for 3 min were liked more by the judges of the sensory panel, taking their texture and chewiness, whereas the samples baked at 370°C for 2.5 min did not score well in comparison to other evaluated samples. The research shows that baking at higher temperatures results in hard bread with reduced consumer acceptability. Thus, even a slight degree of change in temperature and time has substantial effect on the overall quality and acceptance of bread.

Reducing Microbial Activity

The shelf-life of bread is a big constraint for the whole bread industry, as the bread is prone to mold contamination due to its moisture content. Within a matter of days, the microbial contamination spoils the product. There have been various studies done focusing on extending the shelf-life, and it was found that replacement or reduction in NaCl in bread making could affect the growth of *Penicillium roqueforti* and *Aspergillus niger*. The salt added to the bread mix not only improved the flavor but also increased the water activity (a_w), making the bread more susceptible to mold infections. The infection can be prevented by reducing the amount of NaCl or simply replacing NaCl at least partially with other acceptable salts such as calcium chloride, potassium chloride, magnesium chloride, and magnesium sulfate. The growth of *P. roqueforti* and *A. niger* was found to be reduced in the bread dough with 30% less NaCl or with suitable replacers. Mixing wheat flour with other flours (cassava, soybean, etc.) was tried by many bakers to improve the quality of existing bread (Samapundo et al., 2010).

Improving Bread Quality *via* Value Additions

Non-cereal Items

The addition of non-cereal flours in bread dough is very popular these days. It not only improves the texture of bread but also increases mineral nutrition. According to recent studies,

cassava flour (unfermented) is added to bread making due to its high nutritional values. Various combinations of cassava flour with wheat flour have been tried to develop a wide range of food products, *viz.*, pies, rolls, cakes, biscuits, doughnuts, and breads. Due to low setback viscosity and high peak viscosity, yellow cassava flour is considered good for bread dough, as it imparts low tendency to undergo retro gradation, making it suitable for products that require high elasticity and gel strength (Ayeh, 2013). In addition to the improvement in the quality of bread, the addition of cassava flour reduces the time for dough development as compared to all wheat flour dough. Later, Pasqualone et al. (2010) also reported that cassava-enriched bread is suitable for celiac patients, as it is gluten-free, nutritious, and palatable. The desirable loaf volume and crumb firmness of cassava bread can be achieved by using olive oil (extra virgin) and egg white, even if the hydrocolloids and industrial improvers are not added during dough preparation. The Indian bakeries transformed wheat into an Indian bread, known as *chapatti*. On an average, *chapatti* is consumed in every home on a daily basis in India including consumers from weaker economic sections. The recent studies suggested incorporation of 20–50% amaranth seed flour to wheat bread mixture to improve rolling properties, protein content, and mineral availability in the final bread (Mutahi, 2012). The addition of amaranth also increases stickiness, softness, rollability, and elasticity of dough (Banerji et al., 2019). Other non-cereal grains have also been tried to develop multigrain bread, such as buckwheat and quinoa (Gawlik-Dziki et al., 2009), for enhanced protein content, energy, mineral, phytate, and condensed tannin contents; however, when the percentage of wheat was decreased below 70%, the bread quality was found inferior as compared to regular bread (Ayele et al., 2017). The cereal legumes (chickpea, lupin, and soya) are a rich sources of digestible proteins and blend well with wheat dough or other cereals (oat, barley, and rye) for baking purposes. The combination of legume cereals and oats/barley/rye mixed in equal proportions was also found to improve sensory properties and texture of multigrain bread. However, an adverse effect of mixed flours on the technological properties of bread dough was observed that could be corrected by using industrial additives such as ascorbic acid, fungal alpha-amylase, glucose oxidase, xylanase, and vital gluten, alone or in combination (Yaver and Bilgiçli, 2019). Dairy products were also assessed for impact on bread quality (Graça et al., 2019).

Non-plant Ingredients

Monteiro et al. (2018) mixed tilapia-waste flour to bread dough in different proportions (0–20%) and observed that the amount of carbohydrates and total dietary fibers in bread increased with increased levels of tilapia-waste flour, whereas, in sensory evaluation, tilapia-waste flour bread scored low as compared to the traditional bread due to its disagreeable texture, flavor, and aroma. Despite that, the overall acceptance for mixed bread was unaffected as the stickiness in teeth, loaf volume, and cream color of bread did not vary significantly from wheat-based breads. Calcium is another main component that should be adequate in the diet especially for women and children for the health of bones. Recently, to increase the level of calcium in bread, new materials in trend has been used in powder form like skim milk (10%), oyster shell (2%), and eggshell (2%). This also increases set back viscosity, dough stability, percentage of water absorption, the heat of transition, and mixing time. The bread fortified with oyster shell powder showed higher amounts of fiber and ash contents. This bread is also rich in carbohydrates and proteins. It is evident from the latest results that technological properties and nutritional values in bread could be positively increased with the addition of calcium from natural resources. However, the bread fortified with eggshell and oyster shell scored badly in terms of aroma and general acceptability as compared with the bread supplemented with milk powder (Alsuhaibani, 2018). In further continuation with the addition of uncommon substances, seaweed extracts were also tried for improving quality bread for baking purposes. In Indonesia, brown seaweed from coastal areas of Yogyakarta was used to extract alginate, which was proved to be a non-toxic compound with hypo-cholesterolemic effects. Its addition in wheat bread mixture tends to improve proximate values of bread making and is useful for daily consumption (Supartini and Mushollaeni, 2017). However, higher cholesterol levels in alginate added to bread mixture can lead to adverse consequences, which may result in cardiovascular diseases as well. The fortification of wheat bread with plant-based or uncommon additives certainly enhanced the nutritional value, qualities, and texture of the final product. Conte et al. evaluated the effect of bee pollen addition to the flour used for gluten-free bread. They observed that such flour has a higher percentage of total proteins, carotenoids, and minerals, and showed anti-free radical activity (Conte et al., 2020).

Enhanced Aroma Properties

The aroma is among the first few parameters that a consumer is inclined to for bread quality. To date, more than 150 volatile compounds have been characterized in bread loaf, which evolves because of fermentation activities of yeast. Among these, many volatile compounds contribute to the aroma of bread crumb, which is sensed by consumers while eating (Pico et al., 2016). These compounds impart a characteristic odor and flavor to the final baking product. Sensory perception has a major play in the choice of bread by the consumers. The addition of legume flour in gluten-free bread can improve its nutritional value but could harm its sensory properties. Sourdough is often used to improve the sensory properties of bread. Moreover, the addition of sugars and amino acids as precursors of aroma compounds or enzymes that produce them can positively affect bread aroma. In a study, it was established that the addition of pea flour in combination with improvers (fructose, proline, arginine, and protease) helps to enhance sensory properties of bread. The relative amount of pleasant volatiles (key aroma compound 2-acetyl-1-pyrroline) has been found to increase with the addition of quinoa flour (15%) and teff flour (5%) along with wheat and corn starch (40% each). The combination also resulted in lower levels in rancid volatile compounds that originate because of fatty acid oxidation (Pico et al., 2019).

Dough Strength

The starch, which is composed of amylopectin and amylose, is abundantly present in wheat flour and maintains bread stability. However, there are varieties of wheat that are deficient in endosperm amylose. Such wheat is known as "waxy wheat," which can be utilized in the baking industry to alter amylose levels in wheat-based bakery products. The more waxy wheat is added to the dough mix, the lesser is the amylose content in it, and the better is the quality of bread. The quality of Chinese steamed bread was found to be improved with the addition of waxy flour into the bread mixture, although the addition did not improve the bread quality because the firmness of bread was decreased during storage. In experimental trials, flours of waxy wheat and Canadian spring wheat were mixed in varying amounts (0–20%), where 15% addition of waxy flour improved bread stability without affecting the quality (Rustagi et al., 2018). Wheat varieties with different allelic combinations of seed storage proteins were found to be responsible for a better bread loaf and used for production of better end-product variants of wheat bread (Goel et al., 2015, 2017).

Increasing Shelf-Life

The fatty acid salts, when used as surface acting agents and food additives, show antibacterial activity. The mold-proofing activity and improved baking property with fatty acid salts have been studied by many food scientists (Hamaishi et al., 2018). The results have proven that the addition of >5% potassium myristate to dough inhibited fungal growth on bread during storage. The length of the carbon chain of fatty acids contributed to the antifungal activity and antimicrobial effects of fatty acids; it was observed that the activity reduces with an increase in the chain length; also, medium-chain fatty acids showed stronger antimicrobial activity than longer chain fatty acids (Pareyt et al., 2011). Efforts have also been made in the direction of increasing the shelf-life of bread, which is largely affected by molds. As the bread is packed and distributed to several destinations in the world, technological interventions are needed to minimize mold infection bread. Liu et al. (2011) studied the impact of radiofrequency energy in addition to the usual hot air treatment for the control of mold in bread packaging. It was found that the radiofrequency treatments decrease moisture content and water activities in bread, which ultimately reduces formation of *Penicillium citrinum* spores. This method also enhances the storage time by 28 ± 2 days for treated white bread. Some researchers also studied the effect of incorporation of non-plant-based material into bread dough on the final product. Moreover, the addition of marine food products (Kadam and Prabhasankar, 2010), plant extracts such as green tea (Wang and Zhou, 2004), natural antioxidants (Lim et al., 2011), grape seed extracts (Peng et al., 2010), and prebiotics (Korus et al., 2006) to bakery products have been widely proposed to enhance quality and functionality of the bread. Currently, studies are revolutionizing the baking industries and serving in the development of more novel products that are low in calories and cholesterol and suitable for people with celiac disease. There are a lot more opportunities to be tested for ensuring food and nutritional security for the ever-growing population in the world.

MODERN FORMS OF BREAD

Bee Bread

Bee bread is a specialized fermented product comprised of combination of pollens, bee saliva, and nectar that bees pack in the honeycomb to ferment them with the help of many kinds of yeasts and bacteria (Khalifa et al., 2020). It is very important and considered as a key protein source for bee adults and larvae. Apart from this, bee bread is an excellent source of energy and nutrition for humans due to the higher protein concentration of pollens. The biochemical components of bee bread include vitamins, fatty acids, proteins, enzymes, hormones, antioxidants, carbohydrates, and minerals (Kieliszek et al., 2018). Nowadays, bee bread is very popular in the commercial market due to its high nutritional properties. This bread has high antioxidant activity and phenolic content that contribute to its biological and nutritional properties that can be used as beneficial food supplements (Mutsaers et al., 2005). The bee bread is a product with a long history used mainly in folk medicine due to its therapeutic properties. For example, in recent years, numerous studies have been carried out to study the effectiveness of bee bread to treat different illnesses. Bee bread has been exhibited anti-inflammatory, anticancer, antiradical, and antimicrobial activities (Khalifa et al., 2020).

Steamed Bread

Steaming instead of baking is done in some areas for preparation of bread, which is actually a staple food in China. Bread is consumed after steaming in many countries of the East and Southeast Asian regions (Peng and Cheng, 2007). The People's Republic of China grows large quantities of wheat and is a major wheat importer. The wheats that it produce are both hard red (winter and spring) and soft wheat, which are commonly blended to produce basic flours. Hard red spring wheat is used in northern China to produce steamed breads, which are distinctly different in texture from breads produced in southern China from lower protein hard and soft red winter wheat flours (Rubenthaler et al., 1990).

Multigrain Bread

Multigrain bread is made by mixing wheat flour with flours of some legumes, cereals like oats, and some seeds like flaxseeds and sesame seeds. This bread is more nutritious and flavorful than the normal bread. The study conducted found a positive effect of this multigrain on the dough properties and the quality of bread. Multigrain bread with a 15% multigrain mix proved to be effective in increasing protein, fat, and dietary fiber contents of bread (Indrani et al., 2010). There are enough products in the market that can be claimed as gluten-free and can be safely digested by patients affected by celiac disease. Sourdough is a type of foremost fermentation that is commercially used for baking purposes of gluten-free bread. It has also been proven to be ideal for improving the texture, aroma, palatability, shelf-life, and nutritional enhancement in the case of wheat and rye bread. The concept of sourdough in gluten-free baking industry is a new zone of the experimental area to improve the quality

and acceptability of gluten-free bread (Moroni et al., 2009). In addition, the health risk to various celiac diseases has emphasized the focus on gluten-free bread prepared by mixing chestnut, bean flour, and chickpea, with rice flour at different ratios using straight dough bread-making process (Yildrim and Nadeem, 2019). There are challenges even today for optimal formulation when we deal with texture, flavor, and nutrition (Wang et al., 2017). Rye bread is again a variant of ancient bread using rye as a component. A study conducted on rats found that the addition of green tea to rye might help in preventing obesity in rats (Bajerska et al., 2013). High-fiber rye bread was also experimented in menopausal-stage women for insulin secretion and appears to enhance insulin secretion by improving b-cell function (Juntunen et al., 2003). The addition of saffron powder in rye bread showed antidiabetic properties (Bajerska et al., 2013).

CONCLUSION AND FUTURE PERSPECTIVES

After rigorous efforts and interventions by researchers and global food industries, we still have a significant proportion of the chronically undernourished populations in developing countries. Surprisingly, even today, around 80% of the world's growing population is devoid of basic balance diet. The research in food sciences should be directed to focus on the quality of food in addition to the quantity of food that is available to humankind. The application of scientific approaches for the improvement of the baking industry provides the potential solution for resolving the challenges of global food and nutritional security. The fortification of bread dough with more nutritive grains and supplements enhances the quality and digestibility of bread. The recent advancement in bread-making process, namely, addition of enzymes, flours of non-plant origin, antimicrobial supplements, improved yeast strains, tools used in baking, and enhancement of dough rheological properties, have helped to bridge the gap between the nutritional demands and fulfillment to some extent. However, the face of ancient bread changed positively with recent research; there are endless possibilities to explore further. Exploration of novel genotypes with varying wheat proteins suitable for bread making, identification of more stable yeast strains, shelf-life enhancement, attractive color, fiber and flavor enhancement in bread, and development of celiac patient-friendly products are the issues that should be considered in future research. It will undoubtedly give rise to new avenues for food and nutrition research, and such advances will allow the development of better end-products from wheat, which can be utilized to reduce global hunger.

AUTHOR CONTRIBUTIONS

SGr, MS, and SGo: literature survey. SGo, AR, and SW: first draft. SGr, MS, and SW: review and editing. All authors finalized the manuscript before submission.

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REVIEW
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Sustaining Protein Nutrition Through Plant-Based Foods

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Proteins are essential components of the human diet. Dietary proteins could be derived from animals and plants. Animal protein, although higher in demand, is generally considered less environmentally sustainable. Therefore, a gradual transition from animal- to plant-based protein food may be desirable to maintain environmental stability, ethical reasons, food affordability, greater food safety, fulfilling higher consumer demand, and combating of protein-energy malnutrition. Due to these reasons, plant-based proteins are steadily gaining popularity, and this upward trend is expected to continue for the next few decades. Plant proteins are a good source of many essential amino acids, vital macronutrients, and are sufficient to achieve complete protein nutrition. The main goal of this review is to provide an overview of plant-based protein that helps sustain a better life for humans and the nutritional quality of plant proteins. Therefore, the present review comprehensively explores the nutritional quality of the plant proteins, their cost-effective extraction and processing technologies, impacts on nutrition, different food wastes as an alternative source of plant protein, and their environmental impact. Furthermore, it focuses on the emerging technologies for improving plant proteins' bioavailability, digestibility, and organoleptic properties, and highlights the aforementioned technological challenges for future research work.

Keywords: proteins, plants, nutrition, extraction, sustainability

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INTRODUCTION

Since the beginning of life, plants have been utilized for human benefits, providing food, therapeutics, wood, fibers, and many others. Moreover, plants were considered the bioproduction system for valuable substances and provide many primary and secondary metabolites having therapeutic effects. Primary metabolites (protein, carbohydrates, fats, and nucleic acid) are the building blocks of life. Besides these, the secondary metabolites are produced by plants to protect them from predators and pathogens, cope with environmental stress, attract pollinators, and work as their defense system (1). Proteins are molecules with great complexity and diversity that play an important role in maintaining the structure and function of the living form (2). Therefore, it is being used for many applications such as medicine, food, and feed.

By 2050, the world's total population is expected to grow or might exceed 9 billion, and, hence, the demand for food, feed, and fiber around the globe is expected to increase by 70% (3). To meet this increasing demand, new sources must be explored. Nowadays, food derived from plants plays a vital role in the human diet as an important source of bioactive components, such as vitamins, phenolic compounds, or bioactive peptides. Hence, these components benefit human health and

protect against various disease conditions (4). For meeting protein requirements, generally, animals are considered perfect. However, due to many diseases in animals, their consumption is not safer for human health. Also, it replaces animal-based proteins with plant-based proteins due to various limitations, such as increased cost, limited supply of nutrients, hazard for human health, freshwater depletion, and susceptibility to climate change (5–7). Plant-based proteins are considered vegan food, provide an ample number of amino acids, are directly absorbed by the body, and help in treating various disease ailments. Moreover, the proteins derived from plant-based foods are rich in fiber, polyunsaturated fatty acids, oligosaccharides, and carbohydrates. Hence, they are mainly associated with a reduction in cardiovascular diseases, low-density lipoprotein (LDL) cholesterol, obesity, and type II diabetes mellitus (8). Different sources of plant-based protein that include cereals (wheat, rice, millet, maize, barley, and sorghum), legumes (pea, soybean, bean, faba bean, lupin, chickpea, and cowpea), pseudocereals (buckwheat, quinoa, and amaranth), nuts, almonds, and seeds (flaxseed, chia, pumpkin, sesame, and sunflower) were well-explored (5, 9–11) (**Figure 1**). However, the demand for the supply of protein is continuously increasing with the rise of the global population (12), hence the need to search for new sources.

It is hard and expensive to extract an adequate amount of animal proteins; therefore, an alternative for improving the nutritional status of humans is mainly received from plant proteins. Hence, attention has been paid to evaluating the nutritional quality of proteins from different plant species. The best way to increase the supply of proteins is to improve the protein expression and efficiency of protein production in natural resources. The advancement of recombinant technologies of protein production, such as engineering of expression hosts, upstream cultivation optimization (e.g., nutritional, bioreactor design, and physical parameters), and development of methods of protein extraction, as well as purification, supported the growth of the market (13). Also, improving the protein functionality in foods through modification, enhancing the plant proteins proportion in human diets, and improving the bioavailability and digestibility of food proteins in the digestion process (14, 15) could be helpful to increase the overall utilization of plant-based protein.

Along with providing amino acids in food, proteins play a significant role in food formulations due to their diverse properties, such as emulsification, gelling, thickening ability, water holding, foaming, and fat absorption capacity (16, 17). Therefore, several thermal techniques (such as cooking, autoclaving, microwave heating, irradiation, germination, fermentation, extrusion, and drying) used during food processing could be optimized to improve the quality of plant proteins (2). Also, they can be isolated from sustainable and cheap sources such as plant-derived wastes from agriculture and by-products of crop and oil industries, which can also regulate food waste reduction (2, 7, 18).

To provide an overview of plant-based protein that helps sustain a better life for humans and the nutritional quality of plant proteins, this review mainly focuses on the current state of using plants to produce proteins for human health. It mainly focuses on various sources and their alternatives with high-quality protein, factors affecting the nutritional value of plant-based protein, bioactivity and functionality, and its modifications. Also, the information on the nutritional quality of proteins derived from plants and potential health issues linked with plant protein will be elaborated. Finally, the issues and challenges of plant-based proteins from availability, consumption, processing, and functionality will be elaborated, and recommendations were made for sustainable production and better utilization of plant-based proteins for meeting human health requirements.

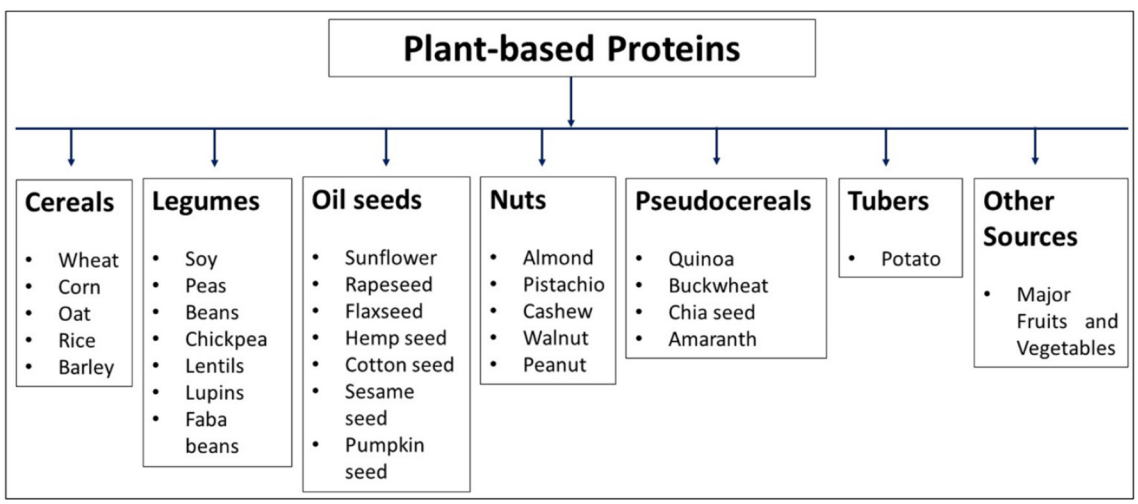

FIGURE 1 | Plant-based proteins derived from different crops.

PLANT-BASED SOURCES AND DEMAND OF DIETARY PROTEIN

Plant-Based Protein Sources

Among all the existing sources of dietary proteins, plant-based sources dominate the supply of proteins (57%), with the remaining 43% consisting of dairy products (10%), shellfish and fish (6%), meat (18%), and other products from animals (9%) (19).

To provide dietary protein supply and overcome the challenges of feeding the population, several sources of proteins from plants have been searched recently (10, 20–22). Based on sources, proteins from plant origin might lack some essential amino acids. For instance, cereals generally contain less lysine, whereas legumes are deficient in sulfur-containing amino acids like cysteine and methionine (23). However, a good amount of lysine is present in pseudocereals (e.g., quinoa and amaranth). Sometimes, the same plants have different nutrients due to differences in soil diversity, climatic conditions, precipitation levels, geographic latitude and altitude, agricultural practices, and different varieties/cultivars (24, 25). Some traditional plants have been utilized by human beings as protein sources, including beans, pea, and soybean. Also, new sources (such as proteins from insects and algae) (14) and unconventional and alternative protein sources (like agro-industry by-products from the extraction of edible oil and those discarded by fruit processing) have been discovered (7). In addition, different meat, milk, and egg analogs from plant-based protein sources have also been identified (**Figure 1**) (26).

Legumes

A diet rich in legumes provides various health beneficial effects for humans (26). Legumes are considered the best dietary options due to their abundant carbohydrates, protein, energy, vitamins, minerals, and fibers. Various commonly known legume crops for protein and other nutritional sources include soybean, common beans, peas, and chickpea. The protein obtained from soybean has been widely studied (22). Common beans are considered the primary source of vegetable protein in developing countries (27). Highly nutritious legumes such as peas can be utilized for different food product formulations to improve the human intake of protein. Food products from chickpea are the major dietary protein source of high-quality protein (22). The protein isolates and defatted flour from lupin fulfill the requirements of essential amino acids (28). Moreover, pigeon pea and its derived isolates of protein are the potential sources rich in sulfur-containing amino acids suitable for the consumption of human beings (29).

Cereals

Cereal consumption, such as wheat, rice, barley, and corn, are the most common staple food throughout the world (30). Globally, in developed and developing countries, rice is one of the most widely consumed cereal crops. Amagliani et al. (30) analyzed the amino acid composition of proteins present in the rice and found that lysine content is highest in albumin, while sulfur-containing amino acids are majorly present in the globulin. Some studies have also been conducted to improve rice protein's extracted yield by using different isolation techniques (31). In one of the studies, it has also been found that lysine is present in significantly less rice protein isolates (32). Mainly consumed in developing countries, millet, and its concentrates of protein are a mostly nutritious source of proteins. It usually contains a high amount of essential amino acids, including lysine. Nutritional profiles of cereal-based proteins have also been extensively used in industrial applications and bakery products. In a study, faba bean flour, and wheat flour bread products showed an increased amount of essential amino acids after fermentation. The mixture of legumes and cereal helps improve the overall nutritional quality (33).

Pseudocereals

Pseudocereals like amaranth, buckwheat, and quinoa are mainly the dicotyledonous plants that are considered false cereals (34). Recently, more interest has been paid to utilize pseudocereals protein, like amaranth and quinoa, to fulfill the high demand for proteins. These sources mainly contain high-quality protein, unsaturated fatty acids, fibers, vitamins, and minerals. They also have a high quality of essential amino acids and increased bioavailability of proteins. Along with these qualities, they are also gluten free, being an alternative in the diet of patients with celiac disease (35). One of the studies also showed that amaranth and quinoa contain a high quantity of lysine, useful as dietary supplements (35).

Seeds

The consumption of plant-derived food components increases continuously, and seeds are an important source that provides good quality of nutrition (30). Flaxseed, one of the richest sources of high-quality protein, also contains phenolic compounds, fibers, and essential amino acids; however, some studies argued that lysine is limiting in flaxseed (36). In their study, Lugo et al. (37) observed that the composition of essential amino acids in chia lacks lysine, whereas the watermelon seeds were found to contain a good amount of leucine and arginine (38). One of the studies has also been identified that the flour of paprika seed mainly contains aromatic amino acids like threonine, lysine, and tryptophan but poor in sulfur-containing amino acids and isoleucine (39).

Almond and Nuts

Almonds and nuts are generally known for their high-quality lipid and fatty acids content and also contain high-quality protein content. The species known as pequi and baru from Brazilian Savanna are non-traditional almonds that are good protein sources and have a complete profile of amino acids (40). Baru almond contains all essential amino acids, whereas pequi almonds are rich in sulfur amino acids and lack lysine, similar to cashew nut (*Anacardiumothonianum*). Peanuts are limited in valine and lysine and are considered as the inferior source of protein.

Meat Analogs From Plant Proteins

Currently, commercial plant-based meat analogs revolutionize the modern food industry. In the US, the market price of plant-based products was ∼$940 million in 2019, which

will increase by 38% in recent years (41). Currently, the food industry helps produce high-quality plant-based meat analogs, such as sausages, burgers, ground meat, and nuggets. However, it is more challenging to make the products that match the properties of whole muscle tissues like connective tissue, muscle fibers, and adipose tissue that form hierarchical structures (42). The arrangement of tissue structure plays a significant role in determining meat products' sensory and physicochemical properties. Plant-based whole muscle products of high quality first require the most suitable ingredients and processing techniques to stimulate muscle fiber, adipose, and connective tissue.

Many reviews have been published on meat analogs from plant proteins (41–44). Ideally, meat analogs should provide adequate structural similarity besides nutrient composition. Meat analogs are mainly produced from plants' macronutrients, including polysaccharides, proteins, and fats, and some micronutrients and other ingredients, such as minerals, vitamins, flavoring, and color agent preservatives, and binders. The components and processing techniques utilized to produce these analogs should be optimized for each meat product. The appearance of the meat analogs' surface should be of opaque texture like real meat. Food industries have used several techniques to maintain the color of plant-based meat alternatives. For instance, Meat™ uses beet juice extract that contains a natural pigment called betalain to recreate the suitable color of meat. Also, Impossible Foods™ uses leg hemoglobin (plant-based heme protein) extracted from soybeans roots to color its products. Various technological and scientific methods, like processing and physicochemical approaches, are being searched to create potential structures of plant-based meat that aim to accurately mimic the texture of real meat. It should also be noted that meat analogs usually simulate the fluid-holding capacity like real meat during cooking. Knowledge about the essential constituents of flavor present in products of real meat is helpful to identify plant-based ingredients that give the meaty flavors in plant-based meat analogs. However, developing plant-based meat analogs is challenging and providing a similar nutritional profile to real meat.

Milk Analogs From Plant Proteins

One of the most consumed food products from plant origin is plant-based milk analogs. Various attributes, such as processing methods, sensory quality, raw materials, physicochemical properties, and nutritional profiles of plant-based milk analogs, have been presented and described in many articles (45, 46). Milk analogs are colloidal dispersions consisting of several particles, such as fat droplets, oil bodies, plant tissue fragments, protein aggregates, and insoluble calcium carbonate particles dispersed in an aqueous solution containing soluble proteins, sugars, salts, and polysaccharides (46). For the formation of high-quality milk analogs, there should be correct information on light scattering theory, techniques of particle reduction, as well as mechanisms of particle instability. Two approaches have been used for producing milk analogs, such as disruption of plant tissue (including soaking, mechanical disruption, enzymatic hydrolysis, separation, formulation, homogenization, and thermal treatment for breaking of plants materials into small particles) and homogenization (including blending of plant-based components that are isolated, such as emulsifiers, oils, and thickeners) (46). The components and processing techniques are generally optimized for creating milk analogs that mimic cow's milk's functional and desirable properties (46). For developing a better quality product, the plant-based milk analogs have been extensively analyzed for features, such as appearance, flavor, color, bio-availability, and nutrition profile.

Egg Analogs From Plant Proteins

The hen's egg consists of 75% water, 12% proteins, and 12% lipids. Also, it includes a variety of constituents that help in different food applications like foaming, emulsification, and gelation (47). Eggs are mainly used in various ways, such as boiled, fried, poached, or scrambled, and part of many other foods, including dressings, mayonnaise, desserts, and baked goods. Generally, plant-based egg analogs should have desirable functional and physicochemical properties. For example, eggs analogs should have the functional ability to transform a solution into a gel when heat is supplied, just like that of real eggs. Plant proteins used in egg analogs have solution temperature in the range of 63–93°C, which shows that higher temperature is needed to mimic the structure and texture of real eggs. Various methods, such as dynamic shear rheometry and differential scanning calorimetry, have been utilized, which provide information on gelation temperatures and denaturation of proteins. The gel nature of plant-based egg analogs depends on the type of protein (e.g., chickpea, pea, sunflower, bean, and soybean), the concentration of protein, and environmental conditions (e.g., pH, ionic strength, and thermal history). Plant-based egg analogs should have the best emulsifying solubility, segregation, separation, and stabilization properties. Like real eggs, they also have a better appearance, flavor, color, bioavailability, and a nutrition profile to produce a better quality of plant-based milk analogs.

Food Waste/By-Products as a Protein Source

Increasing population and industrialization also negatively affected the environmental conditions. Eco-innovation is the term that addresses the essential changes for sustainable development. It is an approach where by-products and waste from plants become an important resource. Food waste/by-products have also been utilized for the extraction of proteins. These mainly include oil meals/press cakes, by-products of cereals, and legume processing.

Oil Meals/Press Cakes

During oil processing, the by-products, such as oil meals/press cakes, have been released from oil-bearing fruits and seeds (48). Oil meals contain 15–50% of protein content and are, hence, considered valuable sources for the extraction of proteins (48). Soybean, cottonseed, peanut, sunflower seed, sesame seed, pumpkin seed, hazelnut, grape seed, walnut, hemp seed, and rapeseed are the major oilseed crops containing a high proportion of protein meal. Also, oil-bearing crops, such as coconut, palm, and olive, have oil in their fruit pulp, and their

residues are useful to isolate proteins. The protein content varies depending on the processing of hulled and dehulled meals of oilseed. Usually, the dehulled meals have higher protein content and lower fiber content, while dehulled meals require an additional fractionation step before they have been used for protein extraction.

By-Products of Cereal and Legume Processing

By-products after cereal and legume processing are important raw materials for the extraction and isolation of proteins. The high content of protein in legumes makes them most important, followed by cereals. Rice bran is the most important protein source among cereals. Along with the rice, several other crops by-products have been used as promising protein sources, such as wheat bran, broken rice, brewer's spent grains, and defatted wheat germ. Commercial milling of pulses also produces ∼25% of by-products consisting of powder, husk, broken, shriveled, and unprocessed seeds. With high nutritional value and a well-balanced profile of amino acids and also various bioactivities, the cereal crops and their by-products are of major attention. Hence, these are considered as appropriate materials for protein extraction due to their quantities, availability, and composition of amino acids (30).

Demand of Dietary Protein

Proteins are molecules with great complexity and diversity that have played an important role in maintaining the structure and function of living cells (29, 49). It is being applied in a number of applications, such as medicine, nutraceuticals, industries, food, feed, etc., and the demand for protein is continuously increasing with the rise of the global population (12). Globally, protein requirements are fulfilled by both plants (80%) (such as cereal grains, beans, soy, pulses, nuts, vegetables, and fruits) and animals (∼20%) (such as meats, milk, eggs, fish, yogurt, and cheese) (50). Along with the increasing nutritious food demand, the protein demand is continuously increasing globally by changing socioeconomic status. Increased urbanization, as well as economic development, has led to various transitions in dietary patterns in the population of low- and middle-income countries, especially the demand for foods derived from animals, which is noticed in developing countries (51). Protein from animal origin causes emissions of greenhouse gases from livestock as well as loss of terrestrial biodiversity by human interventions (52). Therefore, plant-based protein requirements are continuously increasing.

Plant-based proteins play a major role in the human diet as they are rich in a large number of other nutrients, vitamins, and minerals (53). Foods obtained from plants enhance the content of protein that contains various essential amino acids and may also improve the nutritional status of human diets. From the last few decades, interest has been drawn for the search of protein sources with high nutritional quality and functionality and industrial applications (like emulsification, solubility, gelation, foaming, viscosity, oilholding, and water-holding capacities). Furthermore, the development and utilization of novel techniques of food processing enhance the nutritional quality of traditional sources of plant protein. According to the overall status of health, human nutrition is considered an important issue that provides the methods for prevention or development of a number of diseases resulting from excessive, unbalanced, or insufficient nutrient intake (15). Generally, the daily intake of protein is provided by animal-based foods. However, changes in the consumers' requirement led to adoption of alternative sources of proteins for human consumption. And, also, the protein produced from animal sources is costly and environmentally non-sustainable and requires more water (about 100 times) during production than plant protein. Emerging factors in animal proteins, like the growth of world population, climate change, and occurrence of animal diseases, more research is now dedicated to finding various new sources and technologies to produce proteins from plants with high content and resilient to changing climate and thus provide balanced nutrition in humans' diet (51).

The proteins and their amino acid composition play a major role in human health. For instance, sulfur-containing amino acids, such as methionine and cysteine, play a vital role in maintaining the immune system functioning (54) and also the peroxidative protection mechanism in muscles, nervous, and cardiovascular systems (55). Lysine is important for bones calcification, liver activities, nitrogen balance inside the body, and muscle and blood synthesis. While valine helps in the coordination of motor cells, and aspartate and glutamate are essential for hormonal regulation and immunological stimulation, respectively (35). Leucine and isoleucine are assisting as building blocks of other proteins (36). Generally, it has been recommended that, for adults, the protein intake should be in-between 0.8 and 1 g/Kg body-weight/day (56, 57). Pregnant, lactating women, and infants need higher protein ingestion than adults as 1.1, 1.3, and 1.2–1.52 g/kg/day, respectively (57). The intake requirement of proteins and amino acids is determined by various factors linked with genotypic as well as phenotypic characters (age, gender, body weight, lifestyle habits, physical exercises, health conditions, and metabolic capacities) (5).

FACTORS AFFECTING THE NUTRITIONAL VALUE OF PLANT PROTEINS

The protein's nutritional quality can be identified in different ways, but, in a simple way, it is the balance and relative amounts of essential amino acids, as well as digestibility, bioavailability, and bioactivity, which mainly identify its nutritional value. Compared with animal-based protein, the proteins derived from plants are easier to produce; however, when utilized as dietary sources for human consumption, most of the plant proteins are deficient in essential amino acids and are, therefore, nutritionally incomplete. For example, some cereal proteins are low in tryptophan, lysine, and threonine content, while vegetable proteins and legumes have a lower amount of sulfur-containing amino acids, such as methionine and cysteine (58). Due to this deficiency, these essential amino acids become the limiting factor in legumes and cereals. Practically, neither legumes nor cereals can compensate for the deficiency of amino acids for other crops, and, hence, diet feeding regularly provides

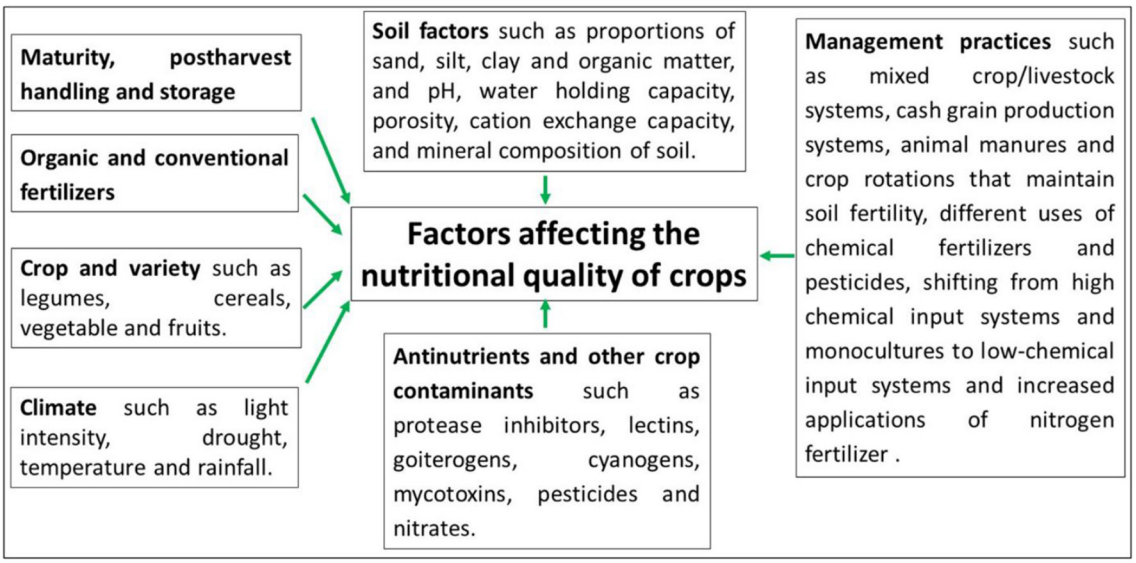

FIGURE 2 | Various factors that affect the nutritional quality of crops.

supplementary amino acids. There are also other factors that affect the nutritional quality of crops, including soil condition, crop maturity, postharvest handling, storage, use of fertilizers and pesticides, crop variety, and climatic conditions (**Figure 2**).

It is important in terms of nutritional as well as economic value to increase the essential amino acids content in plant-based proteins (59). In the past decades, plant breeders and geneticists have done much research for the improvement of the quality and characteristics of plant proteins. For instance, natural mutations, like the high content of lysine in barley and corn, have been recognized and made as elite genotypes (60). But, unfortunately, undesirable characters, like lower yields and susceptibility to pests and diseases, were also linked with these types of natural mutations. Nowadays, the techniques of modern biotechnology as alternative methods help to solve these problems. The method known as the protein digestibility-corrected amino acid score (PDCAAS) is an effective tool for the quality evaluation of protein (49, 61). One of the new methods recommended by FAO in 2013, digestible indispensable amino acid score (DIAAS), has also been used to evaluate protein quality, and, in terms of scientific knowledge, it is considered more accurate than PDCAAS (62).

BIOACTIVE AND FUNCTIONAL PROPERTIES OF PLANT-BASED PROTEINS

Bioactive Properties of Plant-Based Proteins

Several reports have shown the health effects of plant-based proteins as antitumor, antioxidant, hypoglycemic, ACE inhibitory, antimicrobial, and hypolipidemic effects (**Figure 3**) (63, 64). It has been observed that in countries where a high number of pulses are consumed, risk diseases, such as type-2 diabetes, cardiovascular diseases, colorectal cancer, and different types of chronic diseases, have been reduced (65, 66). The bioactivity of small peptides that are mainly released from enzymatic hydrolysis by various proteases, such as pepsin, trypsin, chymotrypsin, alcalase, papain, pancreatin, thermolysin, and flavorzyme, are present in different pulse proteins (67). These peptides exert various bioactivities, such as antioxidant, antifungal, antitumoral, and ACE inhibition activity (67, 68), and are also used for different purposes, like food supplements, functional food ingredients, and nutraceuticals (63) (**Table 1**).

Plant-Based Proteins Against Cardiovascular Disease and Metabolic Risk Factors

A large number of studies showed the potential impact of dietary proteins derived from plants against cardio-metabolic risk factors. The first study for the synthesis and intake of plant proteins as an alternative to animal protein was reported and published in 2017 (83). In this study, the authors reviewed and demonstrated biomarkers for cardiovascular disease from plant proteins consumption (83). They also studied and reported a decrease in the concentration of blood lipids (such as lower apolipoprotein B, low-density lipoprotein cholesterol, and non-high-density lipoprotein cholesterol). The authors also conducted randomized trials, which proved that plant protein is effective in reducing the risk factors associated with cardiovascular diseases in adults. In another study, the impact of proteins derived from plants (mostly soy products) on hypercholesterolemic patients was found superior in lowering the lipid profiles compared with the animal proteins (84). In populations, the adolescent stage, most of the benefits of plant-based proteins and metabolic health concerns have been discussed. Several studies to examine the benefits of plant-based proteins intake have been done for metabolic syndrome,

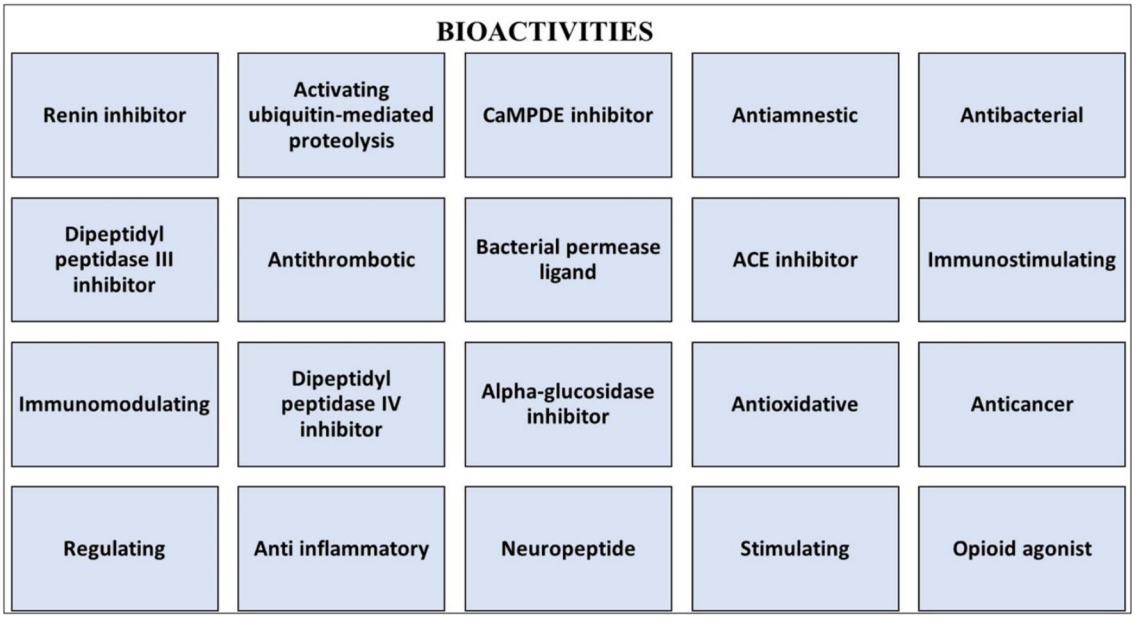

FIGURE 3 | The major bioactivities of plant-based proteins.

weight management, and obesity, as these are the serious and growing health issues globally among adolescents. However, the regulation of protein intake is critical to many physiological development and functions. Therefore, enhancing the proteins derived from plants in adolescent diets as a substitute for animal-based proteins helps in controlling obesity and other cardio-metabolic factors (85). The authors in different studies concluded that there should be the addition of more proteins of plant origin in the human diet for reducing the risks associated with cardiovascular disease as well as metabolic risk factors (86). Also, it was found that consumption of plant-based proteins lowers blood pressure in patients with hypertension (including elderly patients) as compared to animal protein (87, 88).

Most of the studies were also associated with the intake of plant protein sources and mortality. In a recent cohort from the NIH-AARP Diet and Health Study, the authors also observed the effect of choice of dietary protein on mortality (89). In this study, more than six lakh individuals from the U.S. in the age group of 50–71 years were followed from 1996 till December 2011. It was noticed that plant protein intake has led to inverse the mortality rate as well as from stroke in both males and females and cardiovascular disease. They observed the replacing of animal protein with only 3% of plant protein reduced 10% risk of overall mortality in both males and females (89). Therefore, it is beneficial to substitute plant proteins into the diet instead of animal proteins in terms of mortality and longevity. In a recently published review of the 32 cohort studies, it has been interpreted that the plant-based protein diet lowers the risk of all-cause and cardiovascular-associated mortality. Replacement of foods containing animal proteins with plant protein improves longevity (90).

Plant-Based Proteins and Diabetes

Although plant-based diets are mainly linked with reducing the risk of diabetes (91), it is still not clear that substituting the plant-based proteins for animal proteins helps in reducing the risk of diabetes in the population. After studying and analyzing using the dataset from the Nurses' Health Study II, Malik et al. (92) observed that 5% substitution of vegetable protein for animal protein was linked to the 23% reduction in type 2 diabetes risk. In a meta-analysis conducted in 2015, the sources of animal protein were replaced with plant-based protein for ∼35% of the intake of dietary protein for 8-week randomized controlled trials. From this study, the authors found that there are significant improvements in the levels of fasting glucose, fasting insulin, and HbA1c in patients with diabetes (individuals with both type 1 and type 2 diabetes) (93). In a cohort study, individuals were provided a protein-based diet and found that higher protein intake is associated with a lower risk of diabetic and pre-diabetic incidences, and plant-based proteins are the main determinant (94). The plant-based protein diet also contains a variety of bioactive components, which provide beneficial health effects as compared to processed meat products. In another randomized crossover trial, substituting red meat with legumes (lentils, chickpeas, peas, and beans) significantly decreased fasting blood glucose, insulin, and the triglyceride level in patients with diabetes type-2, suggesting the potential role of plant-based proteins over animals (95).

Plant-Based Proteins Against Cancer

Generally, a large number of factors, such as environmental, genetic, dietary, and other habitual features, are associated with the development of cancer. One research group has studied and examined the risk factor of colorectal cancer in individuals with

TABLE 1 | The commonly used physical modification methods of protein and their applications.

Modification methods			Description/Applications	References
Physical modification	Heat treatment	Conventional thermal treatment	Physically modifying plant-based protein structural and functional properties	(69)
			Used in pharmaceutical and food industries	
			Increase thermal stability, gelling properties	
			Reduce or eliminate adverse effects of anti-nutritional compounds	
			Improve digestibility, nutritional, and emulsifying properties of plant-based proteins	
		Ohmic heating	Used for milk pasteurization	(70)
			Result in unfolding, denaturation, and the formation of uniform-sized protein aggregates	
			Having techno-functional properties	
			Decreasing the heating time and improving the emulsifying ability of the protein	
		Microwave heating	Induce protein unfolding by splitting disulphide and hydrogen bonds	(71)
			Improve digestibility, gelling and emulsifying properties	
			Modulate the protein without destroying its primary structure	
			Increase the efficiency of enzymatic modification	
			Used for immunomodulation of different plant-based proteins	
		Radio frequency treatment	Increasing the surface hydrophobicity	(72)
			Improve functionalities such as the oil holding capacity and emulsifying properties	
		Infrared irradiation	Increase digestibility	(18)
			Decrease the amount of anti-nutritional factors	
	Gamma irradiation		Extending the shelf-life of food products	(62)
			Increase thermal stability, digestibility, and surface hydrophobicity	
			Improve antioxidant ability, oil binding capacity, emulsifying, and foaming properties	
			Decrease water binding capacity and immunoreactivity	
	Electron beam irradiation		Sterilize food materials as well as assist in extraction processes	(73)
			Improve solubility, nutritional value, bioactivity, functional properties, and emulsifying activity of peptides	
			Decrease surface hydrophobicity and molecular weight, which positively affected emulsifying and foaming properties	
			Increased efficiency, antioxidant ability, and thermal stability of this plant-based protein	
	Ultraviolet radiation		Induce chemical modification in plant-based proteins which can improve their techno-functional properties	(74)
			Improve the mechanical properties of the formed films	
			Increase sulfhydryl content, surface hydrophobicity, antioxidant activity, solubility, emulsifying and foaming properties	
			Decrease immunoreactivity and allergenicity of plant-based proteins	
	Pulsed-electric field		Increase surface hydrophobicity, antioxidant activity, protein solubility, emulsifying, foaming and functional properties	(75)
	High pressure treatment	High hydrostatic pressure	Inactivate microorganisms, changes texture and emulsification	(76)
			Increase protein surface hydrophobicity and nutritional value of plant-origin proteins	
			Reduce allergenicity	

(Continued)

TABLE 1 | Continued

Modification methods		Description/Applications	References
		Improve techno-functional properties, gelation and aggregation ability, antioxidant activity, emulsifying properties, protein solubility, colloidal and heat stability, water, and oil holding capacity	
	Dynamic high-pressure fluidization	Inactivate microbial cells	(77)
		Improve emulsifying ability, versatility, digestibility, and functionality of plant-based proteins	
		Decrease nanoparticle size and allergenicity of different animal proteins	
		Increase solubility, emulsifying and foaming properties	
	Sonication	Favor water and oil binding capacity, emulsifying and gelling properties	(78)
		Improve solubility, foaming capacity, emulsification properties, antioxidant ability, and digestibility of plant-derived proteins	
		Enhance hydrophobicity	
		Decrease foaming stability, amount of anti-nutrients, and allergenicity	
	Extrusion	Inactivate microorganisms, enzymes, and naturally occurring toxic substances as well as gelatinization of starch or shaping food materials	(79)
		Improve techno-functionality and digestibility of plant-based proteins, and can also generate a texture mimicking that of meat	
		Destroy anti-nutrients compounds	
	Ball mill treatment	Ensure higher solubility	(80)
		Improve gelling properties	
	Cold atmospheric plasma processing	Inactivate microorganism, spores and viruses present on the surfaces of food	(81)
		Improve techno-functional properties of plant-based proteins, solubility, emulsifying and foaming ability, water holding capacity, antioxidant, and gelling properties and surface activity	
		Increase surface hydrophobicity	
	Ultrafiltration	Improve of surface hydrophobicity, emulsifying, foaming, and oil holding capacity	(82)
		Remove anti-nutritional compounds	

the help of analyzing gene-environment interaction, including other factors, such as genetic, lifestyle, and cancer risk factors (96). The authors reported the linkage between colorectal cancer and the genetic diversity of fatty acid metabolism, which are mainly associated with a higher intake of meat, and concluded that those who consume a high diet of meat have a high risk of colorectal cancer (96). Therefore, plant-based protein substitution for animal protein is a better way to reduce the risk of colorectal cancer in humans with certain genetic polymorphisms.

Plant-Based Proteins and Their Renoprotective Effect

The diet, which is lower in vegetables, fruits, healthy oils, and dairy food, but higher in total protein foods, total grains, saturated fats, sodium, and added sugar, has been under trials to know the differences that help to cure chronic disease, especially chronic kidney disease (CKD) (97). Recent studies have suggested that, along with the amount of protein, protein's origin (for example, plant vs. animal) might be a crucial factor that affects the function of the kidney (98). For individuals with chronic kidney disease, on the consumption of plant-based protein, a significant 23% lower mortality rate was reported (99). In a randomized control trial in diabetic adults with macro-albuminuria, the animal protein diet was substituted with soy protein diet (by 50%) and found that it significantly improved proteinuria, cholesterol, and the glucose level (100).

In a crossover study, a diet rich in soy protein reduces glomerular hyperfiltration in individuals having type 1 diabetes with early-stage nephropathy (101). With the increase in glomerular hyperfiltration and the glomerular filtration rate, the incidence of kidney injury has been decreased (102). The plant-based proteins mainly extracted from rice endosperm and soybean have also shown renal protective function in diabetic rat models (103). Also, other factors, like phytochemicals and fiber, also played a significant role in renal protection by consuming whole food from plant-based diets as well as other components of plants. Thus, it is recommended to incorporate high-quality plant proteins for renoprotective effects.

Functional Properties of Plant-Based Proteins

Plant proteins have also been utilized as functional foods. A large number of studies have been done to examine and reduce the risk factors of cardiovascular disease, modulating inflammation and immune system by functional analysis and bioactive properties of soy protein (104). The recent systematic review has focused on the bioactive properties of sources of plant proteins, such as rice, lentil, fava bean, pea, lupin, hemp, and oat (105). Various trials have been done to test the benefits of proteins derived from plants by observing the concentrations of insulin, blood glucose, and hormones regulating the appetite. However, conflicts in results were seen when the study was conducted for determining the beneficial effects of plant proteins on postprandial glycemia regulation. A number of components present in plants, like flavonoids and carotenoids, also confer the benefits of bioactive functionality on human health.

In addition to the nutritional quality of plant proteins and their bioactive properties, these compounds also have functional properties. They play a major role in food processing and formulation, i.e., the production of gluten-free and protein-rich food (106). Chemical and physical properties of protein help during the storage, consumption, processing, and preparation of food products. Properties like solubility of the protein, foaming capacity, absorbing capacity of water and fat, foam stability, gel-forming, and emulsifying activity are involved in protein interaction by combining with other molecules, like proteins, carbohydrates, salts, lipids, water, and volatiles. These functional properties are largely affected by the molecular size of peptides and/or proteins, charge distribution, and structure of the protein. Additionally, different environmental conditions that affect the structural changes of protein during food processing will also affect the functional properties of plant proteins (107). For improvement of nutritional quality and potential health benefits, different protein formulations can be added, such as isolates, concentrates, and protein flours. However, the functional properties of various plant-based proteins were utilized in the industrial production of food products. Briefly, various functional properties such as protein solubility during beverage production lead to solvation of protein; absorption of water molecules and their binding allows entrapment of water in bread, meat, cakes, sausages, etc.; absorption of fat is linked with binding of free fat in meats, doughnuts, and sausages; emulsifying properties of proteins lead to the production and stabilization of emulsions of fats in pasta, cakes, sausages, soups, etc.; protein's foaming properties permit the entrapping of gasses by forming stable films in whipped toppings, bakery products, cakes, and desserts; gelation properties are linked with the formation and maintaining of protein matrix in meats, cheese, and curds (106).

Applications of Plant-Based Proteins in Food and Non-Food Industries

Proteins are the important ingredients of the human diet with great complexity and diversity that play an important role in structural and functional development (29, 49). Plant protein provides many essential amino acids, vital macronutrients and is sufficient to achieve full protein nutrition. Moreover, plants have a high demand for the supply of protein to the increasing population (12). Thus, instead of animals, plants were considered the bioproduction system for useful substances, especially in medicine, which usually provide a large number of secondary metabolites having therapeutic effects. These substances produced by plants mainly help to protect from predators and pathogens, attract pollinators, and have properties like anti-inflammatory, wound-healing, anti-microbial, psychoactive, etc. (1), and hence utilized for protecting and maintaining human and animal health.

Different sources of plants have been widely used as supplements of protein, such as cereals (wheat, rice, millet, maize, barley, and sorghum), legumes (pea, soybean, bean, faba bean, lupin, chickpea, and cowpea), pseudocereals (buckwheat, quinoa, and amaranth), nuts and almonds, and seeds (flaxseed, chia, pumpkin, sesame, and sunflower). Along with providing health benefits, proteins also play a significant role in food formulations because of their diverse functions, such as emulsifying, gelling, and thickening agents, and also have water-holding, foaming, and fat absorption ability (16, 17). In addition, these crops have number of beneficial effects on health and have technological and functional properties with industrial applications in development of food. Thus, these proteins play an important role in circular production systems.

Food derived from plants plays a vital role in human health as an important source of bioactive components, minerals, vitamins, and bioactive peptides (4). In addition, protein obtained from plants provides essential amino acids and improves the overall nutritional status of human diets.

From the last few years, much interest has been paid to search for protein sources with high nutritional quality and functionality in food processing and industrial applications (emulsification, solubility, gelation, foaming, and viscosity oil-holding and water-holding capacities). Recently, the importance and benefits of proteins derived from plants have been trending to provide various health benefits. Many studies have been conducted on the potential impact of dietary proteins derived from plants on reducing cardio-metabolic risk factors, metabolic syndrome, weight management, and obesity (86–88). Most of these studies concluded that there should be an addition of proteins of plant origin in the human diet for reducing the risks associated with cardiovascular disease and metabolic diseases (86). Another interesting area of research to examine the benefits of intake of plant proteins instead of animal protein is reducing cancer risk factors.

Food products containing plant proteins have also been known as functional foods. Various trials have been conducted to test the health benefits of plant-based proteins by observing the concentrations of insulin, blood glucose, and hormones regulating the appetite. Most of the studies were also associated with the intake of plant protein sources and mortality. In a recent cohort from the NIH-AARP (National Institutes of Health-American Association of Retired Persons) Diet and Health Study, the authors also observed the effect of choice of dietary protein on mortality (89). The diet, which is lower in vegetables, fruits, healthy oils, and dairy food, but higher in total protein foods, total

grains, saturated fats, sodium, and added sugar, has been under trials to know the differences that help to cure chronic disease, especially chronic kidney disease (CKD) (97). Recent studies have suggested that, along with the amount of protein, protein's origin (for example, plant vs. animal) might be a factor that affects the function of the kidney (98).

In addition to the nutritional quality of plant proteins and their bioactive properties, they play a major role in food processing and formulation, i.e., the production of gluten-free (GF) and protein-rich foods (106). In addition, however, the functional properties of various plant-based proteins were utilized in the industrial production of food products. Various applications, like protein solubility (bread, meat, cakes, sausages, doughnuts, and sausages; emulsifying properties emulsions of fats in pasta, cakes, sausages, soups, etc.; protein's foaming properties, bakery products, cakes, and desserts; and gelation properties provide stability to the protein matrix in meats, cheese, and curds (106).

Some traditional proteins from plant origin have been utilized by humans as a protein source, such as beans, pea, and soybean. Still, various recent studies have been done for novel (such as proteins from insects and algae) (2) and unconventional and alternative protein sources (like agroindustry by-products from extraction of edible oil) (7).

Gluten-free pseudocereals help in curing of patients with celiac disease (35). The food industry helps produce high-quality plant-based milk, egg, and meat analogs, such as sausages, burgers, ground meat, and nuggets. The proteins derived from plants are considered important and functional ingredients with different roles in food formulations, including gelling and thickening agents, foams and emulsions stabilizers, and binding material for water and fat. Most of the proteins have biological activities, like ACE inhibitory, antioxidant, antimicrobial, and stimulating characteristics (70), and the protein from vegetables is also utilized for synthesizing and extracting bioactive peptides.

HEALTH ISSUES LINKED WITH PLANT-BASED PROTEINS

Antinutrients

There are many health concerns linked with a large intake of dietary proteins derived from plants. Antinutrients, such as tannins, phenolics, saponins, phytates, glucosinolates, and erucic acid, are naturally produced by plants and further interfere with absorption, digestion, and utilization of nutrients present in food, with other side effects as well (108). The adverse effects of antinutrients might be maldigestion of proteins (protease and trypsin inhibitors), carbohydrates (alpha-amylase inhibitors), autoimmune and leaky gut (e.g., some saponins and lectins), malabsorption of minerals (oxalates, phytates, and tannins), inflammation and interfering in thyroid iodine uptake (goitrogens), behavioral effects, and gut dysfunction (when converting cereal gliadins to exorphins) (108). These adverse effects of antinutrients are generally seen in animals when consumed unprocessed proteins of plant origin. However, these antinutrients also showed beneficial health effects. For instance, at a lower level of lectins, phytates, enzyme inhibitors, saponins, and phenolic compounds, there is a reduction in plasma cholesterol, triglycerides, and blood glucose levels (108). Saponins may play a significant role in liver functioning and decrease platelets agglutination. In contrast, some of the saponins and also protease inhibitors, phytates, phytoestrogens, and lignans might help in reducing cancer risk (108). Additionally, tannins also have antimicrobial effects (108). To reduce the concentration of antinutrients in plant proteins and their adverse effects, various treatment processes, such as fermentation, soaking, gamma irradiation, sprouting (germination), heating, and genomic technologies, have been adopted (108). Food processing techniques also remove most of the antinutrients like phytates, glucosinolates, erucic acid, and also insoluble fiber from canola proteins that further improve and increase the digestibility and bioavailability (109).

Isoflavones and Soy Protein

Soy protein is associated with both positive and negative health concerns. The adverse effect on health is due to the presence of isoflavones in soy protein, which are chemically similar to estrogen and could also be bound to estrogen receptors (110). Due to soy isoflavones, the issue of endocrine-disrupting effect is seen on thyroid and reproductive hormones at higher doses in rodent and *in vitro* cell culture studies (111–113). The isoflavones content of different ingredients of soy protein has been reported; for example, isolates of soy protein (88–164 mg/100 g), defatted and whole soy flours (120–340 mg/100 g), textured soy protein isolates that are commercially used (66–183 mg/100 g), and soy hypocotyl and flours' commercial isolates (542–851 mg/100 g) (114). Therefore, consumers mainly avoid taking soy proteins due to various adverse effects on thyroid and reproductive hormones. The study conducted by the European Food Safety Authority in 2015 showed that 35–150 mg daily doses of isoflavones in pre- and postmenopausal women resulted in no significant enhancement in breast cancer risk, uterus's histopathological changes or thickness in the endometrial lining of the uterus, and thyroid hormonal status for about 30 months (115). A meta-analysis has also been done on 15 men of different ages and found that intake of 60 g/day of soy protein has not been linked with sex hormone-binding globulin, changes in testosterone, free androgen index, or free testosterone (116). Also, it did not influence the parameters of semen quality, such as sperm concentration, semen volume, sperm mobility, sperm count, sperm percent motility, sperm morphology, and total motile sperm count in healthy men (117). It has also been reported in the meta-analysis that intake of soy protein might be linked with reducing breast cancer risk in women (118–120).

Plant-Based Proteins and Their Association in Allergenicity

There is an increasing trend of consuming plant proteins, which indicates that different sources of protein from plants influence our health. Such dietary proteins may also have some adverse effects, including allergenicity. An allergy from food is basically an adverse effect that results inactivation of immune response when exposed to a food. According to the literature review, food

allergy is found to affect up to 10% of the population (121). It has been identified that more than 170 foods in the United States of America are responsible for food allergies. Foods commonly causing allergy are tree nuts, soy, wheat, fish, peanuts, milk, shellfish, and egg. Other common food allergens based on the countries are lupines (European Union); sesame seeds (Canada, European Union, and Australia); buckwheat (Japan and Korea), and mustard (European Union and Canada) (122). A higher number of children than adults are sensitive to dietary proteins that mainly cause allergy (123).

Food allergens from plants are mainly categorized into four families, the cupin superfamily, the prolamin superfamily, profilins, and the Bet v 1 family. More than 50% of allergens of plant proteins fall into two categories, i.e., the cupin and prolamin superfamilies (124). The prolamin family has 8 cysteine residues of amino acid that is conserved with pattern CXnCXnCCXnCXCXnCXnC, which mainly stabilizes the structure of protein and contributes proteins allergenicity. The most commonly found allergens are cereal prolamins, α-amylase, 2S albumins, non-specific lipid transfer proteins, and trypsin inhibitor, protein families.

COMPARISON BETWEEN ANIMAL AND PLANT-BASED PROTEINS

Dietary proteins could be derived from animals and plants. Animal protein, although higher in demand, is generally considered less environmentally sustainable. A gradual transition from animal to plant-based protein food may be desirable to maintain environmental stability, ethical reasons, food affordability, greater food safety, fulfilling higher consumer demand, and combating of protein-energy malnutrition. Since the last 20 years, among the alternative sources of protein, the scientific research team and private companies have mainly focused on algae, earthworm or earthworm meal, insects, and other invertebrates (52, 53). Nowadays, food derived from plants plays a vital role in the human diet as an important source of bioactive components, such as vitamins, phenolic compounds, or bioactive peptides. Hence, these components are very helpful to human health and protect against various pathogens (4). Instead of animals, plants were considered the bioproduction system for useful substances, especially in medicine, which usually provide a large number of secondary metabolites having therapeutic effects. These substances produced by plants mainly help protect from predators and pathogens, attract pollinators, and have properties like anti-inflammatory, wound healing, anti-microbial, psychoactive, etc. (1), and hence utilized for protecting and maintaining human and animal health.

The proteins derived from plant-based foods are increasingly used as a health-promoting and economical alternative source in place of animal proteins in human nutrition. However, various limitations, such as increased cost, limited supply, biodiversity loss, hazard for human health in different diseases, freshwater depletion, and susceptibility to climate change, replace animal-based proteins (5–7). Moreover, it is hard and expensive to extract an adequate amount of animal proteins; therefore, an alternative for improving the nutritional status of humans is mainly received from plant proteins.

Globally, protein is produced from both plants (80%), such as cereal grains, beans, soy, pulses, nuts, vegetables, and fruits, as well as animals (∼20%) in the form of meats, milk, eggs, fish, yogurt, and cheese (50). Compared to animal-based proteins, the proteins derived from plant-based foods are rich in fiber, polyunsaturated fatty acids, oligosaccharides, and carbohydrates. Therefore, they reduce the cardiovascular diseases and type II diabetes (8). Increased urbanization and economic development have led to various transitions in dietary patterns in the population of low- and middle-income countries, especially the demand for foods derived from animals, which was seen in developing countries.

Recently, plant-based sources of protein have dominated the supply of proteins throughout the world (57%), with the remaining 43% consisting of dairy products (10%), shellfish and fish (6%), meat (18%), and other products from animals (9%) (3, 19). Generally, the daily intake of protein is provided by animal-based foods. However, changes in the consumers' requirement led to adoption of alternative sources of proteins for human consumption. Therefore, emerging factors for animal proteins like growth of world population, climate change, and production of protein sources that are economically and environmentally sustainable need more research focus, and that is mainly dedicated to proteins from plants with high content, resilient to changing of climate and providing balance nutrition in humans' diet.

Compared with animal-based protein, the proteins derived from plants are easier to produce. Still, when utilized as dietary sources for human consumption, most plant proteins are deficient in essential amino acids and are, therefore, nutritionally incomplete. For example, some cereal proteins are low in tryptophan, lysine, and threonine content. In contrast, vegetable proteins and legumes have lower sulfur-containing amino acids, such as methionine and cysteine (58). Due to this deficiency, these essential amino acids become the limiting factor in legumes and cereals. Practically, neither legumes nor cereals can compensate for the deficiency of amino acids for other crops, and, hence, feed diets regularly provide supplementary amino acids.

Many studies have been done on the potential impact of dietary proteins derived from plants and serve as reducing cardio-metabolic risk factors. The first study for the synthesis and intake of plant proteins as an alternative to animal protein was reported (15). However, the regulation of protein intake is critical to many physiological development and functions. Therefore, enhancing the proteins derived from plants in adolescent diets as a substitute for animal-based proteins help in controlling obesity and other cardio-metabolic factors (85). Although plant-based diets are mainly linked with reducing the risk of diabetes (91), it is not clear that substituting the plant-based proteins for animal proteins helps in reducing the risk of diabetes in the population. After studying and analyzing the Nurses' Health Study II dataset, Malik et al. (92) observed that 5% substitution of vegetable protein for animal protein was linked with the 23% reduction of type 2 diabetes risk. Another interesting area of research to

TABLE 2 | The commonly used chemical modification methods of protein and their applications.

Modification methods		Description/Applications	References
Chemical modification	Glycation	Improve protein functionalities, emulsifying ability, solubility of the protein, foaming ability, thermal stability, and flavor profile	(125)
		Reduce beany flavor in some plant-based proteins	
		Having strong immunomodulatory properties	
	Phosphorylation	Keep nutritive bioavailability	(126)
		Improve solubility, thermal stability, viscosity, viscoelasticity, thermal aggregation functional, foaming, and emulsifying properties	
		Increase *in-vitro* digestibility	
	Acylation	Improve solubility, emulsifying, foaming and functional properties, emulsion stability, and water holding capacity	(127)
		Increasing the molecular weight of some proteins and hydrophobicity will led to improvement or enhancement of thermal stability and gelling properties	
	Deamidation	Mask the bitterness	(128)
		Improve techno-functionality, solubility, water holding capacity, emulsifying, and foaming properties	
		Reduce beany flavor, grittiness, and lumpiness	
		Decrease the allergenicity of plant-based proteins	
	Cationization	Modify techno-functionality	(129)
		Improve solubility, encapsulating, and emulsifying properties	
	pH shifting treatment	Change the structural and functional properties of proteins	(130)
		Improve extensibility and tensile properties of the formed films and also the functionality, such as enhanced solubility, surface hydrophobicity, antioxidant activity, rheological, foaming, and emulsifying ability	
		Induce protein reactivity by promoting its unfolding	

examine the benefits of the intake of plant proteins instead of animal protein is reducing cancer risk factors (96).

THE MODIFICATION APPROACHES OF PLANT-BASED FOOD PROTEINS

Protein modification is the process of alteration of the chemical groups or molecular structure of a protein by specific methods for improving the bioactivity and functionality of proteins. The modification approaches for plant-based proteins help them to make multifunctional food products. The modification of proteins can be classified into physical (18, 62, 69–82), chemical (125–130), biological (131, 132), and other novel methods (133–137) as briefly described in **Tables 1**–**3**. The physical modification approaches include heat treatment (such as conventional thermal treatment, ohmic heating, microwave heating, radio frequency treatment, infrared irradiation), gamma irradiation, electron beam irradiation, ultraviolet radiation, pulsed-electric field, high-pressure treatment (such as high hydrostatic pressure, dynamic high-pressure fluidization), sonication, extrusion, ball mill treatment, cold atmospheric plasma processing, and ultrafiltration. The chemical modification approaches include glycation, phosphorylation, acylation, deamidation, cationization, and pH shifting treatment. The biological modification approaches include enzymatic modification and fermentation. Instead of physical, chemical, and biological modifications, various other modification approaches have been identified, which include complexation (such as protein-polysaccharide, protein-protein, protein-phenolic, and protein-surfactant) and amyloid fibrillization (**Tables 1**–**3**) (138).

PROTEIN EXTRACTION TECHNOLOGIES

The advancement in recombinant technologies of protein production, such as engineering of expression hosts, upstream cultivation optimization (e.g., nutritional, bioreactor design, and physical parameters), and development of protein extraction methods supported the growth of the market (7, 13). The use of protein extraction technologies can help improve the yield of extracted protein and its nutritional and functional properties. Hence, a suitable type of protein extraction method should be selected (**Figure 4**).

Dry Protein Extraction Technique

Sieving and/or air classification techniques, majorly a part of novel dry protein extraction techniques, have been widely used to prepare fiber or protein-rich fractions. Although a high protein yield was generated, however, it utilized more energy than wet protein extraction. Also, the disadvantage of these processes includes the presence of impurity and particle agglomeration (7).

Wet Protein Extraction Technique

In wet protein extraction techniques, the process starts with protein solubilizing in a medium with the pH far from the isoelectric point and then precipitating in that medium where pH

TABLE 3 | The commonly used biological and some other modification methods of protein and their applications.

Modification methods			Description/Applications	References
Biological modification	Enzymatic modification		Improve emulsifying ability, techno-functionality, protein solubility, antioxidant ability, interfacial properties, foaming ability, oil holding capacity, and bioactivity of plant-based proteins	(131)
			Increase the hydrophobicity and surface-active properties of the generated hydrolysates	
			Decrease the bitterness	
	Fermentation		Improve protein solubility, water and oil holding capacity, foaming, and functional properties	(132)
			Promote nutritional and antioxidant properties and also the digestibility	
			Degrade allergens and anti-nutritional compounds	
			Decrease immunoreactivity, bitter, and beany off-flavors of different plant-based proteins	
Others	Complexation	Protein-polysaccharide	Modulate techno-functional properties and address issues such as physical stability around their isoelectric point	(133)
			Improve solubility, susceptibility, stability, emulsifying, and foaming properties	
			Reduce the bitterness of potato protein	
		Protein-protein	Improve techno-functionality	(134)
			Increase water solubility	
		Protein-phenolic	Exhibit different biological activities such as antioxidant, antimicrobial, anticancer, antiallergenic, anti-inflammatory, and also higher thermal stability	135
			Polyphenolic compounds reduce solubility of plant-based proteins	
		Protein-surfactant	Tune the amphipathic properties by modulating hydrophobic or hydrophilic degrees	(136)
			Improve encapsulation efficiency, physicochemical properties, solubility, emulsifying and foaming properties, water dispersibility, pH, salt, physical-, photo-, acid-, and thermal stability	
			Increase stability	
	Amyloid fibrillization		Improve protein functionalities in different applications such as drug and nutraceutical delivery platforms	(137)
			Increase surface hydrophobicity	
			Improve foam, emulsion Pickering stabilizers, degradable films, ultralight aerogels, gels, water purification filters, and rheological properties	

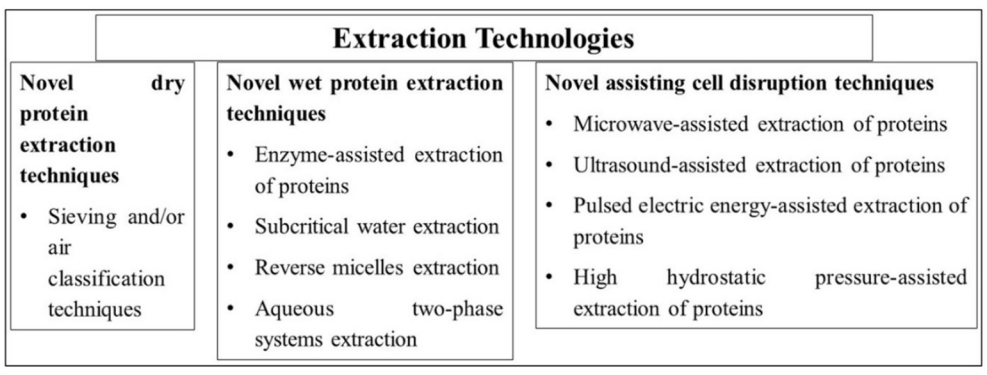

FIGURE 4 | Extraction technologies for plant-based proteins.

is close to the isoelectric point. Several protocols for acidic and alkaline extraction of protein have been reported (7).

Enzyme-Assisted Extraction of Proteins

This method is based on the principle of cell wall disruption with specific enzymes that degrade celluloses, hemicelluloses, and/or pectin, and also proteases that help in the hydrolyzation of protein for solubility enhancement. With the degradation of cell walls, protein bodies released are enabled. This method needs more processing time, high cost, more energy consumption, and suitable conditions like temperature and pH. Still, this method is mostly used with lower environmental impact and superior quality of products for human consumption (7, 139).

Subcritical Water Extraction

In this technique, hot water in the range of 100–374°C with high pressure (for maintaining it into the liquid state) has been used. Biomaterials like carbohydrates and proteins have been hydrolyzed by this method without using an additional number of catalysts. For example, when soy meals were heat denatured, soy protein extraction yield was significantly increased with this method by 59.3% (139).

Reverse Micelles Extraction

This method applies reverse micelles—surfactant molecules aggregate of the nano-meter size that generally contains inner cores of water molecules inside non-polar solvents. The polar molecules of water present in reverse micelles help in solubilizing hydrophilic biomolecules like proteins. The three-phase system called a water–surfactant–organic solvent system has been formed by reverse micelles to protect the denaturation of proteins by organic solvents inside the polar water pools, using forward extraction or backward extraction (7).

Aqueous Two-Phase Systems Extraction

This extraction method is formed when two polymers like two salts or one salt and one polymer are mixed in a suitable concentration at a particular temperature. This method has been considered as the environment-friendly method of protein extraction. It was first reported by Zeng et al. (140) for extracting proteins by an ionic liquid aqueous two-phase system, resulting in proteins extraction with a yield of 99.6% (7).

Novel-Assisting Cell Disruption Techniques

Cell disruption is the initial process in both dry and wet techniques of protein extraction, which helps release protein from protein bodies. Previously, cell disruption was done by mechanical methods like milling, grinding, etc., or chemical or thermal treatments.

Microwave-Assisted Extraction of Proteins

This technology utilizes electromagnetic radiations having a frequency between 300 MHz and 300 GHz, which helps in hydrogen bond disruption, dissolved ions migration, and enhancement of porosity of the biological matrix, which leads to the extraction of protein. For example, one study reported the utilization of this technique to extract proteins from rice bran (141).

Ultrasound-Assisted Extraction of Proteins

This technology utilized sound waves, having a frequency of 20 kHz that induces the phenomenon of cavitation, which enhances the matrix porosity and improves solvent permeation into the matrix. This method has the advantage of effective mixing, selective extraction, faster energy transfer, reduced extraction temperature and thermal gradients, faster response, reduced equipment size, and increased production. Yet, denaturation of protein structure and disruption of functional properties of proteins are reported (7).

Pulsed Electric Energy-Assisted Extraction of Proteins

Several pulsed electric energy technologies for proteins extraction have emerged. This method uses electric pulses of short duration (from several nanoseconds to several milliseconds) of high-pulse amplitude (from 100 to 300 V/cm to 10–50 kV/cm) for the induction of structural changes of the compound of interest. Among a large number of PEE techniques, pulsed ohmic heating (POH), pulsed electric fields (PEF), and high-voltage electrical discharges (HVED) have been widely used in the food industry (7).

High Hydrostatic Pressure-Assisted Extraction of Proteins

High hydrostatic pressure-assisted extraction of proteins is mostly used in the food industry for large-scale microbial cell disruption, meat tenderization, and emulsification. This method is only restricted to bioactive compounds instead of proteins. However, with the application of several HHP iterations, the efficiency of separation and extraction yield has been reduced due to swelling of the cell wall, increase in dynamic viscosity, and size of the particle (7).

ISSUES, CHALLENGES, AND FUTURE PROSPECTS OF PLANT-BASED PROTEINS AND THEIR UTILIZATION IN FOOD PRODUCTS

The proteins derived from plants are considered as important and functional ingredients, having different roles in food formulations as gelling and thickening agents, foams and emulsion stabilizers, and binding material for water and fat (142–145). Most of the proteins have biological activities, like antihypertensive, antioxidant, antimicrobial, and stimulating characteristics (146, 147), and the protein from vegetables is also utilized for synthesizing and extraction of bioactive peptides (148, 149). However, most of the proteins from plant origin are interactable because of their susceptibility and complexity of ionic strength, pH, and temperature, and also have poor water solubility that mainly limits the applications of plant-based proteins (150). Most of the plant-based proteins, like flaxseed, soy, and pea proteins, have the combined nature of various proteins with different fractions, and, hence, they have a wide range of isoionic point (pI). Therefore, modulating the properties of plant-based proteins for improving their

functions and formulation characteristics is essential. A deep understanding of the functional and physicochemical properties of proteins derived from plants is necessary for improving their utilization in food formulation and nutritional value (151–153). The presence of some particular plant residues considered as antinutrients is another challenge of plant-based proteins. These compounds are produced in plants having various biological properties, such as they protect the plants and seeds from insects, fungus, viruses, and other microbes. Therefore, some of the modification approaches discussed have been used to reduce or eliminate the adverse effects of antinutrients (18). Furthermore, some plant-based proteins have challenges in food applications due to their bitter taste, which can be masked by various modulation techniques. The methods of modification for plant-based proteins should be carefully chosen, especially in pharmaceutical and food applications, because these methods have effects on the organoleptic and functional characteristics and nutritional value of plant proteins.

The bio-efficacy of any active compounds generally depends on various factors, like digestibility, solubility, bioaccessibility, food matrix, transporters, metabolizing enzymes, and molecular structures. Therefore, identifying the bioavailability of food constituents is challenging. There are many challenges associated with sustainability and food availability that needs to be solved with different methods of protein modification. The higher amount of essential nutrients found in animal products (meat, milk, egg, etc.) was important and provided a large number of nutrients in the daily diet compared with plant-based proteins (154). Although animal-meat-based products provide a large nutrient component, however, the disease associated with animals, unhygienic conditions, and environmental impact will all provide more attention to the plant-based proteins. Because of that, consumers are also more focused on the health and environmental benefits of plant-based diets, promote the food guidelines on the basis of health and sustainability criteria, produce more attractive plant-based alternative products, and realign their fiscal policy along with environmental and efficiency criteria (155–159).

CONCLUSIONS

People are facing protein and mineral deficiency in their diet throughout the world, especially in developing countries. This challenge is due to lower consumption of pulses and cereals in their diets and other foods that are rich in zinc, iron, calcium, and magnesium. These foods derived from plants also contain higher levels of antinutritional factors that bind to the minerals ions and reduce bioavailability and absorption of plant minerals as well as proteins. Animal protein, although higher in demand, is generally considered less environmentally sustainable and prone to disease conditions, which negatively impact health. A gradual transition from animal- to plant-based protein may be desirable in order to maintain environmental stability, ethical reasons, affordability of food, greater food safety, fulfilling higher consumer demand, and combating of protein-energy malnutrition. Nowadays, products made with proteins from plant origin gain popularity throughout the world. Plant-based proteins have been linked with a number of health-related functionalities. Plant-based proteins are becoming innovative and fast-growing ingredients in various food application industries due to a large number of benefits over animal-derived proteins. Various technologies help in improving the functional and nutritional properties of plant-based proteins. Generally, plant-based proteins have inferior functionality as compared with animal proteins, and also various factors affect their nutrient quality; hence, modification approaches have been required. Different physical, chemical, biological, and other approaches were also mentioned for modification of proteins that induce the structural, chemical, and biophysical changes in protein from plant origins.

This review mainly focuses on the current state of using plants for the production of protein. The potential plants offering various sources and their alternative with high-quality protein demand for future consumption were discussed. Factors that affect protein consumption, bioavailability, and also protein production techniques were covered. Various bioactive and functional properties of plant-based proteins, as well as the factors affecting the nutritional quality of plant-based proteins and the future research strategies, were explained. The modification approaches, protein extraction, purification technologies, along with digestibility, absorption, and bioavailability of plant-based proteins, were discussed. Finally, it gave an idea of issues and challenges as well as future prospects in this emerging area.

AUTHOR CONTRIBUTIONS

SL conceived the idea. SL and FK wrote the manuscript. PY, ZD, RS, and AK edited the manuscript. All authors contributed to the article and approved the submitted version.

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Filling the Protein Gap in Ghana: The Role of Soy

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The study assessed the nutrient value and desirability of eight improved soybean varieties, for use in soymilk, tofu and as an ingredient to enhance staple foods. The soymilk, tofu, and soybean residue (okara) yields were determined across all varieties. The okara was subsequently used in composite with cassava, as a recipe refinement of gari, a popular cassava-based ready-to-eat food. Multiple composite ratios were compared against a control of 100% cassava gari; 80% cassava: 20% okara, 70% cassava: 30% okara, and 50% cassava: 50% okara. The soymilk and tofu from the various varieties and okara enriched-gari were also evaluated for proximate and sensory qualities ($n = 50$) using standard protocols. No differences ($p > 0.05$) existed among soybean varieties in terms of soymilk ($p = 0.55$; 13.0–14.1 L), tofu ($p = 0.05$; 0.12–0.15 kg/L) or okara ($p = 0.08$; 3.17–3.97 kg) yields. The proximate parameters evaluated for soymilk did not vary significantly ($p > 0.05$) among varieties. However, for total solids (3.33–7.93°Brix; $p < 0.01$) there were significant differences. Generally, there was an increasing trend in the crude protein, moisture, crude fat and total ash contents for the okara-enriched gari as the okara inclusion increased from 20 to 50%. Thus, the crude protein content of the 50% okara-enriched gari, the formulation with the highest okara incorporation was almost 11-times higher than the 100% cassava gari. The swelling capacity of the okara-enriched gari ranged from 3.29–5.47 and for water holding capacity 439.7–482.1%. The okara-enriched gari was equally preferred by consumers, except for colour which consumers were mostly indifferent towards. The 50%-okara enriched gari composite was compared equally with 100% cassava gari control. The sensory data showed that the "Favour" soybean variety was desirable for soymilk production while Salintuya 1 was desirable for tofu production. Recipe refinements using the desired varieties and compositing okara with cassava may help fill the protein gap among the vulnerable group in Ghana by improving the protein quality of ready-to-eat foods such as gari.

Keywords: gari, Ghana, okara, protein deficiency, soybean, soymilk and tofu

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INTRODUCTION

Protein undernutrition and other micronutrient deficiencies are serious risk factors for infectious diseases that often coexist and exhibit complex interactions leading to a vicious cycle of malnutrition and infections (Bhaskaram, 2002) among people of all ages. Underprivileged populations living in low-income countries, particularly children, adolescents, and women are the most affected. In children and adolescents, undernutrition leads to poor academic achievements and additional vulnerability to disease (Lam and Lawlis, 2017). In pregnant women, undernourishment affects the mother and normal development and overall health of the foetus (Belkacemi et al., 2010).

Undernutrition continues to undermine development efforts in Sub-Saharan Africa. Although the aetiology of undernutrition is complex and involves many factors, its development may be attributed to low dietary intake of nutrient-rich foods, increased metabolic demands, or increased nutrient losses due to infection (Corish and Kennedy, 2000). Unfortunately, most of the staples consumed by the rural poor in low-income countries are characteristically starchy and limited in quality protein and some essential micronutrients. For instance, in rural Ghana, the overreliance on rapidly digestible carbohydrate staple foods has led to a greater prevalence in stunting, at 22.1% vs. the 14.8% experienced in urban settings (Ghana Statistical Service, 2015), where diets generally have greater diversity and include milk, eggs and meats.

Animal-sourced foods are nutrient-dense and very effective in ameliorating protein undernutrition and micronutrient deficiencies but they are very expensive and have limited affordability by resource-poor households. Additionally, most smallholder farmers in Ghana use their livestock as a source of emergency funds rather than consuming them to improve nutrition in their households (Nyantakyi-Frimpong et al., 2018). Therefore, there is, a need to fully exploit available, affordable, desirable, and sustainable plant-based protein sources such as soybean, to satisfy protein and other micronutrient gaps among the vulnerable population in sub-Saharan Africa. Soybean protein is one of the relatively cheaper sources of complete dietary protein because it contains most of the essential amino acids needed for human and animal nutrition (Hoffman and Falvo, 2004; Michelfelder, 2009) and could be a good alternative for animal-sourced protein (Sacks et al., 2006).

Soybean is a non-traditional staple crop in Sub-Saharan Africa and especially in Ghana (Martey and Goldsmith, 2020). In the recent past, initiatives to support the production of soybeans in Ghana have mainly been donor-driven (Avea et al., 2016), with a larger focus on increasing yields for oil and animal feed. The crop is increasingly attaining commercial attention as more producers and food processors are becoming aware of the opportunities to grow soybean for both its cash crop potential and its benefits to animal and human nutrition (Gage et al., 2012). Moreover, local promotion of soybean processing for human consumption, for instance, can stimulate the demand for nutrient-rich ingredients for food to food fortification and encourage domestic production. Therefore, both nutrient composition and yields are the focus of current efforts to stimulate the production of soybeans in Africa (Khojely et al., 2018). These efforts have led to the development and release of eight soybean varieties (i.e., Afayak, Salintuya 1, Salintuya 2, Songda, Suong-Pungun, Jenguma, Quarshie, and Favour) by the Savanna Agricultural Research Institute (SARI) of the Council for Scientific and Industrial Research (CSIR) in Ghana. Although some information on their nutritional profile exists, the desirability of the varieties for transformation into soymilk and tofu under the constraint of limited resources is scarce. More importantly, like any other industry, the creation of processed products results in the accumulation of food waste, which in the case of soymilk production is called okara. This material still contains a significant amount of protein, fibre and other micronutrients and represents an opportunity for food-to-food fortification if upcycled correctly.

Okara contains significant amount of protein (25.4–28.4%), oil (9.3–10.3%%), soluble fibre (12.6–14.6%) and insoluble fibre (40.2–43.6%) (van der Riet et al., 1989). Okara has minimal beany flavour (Golbitz and Jordan, 2006) often associated with soybean and lower polyunsaturated fatty acids (PUFAs) that could improve the shelf stability of its products compared with full-fat soy flour (FFSF). Earlier work indicates that for every 1 kg of dried soybeans used in the production of soymilk/tofu, an average of 1.1–1.2 kg of wet okara is produced accounting for a large volume of waste (Khare et al., 1995; Guimarães et al., 2018). In Ghana, most companies dispose of okara as animal feed or discarded. Okara has a huge potential of becoming a low-cost functional food ingredient. Nonetheless, drying of okara (i.e., from 80 to 90% moisture to $\leq$12% moisture) could be challenging (Redondo-Cuenca et al., 2008), especially in low-income countries where the power supply is not only expensive but erratic.

Using locally available crops for food to food fortification is considered a sustainable and cost-effective approach in addressing malnutrition among poor populations. A successful food to food fortification program requires that the selected food should be widely consumed by the target population, and its production should be readily available all year round (Dary and Mora, 2002); the product(s) recipes should not extensively vary from existing ones. Gari is a fermented, partly gelatinised, creamy-white granular cassava (*Manihot esculenta* Crantz) product (Oduro et al., 2000; Sanni et al., 2009) widely consumed by individuals regardless of their economic status throughout West Africa sub-region. In Ghana, this economical, ready-to-eat household staple dish is largely consumed by adolescent students (15–25 years) and is a featured menu item in Ghana's school feeding programme (Agbozo et al., 2018). Gari consists of mostly starch and is almost devoid of other nutrients required for growth and development. Therefore, blending protein-rich okara with cassava could significantly bridge the protein and some micronutrient deficiency gaps among the vulnerable in Ghana. Due to gari's low nutritive value, there have been attempts to improve its nutrition using FFSF or okara (Sanni and Sobamiwa, 1994), defatted soybean flour (Twum et al., 2021), and orange-fleshed sweetpotato (Ojo and Akande, 2013; Abano et al., 2020; Atuna et al., 2021). However, the beany flavour associated with FFSF affects the consumer acceptability of products containing FFSF (Iwuoha and Umunnakwe, 1997).

Also, the content of PUFAs in FFSF makes these products susceptible to oxidative rancidity thus producing off-flavours and consequently affecting the overall quality of the product (Park et al., 2018).

In this study, we characterised soymilk and tofu from several soybean varieties in Northern Ghana in terms of proximal composition and sensory acceptability. Then, we explored the proximal composition and desirability of okara originating from only "Afayak" variety and blended with cassava for gari.

MATERIALS AND METHODS

The eight (8) varieties of soybean (i.e., Afayak, Favour, Jenguma, Quarshie, Salintuya 1, Salintuya 2, Songda, and Suong-Pungun) of soybean were obtained from the Council for Scientific and Industrial Research - Savanna Agricultural Research Institute (CSIR-SARI), Nyankpala, Northern Region, Ghana. The production of soymilk and tofu and subsequent determination of their yields was conducted at the Soyplus Facility and Training Centre, Nyankpala. The determination of the total soluble solids content of the soymilk was also assessed at the Spanish Laboratory Complex, University for Development Studies, (UDS) Nyankpala Campus.

Soymilk Extraction

The soybeans of the different varieties were sorted, removing foreign materials, rotten or cracked beans that may affect the taste and quality of the soymilk. Quality soybeans were washed and rinsed with potable water and used to produce the soymilk. About 1 kg from each soybean variety was separately weighed for either soymilk or tofu production. The soybeans were then soaked in 6 L of water for 6 h and then drained and rinsed. About 6 L of water was then added to the soybeans and milled with an electric grinder (F E 05 High-Speed grinding machine). The resulting slurry was cooked by adding another 6 litres of water using Soy-Cow Machine (comprising a boiler, the pressure cooker, the compressor, and a sieve) using steam at a temperature between 110°C and 115°C and pressure of 1 psi. The cooked slurry was then filtered through a filter bag by pressing for about 15 min and the extract (soymilk) was obtained.

Soymilk and Tofu Preparation

Soymilk

The extracted soymilk (5 L) and 150 g of sugar, 20 g of salt, and 15 ml of vanilla essence flavour were added and stirred to obtain a uniform mixture. The soymilk was then refrigerated for about 5 h for sensory analysis, and the samples were obtained for the total soluble solids analysis. Samples of the soymilk were also freeze-dried and milled into powder and packaged for proximate analysis and calcium content determination. All determinations were in triplicate.

Total Soluble Solids Determination

TSS was determined using a Labolan refractometer at room temperature as described by Raja and co-workers (Raja et al., 2014). In determining the TSS, a drop of distilled water was placed on the illumination plate and zeroed. After zeroing, the water was wiped off the illumination plate. A drop of soymilk was placed on the illumination plate. The percent Brix was then read by observing through the eyepiece of the refractometer on a scale of 0 to 32% Brix at 20°C. The process of zeroing was repeated preceding each sample reading.

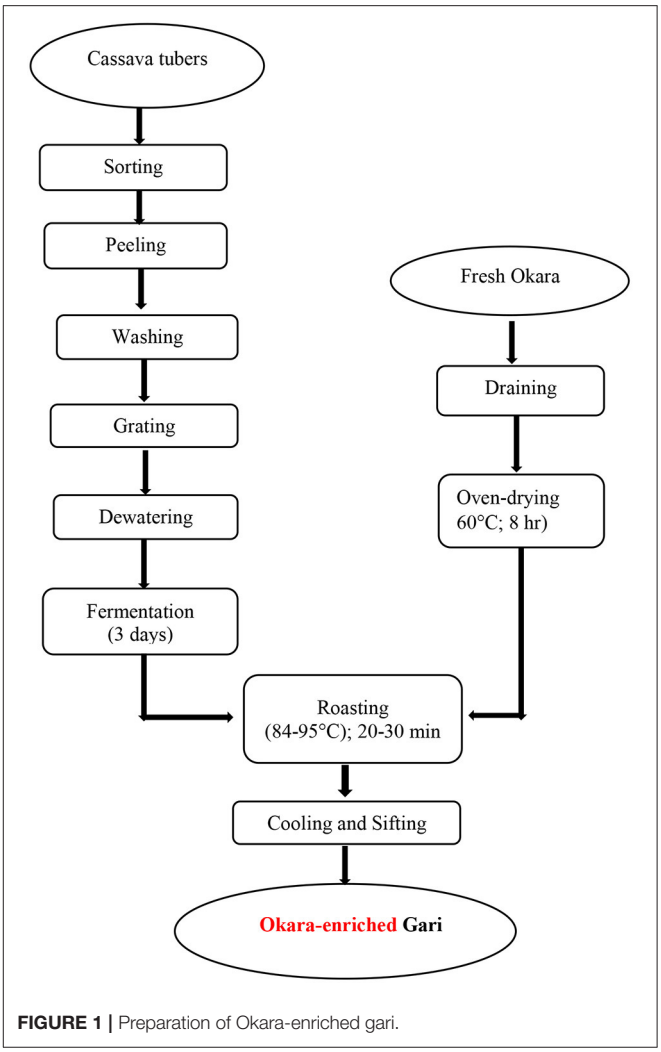

FIGURE 1 | Preparation of Okara-enriched gari.

Tofu

A coagulant solution of citric acid (15 g in 250 L of hot water; 70°C) was prepared and gently poured into 7 L of soymilk while stirring gently with a wooden spatula. The soymilk was allowed to settle for 15 min, and the curds were transferred to a cheesecloth-lined metal box (34 × 23 × 10 cm) and pressed using a compressor for 1 h. The weight was then taken and recorded as the yield of the tofu. The yield of tofu was determined by the procedure of Khatib and co-workers (Khatib et al., 2002).

Gari Recipe Refinement

Sample Collection

Cassava tubers were purchased locally from the Tamale Central market, Northern Region. Okara from Afayak variety was used for the recipe refinement of gari due to its availability.

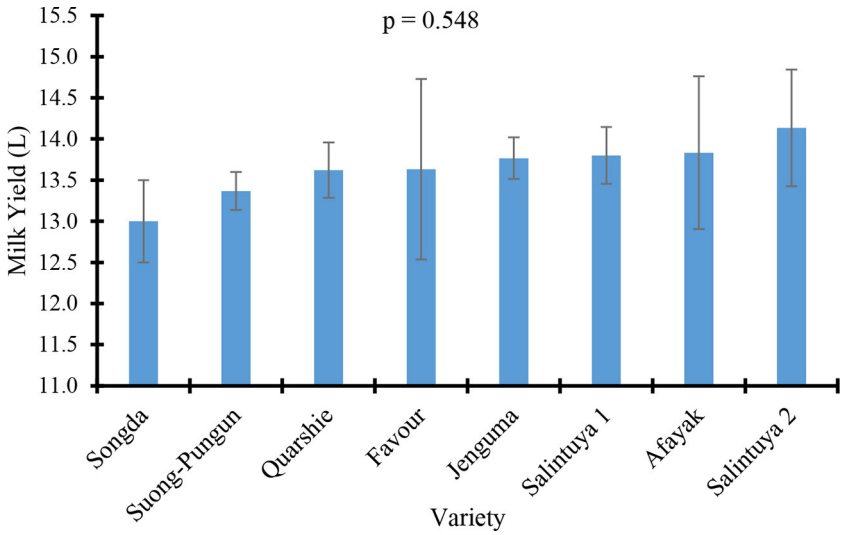

FIGURE 2 | Effect of soybean variety on the yield of soymilk. Bars are means ± SD; $n = 3$. No statistical differences after ANOVA and means compared using LSD test ($p > 0.05$).

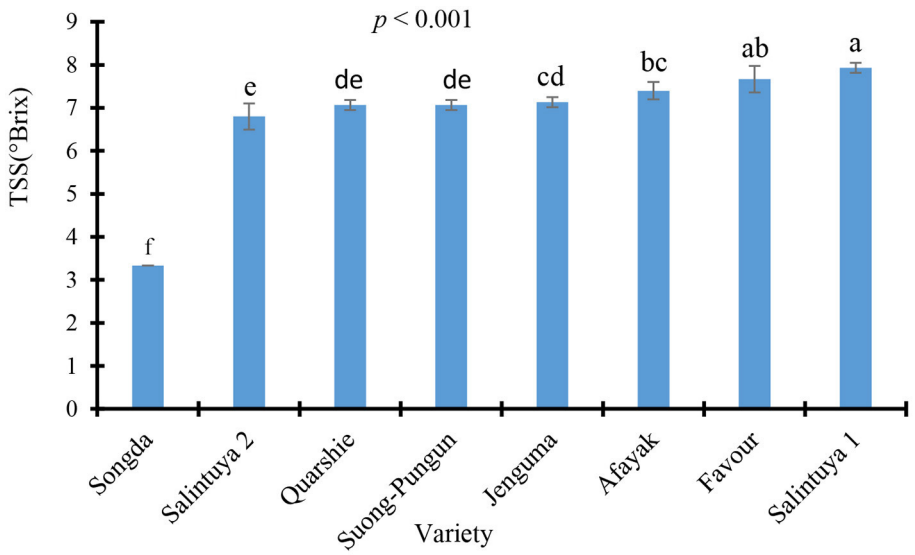

FIGURE 3 | Total soluble solids of soymilk as affected by soybean variety. Bars are means ± SD; $n = 3$. Different letters on top of bars represent statistical differences after ANOVA and means compared using LSD test ($p < 0.05$).

Sample Preparation

Cassava tubers (5 kg) were sorted to remove bruised tubers, washed under running tap water, peeled with a kitchen knife, washed and grated into a mash using a locally fabricated grater. As reported elsewhere, the cassava mash was stored in a porous sack and pressed mechanically on an adjustable press to drain excess water while being allowed to ferment for three (3) days (Oluwamukomi and Adeyemi, 2013).

Processing of Okara-Enriched Gari

The existing 100% cassava gari prepared under the same conditions as the okara-enriched gari was used as the control (**Figure 1**). Fermented cassava and the oven-dried okara were mixed in ratios: 80:20, 70:30, and 50:50 on, as-is basis. The mixtures were then roasted separately at 84–95°C for 25–30 min. The samples were allowed to cool, sifted with a 355 µm sieve, and packaged in high-density polyethylene bags, and kept under refrigerated (4°C) storage until ready for further analysis.

Proximate Composition

The proximate composition and calcium content were determined at the Central Laboratory Facilities, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi. The soymilk and okara-enriched gari

TABLE 1 | Compositional characteristics of soymilk as influenced by variety.

Variety	Proximate parameter (g/100 g)						Ca (mg/kg)
	Moisture	Crude protein	Total ash	Crude fat	Crude fibre	Available CHO	
Afayak	89.35 ±0.02^{e}	29.92 ±0.79^{a}	4.79 ±0.27^{a}	11.13 ±1.86^{a}	0.16 ±0.05^{a}	49.64 ±1.99^{a}	809.0 ±22.4^{c}
Favour	89.21 ±0.02^{f}	31.93 ±6.48^{a}	4.83 ±0.73^{a}	17.88 ±10.17^{a}	0.25 ±0.08^{a}	41.21 ±15.94^{a}	1,013.0 ±35.7^{a}
Jenguma	89.73 ±0.03^{d}	25.70 ±5.86^{a}	4.10 ±0.68^{a}	7.53 ±0.85^{a}	0.30 ±0.14^{a}	57.30 ±7.02^{a}	889.7 ±62.5^{bc}
Quarshie	89.72 ±0.01^{d}	26.70 ±1.86^{a}	4.38 ±0.07^{a}	6.74 ±1.24^{a}	0.26 ±0.11^{a}	57.18 ±2.59^{a}	866.4 ±11.59^{bc}
Salintuya 1	89.90 ±0.01^{c}	33.50 ±3.05^{a}	4.90 ±0.18^{a}	7.63 ±1.26^{a}	0.34 ±0.12^{a}	48.73 ±3.29^{a}	837.7 ±26.9^{bc}
Salintuya 2	89.90 ±0.01^{c}	24.21 ±5.14^{a}	4.25 ±0.46^{a}	8.64 ±0.59^{a}	0.12 ±0.05^{a}	56.23 ±4.01^{a}	1,066.4 ±67.1^{a}
Songda	91.51 ±0.07^{a}	27.25 ±1.78^{a}	4.76 ±0.43^{a}	8.99 ±2.67^{a}	0.27 ±0.13^{a}	53.09 ±3.78^{a}	893.7 ±71.9^{b}
Suong-Pungun	90.04 ±0.06^{b}	28.03 ±1.62^{a}	4.73 ±0.31^{a}	11.32 ±9.41^{a}	0.29 ±0.04^{a}	51.02 ±11.30^{a}	841.4 ±34.9^{bc}
p-value	<0.001	0.124	0.473	0.237	0.147	0.246	<0.001

Values are mean ± SD; n = 3. Means in the same column with the same letters are not significantly different after ANOVA and means compared using LSD test (p > 0.05). Except for Moisture, all values are on dry matter basis.

were analysed in triplicates using standard methods of the Association of Official Analytical Chemists (AOAC, 1995) for moisture (AOAC 925.10) protein (AOAC 960.52), ash (923.03), crude fat (AOAC 922.06) and fibre (AOAC 962.09). Total carbohydrate content was determined by difference as shown in the equation below:

Carbohydrate (%) = 100 – (%Moisture + %Crude protein + %Crude fat + % Crude fibre + %Ash). The calcium content was determined following the oxalate precipitation method as described by Kirk and Sawyer (1991). The amount of calcium present in the samples was calculated using the following formula: Calcium (mg) = 1:2V/W.

where V=volume (mL) of standard KMnO4 solution required to titrate calcium oxalate; W=Weight (mg) of soymilk sample taken to prepare ash.

Determination of Functional Properties of Okara-Enriched Gari

Bulk Density Determination

Bulk density of the okara-enriched gari and the control (100% cassava gari) was determined using the gravimetric method described by Wang and Kinsella (1976) with a modification in the weight of the sample and volume of the graduated cylinder. A sample of 20 g was weighed into a 100 ml graduated cylinder. The cylinder was gently tapped on the benchtop 10 times from a height of 5 m from the ground until a constant volume was observed. The loose bulk density and tapped bulk density were then expressed as the weight per unit volume of the sample (g/ml) after tapping and before tapping, respectively as given in the equations below:

$$\text{Loose Bulk Density} = \frac{\text{weight of the sample (g)}}{\text{Volume of the sample before tapping (ml)}}$$

$$\text{Tapped Bulk Density} = \frac{\text{Weight of the sample (g)}}{\text{Volume of the sample after tapping (ml)}}$$

TABLE 2 | Consumer acceptability of soymilk from different varieties.

Variety	Sensory attributes			
	Aroma	Appearance	Taste	Overall acceptability
Afayak	569.5 (4)^{a}	583.4 (4)^{a}	559.6 (4)^{a}	586.6 (4)^{abc}
Favour	638.0 (4)^{a}	649.4 (4)^{a}	683.0 (4)^{b}	679.0 (4)^{c}
Jenguma	597.4 (4)^{a}	608.0 (4)^{a}	654.3 (4)^{a,b}	628.1 (4)^{a,b,c}
Quarshie	617.6 (4)^{a}	607.4 (4)^{a}	607.0 (4)^{a,b}	577.7 (4)^{a,b,c}
Salintuya 1	553.6 (4)^{a}	570.2 (4)^{a}	559.9 (4)^{a}	579.9 (4)^{a,b,c}
Salintuya 2	669.8 (4)^{a}	639.9 (4)^{a}	592.7 (4)^{a,b}	660.6 (4)^{b,c}
Songda	594.9 (4)^{a}	573.7 (4)^{a}	589.6 (4)^{a,b}	552.6 (4)^{a,b}
Suong-Pungun	563.2 (4)^{a}	572.1 (4)^{a}	558.0 (4)^{a}	539.5 (4)^{a}
p-value	0.054	0.293	0.009	0.002

Values are mean ranks and values in parenthesis are medians. Mean ranks in the same column with the same letters are not significantly different after Kruskal Wallis analysis and compared using the Dunn's test (p > 0.05). 1-dislike extremely, 2-dislike, 3-neither like nor dislike, 4-like, 5-like extremely.

Water Absorption Capacity

The water absorption capacity (WAC) of the okara-enriched gari and the control (100% cassava gari) was determined by using the procedure of Sathe et al. (1982). The procedure was modified by using two (2) grammes of sample and 30 ml of water. Two grammes of the sample were mixed with 30 ml water and shaken for 1 min with a mechanical shaker. The mixture was left to stand undisturbed for 10 min. The mixture was then centrifuged (ROTOFIX 32 A model) at 3,500 rpm for 15 min and the volume of the supernatant was noted. The WAC was calculated using the equation below:

$$\text{WAC} = \frac{\text{Volume of distilled water} \times 100}{\text{Weight of the sample used}}$$

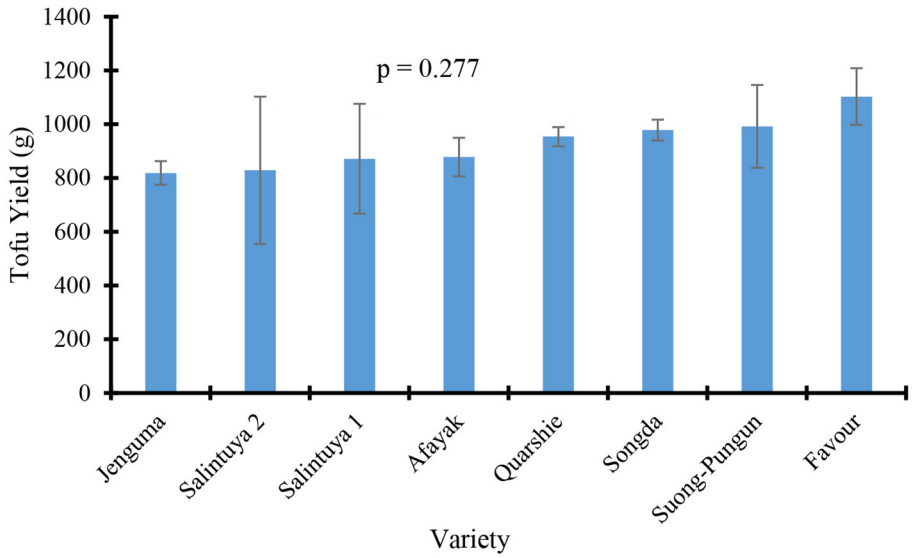

FIGURE 4 | The yield of Tofu as affected by soy variety. Bars are means ± SD; $n = 3$. No statistical differences after ANOVA and means compared using LSD test ($p > 0.05$).

TABLE 3 | Consumer acceptability of Tofu from different varieties.

Variety	Sensory attributes			
	Aroma	Appearance	Chewiness	Overall acceptability
Afayak	611.8 (4)^{a,b}	567.7 (4)^{a}	560.7 (4)^{a}	595.8 (4)^{a,b}
Favour	649.4 (4)^{a,b}	605.7 (4)^{a}	578.5 (4)^{a}	567.7 (4)^{a,b}
Jenguma	586.2 (4)^{a,b}	590.6 (4)^{a}	618.1 (4)^{a}	625.9 (4)^{a,b}
Quarshie	558.6 (4)^{a}	579.5 (4)^{a}	559.6 (4)^{a}	527.2 (4)^{a}
Salintuya 1	688.5 (4)^{b}	647.3 (4)^{a}	646.7 (4)^{a}	666.0 (4)^{b}
Salintuya 2	579.2 (4)^{a,b}	640.8 (4)^{a}	589.8 (4)^{a}	581.2 (4)^{a,b}
Songda	557.5 (4)^{a}	565.8 (4)^{a}	585.4 (4)^{a}	609.5 (4)^{a,b}
Suong-Pungun	572.8 (4)^{a,b}	606.6 (4)^{a}	665.2 (4)^{a}	630.7 (4)^{a,b}
p-value	0.008	0.302	0.063	0.026

Values are mean ranks and values in parenthesis are medians. Mean ranks in the same column with the same letters are not significantly different after Kruskal Wallis analysis and compared using the Dunn's test ($p > 0.05$). 1-dislike extremely, 2-dislike, 3-neither like nor dislike, 4-like, 5-like extremely.

Swelling Capacity Determination

The swelling capacity of the okara-enriched gari and the control (100% cassava gari) was determined by a modification of Onwuka (2005) method. Two (2) grammes of the sample were weighed into a measuring cylinder, and the volume was recorded. Ten (10) millilitres of distilled water was then added to each sample and the solution was allowed to stand for 30 min at room temperature (25°C). The volume of each sample was retaken and recorded. The index of the swelling ability of the sample was calculated using the equation below

$$\text{Swelling capacity} = \frac{\text{Final volume of sample after swelling}}{\text{Initial volume of sample}}$$

Water Activity

The water activity of the gari samples was determined using a water activity metre (model: HBD5-MS2100Wa). The gari sample was weighed to fill about two-thirds of the sample holder. The filled sample holder was placed in the device and was sealed with masking tape after which it was allowed to stand for 10 min before the device was turned on. The temperature and the water activity readings were displayed on the screen of the water activity metre 5 min after it was switched on.

pH

The pH of gari samples was determined as described by the method of AOAC (2005) using the pH metre (JENWAY model: 3510).

Sensory Evaluation

The soymilk, tofu, and okara-enriched gari were separately evaluated by selected undergraduate students of UDS, Nyankpala campus. There were 50-untrained panellists (19–32 years). The panellists were asked to evaluate the *appearance, aroma, taste, sweetness, and overall acceptability* for soymilk and the *aroma, appearance, chewiness, and overall acceptability* of tofu samples and the *colour, taste, texture, aroma, and overall acceptability* of the okara-enriched gari samples by using a 5-point hedonic scale (*1-dislike extremely, 2-dislike, 3-neither like nor dislike, 4-like, 5-like extremely*) that was employed for all evaluations. The soymilk (20 ml) and okara-enriched gari (10 g) samples were served in 3-digit coded disposable cups and evaluated. The fresh tofu from each variety was cut into cubes (2 g) and deep-fried separately in vegetable oil (frytol) for 10 min and then served on disposable plates for the sensory assessment. To minimise sensory fatigue, four (4) products of soymilk and tofu were separately evaluated in a day. However, for the okara-enriched

gari, all the formulations including the existing 100% cassava gari were presented randomly at the same time. The panellist used water in between samples as a palate cleanser.

Statistical Analysis

Statistical analysis was carried out using Minitab statistical software v.16.2.4. (Minitab® Inc. USA). The effect of soybean variety on the yields of soymilk, tofu, and okara, the proximate composition of soymilk and okara-enriched gari, total soluble solids of the soymilk, and the functional properties of okara-enriched gari were analysed using One-way Analysis of Variance (ANOVA). The sensory data of soymilk, tofu and okara-enriched gari were analysed using Kruskal Wallis non-parametric data analysis procedure. The Fisher's Least Significant Difference (LSD) procedure was used to compare differences between means when the ANOVA result was significant ($p < 0.05$). For the sensory data, the Dunn test was used to compare differences among means when the ANOVA result was significant ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Variety on Soymilk Yield

From **Figure 2**, soymilk yield did not vary significantly ($p = 0.548$) among the eight (8) soybean varieties ranging between 13.00 and 14.13 L. This finding supports previous work by Bhardwaj et al. (1999) who observed that genotype had no significant effect on soymilk yield. Thus, all the varieties are equally suitable for milk extraction.

The total soluble solids (TSS) of the eight (8) soybean varieties showed marked ($p < 0.001$) differences with Songda recording the lowest TSS (3.33°Brix) (**Figure 3**). The low TSS recorded in Songda soymilk could be ascribed to the higher moisture content (**Table 1**) of the soymilk. The current findings deviate from earlier works by Khatib et al. (2002) who reported that soymilk produced from soybean varieties did not differ significantly in TSS.

Effect of Soy Variety on Compositional Quality of Soymilk

Generally, soybean variety showed no significant ($p > 0.05$) effect on the proximate parameters evaluated except for the moisture ($p < 0.001$). The moisture content of the soymilk as presented in **Table 1** ranged from 89.21 to 91.51%, similar to those reported in previous studies (Khatib et al., 2002; Ugochi et al., 2015; Abagoshu et al., 2017). Although the protein content did not statistically vary among varieties, it is noteworthy that soymilk produced from Salintuya 1 recorded the highest protein (33.50%) content while soymilk from Salintuya 2 also had the least protein content of 24.21%. The finding in this study contradicts those of Min and Martin who reported that the variety of soybean had a significant effect on the protein content of soymilk (Min et al., 2005a). The total ash content of the soymilk, which represents the total mineral content of soymilk samples in this study ranged from 4.1 to 4.9%. Salintuya 1 recorded the highest total ash content compared to the other varieties.

The crude fat content of the soymilk ranged from 6.74 to 17.88% and did not differ markedly among the soy varieties (**Table 1**). The highest crude fat content was recorded in soymilk produced from Favour. The crude fibre content of soymilk was relatively low varying from 0.12 to 0.34% and did not significantly ($p = 0.147$) vary among the varieties. The available carbohydrate content among the varieties did not vary significantly ($p = 0.246$) and ranged from 41.21 to 57.30%. The calcium content of the soymilk ranged from 809.0 to 1,066.4 mg/kg and varied significantly ($p < 0.001$) among varieties.

Consumer Acceptability of Soymilk

The consumer acceptability scores of soymilk made from the soy varieties evaluated in this study were all within the acceptable limit (sensory score = 4), indicating the soymilk-making potential of all the soybean varieties used in the current study (**Table 2**). Varieties did not differ markedly ($p > 0.05$) in terms of their aroma and appearance. However, Favour recorded a significantly higher score for taste ($p = 0.009$; mean rank = 683) and overall acceptability ($p = 0.002$; mean rank = 679). Based on these sensory scores, Favour could be promoted among varieties for soymilk production. The sensory quality of soymilk has been reported to be affected by genotype, processing method, and environmental conditions (Min et al., 2005b) among other factors.

Tofu

Effect of Soy Variety on Tofu Yield

The yield of fresh tofu did not vary significantly ($p = 0.277$) among varieties contrary to the findings of Wang et al. (1983) who reported significant varietal differences in fresh tofu. The discrepancies in the findings could be attributed to the differences in the water content of the fresh tofu. The yield of tofu ranged from 818.0 to 1102.5 g (**Figure 4**).

Consumer Acceptability of Tofu

The consumer acceptability of tofu processed from the eight (8) varieties of soybean is shown in **Table 3**. Generally, consumers liked tofu prepared from all the eight varieties considering that the median sensory score for all the attributes was 4. Salintuya 1, however, presented significantly higher mean ranks for aroma ($p = 0.008$; mean rank = 688.5) and overall acceptability ($p = 0.026$; mean rank = 666.0) than other varieties. This is an indication that the soybean varieties evaluated in this study are all desirable for tofu production.

Soy Residue (Okara)

Effect of Soy Variety on Okara Yield

The okara yield of soybean varieties did not show marked ($p = 0.082$) differences ranging from 3.17 to 3.97 kg (**Figure 5**). The fresh okara yield obtained in the present study is slightly higher than the 1.1–1.2 kg of fresh okara per kilogramme of soybean processed for either soymilk or tofu reported in other studies (Khare et al., 1995; Guimarães et al., 2018). The amount of water in the fresh okara and the efficiency of pressing to remove the liquid fraction could explain the differences in fresh okara yield in this study relative to the earlier studies.

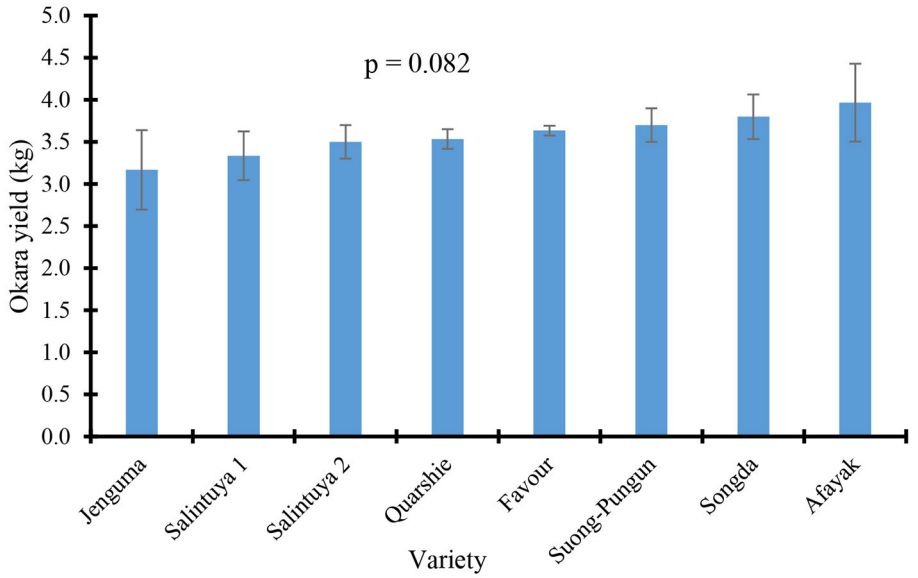

FIGURE 5 | Soy residue (Okara) yield as influenced by soybean variety. Bars are means ± SD; $n = 3$. No statistical differences after ANOVA and means compared using LSD test ($p > 0.05$).

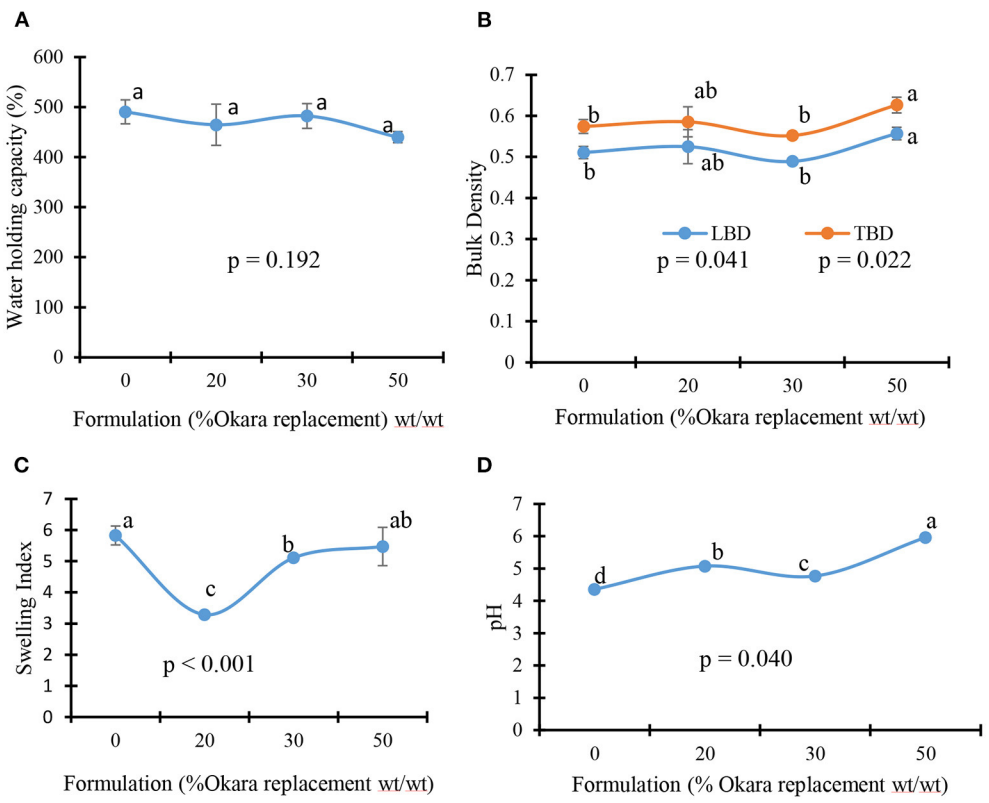

FIGURE 6 | Water absorption capacity **(A)**, Bulk density **(B)**, Swelling capacity **(C)**, and pH **(D)** of okara-enriched cassava grits. Values are means ± SD; $n = 3$. Data points with the same letters are not significantly different after ANOVA and means compared using LSD test ($p > 0.05$). Average pH of fresh okara = 6.3.

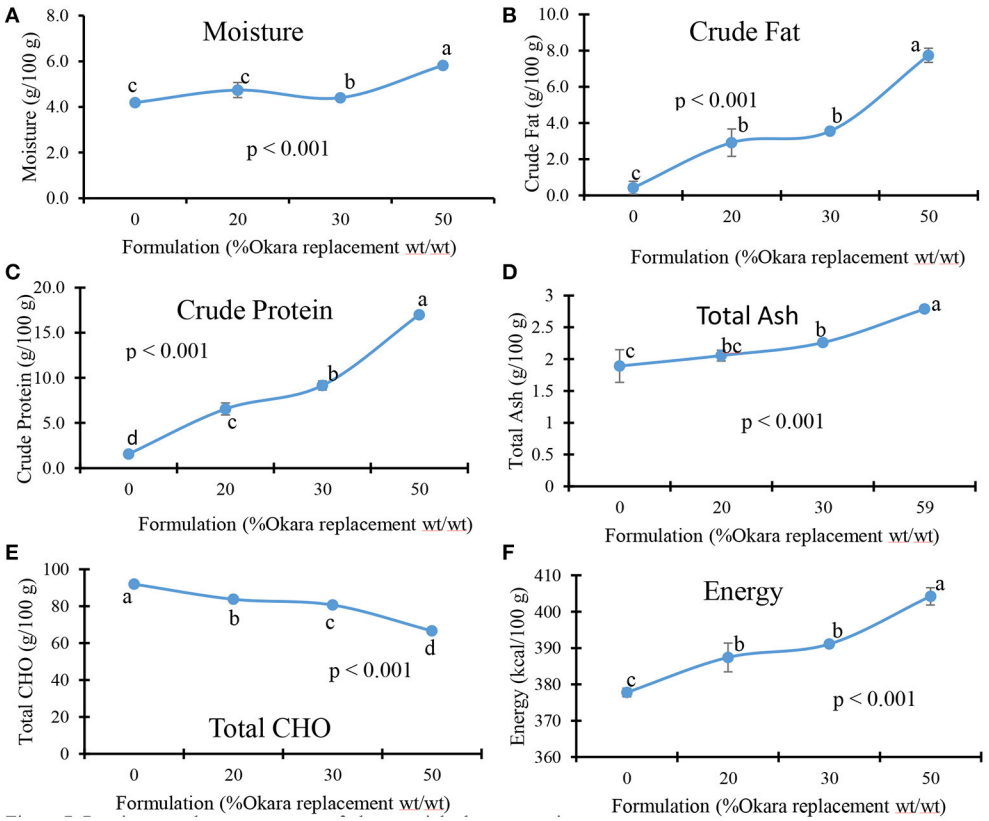

FIGURE 7 | Proximate and energy content of okara-enriched cassava grits. Except for moisture, all determinations are on dry matter basis. Values are means ± SD; $n = 3$. Data points with the same letters are not significantly different after ANOVA and means compared using LSD test ($p > 0.05$). Proximate [Moisture **(A)**, Crude fat **(B)**, Crude Protein **(C)**, Total Ash **(D)**, Total CHO **(E)**] and energy **(F)** content of okara-enriched cassava grits.

Recipe Refinement of Gari With Okara

Functional and Physicochemical Properties of Cassava-Okara Gari

Partial substitution of cassava with okara at 20, 30, and 50% did not significantly ($p = 0.192$) affect the water absorption capacity of the cassava-okara composite gari relative to the 100% cassava gari (**Figure 6A**). The water absorption capacity measures the volume occupied by the starch after swelling in excess water, which maintains the integrity of starch in aqueous dispersion (Bajo et al., 2019). It is an important index for the development of ready-to-eat food products such as gari because a high absorption capacity may assure product cohesiveness (Houssou and Ayernor, 2002).

Bulk densities (tapped or loose) measure the heaviness of flour products and are largely influenced by the particle size and density of the product (Bajo et al., 2019). It is essential in determining packaging requirements and material handling (Huang et al., 2019). In the current study, both the loose and tapped bulk densities of the cassava-okara composite gari followed a similar trend with the 50% okara enrichment recording a significantly higher tapped (0.63 vs. 0.57 g/ml; $p = 0.022$) and loose (0.56 vs. 0.51; $p = 0.041$) bulk densities (**Figure 6B**). This finding lends credence to previous works by Ilelaboye and Ogunsina (2018), who reported increased bulk density with the addition of okara flour. The values obtained for the tapped and loose bulk densities are comparable to those reported elsewhere (Oluwamukomi and Adeyemi, 2013) for okara-melon seed enriched gari.

The swelling capacity measures the ability of particles to imbibe water and swell. It is an essential sensory and mechanical attribute that determines the quality of the product, including mouldability (Udofia et al., 2011). The swelling capacity in the present study (**Figure 6C**) shows that the blend with the highest level of okara incorporation (50% okara-enriched) had a similar swelling capacity (5.5 vs. 5.8) as the existing 100% cassava gari. The higher the swelling capacity, the greater its suitability for use in most West African dishes such as "eba" in which greater mouldability is preferred (Apea-Bah et al., 2009). The decline in the swelling capacity of the composited blends is expected because the swelling capacity of a product is a function of the starch content (ratio of amylose to amylopectin) (Ilelaboye and Ogunsina, 2018) and the extent of gelatinisation of starch (Huang et al., 2019). The low starch content of okara relative to cassava could account for the decline in swelling capacity since starch enhances swelling. The current findings agree with previous works that showed that swelling capacity decrease as the

levels of soy flour increase in cassava-soy-melon composite gari (Oluwamukomi and Adeyemi, 2013).

The cassava-okara composite gari had a significantly higher pH (4.36 vs. 5.96; $p = 0.040$) than the existing 100% cassava gari (**Figure 6D**). The pH of the composited blends steadily increased with increasing incorporation levels of okara. The general increase in pH with okara incorporation could be because the fresh okara with a pH of 6.3 was first dried before compositing with the fermented cassava grits. Hence the okara did not undergo any fermentation. The pH of gari is a function of fermentation and an indicator of its storage life. The lower the pH, the better the storage life as it inhibits microbial activity. The blend with the highest okara incorporation with pH 5.96 is almost similar to those obtained in a previous work when cassava grits were supplanted with soy-melon flour (Oluwamukomi and Adeyemi, 2013).

Proximate Composition and Energy Content

From **Figures 7A–E**, all proximate parameters steadily increased as the level of okara increased in the cassava-okara blends except for total carbohydrates content. The moisture content of the blends ranged from 4.2 to 5.8% (**Figure 7A**) and was all below the 12% threshold per Codex standards for gari (FAO/WHO, 1995). The significantly higher moisture content ($p < 0.001$) of the blend with the highest okara inclusion could be due to the relatively high insoluble fibre and protein contents of okara. Due to the hygroscopic nature of protein and fibre, the blends retained more moisture (Nilufer-Erdil et al., 2012) relative to the existing 100% cassava gari.

The crude fat content of the 50% okara-enriched blend was significantly ($p < 0.001$; 7.7/100 g vs. 0.4/100 g) higher than the existing 100% cassava grits (**Figure 7B**). The blend with the highest okara incorporation (50% okara enrichment) was almost 19-times higher than the cassava-only gari. This was expected because okara contains a considerable amount of fat and could account for the increased fat content of the blends (Grizotto et al., 2010). Similar trends were reported for sorghum-okara composited flours for local staples in an earlier study (Uzo-Peters and Ola, 2020).

The crude protein content increased significantly ($p < 0.001$) with okara substitution (**Figure 7C**) almost 11-fold in the 50% okara-enriched gari compared with the cassava-only gari (**Figure 7C**). The crude protein of the composite blends ranged from 6.5 to 17.0/100 g. The increase in the crude protein content of the composite blends is expected because okara contains protein since it is a by-product of soybean regarded as a powerhouse of quality protein (Grizotto et al., 2010). This increase in crude protein content with okara substitution will complement the protein profile of cassava which is limited in most essential amino acids. Therefore, the okara-enriched gari may be inferred as capable of supporting the growth of school-age children who often consume "gari" as convenience food. For example, to meet the Recommended Daily Allowance of 34 g/day for protein for a school child (11–14 years) (Oluwamukomi and Adeyemi, 2013), he/she would need to consume about 2.13 kg of regular gari per day, but with the 50% okara-enriched gari he/she will need to consume only 0.2 kg.

The total ash content of the blends also steadily increased with okara substitution as presented in **Figure 7D**. Comparatively, the 50% okara-enriched composite blend had a significantly (2.79/100 g vs. 1.89/100 g; $p < 0.001$) higher total ash content, nearly 1.5 times compared with the existing 100% cassava gari (**Figure 7D**). The high total ash content of composite blend with okara substitution indicates that the mineral content of the okara-enriched samples would be enhanced since total ash may represent the mineral content of a food product. The current finding corroborates previous works (Ostermann Porcel et al., 2017) that also observed an increased total ash content of okara-enriched bread.

On the contrary, the total carbohydrates followed a downward trend with the increased substitution of okara. The existing 100% cassava gari was significantly (91.9/100 g vs. 66.7/100 g; $p < 0.001$) higher (**Figure 7E**). The low carbohydrate content of okara (O'Toole, 1999) could account for the decrease in total carbohydrates with increased okara substitution. A similar decrease in carbohydrate was reported in okara-enriched bread (Ostermann Porcel et al., 2017), gari enriched with okara and melon seeds (Oluwamukomi and Adeyemi, 2013), and sorghum-okara composite blend (Uzo-Peters and Ola, 2020).

The energy value of the composite blends increased markedly ($p < 0.001$) with the increased inclusion level of okara (**Figure 7F**). The energy content in the 50% okara-enriched blend increased almost 26.5 kcal/100 g more than the existing 100% cassava gari. This observation is consistent with earlier works that showed increased caloric value in gari enriched with full-fat

TABLE 4 | Consumer acceptability of okara-enriched gari and existing 100% cassava gari.

Formulation (%Okara replacement wt/wt)	Sensory attributes				
	Colour	Taste	Texture	Aroma	Overall acceptability
0	126.2 (4)^{b}	104.2 (4)^{a}	95.2 (4)^{a}	114.0 (4)^{a}	112.2 (4)^{a}
20	93.6 (4)^{a}	109.8 (4)^{a}	106.2 (4)^{a}	97.3 (4)^{a}	107.0 (4)^{a}
30	99.8 (4)^{a,b}	95.5 (4)^{a}	106.1 (4)^{a}	96.1 (4)^{a}	96.0 (4)^{a}
50	82.5 (3)^{a}	92.5 (4)^{a}	94.5 (4)^{a}	94.5 (4)^{a}	86.9 (4)^{a}
p-value	<0.001	0.424	0.592	0.299	0.125

Values are mean ranks and values in parenthesis are medians. Mean ranks in the same column with the same letters are not significantly different after Kruskal Wallis analysis and compared using the Dunn's test ($p > 0.05$). 1-dislike extremely, 2-dislike, 3-neither like nor dislike, 4-like, 5-like extremely.

soy (Oluwamukomi and Adeyemi, 2013). This increase in caloric value with the increased substitution of okara could be attributed to the relatively high-fat content in soybeans and its derivatives such as okara since fat is a high energy-yielding nutrient compared with carbohydrates and protein (Ikya et al., 2013). Higher calorie-dense foods may pose an economic advantage as smaller amounts would have to be consumed to meet the caloric needs of the body compared to lower calorie-dense foods.

Consumer Acceptability

Generally, the okara-enriched blends were all acceptable (sensory score = 4) by consumers for all the sensory attributes assessed (**Table 4**). All the sensory attributes did not significantly differ ($p > 0.05$) except for colour that showed marked significant ($p < 0.001$) difference among the formulations (**Table 4**). The findings in this study suggest that substituting okara up to 50% with cassava is likely to be accepted by Ghanaian consumers in terms of taste, texture, aroma, and overall acceptability. Thus, there is a great potential of improving the nutritional quality of the existing 100% cassava gari using low-cost okara with a minimal effect on most of the sensorial properties. Generally, the colour of gari is creamy-white (Alamu et al., 2019) because it is traditionally processed from white cassava and this has influenced most Ghanaian consumers to perceive that gari must be white to be acceptable. Therefore, compositing okara flour beyond 30% (w/w) with white cassava darkened the product, hence consumers' indifferent posture. The brownish colour of gari could be directly related to the increase in fibre content (Kassahun and Gebremicheal, 2019) as well as caramelisation and Maillard reactions, as the protein contributed by okara may have reacted with sugar during the roasting process. Similar findings have been reported on declined colour scores with increased okara enrichment in bread (Kassahun and Gebremicheal, 2019), cassava-orange fleshed sweetpotato gari, "eba" (Atuna et al., 2021), and okara-enriched biscuits (Grizotto et al., 2010).

The anti-nutritional factors such as trypsin inhibitors and phytate in soymilk and tofu as well as cyanogenic glucosides, in the cassava-based gari, were not considered in the current study and have been acknowledged as the limitation.

CONCLUSION AND RECOMMENDATION

The eight (8) soybean varieties evaluated were generally desirable for soymilk and tofu production based on yield. The sensory data showed that, though consumers generally rated soymilk and tofu from all soybean varieties high (score = 4) for all sensory attributes, Favour and Salintuya 1 were more desirable for soymilk and tofu production, respectively. For every 1 kg of soybean used to produce either soymilk or tofu, almost 3.17–3.97 kg of fresh okara was produced. Recipe refinement of gari with dried okara up to 50% (w/w) inclusion showed increased nutritional composition in the okara-enriched gari. However, beyond 30% (w/w) okara inclusion, the sensory properties of the okara-enriched gari are less preferred by consumers. Thus, recipe refinements using the desired varieties and compositing okara with cassava may help fill the protein gap among the vulnerable group in Ghana and the West African Sub-region by improving the protein quality of ready-to-eat foods such as gari.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

RA, FCA, FKA, and ND conceived and designed experiments. RA, VA, SF, EB, and GK performed the research, analysed, and visualised the data. RA, EG, and MT wrote the manuscript. FCA, AD, FKA, and JL critically revised the draft manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Rediscovering the Potential of Multifaceted Orphan Legume Grasspea- a Sustainable Resource With High Nutritional Values

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The genus *Lathyrus* consists of more than 184 herbaceous annual and perennial species suitable for multifaceted sustainable food and feed production system in the arid and semi-arid regions of the world. The grasspea is a promising source of protein nutrition. However, its potential is not being utilized fully due to the presence of neurotoxin content (β-N-oxalyl-l-α, β diaminopropionic acid, β-ODAP), a causal agent of non-reversible lower limbs paralysis. The high protein contents in seeds and leaves with ∼90% digestibility make it sustainable super food to beat protein malnutrition in future. Therefore, it is desired to breed new grasspea cultivars with low β-ODAP contents. Limited research has been carried out to date about this feature. A draft genome sequence of grasspea has been recently published that is expected to play a vital role in breeding and identifying the genes responsible for biosynthesis pathway of β-ODAP contents in grasspea. Efforts to increase awareness about the importance of genus *Lathyrus* and detoxify β-ODAP in grasspea are desired and are in progress. Presently, in South Asia, systematic and dedicated efforts to support the farmers in the grasspea growing regions by disseminating low β-ODAP varieties has resulted in a considerable improvement in reducing the incidence of neurolathyrism. It is expected that the situation will improve further by mainstreaming grasspea cultivation by implementing different approaches such as the development and use of low β-ODAP varieties, strengthening government policies and improved detox methods. The present review provides insight into the multifaceted characteristics of sustainable nutritious grasspea in the global and Indian perspective.

Keywords: climate-resilient, grasspea, multifaceted, orphan legume, sustainable

INTRODUCTION

The human population growth rate is very fast compared to the food yield per hectare as per the report of World Food Programme, 2018 (1). Therefore, there is a need to boost our food grain production by 70% (taking 2015 as the base year) to feed the 1.66 billion people and meet the Sustainable Development Goal (SDG) targets by 2030. Despite the fact that the hunger has

decreased globally since 2000, the yield plateau in all major crops and increased malnutrition makes hidden hunger severe in many parts of world (2).The number of undernourished people in the world has continued to increase. If recent trends are not reversed, the SDG 2.1 zero hunger target will not be met. Sustainable development is only possible in communities where malnutrition is eradicated. The world may not achieve the global nutrition targets of ensuring access to safe, nutritious and sufficient food for all and eradicating all forms of malnutrition (3). Globally in 2020, the scale challenges in nutritional imbalances amounting to two billion people lacking key micronutrients like iron and vitamin A; 149 million children under age five were estimated to be stunted; 1.9 billion adults are overweight or obese, while 462 million are underweight (4) and out of 141 countries analyzed, 88% of countries face serious burden of more than one form of malnutrition and 29% have high levels all forms of malnutrition (stunted growth, obese and overweight) (5). Utilizing plant genetic resources of various climate-smart species, including underutilized and neglected crops, will be of great significance to achieve SDG's. The present communication reviews grasspea (*Lathyrus sativus* L.) a member of family Fabaceae (*Leguminosae*), subfamily *Papilionoideae*, and tribe *Vicieae,* which is an underutilized and neglected food, feed and pharmaceutically important crop that shows resistance to harsh environmental conditions (6, 7) like drought, heat, soil infertility, floods and many ranges of biotic stresses. It grows either as cultivated crop or weed under natural conditions in South, Southeast Asia, Middle East, Eastern Europe and in many other countries of the world. The cultivation of grasspea requires minimal inputs and cost; thereby, it can be successfully incorporated in the conservation agriculture and breeding programmes for developing climate smart (biotic and abiotic stress-resistant, nutrition rich) varieties. This crop deserves a sustainable and nutritionally rich status and therefore the rediscovery of its potential as food and nutritional security in reference to the global and Indian perspective is desirable.

ORIGIN AND DOMESTICATION OF GRASSPEA

The word "*Lathyrus*" is derived from the ancient Greek word lathuros which means "exciting," and refers to the aphrodisiac properties of grasspea (8). The grasspea is also known by many names (countries in parenthesis) like *chickling vetch, chickling pea, dog toothed pea* (America, Britain); *khesari* (Bangladesh); *san lee do* (China); *fovetta* (Cyprus); *sabberi* (Ethiopia); *gisette* (France); *khesari dal, lang, chural, latri, lakhori, batura, tiwra* (India); *pisellobrettone* (Italy); *kheshari* (Nepal); *matri, mattra, kesari* (Pakistan); *almorta* (Spain); *gilban* (Sudan); *murdumuk* (Turkey) and *pharetta, garbanzo* (Venezuela) (9). The current list of 184 taxonomically accepted names of the genus *Lathyrus* can be accessed through Plants of the World Online, Royal Botanic Gardens, Kew, United Kingdom. The genus *Lathyrus* consists of more than 160 annual and perennial species (10, 11) and subspecies (12) belonging to 15 divisions based on morphological features (13). The common uses of important species in the genus *Lathyrus* is given in **Figure 1**

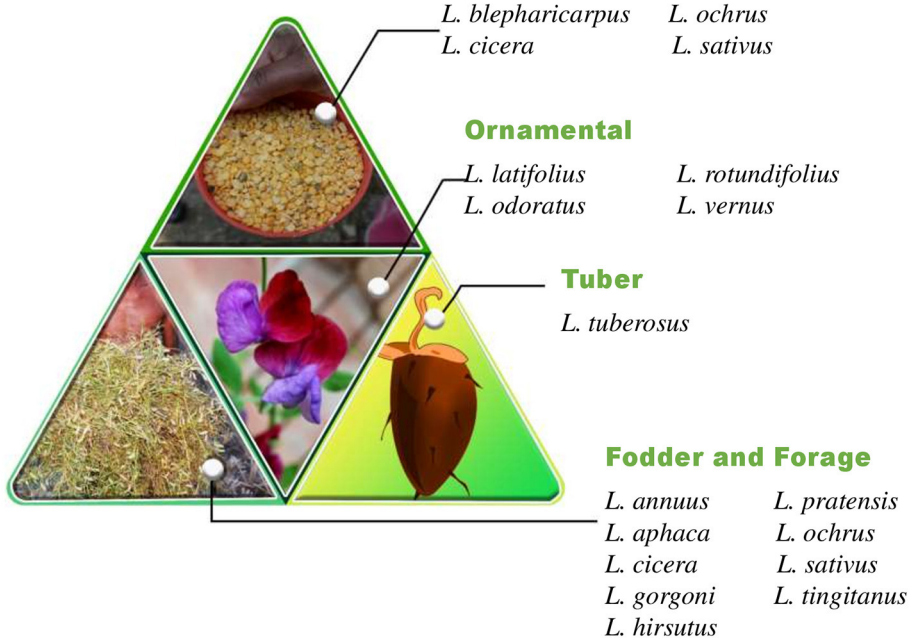

FIGURE 1 | *Lathyrus* species and its uses (14, 15).

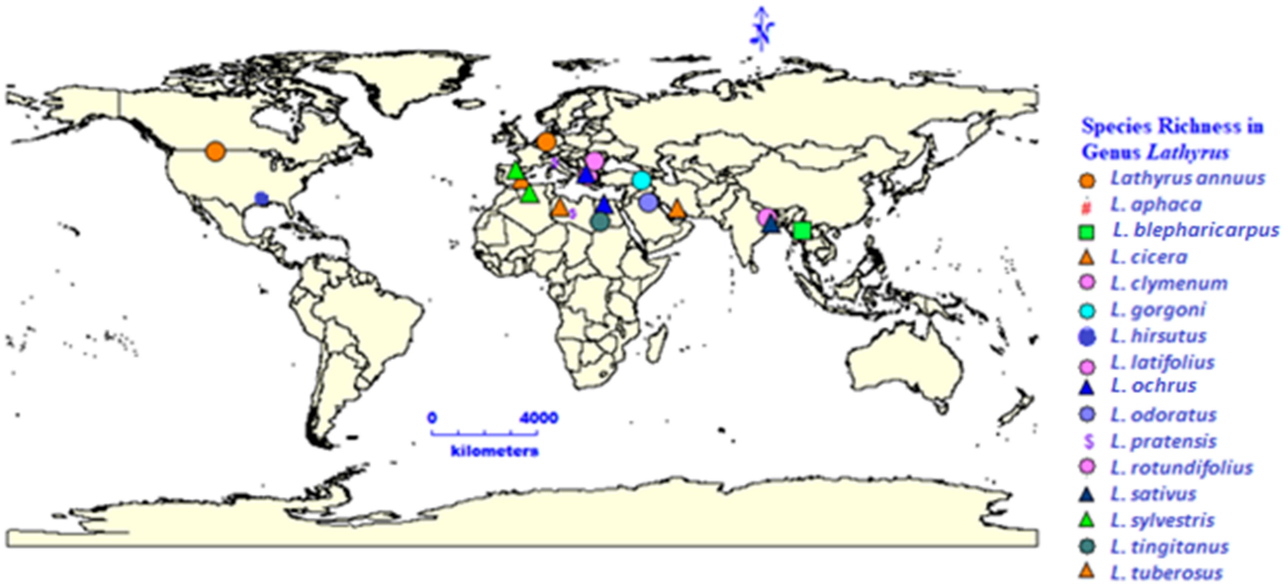

FIGURE 2 | Species richness in genus *Lathyrus* and its distribution.

and the global distribution of species in genus *Lathyrus* in the **Figure 2** (14). *L. sativus*, *L. cicera* L., and *L. hirsutus* L. are the most extensively cultivated species for food and feed whereas *L. latifolius* L. and *L. odoratus* L. are gorgeous looking ornamental plants produced commercially in Europe (14, 15).

The cultivation and domestication of many important annuals such as wheat, pea, grasspea and lentil started during early neolithic era around the 6th millennium Before Common Era (BCE) in the West Asia (Irano-Turanian regions). Later, these crops spread to the temperate Mediterranean region; moving further to tropics and sub-tropics in the northern hemisphere including East Africa, South Asia and naturalized in South America over time (16). Grasspea is grown as a pulse and fodder crop in Southeast Asian countries since time immemorial. The origin and distribution of crops have a long history and in several cases uncertainty is due to the discrepancies in the description of primary and secondary centers of diversity and natural spread in later stages (17). The earliest archaeological evidences dates back from the 8th millennium BCE to the Neolithic age from the village of Jamro lying at the foothills of the Zagros Mountains in Northern Iraq (close to the Turkish and Iranian borders) at an altitude of 800 msl (18, 19). The important archaeological evidences of grasspea are noted in adjacent areas of Tepe Sadz (7500–5700 BCE) and Ali Kosh (9500–7600 BC) in Iran (19) and in the Gangetic plains, India (2000–1500 BCE) (20) with presumption of its introduction from West Asia. Probable remains of *L. cicera* have also been reported at Azmaska Moghila, in Bulgaria 7000 BCE (21). Several archaeobotanical and phytogeographical evidences proved that grasspea was initially domesticated in the Balkan Peninsula during the early neolithic era, around the 6th millennium BCE (22).

BOTANY AND TAXONOMY OF GRASSPEA

Grasspea is a herbaceous annual with a well-developed taproot system that is greatly branched, straggling, or ascending. Small, cylindrical, branching nodules cover the rootlets. The stems are quadrangular and extremely slender, having winged margins. Pinnately opposite leaves have two or three pairs of lanceolate leaflets that terminate in a simple or branching tendril. The leaflets are sessile, entire, and cuneate at the base and acuminate at the top. The stipules are triangular to oval in shape with basal appendage. The flowers are axillary, solitary with varied colors *viz.* blue, violet blue, pink, dark pink, light yellow, white or white with purple stripes. The blue flower is the most common, and the variation in pigmentation is due to four genes (9). The peduncle is relatively long (3–5 cm) with 2-minute bracts. It is primarily a self-pollinated species, but has a high rate of out-crossing, ranging from 9.8 to 27.8%. Insects like honey bees are the main pollinators and also twisted keels with a slight opening in flowers aid in cross pollination (23, 24). Standard petals are erect and clawed. Wing petals are ovate, clawed and obtuse at the top. The keel is somewhat twisted, boat-shaped, completely split dorsally and ventrally near the base, which helps in insect pollination. The colors of the keel have a lighter shade compared to the wings with different color tinges. The stamens are diadelphous (9 + 1) and filiform, having vexillary stamens. The anthers are bright yellow in color and ellipsoid in shape. The stigmas are upturned and enlarged at the tip. The stigma is spatulate, glandular-papillate and terminal. Ovaries are sessile with 5–8 ovules. The pods are oblong, flat, and slightly bulging above the seeds, with a length of 2.5–4.5 cm, with a width of 0.6–1.0 cm, and slightly curled tips (25). The two-winged, short-beaked dorsal regions of the pod contain 3–5 small seeds. The seeds are angled, wedge-shaped, and come

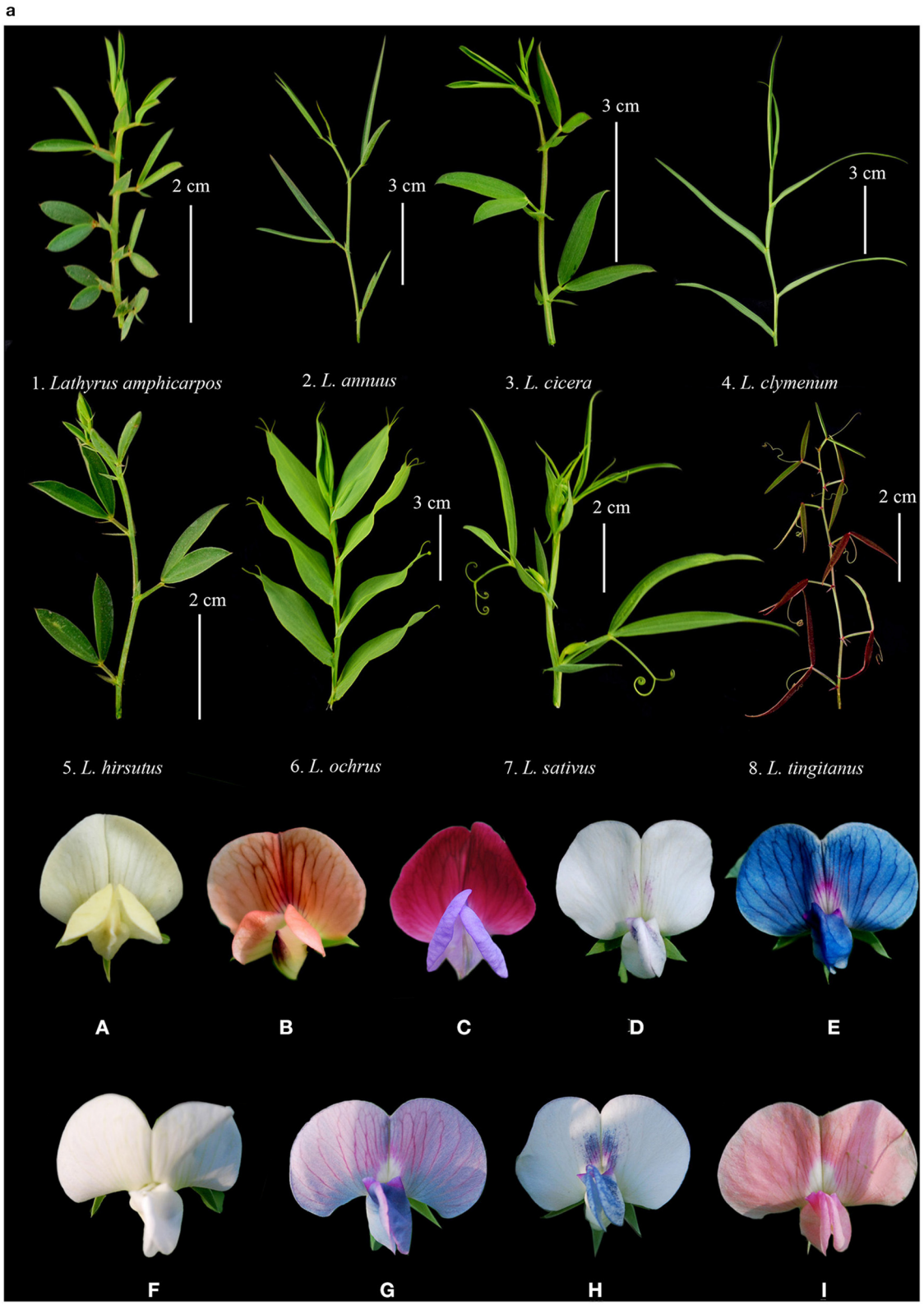

FIGURE 3 | Continued

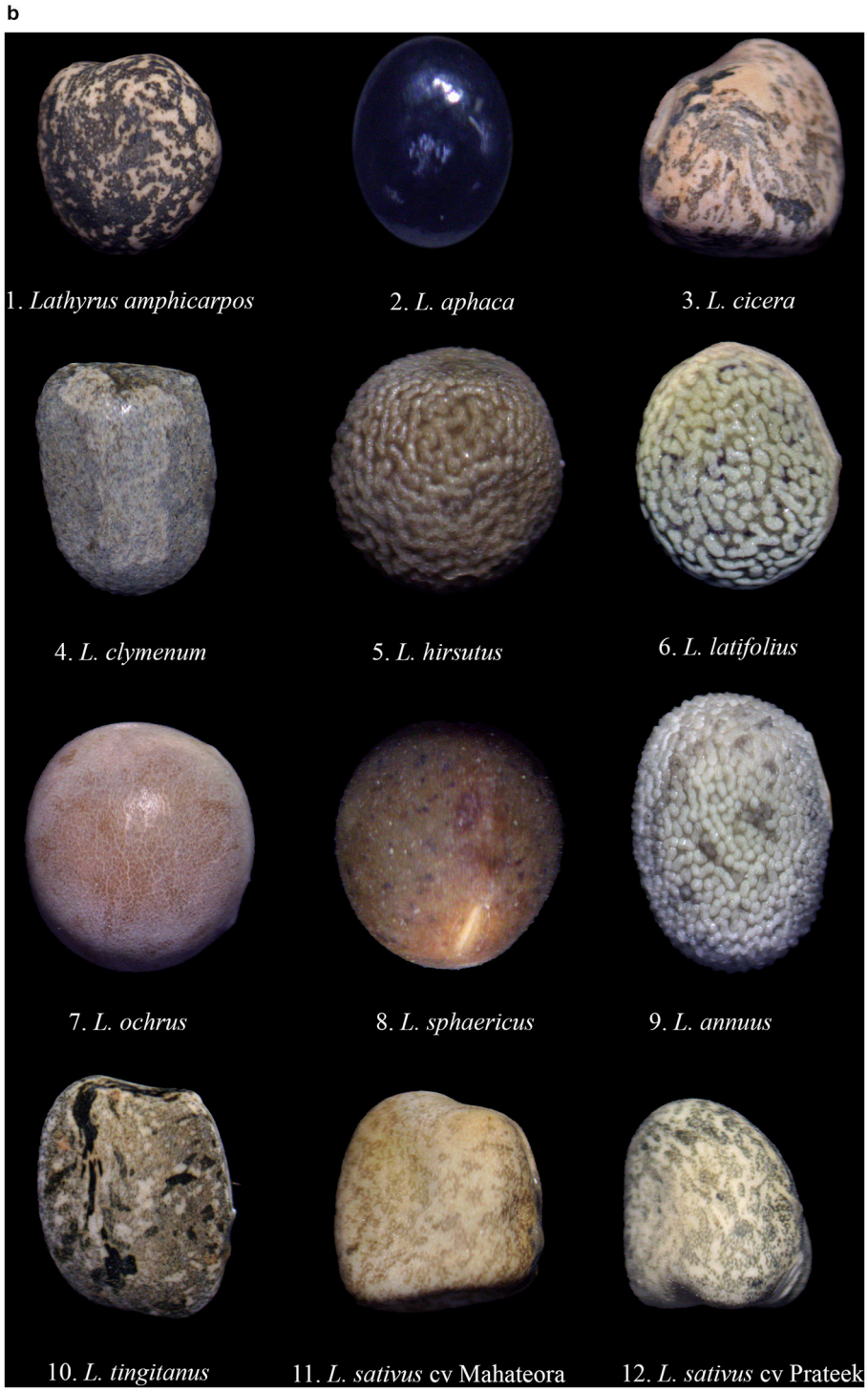

FIGURE 3 | (a) Leaf variation in different species of *Lathyrus* (top) and flower color variation (bottom) - (A) *L. aphaca*; (B) *L. cicera*; (C) *L. odoratus*; (D–I) *L. sativus*. **(b)** Seed color variation in different species of genus *Lathyrus*.

in various colors, including white, brownish-gray, yellow, and are spotted or mottled (26). The hilum is elliptic with yellow to pinkish yellow cotyledons. The seeds germinate hypogeally with purplish-green epicotyls. The morphological variation in leaf, flower and seeds of some species of *Lathyrus* are depicted in **Figures 3a,b**. As described by Hanbury et al. (27); and Jackson and Yunus (19), grasspea accessions are broadly grouped into two groups viz. (1) Blue-flowered accessions with smaller

brown mixed seeds from South West and South Asia and (2) White and mixed-colored accessions from the Mediterranean region. Generally, larger white seeded genotypes yield higher than the accessions from the Indian subcontinent, including those from areas lying in between the Canary Islands to the west of the republics of the former Soviet Union. Small-seeded grasspea accessions are associated with hard seed coats and are considered more primitive like chickpea and lentil of old world.

LATHYRUS GENETIC RESOURCES: DIVERSITY FOR SUSTAINABILITY

The Himalayan region of India has a very rich genetic diversity of genus *Lathyrus* with nine different species viz. *L. aphaca* L., *L. pratensis* L., *L. sphaericus* Retz., *L. inconspicuus* L., *L. odoratus* L., *L. altaicus* Ledeb., *L. luteus* Baker., L. *imphalensis* and *L. sativus* (28–34). In the recent report published by Botanical Survey of India, there are nine taxa (eight species and one subspecies) present in India. These are having their distribution accordingly which include *L. aphaca* present throughout the country; *L. hirsutus* L. and *L. cicera* in Jammu and Kashmir; *L. laevigatus* (Waldst.& Kit.) Gren., *L. pratensis, L. humilis* (Ser.) Spreng. and *L. erectus* Lag. in Himachal Pradesh, Jammu and Kashmir, Uttarakhand; *L. odoratus* in Bihar, Gujarat, Jharkhand, Madhya Pradesh, Maharashtra, Punjab, Tamil Nadu, Uttarkhand and Uttar Pradesh; *L. sphaericus* Retz. in almost throughout India except South and North East India (35). *Lathyrus* germplasm including cultivated grasspea germplasm collections are maintained *ex-situ* at many places in the world. The major genebank collections in the world are given in the **Table 1**. Similarly, 73 taxa of *Lathyrus* have been described in the Flora of Turkey, out of which 22 taxa are endemic (38). The global conservation strategy of grasspea highlights the urgency of upgrading documentation systems, safe multiplication, duplication and adopting international standards for managing existing collections as a means toward a rational and effective conservation system. As a part of global backup or safety duplication, a total of 4,510 accessions of different origin with 45 other species in genus *Lathyrus* from 18 depositors are conserved in the Svalbard Global Seed Vault (39). The establishment of the "*Lathyrus* Genetic Resources Network" (40) propelled foundation for the coordinated international efforts for conservation, collection and other pre-breeding works on the grasspea in the last few decades. South Asia including India is one of the major focussed areas of grasspea cultivation. The geo-referenced map of India highlights the grasspea collecting sites indicating adaptation of crops to eastern part of India which is the most populated region of country (**Figure 4**). If any intervention for grasspea adaptation will be supported by all stakeholders such as farmers, scientists and policy makers in systematic and focussed approach, this crop may alleviate protein malnutrition and food insecurity of the populated region of India and South Asia.

TABLE 1 | The *Lathyrus* holdings in major global genebanks.

S.N.	Major genebanks	Total *Lathyrus* accessions with three major species conserved in different global genebanks
1	Conservatoire botanique national Midi-Pyrénées (CBNPMP), France*	4,477
2	International Center for Agricultural Research in Dry Areas, Lebanon (ICARDA)**	4,417 (*L. sativus*−2,577, *L. aphaca*−339, *L. cicera*−216)
3	Indian Council of Agricultural Research—National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi, India##	2,622
4	Bangladesh Agricultural Research Institute (Plant Genetic Resource Centre (BARI-PGRC), Bangladesh)#	2,422 (*L. sativus)*
5	Instituto Nacional de Investigación Agraria (INIA), Chile *	1,824
6	Australian Grains Genebank, Australia**	1,477 (*L. sativus*- 896, *L. cicera*−201, *L. ochrus*−122)
7	Millennium Seed Bank (MSB), Kew, England**	1,439 (*L. aphaca* - 226, *L. sativus*-156, *L. hierosolymitanus*−97)
8	Ustymivka Experimental Station of Plant Production, Ukraine**	1,215 (*L. sativus*−782, *L. cicera*−73, *L. hirsutus*−70)
9	N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry, Saint Petersburg, Russia**	1,207 (*L. sativus*−824, *L. cicera*−86, *L. hirsutus* – 45)
10	United States Department of Agriculture (USDA) National Plant Germplasm System**	871 (*L. sativus*−294, *Lathyrus. sp.*,−125, *L. odoratus*- 52)

**Patto and Rubiales (36).*
***https://www.genesys-pgr.org/a/overview/v2Vd8B228KX (accessed December 2021).*
#Mathur et al. (37).
##http://genebank.nbpgr.ernet.in/SeedBank/CropSpecieswithICECWise.aspx?CropCode=1641 (accessed January 7, 2022).

NUTRITIONAL COMPOSITION OF GRASSPEA

Grasspea seeds contain about 8.6–34.6%, protein content which is higher than chickpea (18%), field pea (21%), French bean (20%) (41). Grasspea seeds consist of around 60% globulins and 30% albumins in the total seed proteins with 90% digestibility at a 10% level of protein intake (42–46). Analysis of seeds for carbohydrates and crude fiber has shown that these vary between 48–52.3% and 1.1–1.7%, respectively (9, 27, 44). The total amino acids and fatty acids are estimated at 19.69–23.48 g/100 g and 58–80% in the same order in grasspea seeds that are in desirable proportion for animal and human consumption (47–49). Like other legumes, grasspea seeds are rich in lysine (18.4–20.4 mg/kg) but low in sulfur-containing amino acids that range 3.8–4.3 mg/kg in cysteine and 2.5–2.8 mg/kg in methionine (50, 51). Interestingly, one mutant was identified through the research showed 63% more methionine than its parent genotype. However, threonine content in seeds ranged from 10.2 to 11.5 mg/kg (47). The contribution of the total lipids, ascorbic acid and glutathione amount to 1.67 g, 13.50 mg

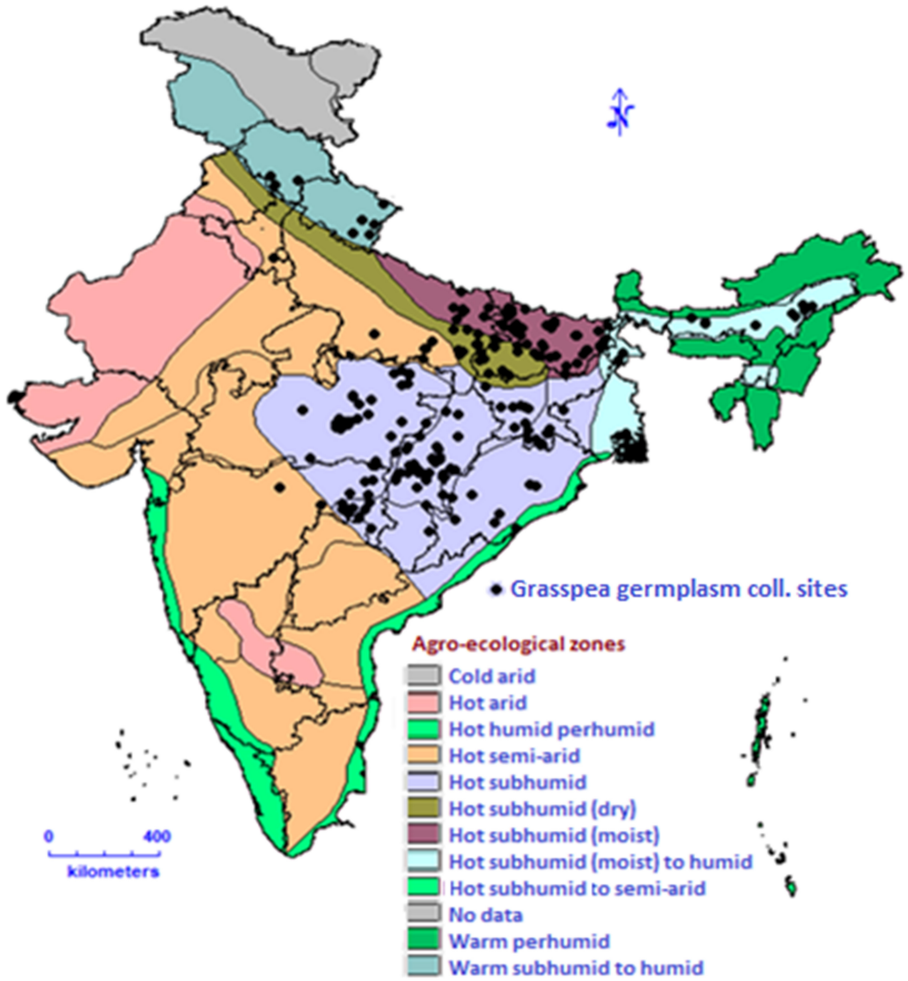

FIGURE 4 | Grasspea germplasm collecting sites in India.

and 15.90 mg/100 g grasspea seeds in the same order. The legume also has higher glutathione and ascorbic acid levels, which contributes to its enhanced antioxidant activity (44, 48, 49). The grasspea seeds contain 4.60 mg thiamine (B1), 2.30 mg riboflavin (B2), 16.40 mg niacin (B3), 18.40 mg pantothenic acid (B5), 5.80 mg pyridoxine (B6) and 5.40 mg/kg folic acid (B9), making them a strong source of vitamin B complex. Furthermore, ascorbic acid (42.5 mg/kg), retinol (34.9 g/kg), and carotene (323.3 g/kg) are all abundant in grasspea seeds (52). The collective nutritional profile of grasspea is given in the **Table 2**.

Utilization of grasspea as food and feed or fodder is limited due to the presence of a plant neurotoxin called β-ODAP that is considered causal agent of muscle atrophy and lower limbs paralysis or neurolathyrism in humans, animals and poultry (50, 53, 61). Recent discovery of some health-promoting nutraceuticals shows some hidden potential of grasspea seeds (18). It is the only known dietary source of L-homoarginine which is useful to treat cardiovascular ailments, hypoxia - Alzheimer's disease and other memory-related disorders (54, 62–64). A wide range of L-homoarginine concentration (6.26–20.97 g/kg) (52–55) reduces excitation of neuronal receptors due to the biosynthesis of nitric oxide (65, 66) in our body. Asparagine content ranged from 0.59 to 5.22 mg/g of seeds desired for young children's healthy brain development and body functions (52). Daily dietary intake of asparagines and L-homoarginine from grasspea seeds could be beneficial to human health and need more systematic research (63). The iron and zinc content in grasspea seeds ranged 6.9–8.7 mg/100 g and 2.46–36.7 mg/100 g, respectively (58, 59). However, large genetic variability for iron and zinc concentration in *Lathyrus* genetic resources were observed at ICARDA breeding programme (46, 67) that can be used for biofortification or genetic improvement of grasspea. The comparative table on nutritive value of grasspea with other cool season legumes are given in the **Table 3**.

TABLE 2 | Nutritional profile of grasspea (*L. sativus* L.).

Nutritional contents	Range	Reference (s)
Protein	8.60–34.60%	(42–46)
Globulins	>60% of the total proteins	(9, 27, 44)
Albumins	>30% of the total proteins	
Carbohydrate	48.0–52.3%	(44, 47)
Crude fiber	1.1–1.7%	
Homoarginine	6.26–20.97 g/kg	(49, 53–55)
Fatty acids (polyunsaturated)	127.39–179.39 mg/100 g	(47, 56, 57)
Amino acid	19.69–23.48 g/100 g	(44, 48, 52)
Total lipids	1.67 ± 0.18 g/100 g	
Glutathione	15.90 ± 0.10 mg/100 g	
Asparagine	0.59–5.22 mg/g seeds	(49, 58)
Retinol	34.9 μg/kg	
Carotene	323.3 μg/kg	
Thiamine (B1)	4.60 mg/kg	
Riboflavin (B2)	2.30 mg/kg	
Niacin (B3)	16.40 mg/kg	
Pantothenic acid (B5)	18.40 mg/kg	
Pyridoxine (B6)	5.80 mg/kg	
Folic acid (B9)	5.40 mg/kg,	(44, 48, 52)
Ascorbic acid	13.50–42.5 mg/kg	
Acid detergent fiber	4.3–7.3%	(47, 50, 51)
Calcium	0.07–0.12 mg/kg	
Phosphorus	0.37–0.49 mg/kg	
Lysine	18.4–20.4 mg/kg	
Threonine	10.2–11.5 mg/kg	
Methionine	2.5–2.8 mg/kg	
Cysteine	3.8–4.3 mg/kg	
Iron	6.9–8.74 mg/100 g	(46, 58–60)
Zinc	2.46–36.7 mg/100 g	
Potassium	644 mg/100 g	
Magnesium	92 mg/100 g	
Vitamin-E	40 IU /kg	(56)

GRASSPEA AND THE CASE HISTORY OF LATHYRISM

Grasspea is a source of debate among agricultural scientists, nutritionists, and farmers for decades due to notoriety for being neurotoxic. Cantani of Naples coined the name "lathyrism" in 1873; however, the history of lathyrism finds its reference way back to ancient times (68). Lathyrism is a crippling disorder and it is more exacerbated when grasspea is the primary component of the human diet accounting for at least 30% of the caloric intake for about 3–4 months as a sole diet (40). Overconsumption of the seed has been linked to neurolathyrism, a neurodegenerative spastic paraparesis disorder due to neuroexcitatory β-ODAP. Zinc deficiency in the soil was found to increase the amount of β-ODAP in the seeds (69). Variable increase in the cases of neurolathyrism was observed among the people of Bangladesh and Ethiopia (70, 71). It is reported that the young human males, cattle and poultry are more affected by the disease (72–74). However, the studies on biosynthesis pathway of β-ODAP have found that it is co-regulated with serine and cysteine of the nitrogen and sulfur metabolism, respectively, that is inversely proportional to the β-ODAP accumulation and key enzyme β-cyanoalanine synthase (75). A novel cysteine synthase gene (LsCSase) has been discovered in grasspea. Under zinc-iron stress and polyethylene glycol-induced osmotic stress, this gene was up-regulated in young seedling tissues and seeds, with an elevated expression level (76). Understanding of the fundamental steps in the regulation and the biosynthesis of β-ODAP are significant in breeding new grasspea cultivars.

ABANDONED, NEGLECTED, AND ORPHAN LEGUME WITH MULTIPLE USES

Grasspea is a promising alternative for sustainable food production because of its inherent qualities, such as minimal water requirements, drought tolerance and disease resistance. Furthermore, it is a highly profitable crop for many developing countries like Bangladesh, Ethiopia, India, Nepal, and Pakistan (77, 78). There are reports claiming that grasspea and several other legumes were used as offerings to kings and in various religious and funeral ceremonies of mummies in the ancient Egypt, in contrast to the modern-day bad reputation of this crop, which makes it as the survival and subsistence food for the poorest of society (18). It is one of the most affordable and the largest source of protein next to soybean. It is a hardy crop, tolerant to both drought and flooding. It fixes 60–124 kg/ha nitrogen under dry conditions (67, 79) and contributes positively to the nitrogen requirements of its subsequent crops. Grasspea is an abandoned, neglected and underutilized crop that can be explored to isolate a number of compounds and metabolites contributing to human health. It has high folic acid that plays a vital role in erythropoiesis (the production of red blood cells) along with nucleic acid and protein synthesis. Therefore, it is essential in preventing congenital disabilities (44). The water-soluble inositol phosphoglycan (IPG) molecules from seeds of grasspea are being used in some traditional medicines to treat diabetic symptoms (80).

A Chinese group has also patented the metabolite β-ODAP from its seeds are used as a hemostatic agent following surgery (81). β-ODAP is also present in the roots of Chinese ginseng (*Panax ginseng* C.A.Mey.), which is believed to promote lifespan and is commercialized as "Dencichine" in the markets and are used in the treatment of hemorrhage and thrombopoiesis (82, 83). Some Chinese toothpaste brands also use its herbal extracts to avoid bleeding gums (18, 84). β-ODAP metabolite has also been shown to have the property of healing wounds naturally (84). Thereby, grasspea seeds have demonstrated range of therapeutic properties, indicating that they may be used as a potential medicinal or pharmaceutical crop plant of the future. The radical scavenging activity is explained by the presence of phenol phytochemicals they contain in their roots. It can be recommended for cultivation on unproductive marginal lands adjacent or close to hill slopes and during droughts to decrease soil erosion (44). Plant antioxidant mechanisms that accumulate ascorbic acid (AC), oxidized forms of AC-dehydroascorbic acid (DAA) and diketogulonic acid (DKGA) in *L. maritimus* (L.)

TABLE 3 | Comparative table on nutritional values of grasspea (*L. sativus* L.) with other cool season legumes.

Composition	Chickpea (*Cicer arietinum* L.) (Whole)*	Lentil (*Lens culinaris* Medik.) (Whole, Brown)*	Dry Peas (*Pisum sativum* L.)*	Field bean, Black (*Phaseolus vulgaris* L.)*	Grasspea
Protein (%)	18.77 ± 0.42	22.49 ± 0.58	20.43 ± 0.79	19.93	8.60–34.60**
Total poly unsaturated fatty acids	2,337 ± 78.2 mg/100 g	277 ± 9.70 mg/100 g	873 ± 41.50 mg/100 g	468 mg/100 g	127.39–179.39 mg/100 g[$]
Total Carotenoids	999 ± 240 μg/100 g	924 ± 89 μg/100 g	933 ± 94.10 μg/100 g	207 μg/100 g	323.30 μg/kg[#]
Iron	6.08 ± 0.27 mg/100 g	7.57 ± 0.67 mg/100 g	5.09 ± 0.45 mg/100 g	4.50 mg/100 g	6.90–8.74 mg/100 g***
Phosphorus	267 ± 21.9 mg/100 g	274 ± 27.40 mg/100 g	334 ± 18.30 mg/100 g	457 mg/100 g	0.37–0.49 mg/kg
Potassium	935 ± 37.9 mg/100 g	756 ± 63.60 mg/100 g	922 ± 67.40 mg/100 g	1,272 mg/100 g	644 mg/100 g***
Calcium	150 ± 18.3 mg/100 g	76.13 ± 9.23 mg/100 g	75.11 ± 13.93 mg/100 g	78.16 mg/100 g	0.07–0.12 mg/kg
Magnesium	160 ± 17.5 mg/100 g	101 ± 13.90 mg/100 g	123 ± 8.10 mg/100 g	197 mg/100 g	92 mg/100 g***
Vitamin B1	0.37 ± 0.04 mg/100 g	0.40 ± 0.07 mg/100 g	0.56 ± 0.05 mg/100 g	0.35 mg/100 g	0.46 mg/100 g[#]
Vitamin B2	0.24 ± 0.01 mg/100 g	0.22 ± 0.03 mg/100 g	0.16 ± 0.01 mg/100 g	0.07 mg/100 g	0.23 mg/100 g[#]
Vitamin B3	2.10 ± 0.06 mg/100 g	2.54 ± 0.12 mg/100 g	2.69 ± 0.15 mg/100 g	1.88 mg/100 g	1.24–2.03 mg/100 g[#]
Vitamin E	1.72 ± 0.07 mg/100 g	0.19 ± 0.02 mg/100 g	0.32 ± 0.02 mg/100 g	0.51 mg/100 g	40 IU /kg[##]
Cysteine	1.27 ± 0.09 g/100 g	1.18 ± 0.04 g/100 g	0.82 ± 0.15 g/100 g	0.59 g/100 g	3.8–4.3 mg/kg[###]
Threonine	3.55 ± 0.31 g/100 g	3.35 ± 0.05 g/100 g	3.65 ± 0.15 g/100 g	4.12 g/100 g	10.2–11.5 mg/kg[###]
Methionine	1.16 ± 0.16 g/100 g	0.84 ± 0.03 g/100 g	0.68 ± 0.19 g/100 g	1.36 g/100 g	2.5–2.8 mg/kg[###]

[#]*Grela et al. (58) and Arslan et al. (52).*
[##]*Grela and Gunter (56).*
[###]*Rotter et al. (47), Hanbury et al. (50), and Lambein and Kuo (51).*
**Indian Food Composition Table-2017 (41).*
***Barpete et al. (42), Tamburino et al. (44), Girma and Korbu (43), Kumari and Kumar Jha (45), and Sengupta et al. (46).*
****Sandberg (60), Urga et al. (59), Grela et al. (58), and Sengupta et al. (46).*
[$]*Arslan (57).*

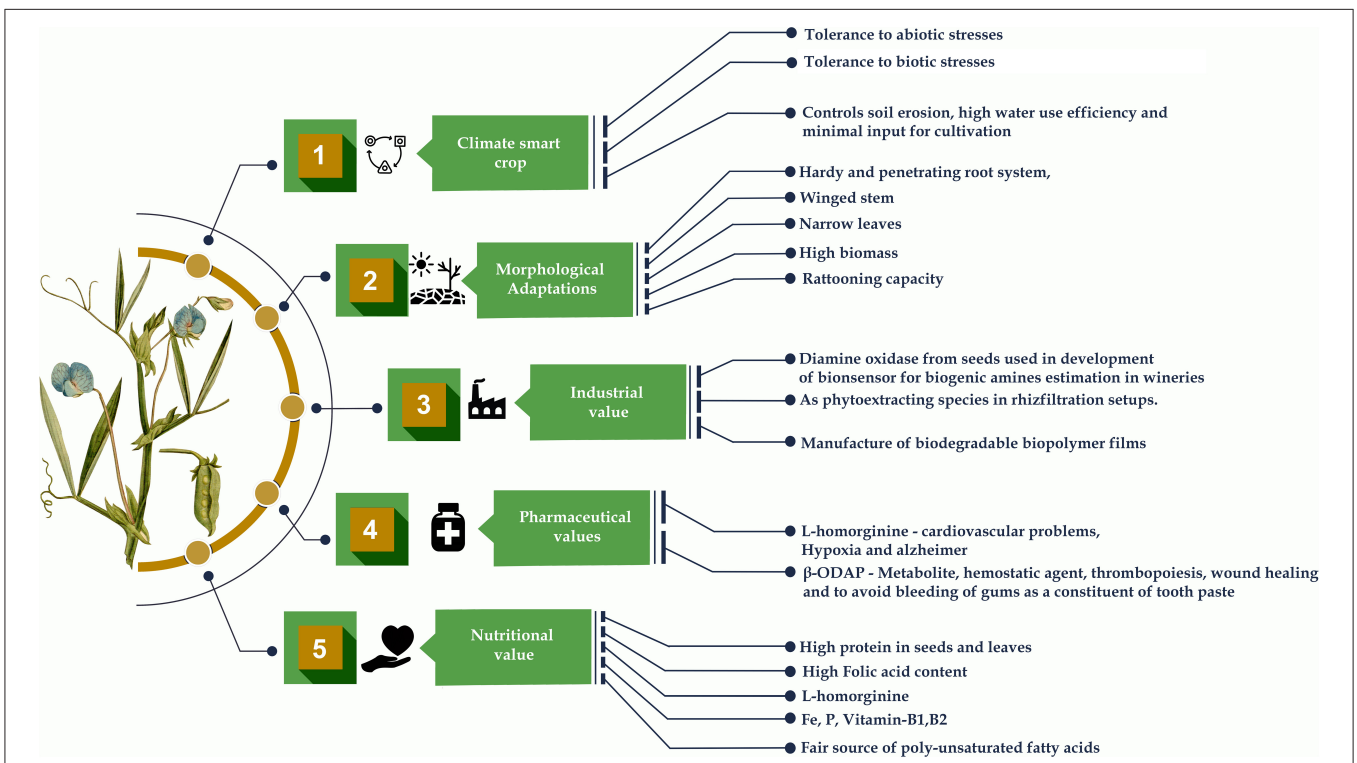

FIGURE 5 | Grasspea as a multifaceted legume.

Fr. contribute to antioxidant activity which helps in adapting to the changing environment (85). A new protein PGIP-Polygalacturonase-Inhibiting Protein from its seeds play an significant role in plant protection against fungal infections by endo polygalacturonases (EPGs), the first enzymes released by phytopathogenic fungi during plant infection (86). It can be used in crop improvement as the effective donor source of resistance to *Ascochyta* blight compared to other field pea cultivars (87, 88). The silver nanoparticles biosynthesized from grasspea species and *Stachys lavandulifolia* Vahl. can also be used as an antifungal agent against *Dothiorella sarmentorum* (89).

Metalloproteases isolated from the dry grasspea seeds are useful in the biotechnology, food, medical and pharmaceutical industries (90). The different proportions of grasspea protein isolates and glycerol were used to combine *Lepidium perfoliatum* L. seed gum to form composite biopolymer films that are biodegradable in nature (91). An effective biocontrol strain of plant growth promoting rhizobacteria obtained from the rhizosphere of grasspea was used as an alternative to conventional fertilizer that could contribute to crop disease reduction and significantly increase crop growth and yield (92). The ability of this crop to withstand nutrient shortage and retain vast amounts of lead in the root tissues has made it a tough species. As a result, it could be incorporated into the phytoremediation and rhizofiltration systems as an effective lead phytoextracting species (93). The characterization,

TABLE 4 | Low-β-ODAP grasspea cultivars released in Australia, South Asia, Central Asian, Mediterranean, East African and Latin American countries.

Breeding method	Variety name	Pedigree/selection method	Country	References
Intraspecific hybridization	Ceora	K33 × 8604	Australia	(67, 96)
	Bari Khesari 1	P-24 × Local cultivar	Bangladesh	(67, 97)
	Bari Khesari 2	P-24 × Local cultivar	Bangladesh	(67, 97)
	Wasie (ILAT-LS-LS-B2)	SC5 × PGRC 46071	Ethiopia	(98)
	Prateek	LS 82046 × A 60	India	(99)
	Mahateora	Ratan × JRL 2	India	(99)
	Studenica	Polish cultivar ×local Serbian landrace (Pedigree method)	Serbia	(67, 100)
	Stinica	Polish cultivar ×local Serbian landrace (Pedigree method)	Serbia	(67, 100)
Mutation breeding	Bina Khesari 1	Mutation	Bangladesh	http://dhcrop.bsmrau.net/binakhesari-1/
Biotechnological approaches	Ratan	Somaclone of cv. Pusa-24	India	(99)
Direct introduction	CLIMA 2 pink	Introduction	Nepal	https://www.scribd.com/document/18528265/Status-of-Grasspea-in-Nepal
	Bari Khesari 2	Introduction	Bangladesh	https://nfsm.gov.in/areacoveragecropsdashboard.aspx
Direct selection from germplasm	Chalus	Selection from IFLA 1279	Australia	(67, 101)
	Bari Khesari 3	Selection from Sel.190	Bangladesh	http://dhcrop.bsmrau.net/bari-khesari-3/
	Bari Khesari 4	Selection from Sel.1337	Bangladesh	http://dhcrop.bsmrau.net/bari-khesari-4/
	Strandja	Local selection (VIL)	Bulgaria	(67)
	LS 8246	Selection from Pusa-24	Canada	(67, 102)
	Luanco-INIA	Selection from LS 0027	Chile	(67, 103)
	Quila-blanco	Selection from germplasm	Chile	(67, 104)
	Ali Bar	Selection from IFLLS- 554	Kazakhstan	(105)
	Pusa-24	Selection from germplasm	India	(99)
	Nirmal	Selection from germplasm	India	(99)
	Bidhan Khesari-1	Selection from LAT-15-6 (BK-14-1)	India	(99)
	19A	Selection from germplasm	Nepal	https://www.scribd.com/document/18528265/Status-of-Grasspea-in-Nepal
	19B	Selection from germplasm	Nepal	https://www.scribd.com/document/18528265/Status-of-Grasspea-in-Nepal
	Derek	Selection from Der	Poland	(67, 106)
	Krab	Selection from Kra	Poland	(67, 106)
	Gurbuz-1	Selection from IFLLS 554	Turkey	(98)

evaluation and utilization of diamine oxidase (DAO) from *L. sativus* species used as biocatalytic component of a novel DAO-based amperometric electrochemical biosensor to determine biogenic amines (BA) index in the wine and beer samples. It can produce a credible assessment of the overall BA's content in production plants or wineries. Food quality commissions around the world are increasingly requesting this metric (94). Several different attractive ornamental species of genus *Lathyrus* like *L. odoratus, L. vernus* (L.) Bernh and *L. latifolius* have potential for increasing diversity in grasspea by interspecific hybridization. In the United Kingdom, a seed bank has been established to preserve the variety of ornamental plants and make the materials freely available to the academic researchers and farmers worldwide (95). Therefore, holistic research on this crop could explore the other possible and sustainable uses of the crop (**Figure 5**).

The seeds are generally used as a pulse, *dahl* and flour, which is used in preparing different types of savories, sweets and snacks. Immature pods and young leaves of a plant are used as a leafy vegetable in India and Bangladesh. Tender leaves and branches of the grasspea are popularly sold by the farmers in Indian and Bangladeshi markets. Apart from its use as a food, it is also used as fodder to cattle during the early vegetative stage to maturity and feed. Split-grains and flour of grasspea are also utilized as feed for lactating animals or bullocks in periods of heavy field work (15). In sustainable agriculture system, this multifaceted species can serve as an insurance crop to the marginal land farmers in drought hit areas with minimum inputs and high output. High protein contents, tolerance against the number of biotic and abiotic stresses and the ratooning (multiple cuts for its foliage) capacity of this crop makes it a good choice for cultivation in the arid regions and semi-arid regions with high scarcity of water.

BREEDING EFFORTS FOR NUTRITIONAL GAIN IN GRASSPEA

Grasspea is recognized as a versatile crop and one of the climate-smart choices for the future and it has attained the status of the multipurpose legume. With the help of conventional breeding and selection strategy from available germplasm, ICARDA and National Agricultural Research Systems (NARS) have developed and released more than 25 improved cultivars of grasspea that can be cultivated in diverse agroecology throughout the world (**Table 4**). Grasspea breeding programs are focussed their efforts toward the improved early maturity, high biomass, plant types, and resistance to both biotic and abiotic stresses along with low β-ODAP cultivars (46, 107, 108). This will incorporate tolerance against major pests, pathogen and induce improved nutritional quality (protein, micronutrients, methionine and homoarginine) using traditional and improved breeding protocols (25).

Characterization and breeding efforts were attempted in grasspea for agro-morphological traits and β-ODAP content (48, 109). Cross compatibility studies were carried out between common peas, *Pisum sativum* and *L. sativus.* It was observed that there was successful isolation, culture and fusion of viable protoplasts from these crop plants. This will allow for the development of genetic novelties with intriguing agronomic properties such as stress tolerance and rusticity from grasspea and grain quality from peas (110). The use of genetically distant grasspea accessions could give possible superior recombination with low β-ODAP content compared to carrying out crosses among or between the genetically closer species (111).

Mutation studies on grasspea with gamma-rays are encouraging and have shown induced salinity (NaCl) tolerance in M2 progeny mutants of grasspea (112). Different kinds of auxins (IBA; IAA; NAA) in tissue culture experiments are carried out to find the factors affecting healthy rooting, acclimatization and the effects of different concentration of sugar on root morphology phenology and developmental attributes of grasspea plants (25, 113, 114).

Grasspea has natural source of resistance to many pulse diseases (36). *Ascochyta* blight can be considered one of the most important diseases in legumes (88). *Ascochyt*a blight resistance was observed in various species of genus *Lathyrus* namely *L. cicera, L. clymenum L., L. ochrus* (L.) DC. and *L. sativus* as in comparison with the field peas (87, 115). The gene expression for creating resistance against *Ascochyta lathyri* in grasspea has also been demonstrated (88).

Limited genetic and genomic research by the public and private sector for the genus *Lathyrus* has resulted in meagre and stagnant data on the desirable aspects of grasspea. In future improvement programmes, the use of molecular markers for the genetic diversity studies and their utilization in marker-trait association for plant phenology and yield-related traits are expected to play a crucial role in understanding the association of novel alleles in trait expression (116, 117). The development and use of simple sequence repeats (SSRs) (45, 118–120), EST-SSR (111), Restriction Fragment Length Polymorphism (RFLP), and Random Amplified Polymorphic DNA (RAPD) (10, 121, 122) markers as a conventional molecular tool and recent development of SSR markers by *In silico* mining of nucleotide sequences (117) has enhanced our understanding in genetic linkage mapping, QTL mapping, association mapping, DNA fingerprinting and genetic diversity studies. The above mentioned research on the topics related to grasspea breeding has given a promising way of exploring the genetic potential of this species. In addition to this information, phylogenetic relationship between different species of the genus *Lathyrus* using chloroplast DNA trnH-psbA -intergenic spacer (123), nuclear ribosomal DNAITS2-nrDNA - Internal Transcribed Spacer 2 (124) and an Inter-Simple Sequence Repeats (ISSR) technique has been carried out (125) to know the better understanding of the existence of genetic diversity among the accessions in their experiments.

A draft genome sequence of grasspea ranged between 6.75 and 7.63 Gbp (126), 7.82 and 8.90 Gbp (127), 6.85 Gbp (128), and 6.52 Gbp (129). This data will help to identify the genes responsible for the gene regulation of biosynthesis pathway of β-ODAP and identify the alleles for different traits that will be helpful in the agronomic and nutritional improvement. It is also expected to allow comparative genomic analyses between different legumes,

which will aid in the development of genetic and physical maps which can be used for the development of marker-assisted and genomic selection strategies through genome editing and tilling platforms (23).

CURRENT SCENARIO OF GRASSPEA CULTIVATION AND LATHYRISM IN INDIA

Grasspea has the immense potential to grow as a rice-fallow pulse crop in eastern India. A study has showed that out of 11.6 million hectares of fallow land in India, ∼0.5 million hectares could be easily brought under grasspea cultivation to improve land productivity and raise revenue for farmers as a second crop (130). The Indian farmers are discouraged to grow grasspea on large scale for commercial purpose, except for family consumption and livestock feed. Commercial production of grasspea is on ban in some Indian states under the Prevention of Food Adulteration Act 1961 (131). This has ended up in reduction of its farming areas from 1.3 million hectares to <850,000 ha in a decade (67). Contrary, the researchers are becoming more interested in grasspea due to its multifaceted importance. Therefore, they are interested to breed zero or low β-ODAP cultivars. It is expected that an interaction among government, breeders, farmers, pharmaceutical professionals will increase awareness about the genus *Lathyrus* and help in developing techniques to detoxify β-ODAP. This will further increase their importance as a new putative functional food (pharmaceutically valuable crop), forage and crop of industry, along with other pulse crops. Hence, abandoning or neglecting this crop may not be a wise decision. Efforts toward developing and popularization of low or zero β-ODAP cultivars would need some detoxification methods to enhance the use of grasspea in the common households. Some of the popular grasspea detoxification methods are described and listed in **Table 5**.

RECENT TRENDS ON LATHYRISM IN INDIA

Presently in India, about 3.62 lakh ha area of land is under grasspea cultivation (138). Several studies about grasspea consumption were conducted by Nagarajan and Gopalan (139) in Bilaspur, Durg, and Raipur districts of Chhattisgarh in India. Previous studies noted that β-ODAP content in most of the lines or cultivars ranged between 0.5 g and 2.5 g/100 g (78). These districts were restudied after 50 years in 2018. The new studies showed that β-ODAP content in local germplasm was significantly reduced and ranged 0.63 ± 0.14 to 0.65 ±0.14 g 100 g^{-1}. No or negligible incidence of neurolathyrism which includes the occurrence of these symptoms in aged persons of 50–60 yrs were reported in these areas (140). Similar findings were reported from Bora and Malgaon villages along with Miraj country (Tehsil) of Sangli district in the Maharashtra, India (141). Chaurasia et al. (142) has reported only three cases of post stroke paralysis from Eastern Uttar Pradesh. However, grasspea consumption cannot be blamed solely for this report. These findings suggested a considerable reduction in the incidence of neurolathyrism compared to its reports in the past primarily due to consumption of low ß-ODAP cultivars that were distributed among the farmers by the state government.

FUTURE PERSPECTIVE

Different species in genus *Lathyrus* including cultivated grasspea have greater potential for nutritional use in the industry

TABLE 5 | Traditional and acquired knowledge based β-ODAP seed detoxifying methods of grasspea.

Detoxification techniques	Methodology	References
Roasting	Seeds are roasted at 180°C for 45 min.	(132)
Roasting after soaking seeds in water	Overnight soaking of seeds then roasting as described in procedure no. 1.	
Boiling in freshwater	Overnight soaking and then boiling next day.	(114, 132–135)
Soaking in alkaline water and boiling	Seeds are soaked for 6 h in a 1% calcium hydroxide solution (1:5 w/v), then wrapped in muslin fabric and boiled for 45 min. Then it is dried and pulverized as flour.	(132, 136)
Soaking in tamarind water and boiling	The seeds are steeped for 6 h in tamarind water (1:3 w/v). Then it is washed in fresh water and cooked for 45 min. After this, it can be dried and powdered to use as flour.	(132)
Germination	Germinated seedlings over a muslin cloth which takes 30–36 h for sprouting can be eaten as microgreens or salads.	
Autoclaving	Soaking seeds overnight followed by autoclaving/ pressure cooked at 15 psi for different time intervals, say 15, 30, and 45 min.	(114, 132, 135)
Frying	Overnight soaking and then deep frying in vegetable oil.	(132)
Fermentation with bacterial and fungal inoculum	Overnight soaking of seeds followed by boiling and then it is crushed in a mixer for 5 s before being placed on Petri dishes. Then this mixture is fermented with *Aspergillus oryzae* spores after sterilization (110°C, 30 min) for 48 h at 30°C. Then it is cooled and again inoculated with *Rhizopus microspores* var *chinensis* and allowed for further and fermentation for about 42 h at 30°C. Both the fungal and bacterial fermentation are inoculated with 108 spores per petri dish of each and ended with steam (100°C, 20 min). The resultant product is called as "tempeh"- a traditional fermented protein-rich product resembling cake slices.	(132, 137)

compared to other legumes. Therefore, Indian government and some International organizations are paying more attention and importance to the conservation and utilization of grasspea genetic resources due to their versatile uses under rapidly changing environmental conditions (78). The coordinated programme with proper methodologies for breeding new low or zero β- ODAP cultivars is desired. Similarly, development of methodologies for detoxification of β-ODAP and antinutrient contents in grasspea are the need of hour for this miraculous crop. These will boost national economy and improve standards of living of the farmers. The ideas to transform grasspea from orphan, neglected and abandoned crop to the multi-faceted mainstream crop would bring additional new sources of income to the farmers along with proper dietary consumption awareness among the people to reduce the incidences of neurolathyrism. The concepts for mainstreaming grasspea are proposed in **Figure 6**.

For enhancing the quality and quantity of pulses seed in the country, an Indian model of creation of seed-hubs can be replicated globally with the mandated objectives and targeted seed production of latest varieties. The farmers should be continuously provided with seeds of improved grasspea cultivars through seed distribution centers in the coming years and should be continuously educated and updated with latest information with diffusion of new dissemination technologies, encouraging them to improve seed production and multiplication technologies following available appropriate agronomic practices. It is expected that the improved government policies highlighting the paramount importance of the grasspea in the South Asian context, as a primary staple pulse for marginal farmers for their subsistence in dietary supplements will make and turn out this crop as the "Golden Pulse Crop of the Future." Recently released draft genome of grasspea by Emmrich et al. (23) will make it easier to harness genomic information for breeding new cultivars to maximize its potential as a high protein pulse and a donor source for multiple resistance to biotic and abiotic stresses. The new and superior cultivars using this technology will facilitate farmers and poor people with small to marginal economies in more sustainable way. Inclusion of grasspea in irrigated and arid agricultural systems and its introduction to marginal lands with low input could

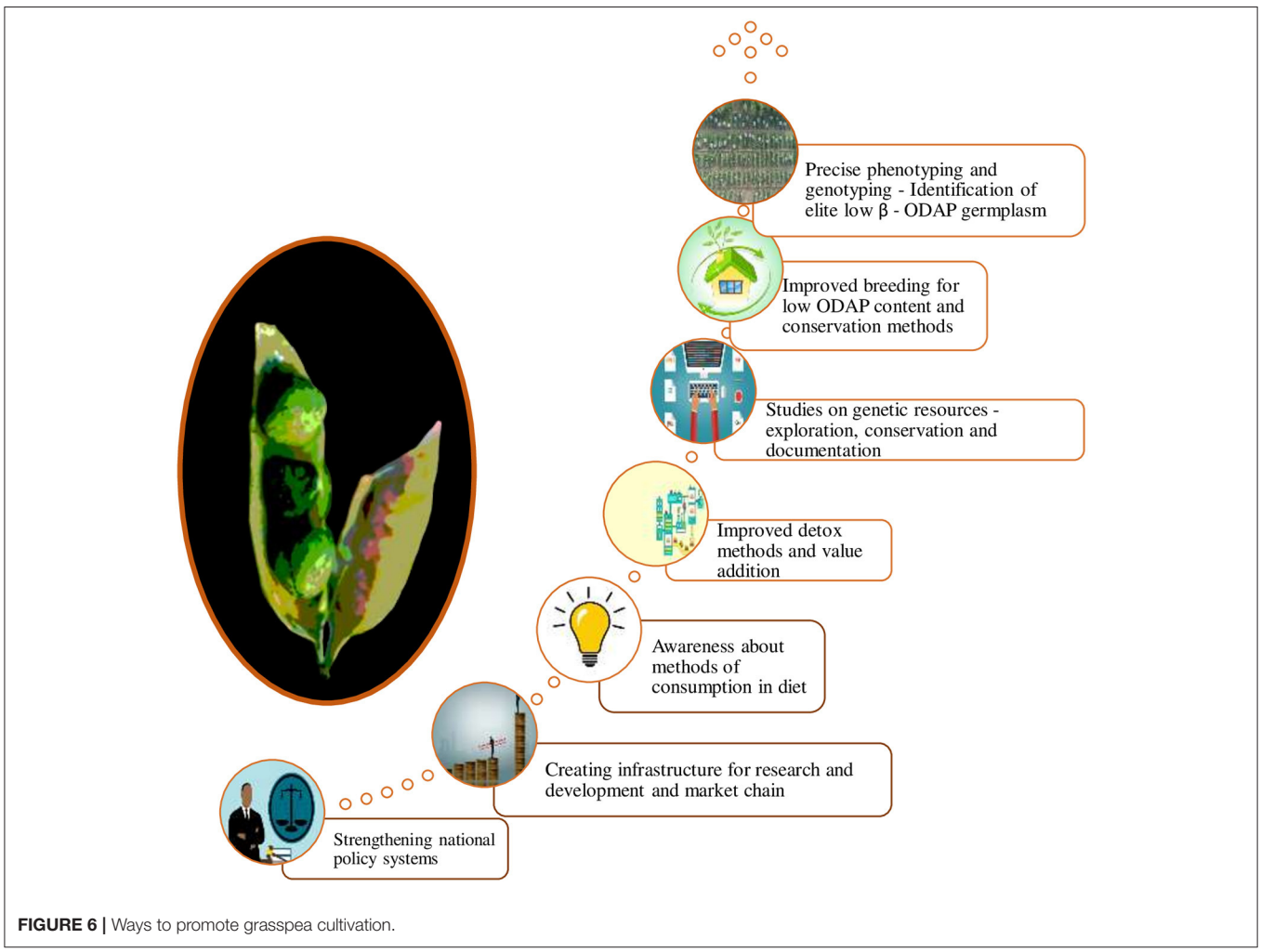

FIGURE 6 | Ways to promote grasspea cultivation.

prove it as a highly resilient, climate-smart crop in times to come.

AUTHOR CONTRIBUTIONS

KT, KR, and AP: conceptualization, writing, and editing of the manuscript. KR, PG, SB, AR, and NS: collection and compilation of information. KT, AP, SB, KK, and AS: conception, writing, review, and final editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Protein for Human Consumption From Oilseed Cakes: A Review

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Oilseed cakes left after the oil extraction for different purposes are chiefly used as cattle feed, compost amendment, or plant conditioner. These oilseed cakes are rich in protein, nitrogenous compounds, and minerals. Beside its conventional usage, studies have been conducted to utilize these protein rich resources for human consumption. Considering the exponentially increasing human population and escalating food prices, these protein rich sources can be a novel food commodity and used to extract protein. The quality and functional properties of extracted oilseed cake proteins not only supplement the existing protein sources for the human consumption but also solve the problem of oilseed cakes disposal along with the additional income to the oilseed crop producers and processers. Production of proteins for human consumption from oil seed cakes may also reduce the carbon and water footprints while producing animal protein. The present review will focused on analyzing the oilseed cake as a protein source, characterization, extraction techniques, and utilization in food products.

Keywords: oil seed cakes, by-products, protein, carbon, water foot print

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INTRODUCTION

In recent decades, a number of snowballing current issues e.g., over-exploitation and mismanagement of resources, the unsustainable consumption behaviors, the degradation of the environment and equilibrium of the ecosystems and climate change, were emerged. To mitigate these challenges, it is necessary to focus on new strategies and ways to make the best use of our natural resources and to eliminate the concept of wastes in supply chain. A new concept has been created where waste has been utilized by transforming into value-added products.

Edible oil industries processed raw seeds to extract oil and leftover solid by-products known as oilseed cakes. For the year 2017–2018, a total of ∼580 million tons production of major oilseed crops was recorded, with the highest recorded for soybean followed by rapeseed, sunflower seed, and cotton seeds. According to FAO Statistics Division (2010), India produces over 25 million tons of oilseed cakes annually, being the world's largest oilseeds producers. Soybean seed cake followed by canola seed cake is the world's largest by-product in terms of quantities. In earlier times, oilseed cakes have been used as animal feed and soil compost, as it was an excellent and economical source to gain benefits. Oilseed cakes are rich source of protein, energy, carbohydrates, and mineral contents. It was found that the oilseed cake increased the weight of the young calves (Hessle et al., 2008). Hempseed cakes have some additional advantages as animal feed because they are rich in amino acids and proteins (Mustafa et al., 1998). Further, oilseed cakes are worthy of research

as they have a potent role in human nutrition; however, they are bean underutilized due to lack of awareness and subsequent research. Evidences have been made on utilizing oilseed cake as dietary sources for human consumption.

Oilseed cakes have a great potential in obtaining various value added products having high nutrient value. To meet the increasing demand of food rich in protein, nutrients, and bioactive compounds, oilseed cake could be a potential candidate. Meyer (1971) reported protein extraction along with processing, functional and nutritional properties of soybean and other oilseed cakes. There is increasing demand for food supply which is rich in protein content. The recommended dietary allowance (RDA) for protein is 0.8 g protein per kilogram of the body weight, which indicates the minimum requirement of the protein for growth and development. Oil cakes/meals being a very rich source of proteins are also suitable alternative for human consumption (Teh and Bekhit, 2015). Plant proteins are versatile, non-toxic; easy to digest, low processing cost, and potent nutritional alternative for replacing animal protein (Sá et al., 2020; Langyan et al., 2022). However, flavor, color, and texture of foods are affected because plant protein lack sulfur amino acids. Although, there is no detailed review of protein from oilseed cakes which can be consumed by human. This paper provides a critical review of the existing literature on the quality and functional properties of extracted oilseed cake proteins to supplement the existing protein sources for the human consumption and also to present the problems associated with oilseed cakes disposal along with the additional income to the oilseed crop producers and processers.

The protein digestibility corrected amino acid score (PDCAAS) preferable method adopted by WHO/FAO to measure the value of protein in human nutrition. The principle of this method is comparing first limiting essential amino acid concentration with reference amino acid concentration pattern. In simple words, it is utilized for evaluation of food's protein quality and is generally used to calculate the %DV for showing on label in R&D Foods. This scoring pattern is taken from requirement of essential amino acids in small age group preschool children. The obtained chemical score by this is corrected for true fecal digestibility of the test protein. PDCAAS values more than 100% are not acceptable and hence it should be truncated to 100%. During adoption of the PDCAAS method, very few research have been done on requirement of amino acid, and therefore there is a need for validating the pattern of scoring, and this patter does not contain conditionally indispensable amino acids (Schaafsma, 2000).

OILSEED CAKES AS PROTEIN SOURCES: TYPES AND CHARACTERISTICS

Protein is isolated from oilseed cakes using an alkaline extraction which has been very well documented. Oilseed cakes are rich source of protein isolates having a wide range of applications in food industry as they differ in functional properties e.g., emulsification, creaming stability, water- and oil-holding capacities. The protein quality of soy protein isolates is very much comparable to those obtained from oilseed cakes such as hemp, canola, sunflower, and palm kernel (Tang et al., 2006; Wang et al., 2008; Tan et al., 2011; Chee and Ayob, 2013); therefore, these can be considered as an alternative to soy protein. Moure et al. (2006) studied in detail the modification process by which functional properties of protein isolates are changed in food products. Although oilseed protein with amino acid content has limited bioactive properties but their biological functions can be improved by chemical or enzymatic hydrolysis to produce protein hydrolysates (Vaštag et al., 2011; Girgih et al., 2013). The angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant activity of oilseed cakes is reported higher as compared to the protein isolates (Marczak et al., 2003; Girgih et al., 2013).

With increasing world population and the demand for food supply and especially protein rich products are increasing. However, the raw material cost, energy consumption, competition for land use, environmental pollution, soil deterioration, and climate changes associated losses are increasing which all ultimately makes the food production process challenging and costlier. Also, the agro-waste generated during food harvesting to its processing create a lot of environmental problems beside problems associated with its proper disposal. Utilization these agro-wastes, including oilseed cakes is a sustainable methods for producing alternative food sources which can be used to meet the increasing protein consumption by the growing population.

Health Benefits of Plant Proteins Over Animal Proteins

There are many health benefits associated with diets high in plant protein e.g. lowering body weight, body cholesterol also with lower blood pressure levels. This is the main reason why these are preferred over animal protein (Wheeler et al., 2002; Craig, 2010; Langyan et al., 2022). Many reports have been documented that intake of diet rich in protein (about half from plants) cause lowering in blood pressure, reduction in cholesterol levels and reduce the risk of various health issues and severity related to heart functioning (Appel et al., 2005). Reduction in cholesterol levels and blood pressure by having a low-carb, high-plant protein diet also attract attention in comparison to a high-carb, low-fat diet (Jenkins et al., 2009). Serving of legumes (plant protein) in diet in place of red meat (animal protein) also reduce the risk of type 2 diabetes which occurs due to change dietary changes. One small study carried out on people with type 2 diabetes found that cholesterol and blood sugar were improved to a significant level when the patients were fed with legumes instead red meat (Hosseinpour-Niazi et al., 2015). Plant protein diets also prove very useful in case of obesity by helping in controlling body weight. Intake of plant proteins such as beans, chickpeas, lentils or peas per day in diet can prove very potent in weight management and weight loss (Li et al., 2014). Mozaffarian et al. (2011) studied 120,000 men and women over 20 years and found that eating more nuts causes a significant reduction in weight loss.

Oilseed cakes rich in polyphenols such as canola and sunflower usually exert a dark color on the protein isolates and also affect their functional properties, e.g., emulsification, foaming, surface hydrophobicity, and water-holding properties, so their removal is recommended before protein extraction (Mansour et al., 1993; Xu and Diosady, 2002; Salgado et al., 2012). Extraction of protein from sunflower seed cake after removal of polyphenols had better functional characteristics such as lighter color, higher surface hydrophobicity, protein digestibility, and solubility as compared to the protein isolate containing polyphenols (Salgado et al., 2012). To determine the optimal use of protein extract, various studies have evaluated the functional properties of protein isolates from different oilseed cakes. For instance, protein isolates from hemp oilseed cakes have similar oil-holding capacity as from soy oilseed cake, but have lower emulsion stability index and water-holding capacity (Wang et al., 2008). Same characteristics also have been reported in gingerbread plum seed protein isolate which exhibited high emulsifying capacity and water-holding capacity, oil-binding capacity, and bulk density than the commercial soy protein isolate (Amza et al., 2011). Canola, flax, and whey proteins are very much comparable in their creaming stability (Karaca et al., 2011). The amino acid profiles of protein isolates from oilseed cakes are better or comparable to soy protein isolate in fulfilling the nutrition requirements of infants and elderly people. For instance, gingerbread plum seed protein isolate offers a better replacement in infant formula as they are rich in arginine, valine, tryptophan, glutamic acid, cysteine, serine and proline than the soy protein isolate (Amza et al., 2011). The gingerbread plum seed protein isolate acts as natural source of calcium supplementation for pregnant and lactating women, children and elderly people because of its high calcium content. As per Food and Agricultural Organization/World Health Organization (FAO/WHO) standards, palm kernel protein isolate have all essential amino acids at concentrations that met the suggested requirements except lysine for 2–5 years old infants. In addition to this, protein isolates from canola, hemp, and flax protein also meet all the requirements of essential amino acid profile when fortified with lysine for both adults and childrens (Tang et al., 2006; Tan et al., 2011; Teh et al., 2014). Methionine and cysteine concentration was found to be very high in protein extract after alkali extraction than soy protein extraction (Teh et al., 2014), which can be advantageous for use in infant formula. Canola seed protein isolates are rich in glutamic acid, aspartic acid, leucine, and proline and contains considerable amounts of phenylalanine, isoleucine, and tryptophan (Shahidi, 1990). The pumpkin seed protein isolate was shown to be nutritionally sounded *in vivo*. LDH, alanine transaminase, aspartate transaminase, and alkaline phosphatase levels are associated with protein malnutrition and can reduced to a considerable level after feeding pumpkin seed protein isolate to rats treated with carbon tetrachloride (CCl4) (Nkosi et al., 2005). Oilcake/ meal obtained after extraction of oil from the oilseeds are rich in protein. The highest content of protein (45–50%) was found in groundnut cake, followed by soybean, cottonseed, rapeseed, sesame, sunflower, palm oil, and olive oilcake. Protein hydrolysates (hydrolysed product of protein), protein isolates (>90% protein), and protein concentrates (30–80% protein) can be prepared by these protein rich oil cake. The preparation of protein isolates includes solubilizing the protein by alkali (0.1N NaOH) followed by isoelectric precipitation using acid (0.1N HCl), and subsequently washing and drying.

There are two categories of oilcakes, i.e., edible and non-edible. Oilcakes have high nutritional value and can be used for the consumption of human as well as animals. They are used as processed ingredients (hydrolysate, protein concentrate, isolate) or as substrate (in the production of amino acids, flavors, bioactive compounds, pigments, antibiotics, surfactants, enzymes, vitamins). The defatted edible cakes can be incorporated in multipurpose supplements, infant dairy products and bakery, and used in the diet of undernourished people. Oilseed cake /meal that consist of toxic compounds and are used as manure are called as non-edible oilseed cake/meal. It includes oilseed cakes obtained from linseed, karanja, castor, neem, and mauha (Gupta et al., 2018). According to the U.S. Department of Agriculture (USDA), the world production of oilseeds in 2018/2019 was 600.47 million metric tons, accounted for a large amount of press cakes and residues. Sustainable means to utilize oilseed cakes includes the use of these by-products and residues to develop new products rich in nutrients (Sarwar et al., 2013). Growing condition, extraction method and variety also determine the composition of oilcakes/meals.

The oilseed cakes have different physiochemical characteristics, depends on the initial raw materials, variety, growing condition, and extraction methods. For example, Walnut oilcakes are yellow to light brown, and sweetish. Pumpkin seeds oilcake color varies from brown to brownish green and has a sweetish, insipid taste. The color of sesame oilcake ranges from cream to light brown and contains an insipid, sweetish taste. Flaxseeds oilcakes show various shades of brown with an insipid, neutral taste (Bochkarev et al., 2016). The highest protein content was found in groundnut cake followed by soybean, almond, chia, rapeseed, sunflower, cottonseed, pumpkin, hemp, safflower, sesame, coconut, flaxseed, and olive oilcakes. The content of oil is 56% in peanut, more than in rapeseed (40%), sunflower (40%), and soybean and cotton (15–25%) (Savoire et al., 2012).

OILSEED CAKE CONTENTS PROFILING

More than 200 species of oilseed plants are cultivated around the world. The most important in food production are soybeans, canola, sunflower, coconut, olive and peanut. Cakes and flours are the residues that remain after the removal of most of the oil from the oilseeds. Desserts and foods based on soybeans, peanuts, cottonseed, rapeseed, sunflower, coconut, palm kernel, linseed and sesame seeds are used. These oilseed cakes and flours are often used in dairy cattle concentrates. The amino acid profile in soybean cake (**Table 1**) and the flow chart of soybean protein extraction process (**Figure 1**) is presented below.

Soybean cake is a rich source of protein and energy with lower fiber content than most oil cakes (**Table 1**). They are widely used as feed ingredients for both animals and humans due to their high digestibility and palatability. Methionine was found to be the main limiting amino acid in soybean cake, while threonine, valine, and lysine were marginal in a chicken performance study (Smith, 1986).

Being a high production and price flour in the cake market, soybean cake is followed by rapeseed cake in production. Rapeseed oil expeller is comparable to soybean expeller in amino acid balance and is richer in sulfur amino acids such as methionine and cysteine. The carbohydrates in rapeseed cake are mainly pectins (14.5%), cellulose, seaweed, arabinan and arabinogalactan. Its use in poultry feed is limited by the presence of antinutrients such as glucosinates, sinapine, tannins, erucic acid and phytates. Rapeseed cultivars that are low in erucic acid and glucosinates that are widely grown in Canada are known as canola. Mustard is an oilseed crop and condiment that belongs to the same genus as canola. The chemical and amino acid composition of mustard oil cake is very similar to that of rapeseed cake, but it contains more glucosinates than canola, although of a different type (Achaya, 1990). Cottonseed oil cake comprises 45% of the seed and is high in protein. But its use as a food ingredient for non-ruminants is subject to severe limitations due to the presence of the toxic metabolite gossypol, high fiber content and low levels of lysine, cysteine and methionine. Genetically glandless cottonseed containing gossypol has been developed and the oil cake obtained from this strain is said to have immense potential as a protein diet. Peanut oil cake, a solid compound with high protein content, is high in arginine, but low in essential amino acids such as lysine. Aflatoxins, toxic fungal metabolites of the *Aspergillus flavu* group, are frequent contaminants in these cakes and their presence has serious implications on animal performance. Sunflower oil expeller has a composition similar to cottonseed expeller and is rich in sulfur amino acids, but remarkably low in lysine. The copra cake or coconut oil cake has a protein content of around 20% with low concentrations of essential amino acids, in particular tryptophan, lysine, methionine and histidine. Palm kernel cake is dry and granulated with high fiber content. This reduces its suitability for monogastric animals. Like sunflower cake, it is deficient in lysine and high in sulfur-containing amino acids. It has a better amino acid index than coconut oil cake. Pie digestibility is low for poultry and pigs due to high fiber content (Ravindran and Blair, 1992).

TABLE 1 | Amino acid profiling of soybean cake.

Amino acid (g/100 g)	Soybean cake
Aspartic acid	4.71
Threonine	1.75
Serine	2.14
Glutamic acid	7.85
Proline	2.43
Glycine	1.56
Alanine	1.67
Cystine	0.57
Valine	2.01
Methionine	0.43
Isoleucine	2.22
Leucine	3.35
Tyrosine	1.42
Phenylalanine	2.18
Histidine	1.16
Lysine	2.64
Arginine	1.41

The individual amino acid content (mg/100 g dry extract) in the extracts of pumpkin, hemp and linseed is shown in **Table 3**. Cystine and cysteine were not detected with this analysis method. A strong correlation ($r = 0.833$; $p < 0.05$) was observed between the results obtained with the Kjeldahl and RP-HPLC methods. In general, the extraction of carbon dioxide into the atmosphere led to higher amino acid recoveries ($p < 0.05$) of all the matrices analyzed. The sum of the total amino acids in the extracts of pumpkin, hemp and linseed was, respectively, 75.09; 409.51 and 65.23 mg/100 g of dry extracts. For extractions in a nitrogen atmosphere with pure water, lower contents ($p < 0.05$) of total amino acids were found: 20.40 (pumpkin); 219.50 (hemp) and 18.84 (flax) mg/100 g of dry extract. The addition of hydrochloric acid in a nitrogen atmosphere enhanced the release of amino acids from the peptides / proteins. The amino acid recoveries (8.48–38.4% of the total protein content) obtained from different subcritical extraction conditions coincided with the values reported for other plant sources (5–53%) [32]. All oilseed extracts had high levels of essential amino acids (EAA: Thr, His, Lys, Val, Met, Trp, Phe, Ile and Leu). Pumpkin seed extracts had a significantly higher percentage of EAA ($p < 0.05$) (51.49–58.58%) than flaxseed extracts (35.91–40.66%) and hemp (22, 60–27.28%). The highest content of essential amino acid Lys (12.37 ± 0.10 mg/100 g dry extract) was observed in extracts of pumpkin seeds obtained in a carbon dioxide atmosphere, while Thr (34.70 ± 0.12 mg/100 g dry extract) and Leu (5.31 ± 0.06 mg/100 g of dry extract) were found to be the most abundant in hemp and linseed extracts, respectively, applying the same extraction method. In general, these amino acids are also dominant in raw materials [33–35]. The high concentrations of EAA in the characterized oilseed extracts suggest their potential use as food ingredients with a beneficial amino acid profile. The hemp seed extracts showed a high content of aromatic amino acids (FAA) Asp, Glu and Ala, with respective average ranges for three extraction conditions of 49.17–72.67 (Asp); 41.98–77.86 (Glu) and 10.77–17.83 (Ala) mg/100 g of dry extract. On the contrary, Gly levels were significantly ($p < 0.05$) higher in extracts of pumpkin seeds (4.82 ± 0.02 mg/100 g for example) and flax (6.08 ± 0.05). Mg /100 g e. Carbon dioxide atmosphere. The % FAA was as follows: 39.85–47.63% (hemp extracts) > 27.95–41.20% (flax extracts) > 16.56–24.88% (pumpkin extracts) high levels of FAA can contribute to the desirable sensory properties of all three oilseed cake extracts (**Table 2**).

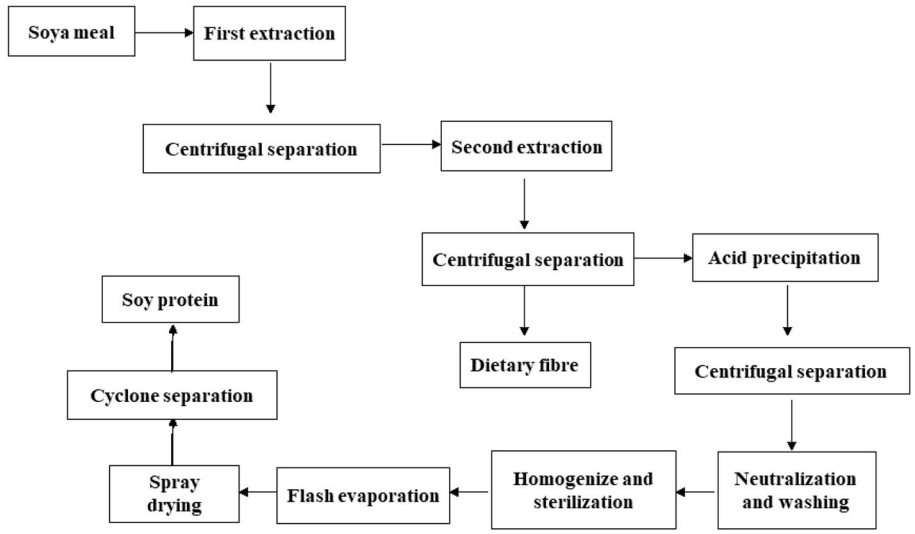

FIGURE 1 | Flow chart of soybean protein extraction process.

METHODS FOR EXTRACTION AND PURIFICATION OF PROTEIN FROM OILSEED CAKES

For preparing protein isolates/concentrates from oilseed cakes, the most widely used method is the two-step process patented by Anson and Pader (1955). After alkaline solubilization of proteins, insoluble materials (mainly starch and fiber) are removed by centrifugation. By adding hydrochloric acid to the supernatant, the protein is isoelectrically precipitated (pH 4.0–5.0), separated by centrifugation and neutralized. The co-product contains the other soluble components, mainly sugars, soluble fiber, fat, and ash. There are other modified versions of this historic 2-step process (Extraction/Isolation) on an industrial scale. In one of the modified method, the extraction was carried out under acidic conditions (Alli et al., 1993), or in water (Klamczynska et al., 2001). In general, the acidic or alkaline extraction can lead to a high level of purity (> 90%). In other method, instead of precipitation with pHi (assisted or not with heat treatment), it is possible to use the ultrafiltration route to purify proteins. This process was developed at the Food Technology Laboratory (Lyngby, Denmark) by Olsen and Hinge Andersen (1978). The main difference between pHi and the filtration process is the solubility and functionality, generally higher in the case of ultrafiltration.

Alternatives to two-step processes (extraction/isolation) have also been proposed on a pilot scale. For instance, the Prairie Regional Laboratory (Canada) has developed a simpler wet process, derived from those described above, to process pea proteins. All the alkaline extract is atomized and the protein content can reach up to 60%. "Micelization," proposed by Paredes-Lopez et al. (1991) is based on the phenomenon of salting in the salting of food proteins. As reviewed by Boye et al. (2010), after protein extraction using a saline solution suitable for the desired ionic strength, the solution is diluted, inducing the precipitation of proteins that can then be recovered by centrifugation or filtration, followed by drying. A purity of 88% has been achieved; however, due to the high consumption of water the scaleup of the process to the industrial scale is challenging.

The Food and Agriculture Organization of the United Nations (FAO) mentioned that protein will be limiting macronutrient in near future, therefore, to sustain the current and future requirements; researchers are adapting different kinds of method and processes to extract protein from oilseed cakes (Arrutia et al., 2020). Researchers extract protein from oil seed cakes using solvent extraction, acid or alkaline aqueous extraction assisted by salt and enzymes. In general, oil cakes are rich in polyphenols and these polyphenols were removed by purification approaches such as isoelectric separation followed by membrane separation (Xu and Diosady, 2002; Ghodsvali et al., 2005), protein micellar formation (Murray et al., 1980), use of ion-exchange resin or/and adsorbate resins (Pickardt et al., 2015). Rehder et al. (2017) reported the extraction of cruciferin rich protein (41.9%) and napin rich protein (58.3%) from rapeseed mustard cake (*Brassica napus* L.) using aqueous acid extraction (pH 2.0) followed by membrane filtration and spray drying technique (**Figure 2**). Similarly, cruciferin and napin protein were extracted and purified by Moreno-González et al. (2021) using ion exchange chromatography through high throughput experimentation technique (HTE). The experimental procedure involves the use of cation exchange resins (CaptoS, POROS 50HS, CM Sheparose and MacroPrep50) and mixed mode resins (CaptoMMC, Nuvia cPrime, PPA HyperCel and Toyopearl MX-Trp-650 M) and standardized procedure under different pH and salt concentration. Among different resins, POROS 50HS showed a promising result with cruciferin (>98%

TABLE 2 | Different oilseed cakes and their protein contents and nutrition characteristics.

Oilseed cake	Protein content	Others	Processing condition	References
Groundnut oil cake	45–60%	22–30% carbohydrate, 3.8–7.5% crude fiber, and 4–6% minerals	Electric expeller	Purohit and Rajyalakshmi, 2011; Srivastava and Mathur, 2018
Soyabean cake and meal	40–50%	Ca, P, Na	Expeller and solvent	Chen et al., 2010
Cotton seed cake and meal	21–57%	amylase	Mechanical/ solvent	Ancut and Sonia, 2020
Sesame oil cake	32%	Ca and P, Vitamin B, Lignan (neutraceutical)	Solvent extraction/ expeller	Yasothai, 2014; Sunil et al., 2015
Flaxseed/Rapeseed	34–42%	Lignan, fiber	Expeller and solvent	Sarwar et al., 2013
Pumpkin seeds	55–56%	Phytosterols, and Zn, K, Ca, Mg, Fe, Cu, and P	Hexane extraction	Nourmohammadi et al., 2017
Grapeseed vinification waste	Antioxidants	–	Supercritical antisolvent extraction	Teh and Bekhit, 2015
Sunflower kernel and shell	Polyphenols	P, Vit. B, Vit. E and fiber	Methanol extraction	Adeleke and Babalola, 2020
Sunflower seed cake	37%	Ca, P and Fe	Mechanical or solvent	Vasudha and Sarla, 2021
Copra cake + rice flakes	Protein and Carbohydrates	Na and K	Industrial samples	Sunil et al., 2016
Corn gluten meal	53–65%	Carotene and Xanthophyll	Mechanical/solvent	Hicks and Verbeek, 2016
Linseed meal	32–35%	Vit. A, and Niacin	Solvent extracted	Hicks and Verbeek, 2016
Copra cake	18%	Ca, P, Na, Fe and Zn	Industrial sample	Sunil et al., 2015
Rice bran pellets	9%	53% carbohydrate, Ca, P, Na, Fe and Zn Oryzanol (neutraceutical)	Industrial sample	Sunil et al., 2015

purity and 99% yield) and napin (98% yield with >99% purity) extraction.

In another approach, Gerzhova et al. (2016) extracted canola protein (58%) from defatted canola meal. using 15% w/w defatted canola meal at pH 12, without salt medium. The increase concentration of salt (0.4–1 M NaCl) tends to decrease the protein concentration (58–43%) under 15% w/w defatted canola meal. The schematic procedure for extraction of canola protein is depicted in **Figure 3**. Similarly, black cumin protein concentrates were extracted by alkaline treatment followed by isoelectric precipitation method.

The application of resin is increasing widely due to rapid adaption of protein extraction approaches from de-oiled cake. Pickardt et al. (2015) standardized the process for extraction of protein isolates and concentrates at laboratory and pilot scale (**Figure 4**). In this approach, cold pressed sunflower cakes were milled and after homogenization, samples were processed into salt solution to remove protein and phenolic fractions. Phenolic fractions were removed through use of adsorbate resin (styrene-divinylbenzene resin Amberlite XAD 16HP), whereas, protein solution were processed through mild acidic extraction followed by isoelectric precipitation. At pilot scale, anion resins were separately used to remove phenolic compounds from the high phenolic protein solution. After spray drying, 94–98% protein isolates and 65–68% protein concentrates were obtained.

Extraction of protein from oilseed cake using novel and advanced extraction technologies and green solvents, have been searched from past decades. As such the innovative techniques, including ultrasonic, microwave, enzyme-assisted, natural deep eutectic solvents, pulsed electric field, and subcritical water extraction were considered for protein extraction from oilseed cake (Phan et al., 2009; Russin et al., 2011; Bardeau et al., 2015; Sicaire et al., 2015).

Ultrasonicaltion-assisted extraction of protein from sunflower meal was performed and tested for its effect on the physicochemical and functional properties of the protein. It was found that under optimal extraction condition of power density (220 W/L), temperature (45°C), extraction time (15 min), the protein yield of 54.26% was obtained. It was observed that under the optimal extraction condition, the protein content, particle size, bulk density, and water and oil holding capacity was 934.92 g/kg, 627.6 nm, 0.372 g/ml, 0.985 g water/ g protein, and 2.06 g oil/ g protein, respectively. The solubility, emulsifying capacity, and emulsion stability was also

TABLE 3 | Benefits of protein from oilseed cake on food security.

Cake	Amino acid profile	Other beneficial nutrients	Antinutritive factors	Negative effect	Solution	Applications	References
Mustard cake	I, L, K, M, T, W, V H, A, R, D, G, S, Y	Gelling and emulsion stabilizing agents	Trypsin inhibitor activity	Reduce protein digestibility	Cooking	Meat and bakery products, INFANT formula and nutritional supplements	Sarker et al., 2015
			Allythiocyanate				
Sun-flower seed cake	L, V, Y, I, R, T, K, F, M, C, H W	Cholesterol-lowering phytosterols	Phytates	Interferes with absorbtion of minerals	Enzymatic treatment	Neutral-ceuticals	Vasudha and Sarla, 2021
Sesame oil cake	R, C, G, H, I, K, M, L, F	Anti-oxidants (resulting in better keeping quality)	Phytates	Interferes with absorbtion of minerals	Enzymatic treatment	Bakery	Yasothai, 2014
			Oxalates	Bitter taste	Decortication		
Almond oil cake	C, H, I, L, K, M, F, Y, T, V, W	Gluten free	Tannins	Inhibit protein adsorption, produce amino acid imbalance	Thermal, biological or solvent extraction	Gluten free cakes or other snacks	Houmy et al., 2020
Sun-flower oil cake	T, V, M, I, L, F, K, D, S, D, P, G, A, C, Y, H, R	Starch	Chlorogenic acid	Protein indigestibility and alter their Organo-leptic properties, storage life and stability in food systems	Thermal, biological or solvent extraction	Tablets coated with starch/honey/ chocolate/caramel	Ancut and Sonia, 2020; Yang and Zhu, 2021
Flax-seed meal	H, I, L, T, V, K, M, C, F, Y, A, R, D, D, G, P, S	Ferulic acid (antioxidant)	Trypsin inhibitor activity	Interferes with absorbtion of minerals	Heat treatment	Bakery and confectionery	Sá et al., 2020; Ancut and Sonia, 2020
			Phytic acid		Fermentation	Fermented beverages	
Pumpkin seed	H, I, L, T, V, K, M, C, F, Y, A, R, N, D, G, P, S	Anti-oxidative peptides	Trypsin inhibitor activity	Interferes with absorbtion of minerals	Thermal, biological or solvent extraction	Tablets coated with starch/honey/ chocolate/caramel	Nourmohammadi et al., 2017; Sá et al., 2020
			Phytic acid				
Rapeseed meal	D, T, G, A, V, M, L, H, K, R	N, P and K	Glucosinolates	Reduce feed intake, impaired thyroid function, liver enlargement	Immersion in water, microwave or acid/ alkali treatment	Protein concentrates, infant products etc.	Nega and Woldes, 2018
			Tannins				
			Phytic acid	astringency, inhibit protein adsorption, produce amino acid imbalance			
Soybean	L, M, C, T, W, R, L, H, F,Y, I, V, A, G, P, S, Q, N	Ca, P, Na	Saponins	Hemolysis, interference in Bl acids, lipid-soluble vitamins, cholesterol and dietary lipids	Thermal, biological or solvent extraction	Nuggets, desserts, fermented beverages etc.	Chen et al., 2010
			Phytoestrogens				
			Trypsin inhibitors				

recorded highest as 74.59, 52.45, and 50.45%, respectively. It was found that the ultrasonication helps in extracting the protein content and providing functional properties and further the product could be used for salad dressing and for meat products (Dabbour et al., 2018). In another study, ultrasound assisted extraction of rapeseed was optimized with a power of 5.6 W/cm^2 and temperature of 45°C which provide protein extraction yield of 4.24 g/100 g. It was found that UAE followed by two stage of

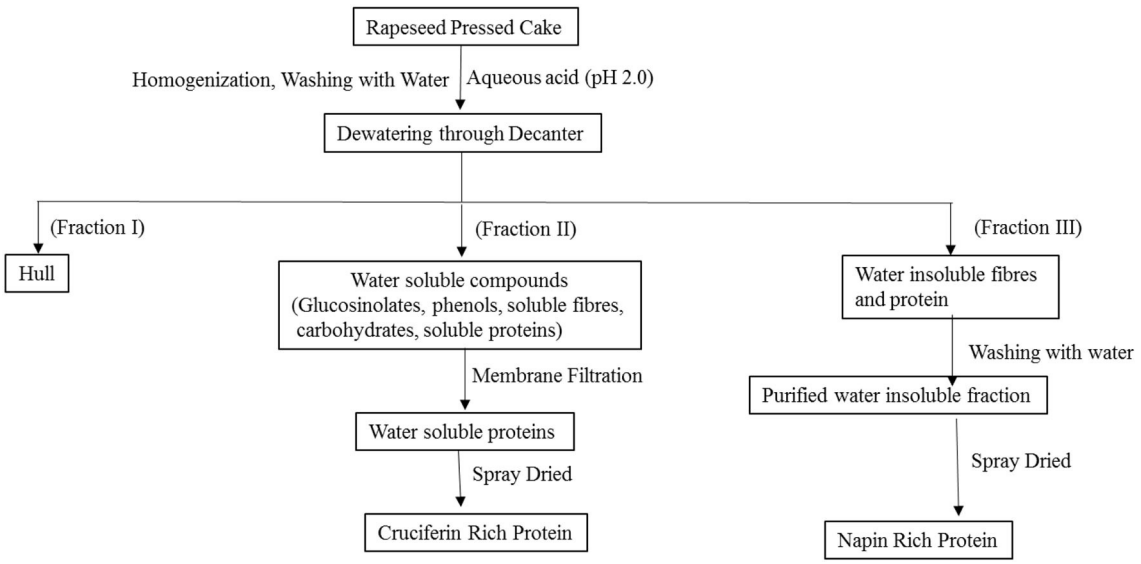

FIGURE 2 | Extraction of cruciferin and napin rich protein from rapeseed mustard cake using acid extraction.

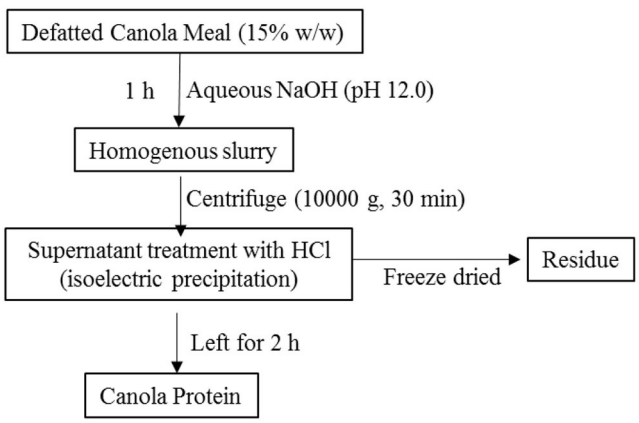

FIGURE 3 | Extraction of canola protein from defatted canola meal using alkaline extraction.

conventional extraction provide the total protein yield of 9.81 g/100 g (Boukroufa et al., 2017).

In a study, high voltage electric discharge was applied on rapeseed and rapeseed press cake for the recovery of protein, antioxidants, and isothiocyanates. The high voltage electric discharge was applied at different energy (0–400 KJ/kg) and different rapeseed press cake water ratio (1:5 to 1:20 w/w). It was found that the optimal energy input for polyphenols was 80 KJ/kg and 240 KJ/g for rapeseed press cake and rapeseed, respectively. For protein the optimal extraction condition is 240 KJ/kg with ratio of 1:20, which provide a protein extraction yield of 9.41 g/100 g and 15.76 g/100 g for rapeseed and rapeseed press cake, respectively (Barba et al., 2015). A multistage counter current extraction process was applied to improve the protein recovery from *Jatropha curcas* seed press cake. The higher protein content was found as 82% under the extraction condition of four stage counter current extraction, 0.055M NaOH at sample to solvent ratio of 10 g/g. It was found that increasing the extraction cycle from one to four significantly increased the extraction yield of protein from 35 to 71% (Lestari et al., 2020). Phenolic compounds and proteins were extracted from sesame cake using pulsed electric fields and high voltage electrical discharges. These were applied as pre-treatment to diffusion. It was found that the polyphenol and protein content increased during extraction with 83 KJ/kg of energy inputs. The electrotechnology reduce the need of organic solvents and high temperature (Sarkis et al., 2015).

In enzymatic extraction of protein from rapeseed press cake treated with pectinolytic, xylanolytic, and cellulolytic enzyme pre-treatments. It was found that these enzyme treatments cause 56 and 7% extraction of the total protein in the intact and dehulled press cake. It was recorded that the enzymatic treatment cause enhancement in the extraction process (Rommi et al., 2014). Enzymatic and non-enzymatic extraction of proteins from defatted rapeseed cold-pressing residue was tested under different pH. It was found that after enzymatic extraction the protein yield was 40–41% at pH 6, which is higher than the non-enzymatic alkaline extraction (pH 10). Also, water extraction provides protein stability, high zeta potential, and smaller particle size (Rommi et al., 2015). Similarly, the alkaline extraction method was compared with the enzyme-assisted method for the recovery of protein from Sacha inchi cake meal. Under alkaline extraction the optimal extraction condition was temperature of 54.2°C, solvent/meal ratio 42:1, NaCl concentration 1.65 M, pH 9.5 for 30 min and obtain 29.7% of protein yield, whereas under enzyme extraction a higher protein yield of 44.7% was obtained at an enzyme concentration of 5.6%, 40.4 min, 50:1 ratio, pH 9.0, and temperature of 50°C (Chirinos-Cuadros and Rosado-Samaniego, 2016).

Two different raw material, i.e., pre-pressed and cold-pressed rapeseed press cake were examined for protein extraction under

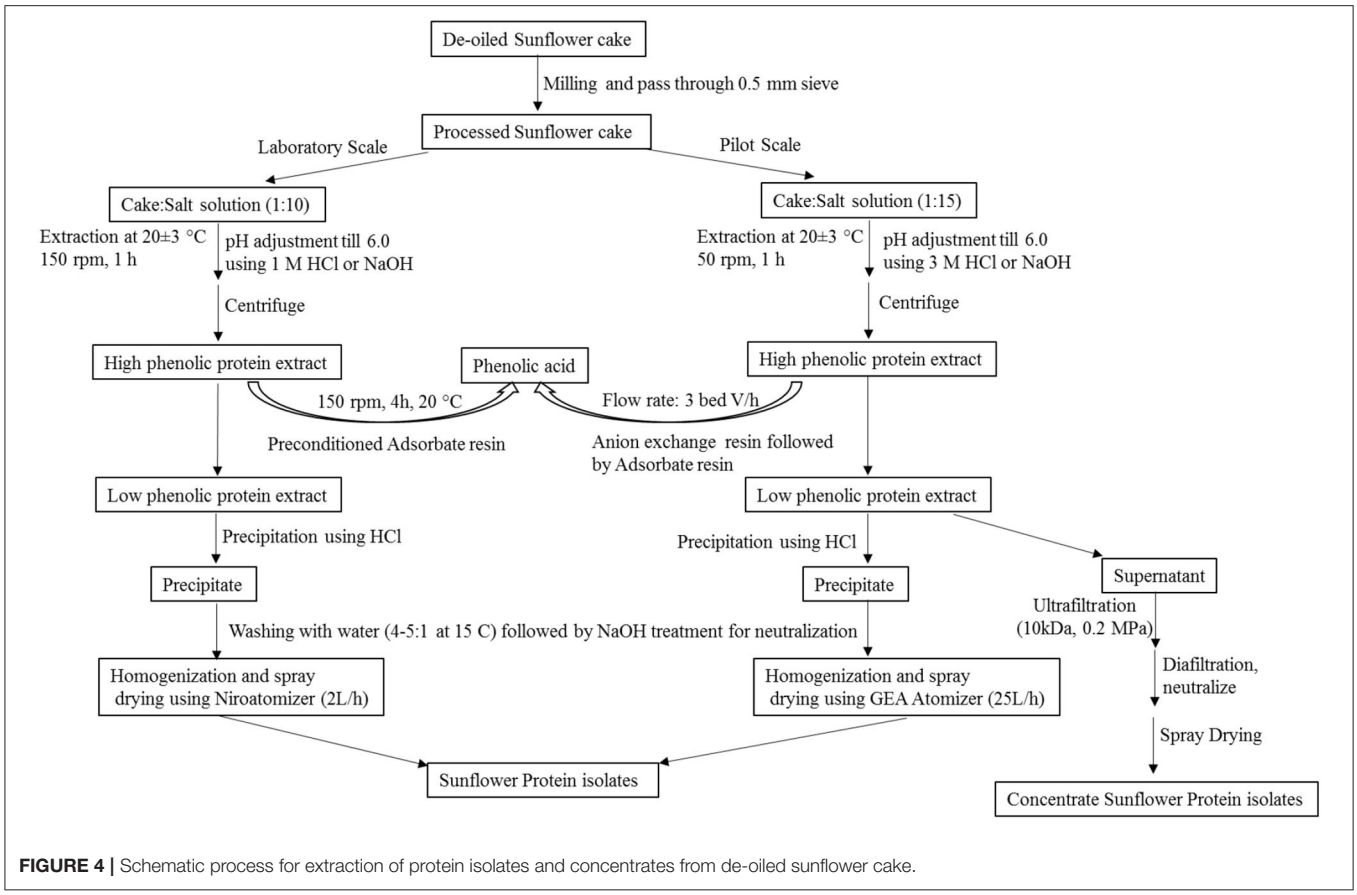

FIGURE 4 | Schematic process for extraction of protein isolates and concentrates from de-oiled sunflower cake.

different factors, i.e., solid to solvent ratio, extraction time, temperature, pH value, NaCl concentration, and number of extraction cycle. The protein yield was obtained as 52.3 and 36.7% for cold-pressed and pre-pressed rapeseed cake, respectively, under mild extraction condition. The protein yield under enzyme assisted extraction condition was 59.5 and 60.6% for cold-pressed and pre-pressed rapeseed cake, respectively, for one step process and 80.7 and 78.3% for three step process (Fetzer et al., 2020).

The use of deep eutectic solvents (DES) for protein extraction from flax cake, camelina cake, and sunflower cake was evaluated. In flax cake and camelina cake the protein yield was higher in DES as compared to n-hexane. Increasing temperature increased the protein extraction yield (Parodi et al., 2021). DES was used to extract protein from rapeseed cake and *Evening primrose* cake using glycerol-choline chloride deep eutectic solvent. It was found that the increasing temperature increase the extraction yield of 20 and 35% at 140°C from rapeseed cake and *Evening primrose* cake, respectively (Grudniewska et al., 2018). The valorization potential for extraction of protein from oilseed cakes of hemp, pumpkin and flax seed by subcritical water were carried out by Švarc-Gajić et al. (2020). In this approach, sample to solvent ratio were kept as 1:30 and subjected to increase of pressure with 10°C/min till 160°C and process was conducted till 1 h. The total protein content in pumpkin, hemp, and flax varied from 1.94–4.87, 4.83–6.83, and 1.30–2.84 g/100 g dry extract, respectively. Among Nitrogen (N_2) and Carbon dioxide (CO_2), CO_2 provide better atmosphere for protein yield.

BENEFITS OF PROTEIN FROM OILSEED CAKE

Oilcake/meal obtained after the extraction of oil from the oilseeds are an alternatively rich source of protein. The highest content of protein was found in groundnut cake (45–50%), followed by soyabean, cottonseed, rapeseed, sesame, sunflower, palm oil and olive oilcake. Protein hydrolysates (hydrolysed product of protein), protein isolates (>90% protein) and protein concentrates (30–80% protein) can be prepared by these protein rich oil cake. A variety of protein rich foods can be prepared using protein isolates. Protein isolates can be used as a functional ingredient in the development of protein beverages, shakes, energy bars, frozen desserts, sour cream, sour cream dips, meat analogs; cheese analogs extruded products, protein rich pasta, convenience beverage powders, and infant and weaning food. Protein hydrolysate can result from the hydrolysis of protein isolate and can be used in food industry. As a result of hydrolysis structural modification can occur in proteins, which improves the hydration and gelling properties, solubility, surface active properties, and the functionality of protein. The type of enzyme used, its specificity as well as degree of hydrolysis determines its functionality. A specific fragment

of protein known as bioactive peptides, are known to have biological activity and positively influences the human health (Langyan et al., 2021). Health benefits of bioactive peptide comprises of immuno-modulatory activity, hypocholesterolemic activity, antioxidant activity, bile acid binding activity and antithrombotic activity. For instance, cleavage of soybean protein hydrolysate under controlled condition followed by hydrolysis with microbial protease results in peptide with good functionality (like enhanced iron chelating activity and improved surface active properties). Hydrolysis of sesame oilcake/meal with alcalse, pepsin, and protein forms a peptide with enhanced functionality comprising of antioxidant activity, enhanced digestibility and antihypertensive property (Chatterjee et al., 2015). **Table 3** listed the benefits of protein from oilseed cake.

CONCLUSION AND FUTURE OUTLOOK

The present review provides a detailed overview on the protein content from oilseed cake, characterization, extraction techniques, and applications in food and health. It was found that a number of oilseed cake have been utilized as a protein rich substitute using both conventional and advanced extraction techniques. The most utilized oilseed cake for protein extraction includes rapeseed press cake and sunflower meal. A large number of aminoacids are present in oilseed cake. However, as plant based proteins have lower concentrations of essential amino acids, like methionine, tryptophan, lysine, and threonine, that limits their use as a complete protein source in human diet, hence research on combining different oilseed cake for meeting the protein requirement are urgently required. For instance, combining corn gluten meal with soybean meal provides a number of amino acids, as the former is a good source of methionins and cysteine but low in lysine and typtophan, and the latter has rich lysine and tryptophan content but deficient in cysteine and methionine. Moreover, research on extraction and purification of proteins from toxic substances by economically efficient method that saves the nutritional quality and/or improves digestibility require further efforts. The utilization of oilseed cake proteins in food and health products are now limited, which require further research on utilization of this enormously rich protein sources in diet.

AUTHOR CONTRIBUTIONS

RS: conceptualization, methodology, investigation, formal analysis, resources, writing—original draft, writing—review and editing, visualization, supervision, project administration, and funding acquisition. SL: investigation, writing—original draft, and writing—review and editing. SS, BR, and AK: writing—original draft, writing—review and editing. MS: conceptualization, methodology, resources, and writing—original draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Nuts as a Part of Dietary Strategy to Improve Metabolic Biomarkers: A Narrative Review

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Background: Nuts are in the spotlight because of their association with improved health outcomes. We aimed to summarize the findings of previous studies to evaluate the impact of nuts consumption on glycaemic and lipid profile, inflammation, and oxidative stress.

Methods: Electronic searches for observational and intervention studies were undertaken in PubMed, Embase, Web of Science, and Science Direct until 2022 for searching the studies aiming the application of different types of nuts and the beneficial effects of nuts in improving glycemia, dyslipidemia, inflammation, and oxidative stress.

Results: Results from 56 interventional, 9 narrative and 3 systematic reviews, and 12 meta-analysis studies, aiming at the evaluating beneficial effects of different types of nuts on metabolic markers, showed that nut consumption could improve metabolic markers, including glycaemic factors, lipid profile, and inflammatory and oxidative stress parameters in both healthy and individuals with metabolic disorders in a type-, dose- and duration-dependent manner. According to their unique nutrient components, nuts can be known as a part of a healthy diet, resulting in improved metabolic biomarkers.

Conclusion: Considering the efficacy of nuts in improving metabolic markers, incorporation of, incorporating nuts the effectiveness of nuts in improving metabolic markers, incorporating nuts in the diet may prevent the incidence or aggravation of chronic metabolic diseases. Considering the health benefits of the nuts' components, including essential micronutrients, if consumed in the appropriate dose and duration to provide the necessary amount of effective micronutrients to improve health, we will see an improvement in metabolic factors. At the same time, more research is required to determine the optimal type, dose, and duration of nut intervention with regards to metabolic control and reducing the risk of developing metabolic disorders.

Keywords: lipid profile, oxidative stress, glycemic control (A1C), metabolic biomarkers, inflammation

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INTRODUCTION

Nuts are known as healthy foods in the Mediterranean diet (MeDiet) because of their unique nutritional contents, and their consumption has been recommended to populations worldwide (1). Tree nuts, such as cashew nuts, hazelnuts, Brazil nuts, walnuts, almonds, pistachios, macadamias, and peanuts, are nutrient-dense foods, each with a unique combination of nutrients. Generally, these foods contain healthy fatty acid profiles including monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, soluble and insoluble fibers, protein, vitamin K, vitamin E, thiamine, folate, minerals such as copper, magnesium, selenium (Se), and potassium, and substances such as antioxidants, phytosterols compounds, and xanthophyll carotenoids, with known health benefits for humans (2, 3). Due to their low levels of saturated fatty acids and high levels of unsaturated fatty acids and bioactive compounds, nuts have been reported to decrease the risk of cardio-metabolic disorders (4). The beneficial effects of nuts consumption on chronic disorders have been studied in previous research. Prior trials have recommended that regular nut consumption can provide beneficial effects on health outcomes and cardio-metabolic disorders, such as hypertension (5), cardiovascular diseases (2), obesity (6), and diabetes mellitus (7), with a reduction in chronic diseases mediators such as inflammation, oxidative stress, hyper-glycemia, visceral adiposity, endothelial dysfunction, and insulin resistance (8). Moreover, several meta-analyses of clinical trials and observational studies support the beneficial effects of nuts consumption on several cardio-metabolic disorders (9–13).

Nuts consumption offers a wide range of health benefits on humans; however, the present review will provide an update for giving an overview of recent findings of focus on metabolic benefits of nuts on glycaemic control, lipid profile, oxidative stress, and inflammatory status, and the appropriate dose and duration of nuts consumption to achieve metabolic benefits.

NUTS AND METABOLIC BIOMARKERS

Evidence of epidemiological and interventional studies suggest that nuts consumption is associated with in reducing the incidence and aggravation of some metabolic diseases (14). Nuts are good sources of fiber, healthy fats, and other beneficial nutrients (**Figure 1**) (15), and each type of nut offers unique nutritional benefits.

The ability of nuts to reduce the risk of chronic diseases and improving the level of metabolic markers is now well recognized (**Figure 2**) (16). Previous studies have shown that nuts consumption is associated with a reduced risk of cardiovascular disease (CVD), diabetes, and other chronic metabolic disorders (**Table 1**).

Glycaemic Control

A significant number of studies showed a link between regular nuts consumption and a reduction in risk of heart and metabolic disorders (73–76). Moreover, studies have shown

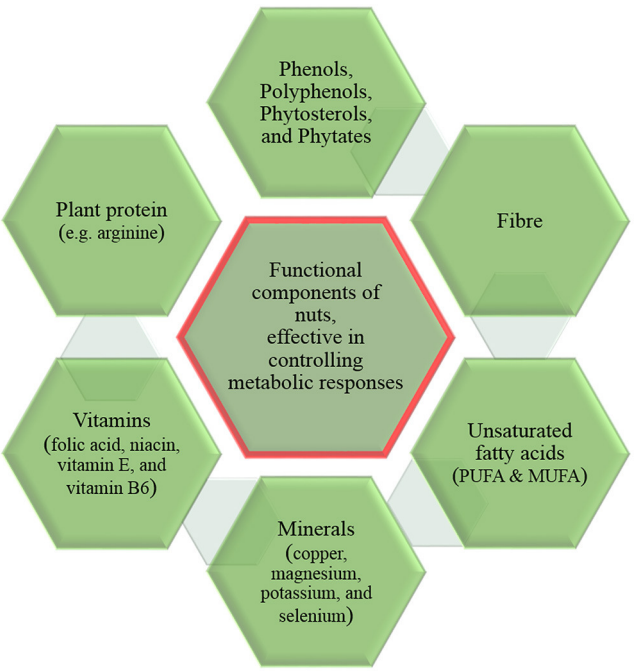

FIGURE 1 | Beneficial components of nuts offering health benefits.

that nuts consumption can improve glycemic responses in healthy and diabetic individuals (73). Nuts affect glycaemic response in a dose-dependent manner (77). According to the previous investigations, nuts help decrease glycaemic excursions; moreover, consuming nuts with carbohydrate-rich foods could reduce the postprandial impact on the insulin demand (77–80). The dose and duration of supplementation in studies that observed a significant effect of nuts consumption on glycaemic factors ranged from 30–60 g/day and 4–24 weeks, respectively (17, 18, 33, 35, 49, 52). The dose-dependent improvement in the glycaemic response to the meal has been revealed in previous investigations. In a study conducted on 10 healthy volunteers, it has been shown that the addition of 28 g of pistachios to white bread could improve glycaemic response, and this improvement was greater with the addition of 84 g of pistachios (79). In another study conducted on normo-glycaemic and individuals with T2D, adding 30, 60, and 90 g nuts to white bread reduced the glycaemic response of the meal by 11.2 ± 11.6% ($P = 0.354$), 29.7 ± 12.2% ($P = 0.031$), and 53.5 ± 8.5% ($P < 0.001$) (80).

It has been shown that people who eat more nuts in their diet have been shown to have a higher cardioprotective profile of glucose/insulin homeostasis (81). Findings from previous studies indicate a significant reduction in fasting insulin levels or an improvement in insulin resistance following the nuts consumption in both healthy and diabetic individuals (82, 83). Results from the Nurses' Health Study cohort indicated that eating more than 5 servings/week of nuts reduces the risk of diabetes compared to rare or no consumption (RR = 0.73, 95% CI = 0.60–0.89; P <.001) (84). Beneficial evidence is also supported

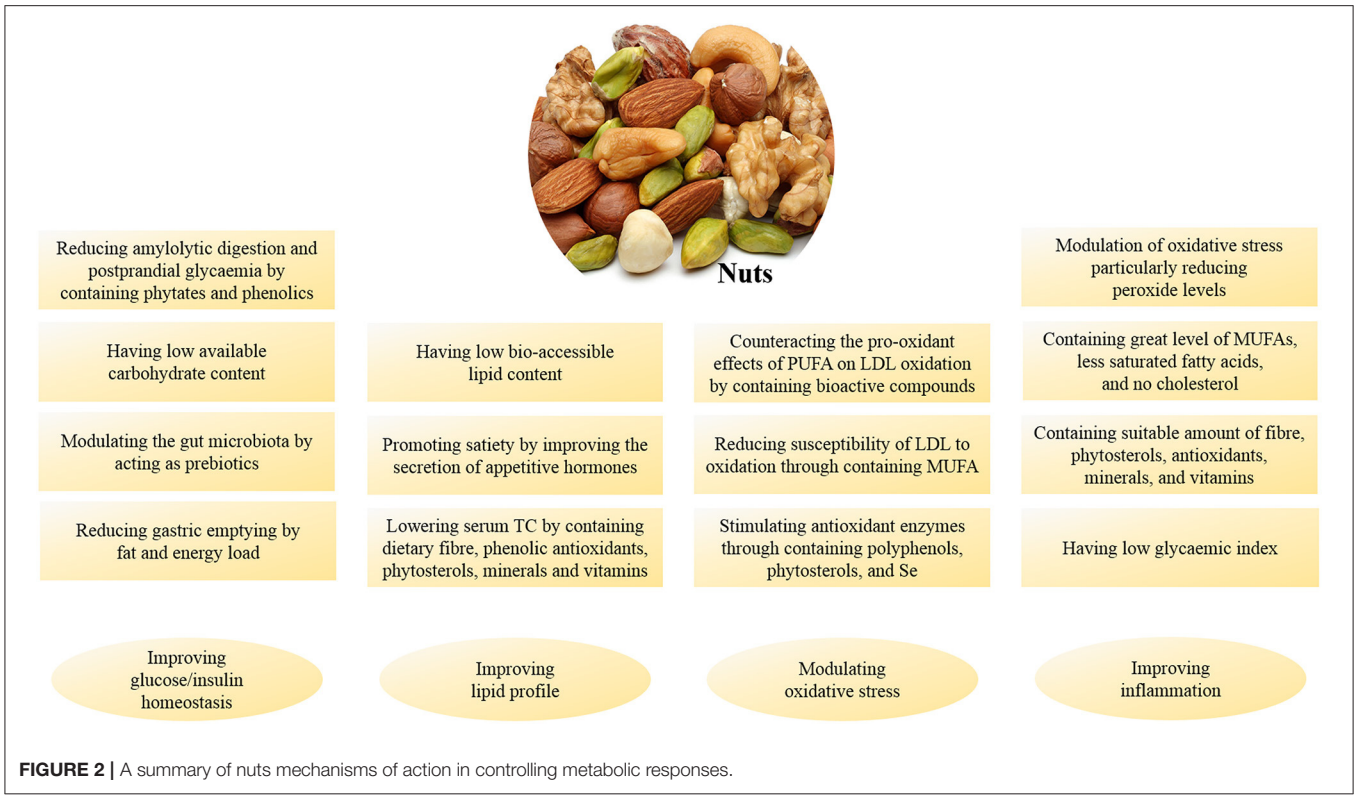

FIGURE 2 | A summary of nuts mechanisms of action in controlling metabolic responses.

by randomized controlled trials (RCTs). A meta-analysis of 12 RCTs with more than a 3-week follow-up period showed that consumption of a median dose of 56 g/day tree nuts could improve glycemic control in individuals with T2D compared to isocaloric diet without tree nuts (7). Additionally, the results of the study, conducted by Kendall et al. (80), showed that nuts eaten alone or with a high glycaemic index (GI) diet can lower postprandial blood sugar (PBG).

In addition to fat and protein, nuts have been reported to be rich sources of phenolics and phytates, both of which can decrease amylolytic digestion and postprandial blood sugar (85). In nuts, abundant fiber and polyphenols (flavonoids and non-flavonoids) may have a prebiotic effect and affect glucose metabolism (86). Several researchers have revealed that prebiotics' modulation of gut microbiota can improve glycemic control in healthy and diabetic subjects (87–89). Moreover, the blood sugar controlling effect of nuts can be attributed in part to the low carbohydrate content of nuts. Additionally, it has been shown that gastric emptying can be reduced by energy and fat load. Therefore, increasing the energy and fat load by increasing the dose of consumed nuts can partly explain the dose-dependent decrease in blood sugar in response to nuts consumption (80). In addition, it has also been shown that nuts can reduce the glycaemic response to a meal compared to a balanced meal in terms of energy and macronutrient contents (78). This can be due to nutsnuts' high unsaturated fat content, unsaturated fat content,and their unique physical structure. Unsaturated fatty acids in nuts in place of saturated fat and carbohydrate appear to, improve fasting, improve fasting and 2-h glucose levels significantly, but further studies should be performed with careful consideration of different types and amounts of nuts (90, 91).

In summary, overall nut intake has been revealed to be inversely associated with glycaemic factors. It may delay the development and progression of chronic metabolic diseases related to impaired glucose tolerance or glycaemic response.

Lipid Profile

The beneficial effects of nuts consumption on lipid profiles, especially total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), have been reported in clinical studies in both healthy and high-cholesterolemic individuals from various geographical areas (92). However, the effects varied based on the type and amount of nut, consumption duration, characteristics of the studied individuals, and study design (93–98). Most studies in this field that have shown a significant effect of nuts consumption on lipid factors have reported a dose of 20–64 g/day and a duration of 4–24 weeks (18, 20, 21, 53, 57, 70).

A Systematic Review conducted by Altamimi et al. (99) evaluated several dietary intervention studies that examined the effect of eating nuts on blood lipid levels. Analyses were performed on different types of nuts. Most of the studies showed improvement in lipid profile, including TC, LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), and total cholesterol/high-density lipoprotein cholesterol (TC/HDL-C) after nut consumption. Tapsell et al. (95) found that following a healthy diet enriched with 30 g of walnuts for 6 months caused a significant reduction in LDL-C and increased HDL-C in subjects

TABLE 1 | A summary of studies evaluating the effect of nuts supplementation on metabolic markers.

N	ID	Type of study	Study population	Dose	Duration	Findings
Almond						
1	Li et al. (17)	Randomized crossover clinical trial	Chinese patients with T2DM	60 g/day	4 weeks	Those in the almond diet had lower levels of fasting insulin, FBS, and HOMA.IR.
2	Abazarfard et al. (18)	Randomized controlled trial (RCT)	Overweight and obese women	50 g/day	12 weeks	TC, TG, LDL-C, and FBS decreased significantly in the almond group compared to the nut-free group.
3	Ashley et al. (19)	Randomized crossover trial	Healthy individuals and individuals with T2DM	One serving per meal	12 weeks	A standard serving of almonds reduced postprandial glycaemia significantly in participants with diabetes but did not influence glycaemia in participants without diabetes.
4	Jung et al. (20)	Randomized, crossover trial	Overweight and obese Korean adults	56 g/day	4 weeks	Almond consumption decreased TC, LDL-C, and non-HDL-C, compared to the control. Of serum inflammatory markers, IL-10 was decreased by almond intake, and IL-1β and IL-6 tended to be lower with almonds, compared to the control.
5	Gulati et al. (21)	Free-living pre-post intervention study	Asian Indians in North India with T2DM	20% of energy intake	24 weeks	TC, TG, LDL-C, HbA1c, and hs-CRP significant improved after intervention.
6	Chen et al. (22)	RCT	Patients with T2DM		12 weeks	Almond decreased post-interventional FBS and HbA1c as compared to that of control.
7	Foster et al. (23)	RCT	Overweight and obese individuals	24 almonds per day	24 weeks	The almond-enriched diet, compared with the hypo-caloric nut-free diet, was associated with greater reductions in TC, total:HDL-C, and TG.
8	Liu et al. (24)	RCT	Healthy adults	56 g/day	20 weeks	Participants in the almond group showed favorable significant changes in including levels of TG, TC, LDL-C, and non-HDL-C after consuming of almond compared with those at baseline.
9	Sabaté et al. (25)	Randomized crossover design	Healthy subjects	10%, and 20% of total energy	4 weeks	Compared with the Step I diet, the high-almond diet reduced TC, LDL-C, Apo B, and ratio of LDL to HDL-C, and increased HDL-C.
10	Berryman et al. (26)	Randomized, crossover, controlled-feeding study	Individuals with elevated LDL-C	1.5 oz./day	6 weeks	The almond diet, compared with the control diet, decreased non-HDL-C and LDL-C
11	Liu et al. (27)	RCT	Young Korean adults	56 g/day	16 weeks	Consuming almonds as a daily snack reduced the levels of TC and LDL-C.
12	Liu et al. (28)	Randomized crossover controlled feeding trial	Chinese patients with T2DM	56 g/day	4 weeks	Compared to the control diet, the almond diet decreased IL-6, CRP, and TNF-α. The almond diet also enhanced the resistance of LDL against Cu^{2+}-induced oxidation compared to the control diet.
13	Jia et al. (29)	Pilot study	Healthy adult male regular smokers	84 g/day	4 weeks	MDA levels in the almond-treated groups were lower than the controls. Almond consumption has preventive effects on oxidative stress caused by smoking.
14	Li et al. (30)	Randomized, crossover clinical trial	Healthy smoker male soldiers	84 g/day	4 weeks	After the almond intervention, serum α-tocopherol, SOD, and GPX increased and MDA decreased significantly in smokers.

(Continued)

TABLE 1 | Continued

N	ID	Type of study	Study population	Dose	Duration	Findings
15	Sweazea et al. (31)	Randomized, parallel-arm controlled study	Individuals with T2DM	1.5 oz/day	12 weeks	The inflammatory biomarker CRP was significantly reduced in the almond-treated group vs. controls.
Pistachios						
16	Sauder et al. (32)	Randomized, crossover, controlled feeding study	Adults with well-controlled T2DM	20% of total energy intake	4 weeks	TC, the ratio of total to HDL-C, and TG were significantly lower following the pistachio diet compared to the control diet.
17	Hern'andez-Alonso et al. (33)	RCT	Prediabetic subjects	57 g/day	16 weeks	FBS, insulin, and HOMA.IR decreased significantly after the intervention period compared with the control group.
18	Parham et al. (34)	double-blind, randomized, placebo-controlled, crossover trial	Patients with T2DM	50g/day	12 weeks	There was a marked decrease in HbA1c, FBS, and CRP in the pistachio group compared with the control group.
19	Gulati et al. (35)	RCT	Individuals with the MetS	20% energy	24 weeks	FBG, TC, LDL-C, hs-CRP, TNF-α, TBARS, and adiponectin levels were significant improved in the intervention group compared with control group.
20	Sari et al. (36)	RCT	Healthy young men	20% of daily caloric intake	4 weeks	Compared with the MeDiet, the pistachio diet decreased FBS, LDL-C, TC, TC/HDL-C and LDL-C/HDL-C ratios, and TG significantly. The pistachio diet significantly decreased serum IL-6, total oxidant status, lipid hydroperoxide, and MDA and increased SOD.
21	Canudas et al. (37)	Randomized crossover clinical trial	Prediabetic subjects	57 g/day	16 weeks	Compared with the control diet, the pistachio diet reduced oxidative damage to DNA and improved FBS and HOMA.IR.
Walnut						
22	Wu et al. (38)	Randomized, controlled, cross-over study	Healthy Caucasian men and post-menopausal women	43 g/day	8 weeks	Walnut supplementation significantly decreased fasting non-HDL-C and Apo B in healthy senior individuals.
23	Hwang et al. (39)	Two-arm, randomized, controlled crossover study	Korean adults with MetS	45 g/day	16 weeks	Significant improvements after walnut intake, compared to control intervention, in HDL-C, FBS, and HbA1c were observed.
24	Ros et al. (40)	Randomized crossover trial	Hypercholesterolemic men and women	32% of the energy from MUFA	4 weeks	The walnut diet significantly reduced TC and LDL-C.
25	Ashraf et al. (41)	Experimental study	Individuals with hyperlipidemia	25 g and 50 g/day	8 weeks	Consumption of walnut showed significant improvements in lipid profile of hyperlipidemic individuals.
26	Zambo'n et al. (42)	Randomized, crossover feeding trial	Men and women with polygenic hypercholesterolemia	35% of the energy obtained from MUFA	6 weeks	Compared with the MeDiet, the walnut diet produced significant changes in level of TC, LDL-C, and lipoprotein (a).
27	Bashan et al. (43)	RCT	Patients with dyslipidemia	40–50 g/day	12 weeks	TC, LDL-C, VLDL-C, and TG levels significantly decreased and HDL-C levels significantly increased in the walnut group at the end of the trial.
28	Torabian et al. (44)	Randomized crossover trial	Subjects with normal to moderate high plasma total cholesterol	12% of total daily energy intake	24 weeks	Significant changes in serum concentrations of TC and TG were seen and nearly significant changes in LDL-C were found by supplementing a habitual diet with walnuts.

(Continued)

TABLE 1 | Continued

N	ID	Type of study	Study population	Dose	Duration	Findings
29	Bamberger et al. (45)	Randomized, controlled, prospective, cross-over study	Healthy subjects	43 g/day	8 weeks	The walnut diet resulted in a significant reduction in fasting cholesterol, non-HDL-C, LDL-C, TG, and Apo B levels.
30	Rock et al. (46)	RCT	Overweight and obese men and women	15% of energy	24 weeks	The walnut-enriched diet group reduced TC and LDL-C.
31	Alibabaie et al. (47)	RCT	Female Undergraduate Students	40 g/day	4 weeks	A significant reduction was observed in the serum levels of LDL-C and TG after the consumption of walnuts.
Peanuts						
32	Liu et al. (48)	Randomized, controlled, crossover postprandial study	Healthy overweight or obese men	85 g		Acute peanut consumption blunted the serum TG.
Hazelnuts						
33	Damavandi et al. (49)	Controlled randomized parallel study	Patients with type 2 Diabetes	29 g/day	8 weeks	Hazelnut consumption non-significantly reduced TG, FBS, TC, and LDL-C levels.
34	Renzo et al. (50)	Prospective pilot clinical trial	Healthy volunteers	40 g/day	6 weeks	Significant up-regulation was detected for SOD, CAT, PPAR-γ, and ACE at the end of the study.
Brazilian nut						
35	Cominetti et al. (51)	RCT	Obese women	One nut per day	8 weeks	Obese people who implement daily consumption of Brazilian nuts could improve lipid profile, especially HDL-C levels
36	Colpo et al. (52)	Randomized crossover study	Healthy individuals	20 or 50 g		A single intake of Brazil nuts caused a significant decrease in serum IL-1, IL-6, TNF-α, and IFN-γ levels.
37	Maranhão et al. (53)	RCT	Obese female adolescents	15–25 g/day	16 weeks	Compared to placebo group, Brazil nuts intake reduced TC, TG, and LDL-ox
38	Macan et al. (54)	RCT	Patients with T2DM	One nut per day	24 weeks	Supplementation with Brazil nuts significantly increased serum Se levels. Furthermore, it was found that the cells were more resistant to H_2O_2-induced DNA damage after the supplementation.
39	Stockler-Pinto et al. (55)	RCT	Hemodialysis patients	One nut per day	12 weeks	The plasma Se and GPx activity increased; moreover, HDL-C levels increased and LDL-C levels decreased significantly after supplementation.
40	Watanabe et al. (56)	RCT	Patients in regular use of statins	One nut per day	12 weeks	Brazil nut decreased levels of CK activity in serum, MDA, and SOD and increased levels of GPX activity. Moreover, the supplementation caused significantly positive changes in plasma and erythrocyte Se concentrations.
Cashew						
41	Mah et al. (57)	Randomized, crossover, isocaloric, controlled-feeding study	Normally active men and women	28-64 g/day (11% of total energy intake)	4 weeks	Consumption of the cashew diet resulted in a significant change from baseline (compared with the control) in TC, LDL-C, non-HDL-C, and the TC:HDL-C ratio.
42	Damavandi et al. (58)	Randomized, isocaloric, controlled-feeding study	Patients with T2DM	10% of total calorie intake	8 weeks	Serum insulin, HOMA-IR, and LDL-C/HDL-C ratio significantly decreased in the cashew group compared with those of the controls.
43	Shidfar et al. (59)	Randomized parallel clinical trial	Patients with T2DM	10% of total daily calorie intake	8 weeks	Mean HDL-C and insulin concentration were significantly improved in intervention group compared with control group.

(Continued)

TABLE 1 | Continued

N	ID	Type of study	Study population	Dose	Duration	Findings
44	Mohan et al. (60)	Parallel-arm, randomized controlled trial	Patients with T2DM	30 g/day	12 weeks	Participants in the intervention group had a greater increase in plasma HDL-C compared with controls.
Pecan						
45	McKay et al. (61)	Randomized, controlled feeding trial	Patients with T2DM	15% of total calories	4 weeks	Changes in serum insulin, HOMA-IR, and HOMA-β were significantly greater in intervention group than those in control group.
46	Campos et al. (62)	RCT	Patients with stable coronary artery disease	30 g/day	12 weeks	The pecan nut consumption exhibited a significant reduction in non-HDL-C levels and in the TC/HDL-C ratio compared to the control group.
Soy nut						
47	Sedaghat et al. (63)	Case-control study	Patients with T2DM	60 g/day	8 weeks	Soy consumption significantly lowered FPG, HbA1c, plasma insulin levels, insulin-resistance, TC, and LDL-C.
48	Sedaghat et al. (64)	RCT	Patients with T2DM	60 g/day	8 weeks	Consuming soy nut significantly decreased the FBS, TC, and LDL-C and increased the capacity of serum total antioxidants.
49	Bakhtiary et al. (65)	RCT	Women with MetS	35 g/day	12 weeks	The soy-nut improved FBG, insulin, HOMA-IR, MDA, and TAC significantly after intervention.
50	Bakhtiari et al. (66)	RCT	Old women with MetS	35 g/day	12 weeks	Soy-nut significantly decreased TC, LDL-C, VLDL-C, Apo B100, FBS, serum insulin, HOMA-IR, and MDA levels. Moreover, the intervention significantly increased TAC compared with the control group.
51	Karamali et al. (67)	RCT	Women with polycystic ovary syndrome	35% daily protein intake	8 weeks	Consumption of soy-nut, compared with the control group, resulted in significant decreases in FBS, insulin, and insulin resistance, as well as a significant increase in quantitative insulin sensitivity check index. In addition, significant decreases in TC, TG, and MDA and significant increases in NO and GSH were seen in the test group compared to the control.
52	Azadbakht et al. (68)	Randomized crossover clinical trial	Postmenopausal women with the MetS	30 g/day	8 weeks	The soy-nut regimen significantly decreased HOMA.IR, FBS, and LDL-C compared with the soy-protein or control.
53	Hematdar et al. (69)	RCT	Subjects with T2DM	a cup of cooked soy beans three days a week	8 weeks	A significant decrease was observed in serum CRP of soy bean group which was significantly more than the controls.
Baru almond						
54	Bento et al. (70)	Randomized, crossover, placebo-controlled study	Mildly hypercholesterolemic subjects	20 g/day	6 weeks	Compared to placebo, supplementation of baru almonds reduced TC, LDL-C, and non-HDL-C.
55	Souza et al. (71)	RCT	Overweight and obese women	20 g/day	8 weeks	The consumption of baru almonds increased HDL-C level compared to baru almond-free diet.
56	Souza et al. (72)	Parallel-arm, randomized placebo-controlled trial	Overweight and obese women	20 g/day	8 weeks	The baru almond group increased the activity of GPx and plasma copper concentration when compared to the placebo group.

FBS, fasting blood sugar; HOMA.IR, homeostatic model assessment for insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; HbA1c, glycated hemoglobin; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; IL, interleukin; hs-CRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor alpha; MDA, Malondialdehyde; TAC, total antioxidan capacity; SOD, superoxide dismutase; GPX, glutathione peroxidase; TBARS, thiobarbituric acid reactive substances; CAT, catalase; ACE, angiotensin-converting enzyme; PPAR-γ, peroxisome-proliferator activator receptor γ; FPG, form-amido-pyrimidine glycosylase; NO, nitric oxide; Apo B, apolipoprotein B; T2DM, type 2 diabetes mellitus; MetS, metabolic syndrome.

with diabetes. A recent study also showed beneficial changes in lipid profile after a MeDiet enriched with 30 g of mixed nuts compared to the control diet in high-risk CVD patients, about half of whom with T2D (96). Compared to the low-fat diet, the average changes in the MeDiet enriched with the nuts group were −6.20 ($P = 0.040$) for TC level, −13.0 ($p = 0.022$) for TG level, and −0.26 ($P = 0.002$) for the TC/HDL-C ratio (96). Lee et al. (97) showed that supplementing 30 g/day of mixed nuts (walnuts, peanuts, and pine nuts) with a usual diet for 6 weeks had beneficial effects on lipid profile in women with MetS. Compared to those in the control group, TC and non-HDL-C levels significantly decreased in the nut group ($P = 0.023$ and $P = 0.016$, respectively). A cross-sectional study conducted to examine the relationship between nuts intake and lipid profile among Iranians showed that regular consumption of pistachios, almonds, hazelnuts, and walnuts is associated with a lower incidence of hyperlipidemia (98). Results of this study revealed that nuts consumption is significantly associated with decreased LDL-C, TG, and Apo B/Apo A in both men and women and decreased TC only in women. In a systematic review, Mukuddem-Petersen et al. (92) found a 2–16% reduction in TC and 2–19% in LDL-C among people who consumed nuts compared to those on controlled diets. In addition, a prospective cohort study with positive quality showed the same results of nuts consumption in reducing TC, non-HDL-C, LDL-C, and Apo B-100 concentrations in women with diabetes (100). Sabaté et al. (25) compared the effects of two different amounts of almonds with the effects of the National Cholesterol Education Program Step I on serum lipids and lipoproteins in healthy adults and those with mildly high cholesterol (Low and High almond diets for replaceing 10 and 20%, respectively, of energy of the Step I diet with almonds). They found that in addition to lowering LDL-C, the high-almond diet significantly reduced Apo B concentrations. Apo B is a component of serum LDL and VLDL and consequently reflects the concentration of atherogenic lipoprotein particles that confer risk of CVD (101). Sabaté et al. (25) observed a decreasing trend in Apo B concentration with increasing amounts of almonds in the diet, indicating a decrease in LDL-cholesterol concentration and the number of LDL particles.

Several claimed reasons explain the biological rationale of consuming nuts and improving the lipid profile. First, the bioavailability of fat content of nuts is low, which means that large amounts of this fat are excreted in the feces (102). Second, the crunchy texture of nuts promotes satiety because the mechanical action of chewing leads to the release of appetite suppressant hormones such as cholecystokinin, which ultimately leads to lower calorie and fat intake (103). Third, it is suggested that nut components other than fatty acids, such as vitamins (e.g., vitamin E, vitamin B6, niacin, and folic acid), minerals (e.g., magnesium, potassium, and copper), dietary fiber, plant protein (e.g., arginine), phytosterols, and phenolic antioxidants are also bioactive in lowering serum TC level (104). Recent evidence suggests that the phytosterols in nuts which are more hydrophobic than cholesterol, impair cholesterol absorption because their hydrocarbon molecule is larger and has a greater affinity for micelles than cholesterol (105). As a result, cholesterol is displaced from the micelles, and the amount available for absorption becomes more limited (106). Nuts are also a rich source of protein (nearly 25% of energy), especially high in L-arginine (107). L-arginine supplementation is usually recommended for people with dyslipidemia. It has been suggested that L-arginine ihelpmproves the lipid profile because of its potential in increasing nitric oxide (NO) production. Production of NO increases the activity of lipoprotein lipase and finally, improving the hydrolysis of TGs reduces its plasma concentration (108). A meta-analysis, conducted by Hadi et al. (109), concluded that L-arginine supplementation could significantly reduce blood TG levels; however, more studies are needed to prove its hypocholesterolemic effects.

Due to their unique nutrient profile, enriching a healthy diet with nuts may positively affecting lipid profile. Lipid-lowering effects of nuts should be considered in future food-based dietary strategies for improving plasma lipid levels.

Inflammation

Inflammation plays a key role in the development of cardio-metabolic diseases, such as CVD and T2D and nuts may moderate inflammation and the development of endothelial dysfunction and cardio-metabolic disorders through their bioactive components such as L-arginine, alpha-linolenic acid (MUFA), polyphenols, and fiber (110). Cross-sectional studies in this field showed that people with regular nut consumption had lower serum concentrations of proinflammatory cytokines or endothelial cell adhesion molecules. Effective interventional periods ranged from 4 to 24 weeks, with doses ranging from 20 to 56 g/day (20, 21, 34, 52).

It has been shown that α-linolenic acid [18:3(n-3)], extracted from nut is inversely associated with interleukin-6 (IL-6), soluble tumor necrosis factor (TNF) receptors 1 and 2, fibrinogen, and C-reactive protein (CRP) levels in both healthy subjects and individuals with coronary artery disease (111, 112). Furthermore, subjects who ate more nuts showed lower levels of intercellular adhesion molecule 1 (ICAM-1)-1, vascular cell adhesion protein 1 (VCAM-1), CRP, and IL-6 (113). In a cross-sectional analysis of the Multi-Ethnic Study of Atherosclerosis, consumption of seed and nut was inversely associated with IL-6, CRP, and fibrinogen (114). Yu et al. examined the association of common consumption of nuts with inflammatory biomarkers in two large groups of American men and women (115). They showed that nuts consumption was inversely associated with the concentration of IL-6 and CRP. The health effects of nuts on inflammatory markers have also been investigated in clinical trials. In a randomized trial, patients with MetS were advised to follow a healthy diet with 30 g of daily supplementation of raw nuts for 12 weeks (7.5 g hazelnuts, 15 g walnuts, and 7.5 g almonds) (82). Among inflammatory markers, the diet of nuts significantly resulted in changes in plasma IL-6. In another clinical trial, almond diet (56 g/day) for 4 weeks reduced CRP by a median 10.3 % (95% CI: −24.1, 40.5), TNF-α by a median 15.7 % (95% CI: −0.3, 29.9), and IL-6 by a median 10.3 % (95% CI: 5.2, 12.6 %) in comparison with the control diet (28). Gulati et al. (35) showed that supplementation of pistachio (20% energy)

for 24-wk significantly improved hs-CRP ($P < 0.05$) and TNF-α ($P < 0.03$) in individuals with MetS. A meta-analysis of 32 RCTs showed that consumption of nuts resulted in small and non-significant differences in CRP levels (10).

The beneficial effects of nuts on inflammatory markers are attributed to their composition, which is recognized by lesser saturated fatty acids, a greater level of mono-unsaturated fatty acids (MUFAs), no cholesterol, and a suitable amount of phytosterols, fiber, protein, antioxidants, and numerous vitamins and minerals (116, 117). An increasing number of studies have examined the effect of these nutrients on inflammation. As suggested in cross-sectional studies, the high content of phenolic compounds in nuts may anticipate the anti-inflammatory effect of regular consumption of nuts (100). Furthermore, nuts are rich sources of fiber and therefore have a low glycaemic index (GI). It has been reported that diets with low GI can reduce CRP levels (118). Nuts are an important source of antioxidants essential for human health (8). Modulation of oxidative stress (particularly reducing peroxide levels) can decrease inflammation. The fatty acid composition of nuts is beneficial because the content of saturated fatty acids (SFAs) is low (4–16%), and almost half of the total fat content is composed of unsaturated fats. In most nuts MUFA (oleic acid) and different amounts of PUFA (119). Earlier data indicate that MUFA and PUFAs can exhibit anti-inflammatory properties in various experimental models of inflammation (120). The mechanisms by which dietary fatty acids inhibit cytokine production are unknown but may be related to inhibition of the inflammatory cascade at the level of lipoxygenase (LOX) and cyclooxygenase (COX) (121). Regarding the protective effect of nuts, it has been reported that the MeDiet enriched with nuts can lower the incidence of major cardiovascular events among people at high risk of cardiovascular diseases (122).

Considering existing evidence regular consumption of nuts can be associated with a nutritional profile of inflammatory biomarkers. However, further studies are needed to definitively address the important question of the anti-inflammatory effects of nuts.

Oxidative Stress

Consumption of nuts as food sources of antioxidants can prevent pro-oxidation and excessive oxidation of LDL (123). Consumption of antioxidant foods as components of the diet playss an important role in managing cardio-metabolic disorders (124). Vitamin E and phenolic compounds are among the nutrients with antioxidant activities that nuts are a rich source of both. (125). *In vitro* studies (126, 127), animal models (128, 129), observational studies (130), and randomized trials (35, 72) suggest the potential benefits of including various nuts in the diet with regards to oxidative stress biomarkers. Most of the studies that have reported the potential beneficial effects of eating nuts on oxidative stress have focused on pistachios, almonds, peanuts, or walnuts, all of which are rich sources of MUFA. For instance, a sub-analysis of the PREDIMED (Prevención con Dieta Mediterránea) study, which assessed biomarkers associated with oxidative stress, identified higher superoxide dismutase (SOD) and catalase activity and lower plasma xanthine oxidase activity in the intervention group (the MeDiet + extra-virgin olive oil and the MeDiet + nuts) (131). The effective dose and duration observed in clinical studies were 20–84 g/day and 4–16 weeks, respectively (28, 30, 50, 53, 65, 72).

The nutritional intervention of 1 unit/d (20 g/day) of Brazil nuts for 3 months in hemodialysis patients resulted in improved plasma glutathione peroxidase (GPx) levels and a reduction in 8-isoprostane and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels ($p < 0.001$) (55). A clinical trial on 46 overweight and obese women found that after 8 weeks of intervention, the Baru nuts group (20 g/day) showed a significant increase in GPx activity compared to the placebo group (72). Jenkins et al. (132) showed that a full dose of almond supplement (73 ± 3 g/day, 22% of energy) or half a dose over 4 weeks reduced oxidized LDL in subjects with dyslipidemia. Liu et al. (28) found similar results after 4 weeks of supplementation with almond (∼ 6 g/day) in individuals with dyslipidemia and T2D. Pistachio supplementation (10 or 20% of energy) for 4 weeks was also effective in reducing oxidized LDL in people with hyperlipidemia (133).

It has been revealed that bioactive compounds (phytosterols, polyphenols), MUFA, Se, and tocopherols are the main factors in the beneficial effects of nuts in modulating oxidative stress. These agents can also reduce the pro-oxidant effects of PUFA on LDL oxidation and DNA damages (134). The main fat compounds in hazelnuts and almonds are MUFA and are associated with decreased LDL sensitivity to oxidation (119). Therefore, differences in the fat content of different nuts may partly explain why pistachios, walnuts, almonds, and other MUFA-rich nuts can reduce oxidative stress, whereas walnuts (PUFA-rich) Do not have. Se, phytosterols, and polyphenols can activate the Nrf2 (nuclear factor erythroid 2-related factor 2) pathway (134). Nrf2 stimulates the antioxidant response element (ARE) genes transcription and encodes the antioxidant (e.g., GPx) and detoxifying enzymes (55). The Nfr2 pathway also activates NQO1, stabilizing proteins and protecting them against oxidative degradation (135).

Consumption of antioxidant food sources and/or antioxidant supplements seems to contribute to the prevention and/or treatment of metabolic disorders through widely known mechanisms; nuts are a good example of such food sources because of their good taste and dose-effect that allows them to be included in diets.

Based on the findings of evaluated studies, depending on the dose and duration of consumption (**Table 2**), mixed nuts can

TABLE 2 | Appropriate dose and duration of mixed nut consumption to insert metabolic efficacy.

Metabolic response	Optimal dose (g/day)	Optimal duration (weeks)
Glycaemic response	28–60	4–24
Lipid profile	20–64	4–24
Inflammatory response	20–56	4–24
Oxidative stress markers	20–84	4–16

have health effects on metabolic markers, including glycaemic response, lipid factors, oxidative stress, and inflammation. In contrast, no adverse effects of nuts intake on metabolic biomarkers have been found in clinical trials (136).

CONCLUSIONS

As ready-to-eat snack foods, nuts have healthy lipid components and are excellent sources of fiber and some micronutrients. The US FDA has approved a qualified health claim for nuts suggesting that daily consumption of nuts may help to reduce the risk of some chronic disorders purportedly through the improvement of metabolic markers. However, the intervention's selected type, dose, and duration should be based on the therapeutic goals. For example, the effectiveness of different types of nuts including, Almond, Pistachios, Walnut, Hazelnuts, Brazilian nut, Peanuts, Cashew, Pecan, and Soy nut on glycaemic response and lipid factors has been shown in several clinical trials. But, in the case of inflammation, the effectiveness of walnuts has been more supported due to their high ALA and phenolic compounds. Additionally, in the case of oxidative stress, the effectiveness of almonds, pistachios, pecans, or peanuts due to their rich sources of MUFA has been supported. Further research is needed to establish the intakes of different varieties of nuts to deliver optimal metabolic benefits.

AUTHOR CONTRIBUTIONS

All authors conceived the idea, participated in the study design, drafted the manuscript, reviewed, edited, and approved the final manuscript.

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Optimization of Protein Quality Assay in Normal, *opaque-2*, and Quality Protein Maize

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The development of quality protein maize (QPM) was considered a significant leap toward improvement in the nutritional status of rural masses in developing countries. The nutritional quality of QPM is attributed to the higher concentration of essential amino acids, particularly lysine and tryptophan, in its kernel endosperm. However, the similarity in the grains of QPM and normal maize necessitates the development of a standard protocol to assess the protein quality of maize. The present study aimed at improving the protocol of protein quality assessment in QPM. For this purpose, endosperm defatting and protein estimation procedures were restandardized and optimized with respect to the protocol duration and its amenability for high-throughput analysis. Unlike normal maize, QPM and *opaque-2* mutants were completely defatted within a 48 h period. It was observed that the tryptophan content, calculated at each defatting interval, increased in the samples defatted for a longer duration. No significant differences were observed in the tryptophan content analyzed in the samples defatted for 48 and 72 h. Moreover, the endosperm protein estimated by using the Bradford method with certain modifications strongly correlated with the micro-Kjeldahl method ($r = 0.9$). Relative to the micro-Kjeldahl method, the Bradford method was found to be precise, rapid, and hazard-free. The present findings enable a testing protocol of reduced time duration that can be used in resource-poor settings for the determination of a protein quality assay in QPM. Overall, the present study effectively helped in reducing the defatting time by 24 h and protein estimation by 3 h as compared to the already established International Maize and Wheat Improvement Center protocol. This is expected to enable the aggregation of high-protein-quality maize to facilitate its commercialization.

Keywords: quality protein maize (QPM), *opaque-2*, defatting time, tryptophan, Bradford, Kjeldahl

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INTRODUCTION

Maize is a globally important crop and the third most important cereal in India, in terms of production (FAOSTAT, 2020). It played a major role in alleviating protein-energy malnutrition, micronutrient deficiencies, and vision impairment worldwide. Generally, plant-based foods are economically and environmentally suitable for human protein nutrition (Langyan et al., 2022). Due to its use in the staple diet and processed food, it is a good candidate to manage the health

of vulnerable populations. A number of technologies, including molecular breeding (Olsen and Phillips, 2009, Prasanna et al., 2020), genetic engineering (Pérez-Massot et al., 2013; Guo et al., 2018), and gene editing, have the potential to deliver nutritious maize (Ku and Ha, 2020; Singh et al., 2020). Quality protein maize (QPM) is a nutritionally improved commodity and is reported to be equivalent to the milk protein casein in terms of nutritional composition (Arsenault and Brown, 2017). In the presence of a specific *opaque*-2 gene, the protein profile of QPM is improved. Thus, QPM has about twice the level of lysine and tryptophan (4.15 and 1%) than normal maize (2 and 0.5%) and also increased levels of histidine, arginine, aspartic acid, and glycine but a reduced level of leucine (Wu et al., 2010; Onireti and Ikujenlola, 2020). The enhanced levels of lysine and tryptophan in QPM, which are otherwise limiting in normal maize, make them a good source of biologically usable protein along with several nutritional benefits. QPM is thus a valuable resource for philanthropic efforts toward the nutritional upliftment of vulnerable populations, as various studies on children and adults in a controlled manner have established its nutritional superiority. These studies demonstrated that children fed with QPM had fewer sick days, were more probable to escape death due to diarrhea, and had reduced stunting and better growth-enhancing capabilities (Nedi et al., 2016). Furthermore, these studies also revealed that as animal feed, QPM is more economical and productive than purchased rations (Nurit et al., 2009).

QPM is a product of conventional breeding. The QPM breeding program involves the introduction of three distinct genetic systems, *viz*., (i) the homozygous recessive and mutated allele of the *opaque-2* gene that downregulates the expression of 22-kDa α-zein and 15-kDa β-zein genes, leading to a decrease in nutritionally poor zein protein, (ii) endosperm hardness modifier genes to eliminate the pleotropic effects like a soft and chalky kernel appearance associated with *opaque-2* mutants, and (iii) amino acid modifier genes (Arruda et al., 2000; Gavazzi et al., 2007; Tripathy et al., 2017). The *opaque-2* mutation has been extensively studied at the biochemical, genetic, molecular and phenotypic levels. Due to the unavailability of the rapid protein assay, the nutritional potential of QPM could not be harnessed as the farmers were unable to fetch any remunerative prices for their QPM produce as it becomes difficult to distinguish QPM from normal maize at grain procurement centers. The similarity in the kernel texture between QPM and normal maize, therefore, necessitates the development of a rapid assay methodology to assess the protein quality of QPM. As per the standard protocol devised by the International Maize and Wheat Improvement Center (CIMMYT), the concentration of tryptophan in the kernel endosperm of QPM should be more than 0.6% of endosperm protein. The method involves the defatting of the maize endosperm for 72 h, followed by protein hydrolysis for 16 h using papain. The tryptophan is then measured spectrophotometrically and expressed as a quality index by calculating the ratio of tryptophan in the endosperm protein. Although the method is precise and accurate, it is lengthy and time consuming. Presently, researchers are seeking a concise and precise method for the protein quality assay. The objective of the present study was, therefore, to develop a rapid laboratory assay to expedite the tryptophan estimation process by optimizing the defatting process and protein estimation in maize.

MATERIALS AND METHODS

Chemicals and Reagents

For the defatting of samples, petroleum ether (40–60°C) was procured from Sigma-Aldrich (3050 Spruce street, St. Louis, MO 63103 USA 314-771-5765 SIGMA ALDRICH CHEMIE GmbH, Riedstr, 2D-89555 Steinhelm 497329970). For the analysis of tryptophan content, the glacial acetic acid, ferric chloride, papain, DL-tryptophan, and sulfuric acid were obtained from Merck (Merck Lifescience Private Limited, Godrej one, 8th floor, Pirojshanagar, Eastern Express Highway, Vikhroli (East), Mumbai 400079). For the extraction of the maize protein, sodium acetate was procured from Fischer Scientific (Thermo Fischer Scientific India Pvt. Ltd. 403-404, B wing, Delhi, Hiranandani, Business park Powai, Mumbai-400076); ethanol and the Bradford reagent were procured from Himedia (HiMedia Laboratories Pvt. Ltd. 23, Vodhani Ind. Est., LBS Marg, Mumbai-400086, India).

Experimental Material

The genetically pure seeds of a diverse panel of inbreds consisting of 25 lines of normal maize and 13 lines each of *opaque-2* maize and QPM were used for the present study. Each plot consisted of 5 rows of 3 m with a row-to-row distance of 60 cm and plant-to-plant distance of 20 cm, resulting in 75 plants of each genotype. The experimental lines varied in kernel color, ranging from deep yellow to deep orange. The complete set of experimental material was grown in a single block at the experimental fields of Indian Council of Agricultural Research (ICAR)-Indian Institute of Maize Research, Ludhiana, India. All the individual entries were pollinated separately. Proper care was taken to maintain the genetic purity of each genotype.

Sample Collection and Screening

The samples from individual entries were collected from four different ears. An equal number of kernels were pooled and treated as one sample in order to reduce the effect of biological variation between ears on gene expression. A minimum of three technical replicates were used for each experiment. Further samples were screened using a light box and assigned a modification score ranging from 1 to 5 as per the percent opaqueness observed in the kernel (Sethi et al., 2020). It is a preliminary measure to segregate *opaque-2,* QPM, and normal lines on the basis of their endosperm textures. Following screening, the endosperms were extracted from the maize kernels and dried in a hot air oven; the dried samples were ground using a Cyclotec sample mill.

Oil Estimation

The oil content was estimated as per the Ankom method with certain modifications (Ankom, 2005). For this purpose, 2 g of milled maize flour was encapsulated in a filter bag and completely demoisturized prior to defatting. To optimize the defatting time, the dried filter bags containing samples were defatted for different time durations, *viz.*, 6, 12, 24, 48, and 72 h using a non-polar solvent such as petroleum ether (40–60°C).

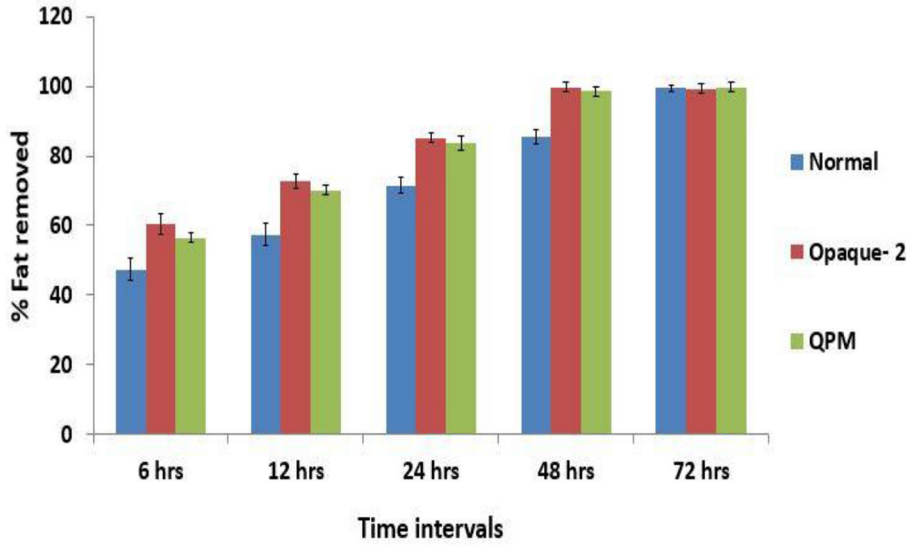


FIGURE 1 | Comparative evaluation of rate of oil extracted (%) at different time intervals in the experimental genotypes. Error bars denote overall ±SD of the genotypes at different time intervals. The difference in the extracted oil (%) within the experimental materials at different defatting time was significant at $P \leq 0.05$.

Tryptophan Estimation

The tryptophan content was estimated in the samples defatted for different time intervals (Hernandez and Bates, 1969). For this purpose, 100 mg of defatted sample was digested using papain. After incubation (65°C for 16 h), the supernatant was treated with reagent C. The colored complex was measured at 545 nm in a UV-Vis spectrophotometer.

Protein Estimation

Protein was estimated using both micro-Kjeldahl and Bradford methods. The micro-Kjeldahl method measures the crude protein content (AOAC, 1975). The protein estimated by the Bradford method (Bradford, 1976; Affolter et al., 2021) involves protein extraction *via* sonication for 30 min with 0.1 N NaOH (pH-12.5), followed by 55% ethanol (Shukla and Cheryan, 2001), and the final extract was estimated as per the Bradford method.

Statistical Analysis

The F test and ANOVA for multivariate data were conducted to check the level of significance for the time course evaluation of % oil removed in the experimental lines. Similarly, the level of significance, i.e., *p*-value, was calculated for the tryptophan content estimated at different time intervals. Further, the Pearson's correlation coefficient (r) and coefficient of determination (R^2) were calculated for the total protein content estimated *via* the Bradford method and micro-Kjeldahl method.

RESULTS

Optimization of Endosperm Defatting in the Experimental Lines

Defatting for different time intervals was done to optimize the oil extraction protocol. The results revealed that the proportion of oil content extracted is directly proportional to the duration of defatting in all the experimental genotypes (**Figure 1**). As the duration for defatting is increased, the extracted oil content also increased. However, the rate of defatting is maximum in *opaque-2*, followed by QPM and normal genotypes. The ANOVA for the multivariate data for defatting revealed that a significant difference exists between normal, *opaque-2*, and QPM with $P \leq 0.005$. It is also observed that for complete defatting, normal lines require 72 h, whereas *opaque-2* and QPM require only 48 h of extraction. The vitreous and hard kernel texture in normal maize may be primarily responsible for slower defatting, whereas the softness in the *opaque-2* kernel assists the fat component to leave the kernel at a faster rate. The delayed extraction of oil in normal maize is also evident from the heat map of defatting (**Figure 2**).

Effect of Defatting on Tryptophan Estimation

A significant difference exists in the tryptophan content estimated in triplicates in all experimental lines, which were defatted for different time intervals ($p = 0.0011$; **Figure 3**). The tryptophan content increases as the time duration for defatting increases. The maximum tryptophan content was observed in the samples defatted for 72 h, and the minimum tryptophan content was observed in the samples defatted for 6 h in all 51 lines taken together (**Figure 3**), indicating that the oil content has a profound effect on the tryptophan estimation process. It was also observed that the tryptophan content is almost similar in non-defatted samples and the samples defatted for 6 h. The comparisons in the tryptophan content among normal, *opaque-2*, and QPM lines defatted for different time intervals is presented in **Figure 3**. Although the estimated tryptophan content increases as the time duration of defatting increases, no appreciable differences were observed in the experimental lines defatted for 48 and 72 h. The mean values of tryptophan content at 48 and 72 h was observed

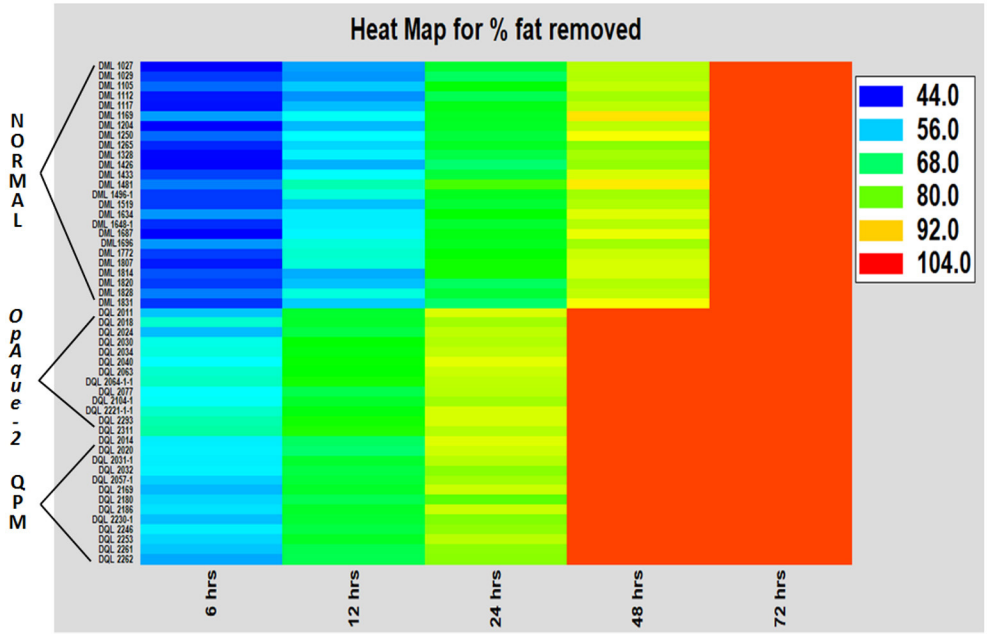
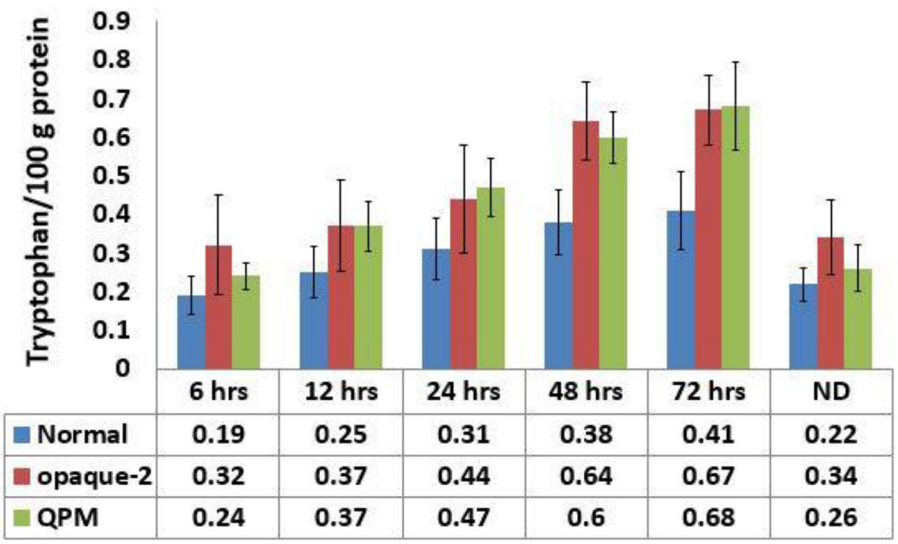


FIGURE 2 | Heat map depicting rate of extracted oil (%) at different time intervals.



	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	ND
Normal	0.19	0.25	0.31	0.38	0.41	0.22
opaque-2	0.32	0.37	0.44	0.64	0.67	0.34
QPM	0.24	0.37	0.47	0.6	0.68	0.26

FIGURE 3 | Comparative tryptophan content in the experimental genotypes estimated at different defatting time. Error bars denote overall ±SD of the genotypes at particular time interval. The difference between the tryptophan content (%) among all experimental lines at different defatting time was significant at $P \leq 0.05$. ND, non-defatted samples.

to be 0.38 and 0.41%, respectively, for normal, 0.64 and 0.67% for *opaque-2*, and 0.62 and 0.65% for QPM.

Comparison of Total Protein by Micro-Kjeldahl and Bradford Method

The total protein estimation in the kernel endosperm is significant in the analysis of the protein quality index. The protein quality index is the ratio of the concentration of tryptophan to the endosperm protein. The protein content was estimated in triplicates in all 51 experimental lines by micro-Kjeldahl as well as Bradford methods. The comparative means of protein content estimated through Bradford and micro-Kjeldahl methods in 25 normal, 13 *opaque-2*, and 13 QPM lines is depicted in **Figure 4**. A significant difference was observed between the protein content estimated by both the methods ($p < 0.05$). The total protein content determined by the Bradford method was

found to be underestimated as the mean values of the protein content in normal, *opaque-2*, and QPM lines are around 20% less as compared to the samples analyzed through the micro-Kjeldahl method (**Figure 4**). However, the protein estimations by the modified Bradford method and micro-Kjeldahl method are highly correlated. The linear regression coefficients of 0.89, 0.72, and 0.89 were observed between the protein estimated by the modified Bradford method and micro-Kjeldahl method in normal, *opaque-2*, and QPM lines, respectively (**Figures 5A–C**). However, a strong positive correlation ($r = 0.9$) with coefficient of determination 0.81 was observed between the protein content estimated through micro-Kjeldahl and Bradford methods in all the 51 lines taken together (**Figures 5D**, **6**).

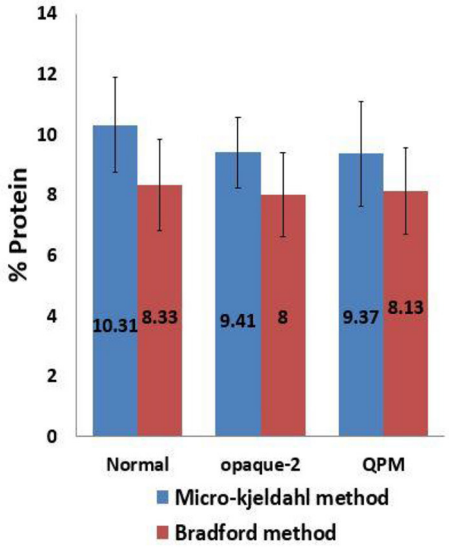


FIGURE 4 | Comparative evaluation of protein content estimated by micro-Kjeldahl and Bradford methods. Error bars denote overall ±SD of the genotypes at particular time interval.

DISCUSSION

Maize kernels usually contain between 2 and 4% of oil, which is primarily located in the germ portion (Chaudhary et al., 2012). It has been shown that maize protein fractions accumulate in the developing kernel differentially (Sethi et al., 2020). With the progressive accumulation of zein/prolamin and prolamin-like protein fractions, the overall kernel hardness increases, but this affects the amount of lysine and tryptophan (Sethi et al., 2021). Oil interferes with the estimation of other components, including tryptophan. The tryptophan estimation of maize requires a papain hydrolysis of the protein to release the amino acids, subsequently specifically estimating the tryptophan content using the acetic acid method (Hernandez and Bates, 1969; Bjarnason and Vasal, 1992). For the proper enzymatic hydrolysis of maize protein using papain, the acetic acid method for tryptophan estimation requires prior defatting of samples. The tryptophan content was analyzed in the samples defatted for different time intervals using the papain hydrolysis method. As mentioned above, the tryptophan content increased as the duration for defatting is increased. It was observed that QPM and *opaque*-2 samples were completely defatted in 48 h (**Figure 1**). As per the standard protocol, 72 h of defatting is required for an accurate estimation of tryptophan in maize flour (Hernandez and Bates, 1969; Shetti et al., 2020). A reduction of 24 h for the complete defatting of *opaque-2* and QPM and without the use of special equipment would immensely help toward the fast screening of high-protein-quality material for the benefit of farmers as well as seed procurement agencies involved in QPM aggregation on a commercial scale. Unlike *opaque-2* and QPM, normal maize has a hard endosperm. The positive correlation between the tryptophan content and kernel opacity has already been established by a previous study, which reported that *opaque-2* mutants retained higher tryptophan content as compared to normal lines (Gupta et al., 2013). *Opaque-2* mutation is also responsible for the increased concentration of free amino acids as reported by earlier studies (Brochetto-Braga et al., 1992; Wang et al., 2001; Manicacci et al., 2009; Sethi et al., 2021). The softness of the endosperm due to *opaque-2* mutation leads to a distorted protein–starch matrix in *opaque-2* and QPM germplasm. This results in a chalky and soft kernel texture, which is hardened through the action of endosperm modifiers. Despite this, the differences in the organization of the starch–protein matrix remain to some extent (Holding, 2014). These difference in starch–protein complexation in normal maize vs. the *opaque-2* and QPM may be responsible for the enhanced defatting rate of the *opaque-2* and QPM as compared to the normal maize. We have demonstrated that a defatting time of 48 h, without the use of specialized equipment, is sufficient to screen for high-protein-quality material in maize (**Figure 3**).

In order to select high-protein-quality maize, a parameter termed "protein quality index" needs to be analyzed. A protein quality index is analyzed by determining the ratio of tryptophan to protein content in the kernel endosperm. The protein content is usually measured by the micro-Kjeldahl method, whereby the total nitrogen content is measured through digestion, distillation, and titration. This requires expensive instrumentation, skilled workforce, a constant running cost to cover maintenance charges, and certain consumables that are hazardous in nature. Moreover, it is not very amenable for high-throughput analysis. The whole process requires 4–5 h, and a maximum of 16 samples can be analyzed in a day by this protocol. This is in contrast to the spectrophotometric tryptophan estimation described above, which can be done in a high-throughput format. In order to screen for high-protein-quality material, which requires the determination of both tryptophan and protein contents, the necessity of a rapid, inexpensive, and high-throughput method for maize protein estimation was felt. Keeping this consideration in mind, we standardized the Bradford method for the estimation of maize protein. A maize endosperm contains the prolamin zein, which accounts for 60% of the total protein, glutelins (26% of total protein), and albumins and globulins (6% of

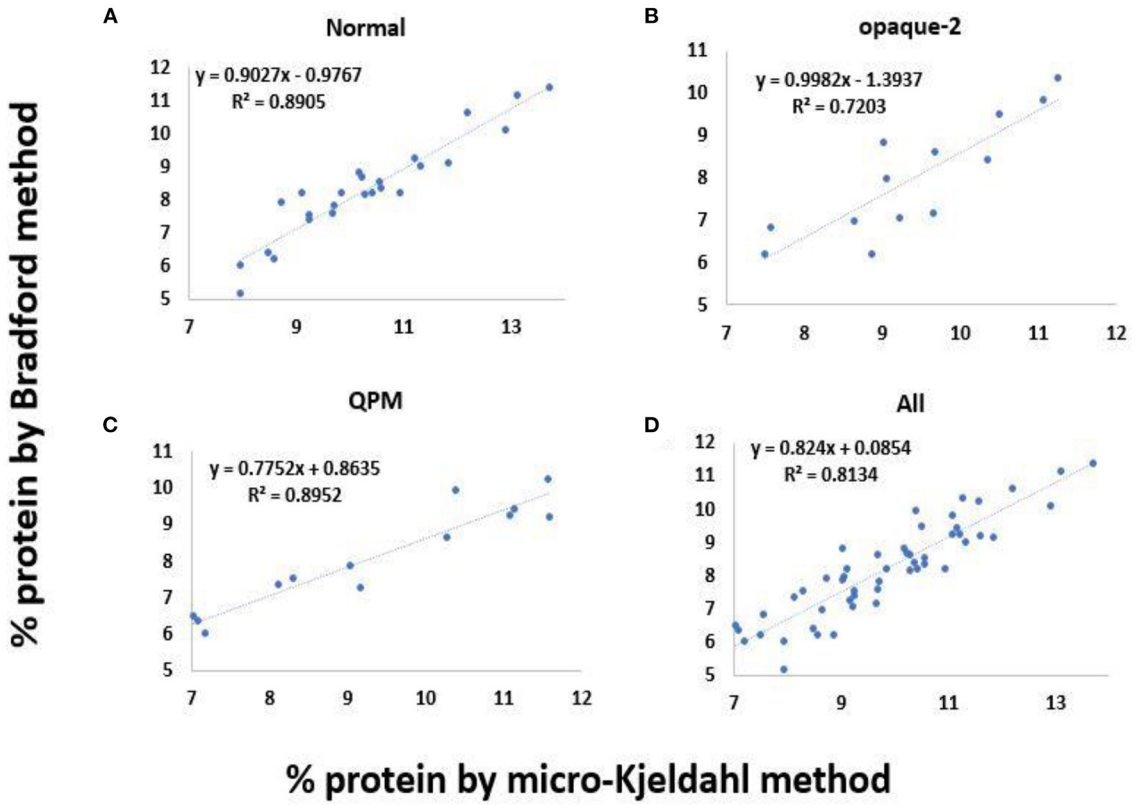


FIGURE 5 | Correlation between protein content estimated by micro-Kjeldahl and Bradford methods. **(A)** Normal maize genotypes (sample size = 25), **(B)** *opaque-2* maize genotypes (sample size = 13), **(C)** QPM genotypes (sample size = 13), and **(D)** Composite of above three samples (sample size = 51).

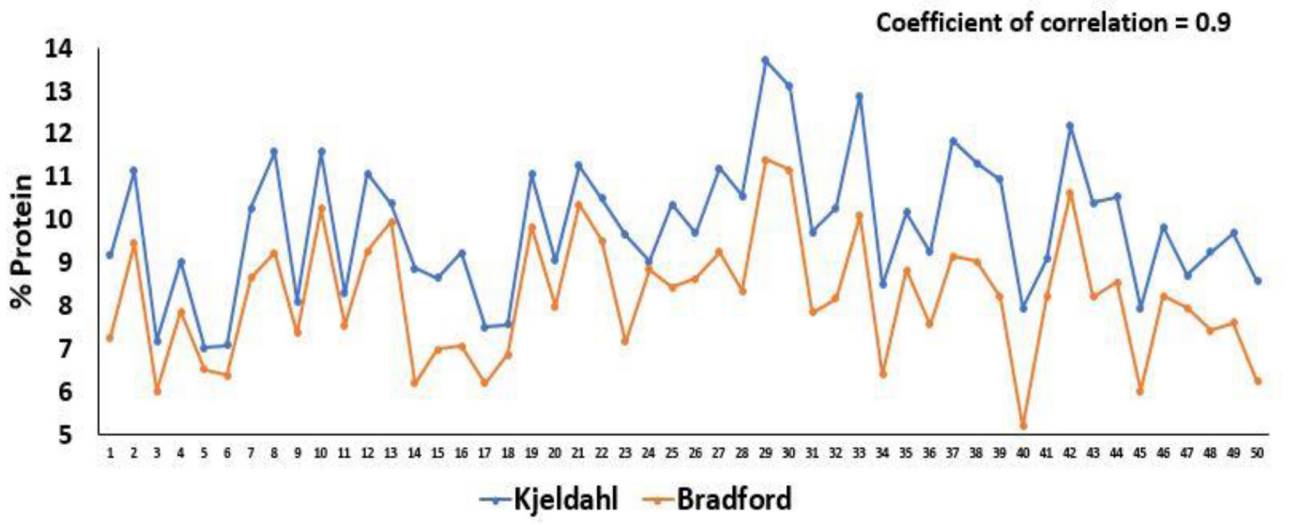


FIGURE 6 | Protein content estimated by micro-Kjeldahl and Bradford methods in 51 experimental lines.

total protein) (Anderson and Lamsal, 2011). In our study, the extraction procedure mainly focused on the extraction of the major component of maize protein, i.e., glutelins and zeins. To solubilize the glutelins and zeins, a sequential extraction using alkali and alcohol was employed. The extraction protocol involved sonication for 30 min with 0.1 N NaOH (pH 12.5)

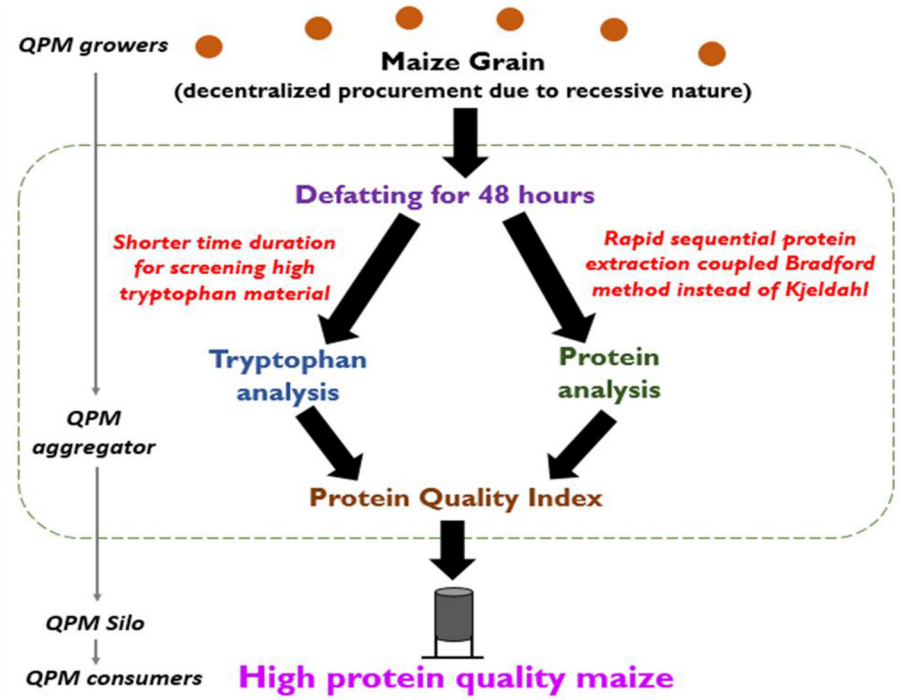


FIGURE 7 | Workflow to screen high-protein-quality maize for aggregation of QPM material. Shorter duration of defatting for tryptophan estimation coupled with the use of defatted maize for protein estimation by the modified Bradford method would enable the resource-poor laboratories to screen QPM and extend the technology for societal benefits.

followed by 55% ethanol (Shukla and Cheryan, 2001), and the pooled extract was used for protein estimation *via* the Bradford method. This procedure requires only 2 h, and then the protein content is estimated within a few minutes in a large number of samples.

The Coomassie dye-based Bradford protein assay is suitable for the rapid estimation of protein extracts. The protein content determined through the Bradford method was found to be slightly reduced as compared to the protein content estimated through the micro-Kjeldahl method (**Figure 4**). A correlation of 0.94, 0.84, and 0.94 was observed between the protein content estimated by modified Bradford and micro-Kjeldahl methods in normal, *opaque-2*, and QPM lines, respectively, with the coefficient of determination as 0.89, 0.72, and 0.89 (**Figures 5A–C**). An overall significantly positive correlation of 0.9 ($R^2 = 0.81$) was observed for the protein estimations done *via* the Bradford method and micro-Kjeldahl method in all the 51 lines (**Figures 5D**, **6**). The biggest advantage of the Bradford protein assay is its inexpensive nature and amenability for high-throughput analysis. Once the test has been set up, each sample requires only 5 min of incubation to form a stable blue color required for estimation. This allows several samples to be analyzed in a short span of time, and the retesting of a sample can be quickly performed. Although Nurit et al. (2009) described a rapid tryptophan protocol, they used specialized equipment. Further, as mentioned above, in the absence of a rapid protein estimation method, the protein quality index cannot be determined, which is the international standard for screening high-protein-quality maize.

We propose a workflow for the high-throughput screening of high-protein-quality maize using the standardizations done in the present study (**Figure 7**). For the screening of high-protein-quality material containing higher tryptophan per unit protein, a defatting of 48 h is sufficient. The defatted sample is then analyzed for the protein content using the modified Bradford method, which does not require expensive equipment and hazardous chemicals. Both the tryptophan and the protein content are used to calculate the protein quality index, which is an important parameter of the protein quality of a particular maize sample. This workflow can be utilized by resource-poor laboratories to enable the aggregation of the high-protein-quality material and extend the benefits of science and technology for the societal benefit.

CONCLUSION

The present study has helped in optimizing the protein quality assay in maize. It is concluded that the rate of defatting is maximum in *opaque-2* followed by QPM and normal maize. QPM and *opaque-2* maize defats completely in 48 h of extraction. Since no significant difference is observed in the samples defatted for 48 and 72 h, therefore, 48 h of defatting is optimized for

the precise estimation of tryptophan in the maize endosperm. Defatting for 48 h would be sufficient for screening high- protein-quality material (QPM and *opaque*-2) in the bulk material. Further, the present study optimized the Bradford method for protein estimation in the kernel endosperm of maize, which shows a strong positive correlation ($r = 0.90$) with the micro-Kjeldahl method with a regression coefficient ($R^2 = 0.81$). Thus, the present study effectively helped in reducing the defatting time by 24 h and protein estimation by 3 h as compared to the already established CIMMYT protocol. In addition, the requirement of sophisticated equipment involving a significant capital cost is avoided by the use of the modified Bradford method. This would enable the agencies with low resource settings to take advantage of the developed protocols for the assessment of protein quality. This is expected to facilitate the aggregation of high-quality material for empowering nutrition with the utilization of QPM material.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CK conducted the experiment, analyzed the data, interpreted the results, and wrote the manuscript. AS envisaged the concept, analyzed the data, interpreted the results, and revised the manuscript. MS conducted the experiments. VD conducted the experiments RP contributed in plant material acquisition of samples at regular intervals. BB and SL revised the manuscript. SR envisaged the concept and revised the manuscript. DC envisaged the concept, analyzed the data, interpreted the results, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Development of Protein Rich Pregelatinized Whole Grain Cereal Bar Enriched With Nontraditional Ingredient: Nutritional, Phytochemical, Textural, and Sensory Characterization

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This study was aimed to use extrusion cooking as a pretreatment for non-conventional seeds (Indian horse chestnut flour) to blend them with whole grain flours (whole wheat flour, whole barley flour, and whole corn flour) for the development of a pregelatinized cereal bar (PCB). In this study, date paste (7.5–17.5%) and walnut grits (2.5–12.5%) were incorporated at varying levels to prepare PCB. The PCB was evaluated for its nutritional, color, textural (both three-point bending test and TPA), antioxidant activity, and sensory attributes. The flexural modulus, rupture stress, and fracture strain of PCB increased with the incorporation of a higher proportion of date paste. The protein and fiber content in PCB increased from 7.74 to 9.13% and 4.81 to 5.59% with the incorporation of walnut grits and date paste, respectively. The DPPH, total phenolic content, and water activity of PCB were determined, which progressively enhanced with increased levels of walnut grits and date paste. The correlation between sensory attributes and instrumental texture on PCB was also investigated. The correlation results showed a significant ($p < 0.05$) positive correlation between texture analysis and sensory hardness, springiness, adhesiveness, and negatively correlated to instrumental and sensory cohesiveness. For sensorial attributes, all PCB samples presented average scores of 7/10 and 4/5 for buying intention. Therefore, whole grain extrudates, date paste, and walnut grits can be efficiently used to develop PCB with improved nutritional, nutraceutical, and economic values.

Keywords: pregelatinized, antioxidants, textural properties, nutraceuticals, sensory attributes, dates

INTRODUCTION

The demand for ready-to-eat products has increased tremendously due to the change in consumers' lifestyles. In this context, a nutritious, healthy, balanced, and safe diet is always endorsed to reduce disorders such as diabetes, obesity, cardiovascular diseases, and malnutrition (1). Cereals play a vital role in the development of ready-to-eat snacks such as instant bars, energy bars, and cereal bars. The consumption of cereal products has been elaborated from the breakfast table till dinner in the form of flakes, rice, chapatti, or cereal bar as a snack (2). The increased intake of refined cereal-based products is due to their wide availability and low cost. However, during the milling process, bran and germ fractions are being removed, which results in the loss of many essential phytochemicals and macro as well as micronutrients in the human diet that have a direct relation with human health (3, 4). For this reason, consumers have valued the consumption of whole grains and their products as they contain all three fractions in the same proportion as present in the intact original grain (5). Cereal-based bars have become a significant part of the human diet, especially among children and can influence overall nutrition (6). They are convenient to carry, light in weight, available in small pouches, and can be consumed easily. Cereal bars are made primarily with whole grains such as whole wheat flour (WWF), whole corn flour (WCF), and whole barley flour (WBF). WWF contains high levels of dietary fiber and phenolic compounds including benzoxazinoids, lutein, zeaxanthin, and β-cryptoxanthin (7). WCF possesses a high concentration of zein and a prolamin protein fraction (8) and contains several bioactive constituents such as ferulic acids, anthocyanins, flavonoids, carotenoids, and phenolic compounds that have many different disease-preventing properties and potential health-promoting benefits (9). WBF is the richest source of tocols and is high in viscous soluble fiber especially β-glucan that lowers blood glucose serum, blood pressure, and low-density lipoprotein cholesterol (10) and also increases the intraluminal viscosity, thus extending gastric emptying time and absorption of nutrients in the small intestine (11). However, whole grain flours tend to have low mineral content and many other nutrients. As a result of this, the addition of Indian horse chestnut seed (non-conventional seeds), date, and walnut enhances the nutritional profile and the therapeutic value of developed whole grain-based cereal bars. Indian horse chestnut (*Aesculus indica*) has high levels of dietary fiber, starch, minerals, vitamins, and bioactive compounds (12). However, seeds are bitter in taste and poisonous, if consumed raw or without processing, due to anti-nutritional factors such as aesculin (saponins) and tannin. These anti-nutritional factors can be eliminated by carefully washing the seeds under running water (13). Dates are prolific with dietary fiber, protein, and carbohydrates mainly in the form of natural sugar (glucose, fructose, and sucrose) and are a rich source of minerals, vitamins, and bioactive compounds (14). It has potential medicinal values such as control or prevention of diabetes mellitus due to the presence of minerals and antioxidants. Children and women are more susceptible to the deficiency of nutritious food due to growth and reproduction, respectively (15). Phenolic compounds found in date paste can retard the α-amylase and α-glycosidase activities that also reduce the digestion rate of carbohydrates, resulting in less absorption of glucose into the blood circulation (16). Moreover, the texture of date paste is sticky and dense, which has a property of binding with other ingredients used during the product development. Walnut kernel stands out for high minerals, protein, vitamins, fat, and polyphenols. They also contain essential dietary fatty acids such as omega-3 and 6 polyunsaturated fatty acids that lower the risk of various disorders such as cholesterol, cardiovascular disease, and inflammation (17).

To develop nutritious healthy cereal bars, some preliminary trials were carried out. Among them, extrusion processing is recommended because of its versatility, which combines several different unit operations such as conveying, mixing, cooking, shearing, shaping, and forming in a single system that converts native ingredients present in cereals into a new functional product with unique shape and size (18). This technique manages to alter the molecular configuration of starch, leading to an increment in its functional characteristics (19). Recent literature has reported that extrusion cooking favors the synergic effect with starch that increases the viscoelasticity property of dough and also promotes the structural changes in ingredients such as in corn protein (zein) (20). In addition, there is ample research reporting other beneficial effects of extrusion cooking such as reduction or elimination of anti-nutritional factors, conversion of insoluble to soluble fiber, escalation of bioavailability of minerals and proteins, and increase in bioactive activity by releasing the phenolic compounds bound in insoluble fibers (21–23). In contrast, one drawback of pregelatinized cereal bar (PCB) developed from whole grain flours and Indian horse chestnut flour (IHCF) is texture and taste, but this can be reduced with the addition of date paste and walnut grits. These two natural ingredients additionally contribute to sweet taste and gritty mouthfeel and eliminate the filthy taste and texture of PCB. Therefore, this study was proposed to develop a whole grain-based cereal bar (from pregelatinized extrudates) incorporated with date paste and walnut grits. The functional cereal bar was analyzed for the nutritional, antioxidant, sensory, color, and textural characteristics. In addition, the correlation between instrumental texture and sensory attributes was established.

MATERIALS AND METHODS

Procurement of Raw Materials

Whole grain wheat (SW-2), whole white corn (DT-2), and whole grain barley (PL 807) were obtained from Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Jammu and Kashmir (J&K), and Kargil, India, respectively. Milling was done to obtain WWF, WCF, and WBF. The whole grain flours were then packed and stored at −21°C until further use. Indian horse chestnut seeds (*Aesculus indica*) were collected manually in October from the local area of Shalimar, J&K. Dates and walnuts were procured from a local market in Srinagar, J&K, India.

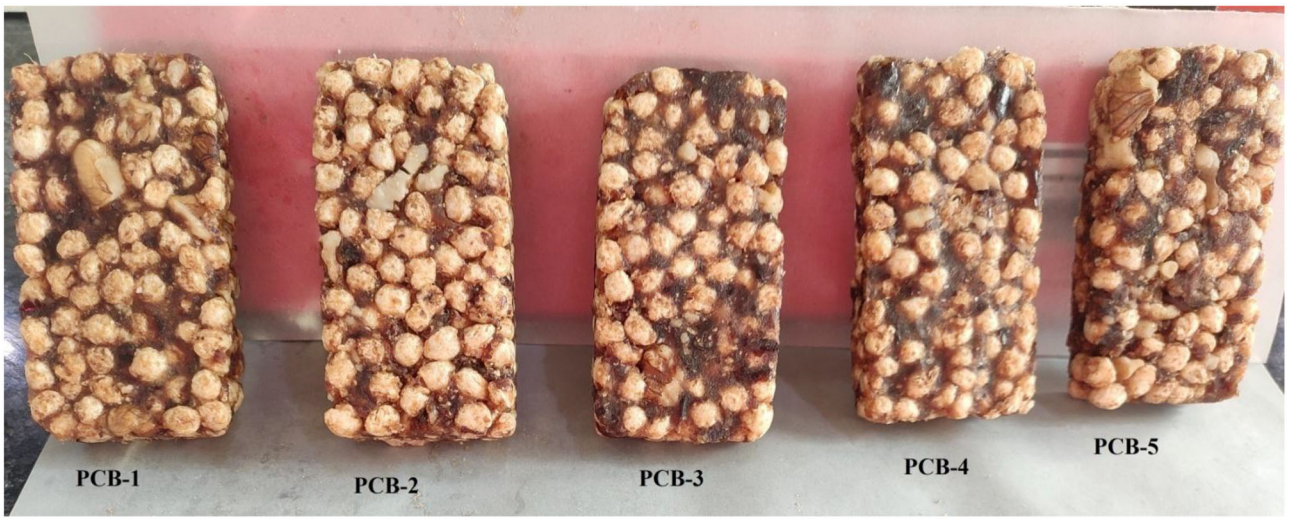

FIGURE 1 | Images of pregelatinized whole grain-based cereal bar enriched with Indian horse chestnut flour, date paste, and walnut grits.

Preparation of Indian Horse Chestnut Flour

The selected seeds were manually peeled, and the kernels were cut into two halves and then sliced using a vegetable slicer. The slices were blanched for 15 min and then soaked in water for 56 h followed by continuous washing and changing the water after every 2 h till the lather or foam comes down to remove the anti-nutritional factors (24). The slices were dried in a single layer in a tray drier (MFG, SSI-103C, Sood Steel Industries, India) at 60°C followed by cooling at room temperature and then fed to a laboratory grinder to obtain flour, which was passed through 60 mesh sieves.

TABLE 1 | Raw material and proportion used in the development of pregelatinized cereal bars.

Ingredients	Formulations				
	PCB-1	PCB-2	PCB-3	PCB-4	PCB-5
Extrudates (%)	80	80	80	80	80
Date Paste (%)	7.5	10	11.5	12.5	17.5
Walnut Grits (%)	12.5	10	8.5	7.5	2.5

PCB, pregelatinized cereal bars; extrudates, made of 10% WWF, 10% WBF, 2.5% IHCF, and 77.5% WCF.

Preparation of Pregelatinized Cereal Bar

Cereal bars were prepared from extrudates made of WWF, WBF, WCF, and IHCF. Date paste and walnut kernels as walnut grits were also used for the development of whole grain PCBs. Extrudates were extruded through a corotating twin-screw extruder (Basic Technology Pvt. Ltd., Kolkata, India) with a die diameter of 3.0 mm and length to diameter ratio of 8:1. The process conditions of extruder and proportion of feed composition in the composite flour were designed by using Central Composite Rotational Design (CCRD). Feed moisture content (12–16%), barrel temperature (90–130°C), screw speed (320–380 rpm), and IHCF (2.5–4%) were the independent variables. The proportion of WWF and WBF was kept constant (10%, from preliminary trials) in all treatments. The remainder of 80% of the feed formulation was made of WCF in the control sample. IHCF (2.5–4%) was used to substitute WCF in the feed mixture. Numerical optimization was done to optimize the independent variables [IHCF (2.5%), moisture content (16%), barrel temperature (130°C), and screw speed (380 rpm)] to obtain the highest desirability and good quality extrudates. The novelty of this study was to use extrudates with their intact shape and size instead of using the flours as presented in **Figure 1**. Pitted dates were ground in a laboratory grinder (Usha-3345, New Delhi) to obtain date paste. Walnuts were deshelled and crushed to form walnut grits.

Extrudates were mixed with different percentages of date paste and walnut grits to obtain five different PCBs (PCB-1, PCB-2, PCB-3, PCB-4, and PCB-5) as shown in **Table 1**. The ingredients were individually weighed and mixed properly to get a uniform mixture. Molds of uniform size (8 × 4 × 1.5 cm) were used to develop a uniform PCB of different treatments (**Table 1**). Butter paper was used before filling up the mold. The bars were then dried in an oven at 40°C for 3 h and cooled to room temperature. PCBs were packed in aluminum foil polyethylene laminate and stored at 25°C for further analysis.

Nutritional Properties of Raw Material and Developed Pregelatinized Cereal Bars

The nutritional value of cereal bars, i.e., moisture, ash, fat, protein, and fiber, was evaluated according to the AOAC (25)

and Sapna et al. (26) procedure. Carbohydrate content was determined by the difference method.

$$Carbohydrate\ (\%) = 100 - (\%\ moisture + \%\ fat + \%\ protein + \%\ ash + \%\ fiber)$$

The calorific value was calculated using the equation:

$$Energy\ value\left(\frac{Kcal}{100\ g}\right) = (4 \times \%\ CHO) + \left(9 \times \%\ fat\right) + (4 \times \%\ protein)$$

Water Activity (a_w)

The water activity (a_w) of cereal bars was evaluated using a water activity meter (Novasina AG CH-8853, Lachen) at 25°C. The analysis was done in triplicates.

Color Evaluation

The color differences of cereal bars were analyzed by measuring the CIELAB space parameters using a Hunter Lab colorimeter (CR 300, Konica Minolta, Japan). Cereal bars were set in optical glass cells to measure the reflected color, represented as L^* (lightness/darkness), a^* (redness/greenness), and b^* (blueness/yellowness) values. Each value is an average of five different independent measurements.

Antioxidant Activity

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was estimated by a method discussed in the study by Kaur et al. (27). Extraction of samples was done in 80% methanol at 25°C for 120 min. After extraction, the samples were filtered through Whatman No. 1 filter paper. From the extracts, an aliquot of 100 μl was taken and added to 3.9 ml of DPPH. After 30 min of incubation, absorbance was measured at 517 nm at room temperature.

$$DPPH\ radical\ scavanging\ activity\ (\%) = \frac{A_C - A_e}{A_C} \times 100$$

where A_c is the absorbance of control at 0 min time and A_e is the absorbance of the sample at 30 min.

Total Phenolic Content

The procedure developed by Zahoor and Khan (28) was used to calculate the TPC in raw and PCB samples. Methanol as solvent was used for the extraction process. Samples of 2 g were homogenized in 20 ml of methanol. The homogenate was then kept undisturbed for 12 h. The obtained mixture was centrifuged at 10,000 × *g* for 15 min. After centrifugation, 0.2 ml of aliquot is mixed with 1.5 ml of Folin-Ciocalteu reagent and 1.2 ml of 7.5% of Na_2CO_3. The mixture was then kept aside for 2 h at 25°C. Finally, the absorbance was measured using a spectrophotometer at 765 nm. A calibration curve was made by gallic acid, and the TPC was expressed as mg gallic acid equivalents (GAE)/g of dry sample.

Texture Analysis

Texture analysis was done using the following two methods.

Three-Point Bending Test

The texture of the cereal bars was evaluated using TA-HD Plus, texture analyzer (Stable Micro System, Godalming, Surrey, UK) equipped with a load cell of 50 N and a three-point bending rig (**Figure 2**). A PCB is placed between two supports of width 3.25 mm with a fixed distance between the two supports (L) that is predetermined based on the sample size. The sample is compressed vertically downwards at the center of the cereal bar, with a blunt blade probe (HDP/3PB) (40 mm) at a crosshead speed of 1 mm/s to obtain maximum compression force (F) (N, Newton) or force deformation curve and is termed as "Force of rupture" (29).

The flexural modulus (E), rupture stress (σ), and fracture strain (r) of the samples were derived using the following equation:

$$\sigma = F\frac{3L}{2h^2w}$$

$$E = \frac{F}{d}\frac{L^3}{4h^3w}$$

$$r = \frac{6Dw}{L^2}$$

where F (N) is the force of rupture, h (mm) is the thickness of the bar, w (mm) is the width of the bar, F/d (N/mm) is the slope of the linear part of a force-displacement curve, and D (mm) is the deflection of the center of the bar at the point of the break.

Texture Profile Analysis

Texture profile analysis of PCB samples was determined by Carvalho and Conti-Silva's (30) method with a slight modification. The TPA technique involves a two-cycle compression test, which imitates two bites. The instrument is equipped with a 30 mm cylindrical probe (P/36 R) and operated at a pretest speed of 1 mm/s, test speed of 0.50 mm/s, posttest speed of 10.00 mm/s, trigger force of 5.0 g, and time of 5 s were kept in between the two compressions. The samples were compressed to 50% of their original height. The result represented the hardness, springiness, gumminess, adhesiveness, and cohesiveness parameters.

Sensory Evaluation

The sensory evaluation was performed in the Department of Food Science and Technology, Islamic University of Science and Technology, Srinagar, India, in individual cabins. A total of 35 judges, who were potential consumers of the product, were randomly selected from the campus. Samples were given in a coded form with four-digit numbers and a 9-point hedonic scale (1 = dislike very much and 9 = like very much) was used to carry out the acceptance test (31) for aroma, color, texture, taste, and overall acceptability. After every sample, the consumers were guided to rinse their mouth with water, to differentiate the taste of different treatments of cereal bars. Acceptance results were complemented by questioning the purchase intent regarding

FIGURE 2 | Three point bending rig test.

each sample, using the 5-point scale (5 = definitely would buy, 3 = might or might not buy, and 1 = definitely would not buy). The acceptability index (AI) was evaluated using the following equation:

$$AI\ (\%) = \frac{A}{B} \times 100$$

where A and B denote the average score given to the product and the maximum score obtained for the product, respectively. AI with a 70% score is considered to be a good product (32).

Statistical Analysis

The data collected from the experiments were subjected to SPSS (version 20) statistical software package. Values were expressed as mean ± SD. The significant difference at ($p < 0.05$) was performed by one-way analysis of variance (ANOVA) and Duncan's multiple range test.

TABLE 2 | Proximate and color analysis of extrudates, date paste, and walnut grits.

Parameters analyzed	Extrudate	Date paste	Walnut grits
Moisture (%)	3.59 ± 0.08^{c}	18.3 ± 0.26^{a}	3.91 ± 0.27^{b}
Protein (%)	9.01 ± 0.12^{b}	2.4 ± 0.12^{c}	14.38 ± 0.28^{a}
Fat (%)	4.5 ± 0.08^{b}	0.8 ± 0.02^{c}	10.03 ± 0.16^{a}
Crude fiber (%)	4.91 ± 0.09^{b}	9.6 ± 0.5^{a}	2.37 ± 0.16^{c}
Ash (%)	1.67 ± 0.07^{a}	1.61 ± 0.05^{a}	1.66 ± 0.09^{a}
Carbohydrate (%)	76.32 ± 0.21^{a}	67.3 ± 0.33^{b}	67.65 ± 0.14^{c}
DPPH (%)	18.33 ± 0.17^{b}	3.56 ± 0.21^{c}	23.71 ± 0.17^{a}
TPC mg GAE/g	5.87 ± 0.22^{c}	232.01 ± 0.15^{a}	44.53 ± 0.09^{b}
L^*	64.9 ± 0.15^{a}	43.31 ± 0.13^{c}	58.5 ± 0.07^{b}
a^*	3.68 ± 0.02^{b}	12.03 ± 0.17^{a}	1.78 ± 0.13^{c}
b^*	14.97 ± 0.33^{b}	5.62 ± 0.24^{c}	35.2 ± 0.22^{a}

Extrudates, made of 10% WWF, 10%WBF, 2.5% IHCF, and 77.5% WCF; TPC, total phenolic content; DPPH, radical scavenging activity; L, lightness; a*, redness; b*, yellowness.*

Values are an average of triplicate observations (± SD). Values followed by similar superscripts in rows do not differ significantly ($p \leq 0.05$).

RESULTS AND DISCUSSION

Proximate Composition and Color Analysis of Raw Materials

The proximate composition of whole grain-based extrudates (WGE), date paste, and walnut grits are shown in **Table 2**. The moisture, protein, ash, fat, crude fiber, and carbohydrate content of WGE utilized in this study were 3.59, 9.01, 1.67, 4.5, 4.91, and 76.32%, respectively. The protein content of walnut grits (14.38%) and date paste (2.4%) plays a significant role in improving the nutritional quality of PCBs (33, 34). Walnut grits had higher fat content (10.03%) but lower fiber content (2.37%) than date paste (9.6%) and WGE (4.91%). The DPPH and TPC of raw materials, i.e., whole grain extrudates, date paste, and walnut grits ranged from 3.56 to 23.71% and 5.87–232.01 mg GAE/g, respectively. Therefore, the addition of date paste and walnut grits to WGE will increase the antioxidant activity, dietary fatty acids, amino acid profile, and dietary fiber of the developed product.

The color characteristics of extrudates, date paste, and walnut grits are depicted in **Table 2**. The L^* value indicates the lightness was higher for WGE (64.9) and walnut grits (58.5) as compared with date paste (43.31). Date paste showed more redness ($a^* = 12.03$) than WGE (3.68) and walnut grits (1.78). The yellowish color was more prominent in walnut grits (35.2) indicated by its b^* values followed by WGE (14.97) and date paste (5.62).

Nutritional Composition of Pregelatinized Cereal Bar

Results for the nutritional composition of PCBs are provided in **Table 3**. The study reported that with the substitution of date paste and walnut grits, the nutritional profile of formulated PCB improved. Moisture content in PCB is an important parameter as it not only affects the shelf life but also affects the quality of a product such as taste, texture, and appearance. Water content values of PCB were significantly ($p \leq 0.05$) lower than date paste (18.3%) but higher than walnut grits (3.9 %). This result follows the previous observations of Nadeem et al. (35) and Yerlikaya et al. (36) who have worked on date bar and walnut composition, respectively. It was found that moisture content ranged between 8.7 and 11.25%. The highest moisture content was found in PCB-5 (11.25%), while the lowest value was found in PCB-1 (8.7%). The increase in moisture content reflects the increased residual water content of the product's development from the protein enrichment ingredient (37, 38). PCB-2 showed significantly higher protein (9.13%), fat (4.71%), and ash content (1.76%) with an increase in walnut grits. Walnut proteins contain a relatively higher quantity of arginine and also contain a myriad of essential amino acids such as albumin, glutelin, and globulin (39). Thus, walnut proteins could be a good source of essential amino acids for both kids and adults. In date paste, the amount of protein content is too low to be considered, but still, it can fulfill the daily requirements of the human body (40). Generally, walnut kernels contain 62–68% of oil mainly composed of linoleic, oleic, and linolenic acids. These fatty acids have different health-promoting benefits and are vital to the nutritional and economic value of food products (41). Walnut grits are perfect ingredients for products that do not need further cooking such as bars, muffins, and cakes as high content of linoleic acid on cooking are more prone to charring (42).

Concerning crude fiber, PCB-5 prepared from a higher percentage of date paste showed significantly higher content (5.59%) compared with PCB-4, PCB-3, PCB-2, and PCB-1. A prominent increase in crude fiber may be ascribed to the extrudates made of whole grains and date paste as it contains a higher number of polysaccharides composed of galactan, glucan, arabian, xylan, cellulose, hemicelluloses, pectin, etc. (43).

The carbohydrate content of PCB varied from 69.11 to 71.09% (**Table 2**). In this study, the results were comparable with the high carbohydrate content generally present in bars developed from cereal and fruits, cereal bars with fruit by-products such as guava peels and cashew, cereal bars with tonka beans, and gluten-free cereal bars. A previous study has shown that cereal bars prepared from puffed rice, fruits, and cereals contain increased content of carbohydrates (44). Additionally, cereal bars made with the substitution of sugar and honey as a binder reported high content of carbohydrates (45). The gross energy values of PCB ranged between 345.67 and 362.28 kcal/100 g (**Table 2**). The increase in energy values of developed PCB can be possibly due to the subsequent increment in its carbohydrate content. These results coincide well with the results reported by Samakradhamrongthai et al. (31).

Antioxidant Activity and TPC

The DPPH radical scavenging activity and TPC of PCB are presented in **Table 4**. PCB -1 had the highest DPPH content among all the tested samples with a DPPH of 15.48%. Similarly, PCB-5 showed the highest amount of TPC (127.23 mg GAE/g). After extrusion, the DPPH and TPC of samples were reduced since the phenolic compounds are heat sensitive. In addition, the higher shear force might cause a breakdown of the molecular structure of bioactive compounds, resulting in the decrease of

TABLE 3 | Nutritional values of developed PCB.

Sample	Moisture (%)	Protein (%)	Fat (%)	Crude fiber (%)	Ash (%)	Carbohydrate (%)	Energy value (Kcal/100 g)
PCB-1	8.7 ± 0.25^{e}	9.04 ± 0.05^{b}	4.64 ± 0.03^{b}	4.81 ± 0.07^{e}	1.72 ± 0.03^{b}	71.09 ± 0.09^{a}	362.28 ± 0.21^{a}
PCB-2	9.1 ± 0.03^{d}	9.13 ± 0.12^{a}	4.71 ± 0.06^{a}	5.30 ± 0.09^{c}	1.76 ± 0.02^{a}	70.06 ± 0.27^{b}	359.15 ± 0.33^{b}
PCB-3	10.2 ± 0.29^{c}	8.36 ± 0.08^{d}	4.31 ± 0.02^{d}	5.12 ± 0.07^{d}	1.67 ± 0.04^{c}	70.34 ± 0.22^{b}	353.59 ± 0.16^{c}
PCB-4	10.62 ± 0.08^{b}	8.59 ± 0.05^{c}	4.58 ± 0.08^{c}	5.40 ± 0.08^{b}	1.70 ± 0.02^{b}	69.11 ± 0.31^{c}	352.02 ± 0.22^{d}
PCB-5	11.25 ± 0.14^{a}	7.74 ± 0.11^{e}	3.95 ± 0.07^{e}	5.59 ± 0.08^{a}	1.68 ± 0.04^{c}	69.79 ± 0.29^{c}	345.67 ± 0.11^{e}

PCB, pregelatinized cereal bar.
Values are an average of triplicate observations (± SD). Values followed by similar superscripts in columns do not differ significantly ($p \leq 0.05$).

TABLE 4 | Water activity, color analysis, DPPH, and total phenolic content of pregelatinized cereal bar enriched with date paste and walnut kernels.

Sample	a_w	Color analysis			Antioxidant activity	
		*L**	*a**	*b**	DPPH (%)	TPC (mg GAE/g)
PCB-1	0.344 ± 0.001^{e}	57.22 ± 2.5^{a}	11.15 ± 0.05^{a}	22.80 ± 0.07^{b}	15.48 ± 0.19^{a}	49.27 ± 0.23^{e}
PCB-2	0.411 ± 0.009^{d}	47.64 ± 0.4^{b}	10.64 ± 0.11^{b}	23.91 ± 0.11^{a}	10.32 ± 0.17^{c}	82.34 ± 0.33^{c}
PCB-3	0.438 ± 0.016^{c}	44.80 ± 2.6^{c}	9.12 ± 0.11^{c}	21.36 ± 0.11^{c}	8.53 ± 0.27^{d}	66.26 ± 0.024^{d}
PCB-4	0.500 ± 0.004^{b}	43.62 ± 1.6^{d}	9.07 ± 0.08^{d}	21.45 ± 0.06^{d}	12.11 ± 0.15^{b}	109.77 ± 0.17^{b}
PCB-5	0.593 ± 0.006^{a}	40.02 ± 0.1^{e}	8.37 ± 0.08^{e}	20.08 ± 0.08^{e}	5.32 ± 0.21^{e}	127.23 ± 0.11^{a}

PCB, pregelatinized cereal bar; a_w, water activity; L, lightness; a*, redness; b*, yellowness.*
Values are an average of triplicate observations (± standard deviation). Values followed by similar superscripts in columns do not differ significantly ($p \leq 0.05$).

phenolic content. This result was consistent with the findings of Bhat et al. (46) and Cheng et al. (47). The addition of date paste and walnut grits to extrudates increased the DPPH and TPC up to 3 and 2.5 times, respectively. The higher antioxidant potential and TPC in PCB may be ascribed to the bioactive rich compounds in date paste such as phytosterols, anthocyanin, phenolics, tocopherols, carotenoids, tocotrienols, and dietary fiber (48) and walnut grits such as ellagitannins, melatonin, and serotonin (49). Similar results of elevated antioxidant properties were reported by Kaur et al. (27) for pasta products incorporated with orange peel powder and cucumber peel powder.

Water Activity

The water activity (a_w) of PCB varied from 0.34 to 0.59 (**Table 3**). It measures the free water in foods. PCB-5 reported the highest a_w (0.593), which contained the highest percentage of date paste (17.5%). The lowest a_w (0.34) was observed in PCB-1, which had the lowest quantity of date paste (5%). The a_w below a critical value of 0.6 is recommended for safe storage (50). Generally, cereal bars developed with the addition of sugar observed a_w in a range of 0.1–0.6, whereas cereal bars formulated without the substitution of sugar reported $a_w > 0.7$ (31). These results indicate that the food products can be stored for a long time without the risk of production of mycotoxins and microbial growth, which would lead to spoilage (51).

Color Analysis

Color is an important parameter to decide the acceptability of a food product. The results obtained for color analysis were found to be ranged from 40.02 to 57.22, 8.37 to 11.15, and 20.08 to 23.89 for L^*, a^*, and b^* values, respectively. The color values (L^*, a^*, and b^*) were mainly influenced by the walnut grits and date paste. All the color values showed statistically significant ($p < 0.05$) variations among different formulations (**Table 3**). The highest value (57.22) for L^* was observed in PCB-1, while the lowest value (40.02) was reported in PCB-5. L^* values differed significantly with an increased percentage of date paste that showed a slightly darker color (lower lightness values). a^* and b^* represent the red-green axis and blue-yellow axis, respectively. All the samples showed positive $a^*$and b^* values and therefore indicate the reddish and yellowish color of the developed product due to the presence of date paste and walnut grits (52).

Textural Analysis

Three-Point Bending of Pregelatinized Whole Grain Cereal Bar

Three-point bending parameters such as flexural modulus (E), rupture stress (σ), and fracture strain (r) are shown in **Table 5**. Flexural modulus (E) also known as bending modulus indicates the stiffness of a product, i.e., the higher the flexural modulus of a product, the harder is to bend. Generally, samples with higher moisture content showed increased modulus, rupture stress, and fracture strain (29) as excess water acts as a plasticizer in amorphous regions of starch molecules, leading to the breakdown of hydrogen bonds and the formation of bonds between associated starch chain and water molecules (53). In addition, an increase in water content decreases the viscosity, resulting in a hard, compressed, and dense structure of the product (54). Sample PCB-5 showed higher bending modulus (15.86 MPa) due to the high percentage of date paste (17.5 %),

TABLE 5 | Textural characteristics of developed PCB.

Samples	3-point bending test			Textural profile analysis				
	σ (MPa)	E (MPa)	r (MPa)	Hardness (N)	Springiness	Adhesiveness	Cohesiveness	Gumminess (N)
PCB-1	4.33 ± 0.11^{d}	8.05 ± 0.09^{e}	0.67 ± 0.04^{e}	82.64 ± 0.22^{e}	0.16 ± 0.17^{d}	-835.77 ± 0.07^{a}	0.126 ± 0.34^{d}	14.41 ± 0.22^{e}
PCB-2	6.04 ± 0.3^{c}	9.23 ± 0.25^{d}	0.71 ± 0.27^{d}	109.15 ± 0.19^{d}	0.18 ± 0.08^{c}	$-1{,}107.3 \pm 0.05^{b}$	0.136 ± 0.11^{c}	18.84 ± 0.18^{d}
PCB-3	6.07 ± 0.14^{c}	10.73 ± 0.22^{c}	0.94 ± 0.31^{c}	122.34 ± 0.35^{c}	0.21 ± 0.26^{b}	$-1{,}248.3 \pm 0.17^{c}$	0.139 ± 0.16^{c}	21.0 ± 0.09^{c}
PCB-4	9.12 ± 0.09^{b}	12.34 ± 0.17^{b}	1.44 ± 0.24^{b}	134.62 ± 0.32^{b}	0.24 ± 0.09^{b}	$-1{,}777.9 \pm 0.04^{d}$	0.147 ± 0.11^{b}	29.78 ± 0.04^{b}
PCB-5	10.95 ± 0.16^{a}	15.86 ± 0.19^{a}	1.78 ± 0.23^{a}	147.74 ± 0.35^{a}	0.42 ± 0.27^{a}	$-3{,}171.1 \pm 0.11^{e}$	0.153 ± 0.17^{a}	32.6 ± 0.21^{a}

PCB, pregelatinized cereal bars; flexural modulus (E); rupture stress (σ); fracture strain (r).
Values are an average of triplicate observations (± SD). Values followed by similar superscripts in columns do not differ significantly ($p \leq 0.05$).

i.e., as the date paste contains natural sugar that has a plasticizing effect even at a low percentage led to lower melt temperature and decreased water vapor pressure resulting in the hard texture of the product. Another explanation results from the difference in the properties of ingredients used during processing such as walnut tend to return to their original shape after compression (55). Thus, the ingredients exhibited different functional and mechanical characteristics depending on the process conditions.

Additionally, a high amount of date paste and walnut can increase the rupture stress (σ) and fracture strain (r) of whole grain PCB from 4.33 to 10.95 (MPa) and 0.67 to 1.78 (MPa), respectively, due to the increment in moisture absorption of sucrose and liquid sugar from date paste that shows elevated hygroscopic nature in a cereal bar. The incorporation of sweetener or binder can influence the solid cohesion between the ingredients used during the development of cereal bar due to strong interaction between sugar networks that needs high penetration forces. Similar results were found in the development of granular and cereal bars by using sucrose as an alternative sweetener (56).

Texture Profile Analysis

Pregelatinized whole grain cereal bar contains different percentages of protein-enriched ingredients date paste and walnut (**Table 5**). Results obtained by TPA showed significant variation ($p < 0.05$) for the formulation of PCB-1, PCB-2, PCB-3, PCB-4, and PCB-5. Variation in different parameters of TPA could be ascribed to different moisture content and concentration of ingredients.

Hardness values increased with an increasing percentage of date paste. Highest value for hardness was found in PCB-5 (147.74 N) followed by PCB-4 (134.62 N), PCB-3 (122.34 N), PCB-2 (109.15 N), and PCB-1 (82.64 N). These values were measured from the maximum force obtained during the first probe penetration. The higher amount of date paste can increase the hardness due to the moisture migration between carbohydrates such as starches, sugars, dietary fibers, and proteins that make food products less elastic and more prone to rupture upon compression (57), i.e., the transformation of rubbery behavior (easy to deform) of date paste to leathery state (tough to deform) as the water molecules in date paste migrates from protein toward sugars, which requires higher penetration force. When all the ingredients such as extrudates (made of a mixture of WWF, WBG, WCF, and IHCF), walnuts, and date paste are added together, it caused the stickiness and hardness to increase as shown in **Table 5**. Thus, the result suggested that the amount of protein and type of fiber also affects the hardness of the cereal bars (58).

Cohesiveness and adhesiveness are related to the probe withdrawal force within the PCB. Cohesiveness values were 0.126, 0.136, 0.139, 0.147, and 0.153 for PCB-1, PCB-2, PCB-3, PCB-4, and PCB-5, respectively. Cohesiveness indicates the strength of intrinsic interactions that shows the degree of mass of ingredients sticks together after chewing (59). Thus, when an increased percentage of date paste is mixed with other ingredients, more cohesion was observed. This result suggests that increased heterogeneity between raw materials leads to more interactions and thus higher cohesiveness strength. Similar results were reported by Conte et al. (60) for gluten-free bread. Adhesiveness determines the ratio of work to force that overcomes the attractive force between the surface of a probe and the product (61). Negative values of adhesiveness (**Table 5**) show that the bars made from date paste are very sticky or adhesive (33). Bars from the PCB-5 recipe were more cohesive and adhesive, but the sample PCB-1 and PCB-2 had a gritty mouthfeel as it contains more walnut grits than other bars.

Springiness values of PCB varied from 0.16 to 0.42, this means that the values for springiness are <1, suggesting that the bars developed from date paste do not come back to their original shape once the force is applied to them (33). Gumminess values were calculated between cohesiveness with hardness and are defined by the force needed to break the sample completely into a steady state of swallowing (62). Gumminess values were 14.41 N, 18.84 N, 21, 29.78 N, and 32.6 N for PCB-1, PCB-2, PCB-3, PCB-4, and PCB-5, respectively. Since PCB-5 and PCB-4 have the higher values due to the inclusion of a higher percentage of date paste and walnut that increased the number of chews required before swallowing.

Sensory Evaluation

The mean values for sensorial evaluation are depicted in **Table 6**. All the samples showed good scores for taste, texture, color, and aroma of all the parameters analyzed. The average values ranged

TABLE 6 | Sensory and average acceptance of PCB.

Samples	Taste	Texture	Color	Aroma	Overall acceptability	Intention to purchase	Acceptability index (%)
PCB-1	7.55 ± 0.23^{d}	7.10 ± 0.11^{c}	8.00 ± 0.05^{a}	7.00 ± 0.03^{b}	7.54 ± 0.04^{d}	3.00^{b}	75.22^{d}
PCB-2	7.92 ± 0.14^{b}	7.05 ± 0.15^{c}	7.56 ± 0.18^{c}	7.10 ± 0.02^{b}	7.88 ± 0.21^{b}	4.00^{a}	77.23^{c}
PCB-3	7.77 ± 0.07^{c}	7.05 ± 0.23^{c}	7.94 ± 0.25^{b}	7.10 ± 0.16^{b}	7.63 ± 0.23^{c}	4.00^{a}	70.11^{e}
PCB-4	7.8 ± 0.05^{c}	7.81 ± 0.19^{b}	7.00 ± 0.33^{d}	8.00 ± 0.19^{a}	7.9 ± 0.19^{a}	4.00^{a}	79.45^{a}
PCB-5	8.00 ± 0.09^{a}	8.00 ± 0.25^{a}	6.90 ± 0.36^{d}	8.04 ± 0.09^{a}	8.00 ± 0.07^{a}	4.00^{a}	79.01^{b}

PCB, pregelatinized cereal bars.

Values are an average of triplicate observations (± SD). Values followed by similar superscripts in columns do not differ significantly ($p \leq 0.05$).

TABLE 7 | Correlation coefficient between human assessment and mechanical variable.

Mechanical variable	Human assessment	Pearson's coefficient correlation	*p*-value
Hardness	Firmness	0.945	0.004
Cohesiveness	Breakdown rate	−0.932	0.017
Adhesiveness	Adhesive (mouth)- degree to which PCB sticks to the teeth surface after swallowing	0.967	0.002
Springiness	Sample recovery –recoverable strain	0.8	0.24

between 7.54 and 8.0, which means "like slightly" to "like very much" in terms of hedonic scale. The test for an index of purchase indicates the probable buying of a product. PCB-2, PCB-3, PCB-4, and PCB-5 showed non-significant variations, and the panelist suggested that the products are recommended to buy (4 scores). The acceptability index (AI) was determined based on the mean scores given by the judges, where PCB-5 (containing 12.5% of date paste and 7.5% of walnut grits) reported the highest AI (79.45%), but other treatments showed AI above 70% (**Table 6**). A product having at least 70% of approval is considered to be acceptable (63).

Correlation Between Human Assessment and Mechanical Variable

The Pearson correlation coefficient was used to analyze the correlation between two variables, i.e., human assessment (sensory analysis) and mechanical variable (instrumental texture). In a correlation coefficient, a perfect linear relationship indicates an absolute value of 1, and a p-value < 0.05 shows that the parameters were significantly related to each other. The behavior of date paste and walnut grits in PCB were achieved through developing the correlations of the parameters obtained by human assessment and mechanical variable as given in **Table 7**.

From the analysis, hardness and springiness (mechanical) showed an excellent correlation with firmness and sample recovery (human assessment). There is a significant positive ($p < 0.01$) correlation between sensory and instrumental hardness as well as sensory and instrumental springiness. These results confirm the usefulness of TPA in predicting the human perception (sensory) of cereal bars, as reported in a previous study (64). A PCB sample with minimum hardness value obtained by mechanical variables could be translated to less firmness in the mouth, which needs minimum efforts to break down the sample. In the instrumental texture test, a sample is first compressed where the sample surface is less than the instrumental surface. In sensory, deformation of sample is done either by fingers or teeth, i.e., the force required to compress the food product between molar teeth during mastication (65). Springiness can be defined as the recovery deformation. It is the function of test time, less test time means an elastic property, and therefore, the textural values are close to 1. Mechanical springiness is a good evaluator of sensory recovery deformation (66).

Sensory adhesiveness (mouth) shows a positive correlation with mechanical adhesiveness (the rate at which the sample adheres to the probe surface after the first compression), while a negative relationship was observed between sensory cohesiveness (breakdown) and instrumental cohesiveness. **Table 7** depicts that adhesiveness values from both human perception and mechanical were strongly correlated ($r = 0.96$) and poor correlation was between sensory and instrumental cohesiveness ($r = -0.932$) (67). For dry samples such as cereal bars, an adequate amount of saliva is mixed during chewing and before swallowing. Thus, it would be difficult to determine the accurate cohesiveness by instrumental test unless the effect of incorporation of saliva is added to it. Cohesiveness increases with the presence of saliva as it enhances the viscoelastic characteristic of food due to mucin components (68).

The results obtained for PCB incorporated with date paste and walnut grits showed that instrumental measurements are one of the best methods to evaluate most of the human assessment or sensory attributes. It is a cost-effective and rapid tool that mimics oral processing or sensory evaluation.

CONCLUSION

Utilizing extrudates, date paste, and walnut grits to develop PCB is an excellent way to attract consumers. Incorporating date paste and walnut grits enhanced the three-point bending test and textural properties. The antioxidants, protein, ash, fiber, and dietary fatty acid content progressively increased with increased

levels of date paste and walnut grits. High acceptability index and appealing color for all PCB were obtained. Instrumental measurements and sensory attributes were positively correlated. Hardness, adhesiveness, and springiness attributes from the instrument and sensory attributes were positively correlated. Cohesiveness obtained from TPA was negatively correlated to the breakdown rate. It indicates protein-rich PCB is easy to chew into a desirable state before swallowing, which makes it suitable for kids and elderly people. This novel PCB can be consumed as ready-to-eat food, a healthy snack bar, and as a dessert (if kept chilled). Developing a healthy product from date paste and walnut kernels rich in bioactive compounds, proteins, and dietary fiber is a practical approach that enhances the potential health-promoting benefits.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

FA: conceptualization, writing—original draft preparation, data curation, investigation, and methodology. BD: data analysis, validation, and visualization. KG: formal analysis, supervision, investigation, and validation. MA: data curation, validation, and writing—review and editing. SA: methodology and writing—review and editing. MH: conceptualization, writing—original draft preparation, and investigation. VP and ZA: conceptualization, data analysis, validation, project administration, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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A Quick Analysis Method for Protein Quantification in Oilseed Crops: A Comparison With Standard Protocol

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Protein is one of the most abundant substances in plants and plays a major role in human health hence standardization of its analytical quantification method is essential. Various methods for protein quantification exist, such as Kjeldahl, Bradford, Lowry, bicinchoninic acid assay (BCA), Biuret, and total amino acid content methods. These methods are widely applied; however, the development of the rapid and efficient method is the need of the time hence the objective of this research was to analyze and comparing compare the modification of the Kjeldahl method for the determination of protein content in oilseed crops. The study was performed to improve the sample preparation method (processing and digestion) for protein quantification. Generally, the method initially requires homogenization of grains to a fine flour, which involves time and increases the risk of sample cross-contamination and partial loss of oil from the sample during grinding. Moreover at times, it becomes challenging to homogenize oil seeds to fine flour due to high oil content. However, in the present research, the whole grain was digested in place of grounded flour to accomplish quick protein quantification and compared it with the flour matrix of different oil seeds. To further reduce the digestion time and avoid frothing, we have used the modified digestion mixture. The developed method was statistically validated using analysis of variance (ANOVA), Pearson correlation reliability test, paired *T*-test, and different types of plot analysis. The validation of the sample preparation method in protein quantification demonstrated non-significant differences that the protein content from whole grain of all the five oilseed crops shows 100% non-significant results compared with the flour matrix in both the digestion mixtures. The developed novel method could be used to prepare the sample for protein analysis and reduces the overall analysis time while ensuring the accuracy of the results.

Keywords: Kjeldahl method, protein quantification, digestion, distillation, titration, oilseed crops, whole grain, flour

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INTRODUCTION

The quality of protein and its type is important to determine its overall health impact upon consumption (1, 2). Protein provides energy, and it is also a vital component for other purposes, including enzyme activity and bio-chemicals passage in cellular membranes (3, 4). The accuracy in quantification of protein content is important to further determine its food's economic value (5, 6). To determine protein's quantity in food matrices, several

methods are used and reported such as Kjeldahl nitrogen estimation, colorimetric assays (Bradford, Lowry, Bicinchoninic acid assay, and Biuret assay), total amino acid content analysis, chromatographic, and radiolabelling methods. Among them, Kjeldahl nitrogen method is the most widely used method for protein quantification in samples. The Kjeldahl method was discovered in 1883 by the Danish chemist Johan Gustav Christoffer Thorsager Kjeldahl and used to determine the nitrogen and protein content. Publicly, it was made available on March 7, 1883, during the meeting held by Danish Chemical Society (Kemisk Forening) (7–9). Initially, this method was designed to aid in grain's protein changes at the time of fermentation and germination in brewing industries (10). This method indirectly quantifies the total content of protein present in food by direct measurement of nitrogen (5, 11). Today, the Kjeldahl method of protein quantification is universally accepted and used in many laboratories for various food samples. It consists of three major steps, i.e., digestion of organic nitrogen-containing samples with sulfuric acid to ammonium sulfate, distillation of digested sample solution at an elevated temperature and pH to release ammonia which is trapped in boric acid solution, and finally titration of boric acid solution with standard acid (10). Although the method has various advantages, such as its universality, high precision, and reproducibility, it is time-consuming to prepare the sample (digestion) for the analysis (12). There is also a limitation w.r.t. analytical selectivity as it cannot differentiate between protein-based nitrogen and non-protein nitrogen. Some modifications in the standard Kjeldahl method have enhanced the versatility and decreased the procedural time for analyzing different samples simultaneously (11, 13–15). Lee and collaborators (16) compared the standard Kjeldahl method with three modified Kjeldahl procedures by the addition of salicylic acid prior to digestion, the pre-reduction of nitrate to ammonium using $CrK(SO_4)_2$, and the addition of phenyl-acetate to the standard digestion mixture. This last procedure yielded the best nitrogen measuring results in plant tissue, but the salicylic acid method performed better in the presence of water. Amin and Flowers (17) reported the use of salicylic acid dissolved in concentrated sulfuric acid to recover other nitro compounds. A Kjeldahl method guide (18) suggests the use of salicylic acid followed by sodium thiosulfate for nitrate reduction. Further modifications made by researchers have improved the digestion process. Studies have reported the use of ultrasound and microwave energy in the Kjeldahl procedure. Domini and collaborators (19) concluded that a combination of ultrasound and microwave energy in the digestion process improves its performance by reducing digestion time to 7 min compared to 30 min for the classical Kjeldahl. Ultrasound energy has been used to substitute the distillation system in classical Kjeldahl for a purge-and-trap system in order to stimulate a chemical reaction between the alkaline reagent and the digestion mixture (20). Although these methods provide rapid analysis of protein in the sample; however, they may require an additional instrument. Routinely, in laboratory analysis, there is a need for a fast, robust, accurate, and reliable method for estimating protein content.

The introduction of novel analytical approach in laboratory analysis requires comparison and validation with standard methods being followed for quality assurance. In the validation process, two important and complementary stages involves single- and inter-laboratory validations (21). Additionally, the protein's quantitative analysis is important for accurate labeling of food as well as its quality control (22). Primarily used methods for protein nitrogen determination in food samples has also been utilized for determining various other nitrogen forms in plant materials, soils, wastewater matrices, and biological tissues (23, 24). To date, many methods have been developed for determining protein in food samples. However, Kjeldahl digestion and distillation 'method is most frequently used method (25). For successful analysis, proper handling of samples and sample preparation are the major steps, affecting the overall analysis time of the sample, thus need to redefine the analysis by developing novel methods.

Generally, the method initially requires a flour matrix for the digestion process that needs lots of time, especially when dealing with a large sample set. Mainly, homogenizing oilseeds is difficult due to high oil content; flour is often sticky, cleaning the homogenizer is complex, and carries a high risk of cross-contamination compared to cereals and pulses. During digestion, frothing occurs in the oilseed crops when using standard digestion mixture and to avoid this issue, we developed a modified digestion mixture. To accomplish quick protein quantification, we have used this modified digestion mixture to digest the whole grain of five oilseed crops and compared it with their flour matrix. The aim of this research was to evaluate and validate the developed novel analysis method to reduce the homogenization time, cleaning, and avoid the frothing. The method demonstrated that the protein content from whole grain of five tested oilseed crops shows 100% non-significant differences when compared with their flour matrices in both the digestion methods. Several statistical analyses such as descriptive statistics, correlations, paired *t*-test, reliability tests, and different types of plot are employed to validate the results. We hope that this method if utilized can help save sample processing time, costs, and minimize the risk of cross-contamination with accurate results.

MATERIALS AND METHODS

Chemicals and Reagents

A high purity analytical grade chemicals such as methyl red, bromocresol green, lithium sulfate, sodium hydroxide pellets, hydrochloride salt, and selenium metal powder from Sisco Research Laboratory, India. Boric acid from Brunswick Scientific (United States), sulfuric acid from Qualigens (India), and hydrogen peroxide from Rankem Laboratory (India) was purchased.

Plant Material and Preliminary Screening of Seeds

A total of five oilseed crops, i.e., safflower (12 accessions), sesame (10 accessions), linseed (10 accessions), mustard (10 accessions), and niger (10 accessions) were chosen for the study (**Table 1**) at ICAR-National Bureau of Plant Genetic Resources

(NBPGR), New Delhi, India. Initially, the seeds were oven-dried to reduce the moisture content and grinded to fine flour using mixture grinder. Flour as well as seed samples were kept in air-tight sample containers for analysis of protein content. The samples were processed in two ways, first as whole-grain (WG1) and second as flour (FL1) samples using traditional digestion mixtures, and whole grain 2 (WG2) flour 2 (FL2) using modified digestion mixture (**Figure 1**). There were two main hypothesis of the study. First, The difference in estimated protein content in all the five oilseeds of whole grain (WG) and flour samples (FL) were non-significant with both digestion methods. The method of directly digesting whole grains can be used for small-seeded grains, as sample representativeness can be ensured; on the contrary, bold seeds limit sample representativeness and will reduce grinding time and fasten the protein quantification. Second, the modified digestion mixture is less time consuming over the traditional digestion mixture. The traditional digestion mixture contains sulfuric acid (specific gravity 1.84), nitrogen-free catalyst mixture (K_2SO_4 and $CuSO_4$), whereas the modified digestion mixture protocol is given below.

Modified Protocol Development

Digestion Solution I

Briefly, 1.6 g of selenium powder was added to 450 mL of concentrated H_2SO_4, and the mixture was heated till H_2SO_4 became pale yellow. The sulfuric acid-Se solution was cooled down to room temperature and, after that placed in a deep freezer at −20°C.

Digestion Solution II

A 14.0 g of lithium sulfate or sodium sulfate was dissolved in 350 mL of hydrogen peroxide solution (30% AR grade), and the solution was cooled in an ice bath.

Digestion Mixture

To the chilled hydrogen peroxide solution (Digestion solution II), which was kept in an ice bath, the chilled sulfuric acid solution (Digestion solution I) was added slowly. The digestion mixture was thus prepared is fit for use till one week if stored at 5–8°C in the refrigerator and for one month at −20°C in deep freezer without any loss of activity.

Protein Analysis

Analytical micro-Kjeldahl method for determining crude protein was conducted by estimating total nitrogen (11, 12) using FOSS autoanalyzer (Model 2300 Kjeltec unit).

Statistical Analysis

The results were analyzed statistically using univariate and multivariate statistics, two-tailed Pearson correlations at a significance level of 1 and 5%, reliability test, and paired *t*-test and different types of plot analysis have been conducted (26–32).

The analysis was performed using SPSS 17 (International Business Machines, United States).

TABLE 1 | Accession number and protein concentration of all the five oilseed crops for flour and whole-grain for both digestion mixtures.

Sr. no.	Accession no.	Traditional DM		Modified DM	
		WG1	FL1	WG2	FL2
Safflower					
1	NIC594327	14.78	15.01	15.26	15.28
2	IC138882	17.33	16.91	16.99	16.95
3	NIC7094	18.6	17.99	18.35	18.39
4	IC96004	14.38	14.86	14.21	14.62
5	IC95994	15.39	15.68	15.10	14.99
6	PI250202	18.1	17.89	17.99	18.01
7	IC305162	16.3	16.46	16.06	15.99
8	LSRM-14-38	16.2	15.68	15.89	16.01
9	IC95966	19.35	19.87	18.96	19.35
10	IC11122	12.39	12.45	12.45	12.25
11	IC95980	19.25	18.98	19.02	18.99
12	IC96017	19.36	18.99	18.87	18.97
Sesame					
13	IC0430504	21.94	22.07	21.67	21.83
14	IC0430512	19.37	18.97	19.87	19.01
15	IC0430614	23.28	23.68	23.50	23.68
16	IC0500438	26.12	25.87	26.10	25.97
17	IC0500837	23.99	24.22	24.01	24.22
18	IC0501037	23.47	22.98	23.35	23.06
19	IC0510962	21.22	21.79	21.20	20.97
20	IC0510964	17.71	17.61	17.57	17.72
21	IC0510975	18.19	18.40	18.23	18.32
22	IC0510980	19.47	18.93	19.34	18.9
Mustard					
23	IC491078	25.14	25.24	24.89	24.99
24	IC122369	23.83	23.63	24.03	23.89
25	IC122423	23.72	23.11	23.78	23.67
26	IC122032	24.14	23.93	24.24	24.45
27	IC10976	25.87	25.34	25.67	25.45
28	IC11037	22.3	22.31	22.42	23.01
29	IC122441	24	24.25	23.98	23.78
30	IC73236	26.5	25.89	26.38	26.43
31	IC491280	23.73	23.16	23.59	23.19
32	IC491181	27.4	26.99	27.57	26.89
Linseed					
33	IC0096509	19.13	19.37	19.72	20.09
34	IC0096510	19.34	18.97	19.11	18.99
35	IC0096520	21.1	20.98	20.97	21.01
36	IC0096524	19.13	19.25	18.99	18.94
37	IC0096525	20.6	20.31	20.79	20.88
38	IC0096526	17.71	17.05	17.80	16.89
39	IC0096527	17.69	17.71	17.67	17.01
40	IC0096528	19.34	18.79	19.27	18.99
41	IC0096529	18.59	18.51	18.68	18.71
42	IC0096530	19.11	18.97	19.01	18.99
Niger					
43	IC305116	20.3	20.49	19.45	19.34
44	IC412911	19.01	19.03	18.97	18.89
45	IC510922	15.06	14.90	15.1	15.3
46	IC545083	22.01	21.67	21.89	22
47	IC552739	17.49	17.54	17.56	17.7
48	IC552811	15.21	15.76	15.1	15.6
49	IC564781	18.04	17.78	17.89	17.95
50	IC565448	20.12	20.21	20.32	20.41
51	IC617173	16.97	16.85	16.96	17.06
52	IC618584	20.02	21.26	20.00	20.09

DM, digestion mixture

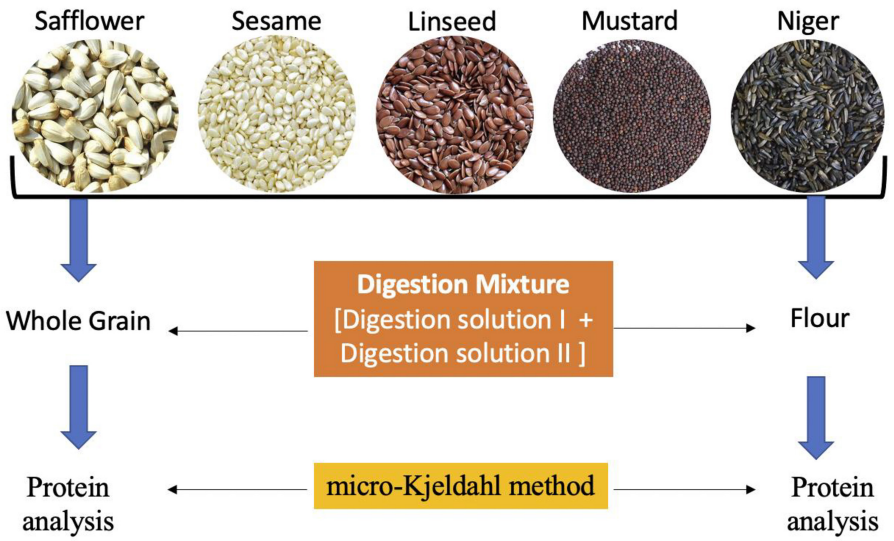

FIGURE 1 | The modified sample preparation method with digestion mixture for protein analysis using Kejdhal method.

TABLE 2 | Descriptive statistical analysis showing range, mean, standard deviation, variance, skewness, and kurtosis for flour and whole-grain of the five oilseed crops.

	Descriptive statistics								
	N	**Range**	**Min**	**Max**	**Mean ± SE**	**Std. Dev**	**Variance**	**Skewness**	**Kurtosis**
WG1	52	15.01	12.39	27.40	19.97 ± 0.47	3.42	11.73	0.21 ± 0.33	−0.43 ± 0.65
FL1	52	14.54	12.45	26.99	19.89 ± 0.46	3.35	11.25	0.20 ± 0.33	−0.58 ± 0.65
WG2	52	15.12	12.45	27.57	19.91 ± 0.47	3.44	11.87	0.24 ± 0.33	−0.47 ± 0.65
FL2	52	14.64	12.25	26.89	19.88 ± 0.47	3.41	11.64	0.22 ± 0.33	−0.56 ± 0.65

RESULTS

The present study was designed to modify the standard Kjeldahl method to quantify the protein content in five oilseed crops. Sample processing/preparation is the first major step required to successfully analyze chemical content. By directly digesting the whole grain of oilseed crops, protein quantification was accomplished quickly, and the results are comparable. The validation of this novel analysis demonstrated that the protein content from whole grain of oilseed crops showed 100% non-significant results compared with the flour matrix.

Statistical Analysis

Descriptive Statistics

By using a total of 52 accessions of five oilseed crops, various descriptive statistical analyses were performed to validate the findings. Four categories were made, including whole grain (WG1) and flour (FL1) with traditional digestion mixture and whole grain (WG2) and flour (FL2) with modified digestion mixture. For each sample, the statistical analysis such as range, mean, standard deviation, variance, skewness, and kurtosis was calculated (**Table 2**). The Box-plot represents the locality, spread, and skewness groups of numerical data through their quartiles (33). For each category, there is no major difference in the mean, standard deviation, variance, and skewness values for the flour and whole grain samples under traditional and modified digestion mixture (**Figure 2**). It showed that the developed method is robust and provides meaningful results without any variance in the data compared to the traditional method.

Two-Tailed Pearson Correlation

The Sig (2-tailed) Pearson correlation of *p*-value describes significant correlation at a particular level. Smaller the *p*-value, significant is the correlation (26). By using a total of 52 accessions of five oilseed crops two-tailed Pearson correlation was conducted for all the four categories, i.e., WG1, FL1, WG2, and FL2 in order to identify the correlations between the flour and whole grain protein as well as for both the digestion mixtures. Correlation is significant at the (0.01 level) (2-tailed), i.e., the value will be considered significant if it lies between 0.001 and 0.010. For each sample the correlation analysis has been done, and showed the 100% significant result in whole grain and flour samples (**Table 3**). We can easily identify the bivariate Pearson correlation coefficient (with a two-tailed test of significance) using paired samples correlations for each pair of entered variables, i.e., pair 1 (WG1 and FL1), pair 2 (WG2 and FL2), pair 3 (WG1 and WG2), and pair 4 (FL1 and FL2). Therefore, our result supports both the hypothesis that there is no significant difference in the protein quantity for samples

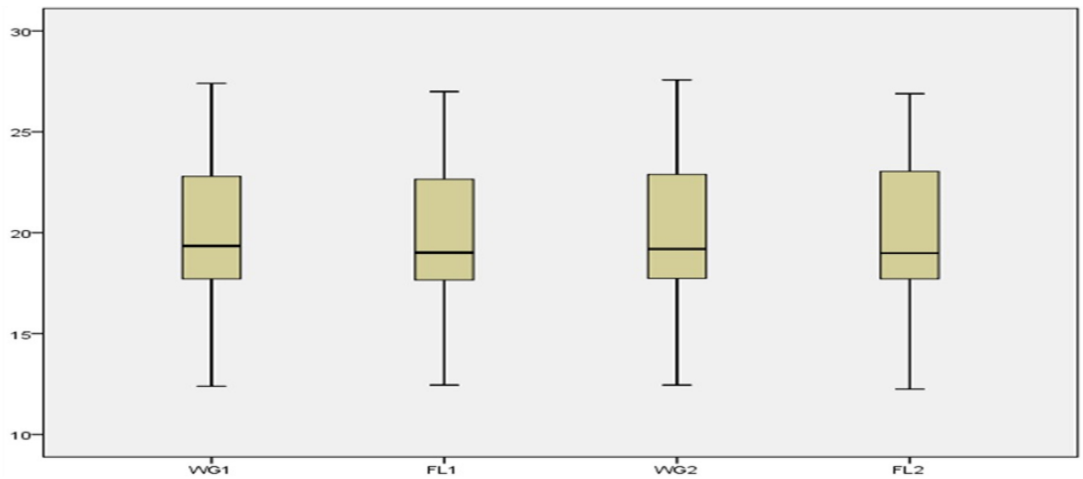

FIGURE 2 | Box plots of WG1, FL1, WG2, and FL2.

(flour and whole- grain) treated with traditional or modified digestion mixtures.

Reliability Tests

The reliability tests played a significant role in quantitative research and are considered instruments for measurement. Reliability provides consistency and accuracy in a given method (27). By using a total of 52 accessions of oilseed crops, reliability tests have been conducted (**Tables 4, 5A–C**) taking all variables as scale, "Strict parallel model" to test the goodness of fit model, analysis of variance (ANOVA) with Tukey's Test for Non-additivity, Hotelling's *T*-squared test, and inter-class correlations.

Based on the statistical analyses, the reliability of the scale was 0.999, and the common Inter-Item correlation was 0.994, which is considered as "the best" and well supported our hypothesis as well.

The Intraclass correlation coefficient (ICC) is utilized in assessing agreement if there are two or more independent raters (which should be independent). In this, the resultant outcome is determined at a continuous level. It is considered as the more powerful reliability test due to utilization of continuous measurement (**Table 5C**).

Test for Goodness of Fit Model

A chi-square test is used to test the relationship between two categorical variables, and is generally known as the test for goodness of fit model. It generally shows the difference between observed counts and expected counts of the dataset if no relationship is found in the population (28) McHugh. The chi-square test value for all the 52 accessions of oilseed crops was 38.278 with an 11 degree of freedom at $p < 0.000$ significance level under the strictly parallel model assumption. The log of the determinant of an unconstrained matrix was –5.004, and for constrained matrix, it was –4.234. The results strongly favor both hypothesis.

A P-P plot is generally used for comparing the empirical cumulative distribution function of data to that with specified theoretical cumulative distribution function $F(\cdot)$. In contrast, a Q-Q plot compares quantiles of the distributed dataset with the standardized theoretical distribution quantiles from specific family distributions. The P-P requires the location and scale parameters to visualize the linear pattern intercept and slope, whereas Q-Q plot does not require these parameters (31, 32; **Figures 3**–**5**).

According to the null hypothesis, the expected and observed protein concentration is the same across all the treatments (whole grain and flour) for both the digestion mixtures. For plotting P-P plots, Bloom's Fractional Rank Estimation Method is applied (**Figure 3**). The model indicates that protein concentration is significantly associated with the treatments ($p < 0.001$). The P-P plots showed the observed proportion of protein concentration in the data and the expected proportion, as predicted by the model. Ideally, all the points fall on the diagonal line producing the best fit and supporting the null hypothesis.

For plotting Q-Q plots (**Figures 4**, **5**), Kolmogorov–Smirnov and Shapiro–Wilk tests were applied. The higher *p*-value suggested that the differences in protein concentration of the whole grain and flour; and for both the digestion mixtures are non-significant and strongly supporting the proposed hypothesis (**Table 4**). The Q-Q plots for WG1 and FL1, and WG2 and FL2 are similar and there is no significant deviation for the treatments (**Figures 4**, **5**).

TABLE 3 | Two-tailed Pearson correlation analysis in flour and whole-grain digested with traditional and modified digestion mixtures.

	FL1	WG2	FL2
WG1	0.994** $p = 0.00$	0.998** $p = 0.00$	0.994** $p = 0.00$
FL1		0.992** $p = 0.00$	0.993** $p = 0.00$
WG2			0.996** $p = 0.00$

***Correlation is significant at the 0.01 level (2-tailed).*

TABLE 4 | Tests of normality.

	Kolmogorov–Smirnov[a]			Shapiro–Wilk		
	Statistic	***df***	**Sig.**	**Statistic**	***df***	**Sig.**
WG1	0.136	52	0.018	0.976	52	0.361
FL1	0.121	52	0.055	0.976	52	0.379
WG2	0.112	52	0.123	0.975	52	0.343
FL2	0.140	52	0.013	0.971	52	0.226

[a]*Lilliefors significance correction.*

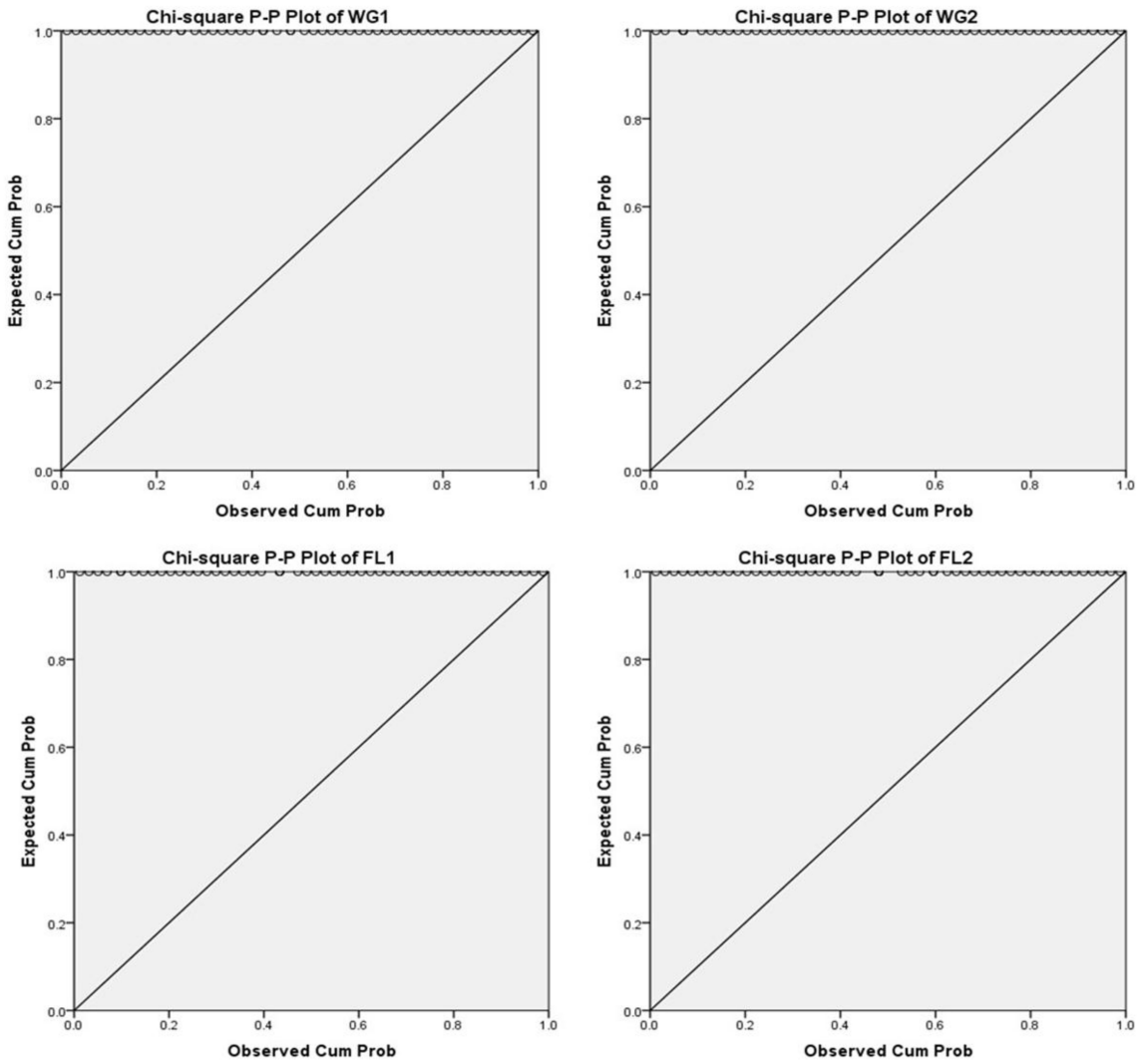

FIGURE 3 | Chi-square P-P plots of WG1, WG2, FL1, and FL2.

Hotelling's *T*-Squared Test

In statistical analysis, particularly in hypothesis testing, one of the methods developed by Harold Hotelling, known as the Hotelling's T-squared distribution (T^2), is a multivariate probability distribution tightly related to the F-distribution and a generalization of Student's t-test (30). Results showed that the Hotelling's T-Squared value is much higher (5.206) than the F-value (1.667) with a significance level of 0.186 (**Table 5A**), hence supporting the hypothesis.

Analysis of Variance With Tukey's Test for Non-additivity

Tukey's test of non-additivity shows an interaction between different factors with no replication. Hence, it has been utilized

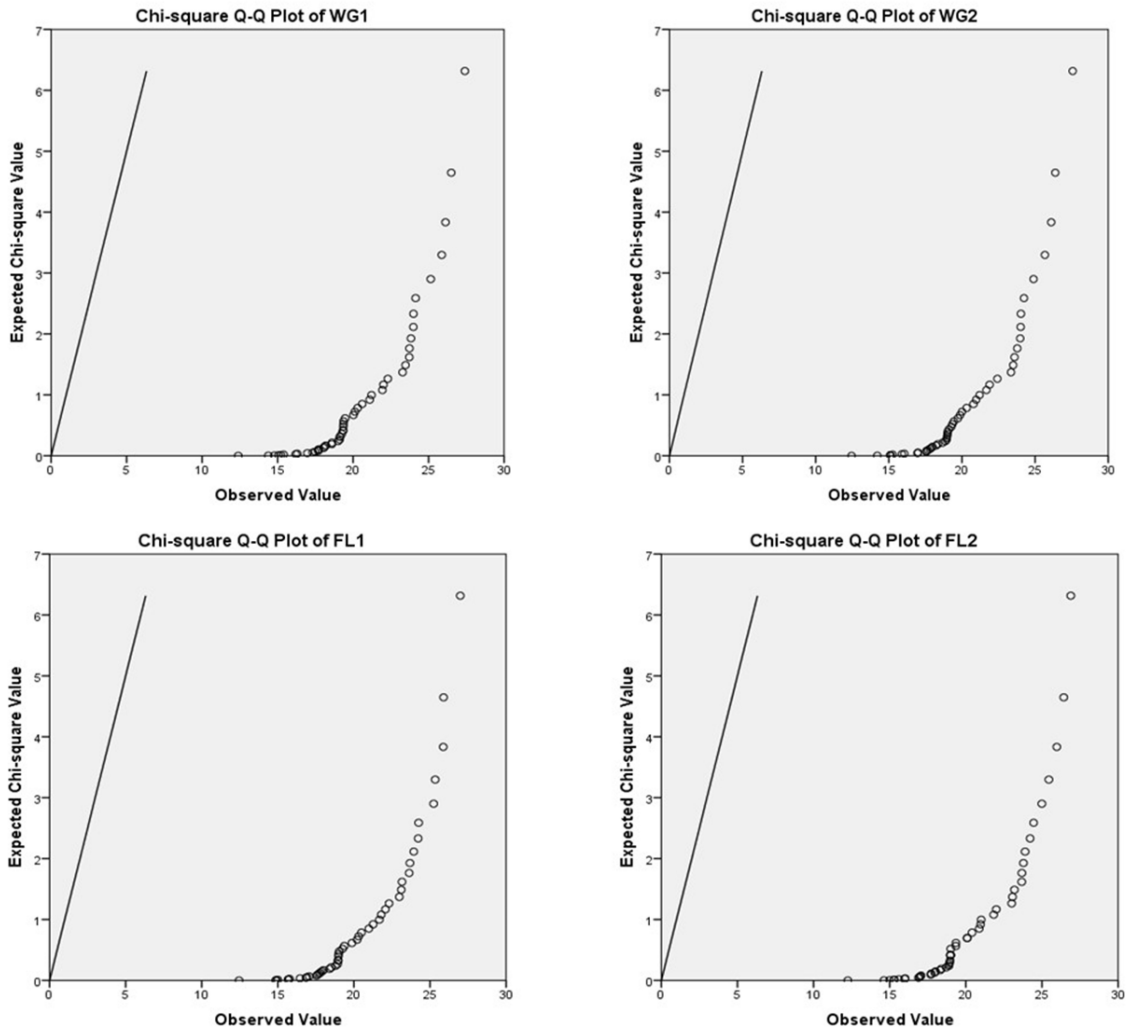

FIGURE 4 | Chi-square Q-Q plots of WG1, WG2, FL1, and FL2.

to test the interaction between the treatment and block factors in a randomized block design. Each block shows the interactions involving different magnitudes but not different directions of treatment effects (29; **Table 5B**).

Interclass correlation coefficient at 95% confidence interval was 0.994 for single measures and 0.994 for average measures (**Table 5C**), providing a robust correlation and supporting both the hypothesis.

Paired *T*-Test

The paired sample test, commonly known as the dependent sample test, is a statistical method utilized to determine the mean difference between two datasets and is thought to be zero. Each entity is measured two times and observed as a pair of items. Generally, it gives the hypothesis test results. In this research, four pairs, i.e., pair 1 (WG1 and FL1), pair 2 (WG2 and FL2), pair 3 (WG1 and WG2), pair 4 (FL1 and FL2) has been tested. For each pair, the differences in the mean and standard deviation are near to zero (**Table 6A**). The t-score ranges from 0.15 to 1.73, for all the four categories under investigation and supports the null hypothesis (**Table 6B**).

DISCUSSION

The health benefits of plant-based protein from different sources are recognized and much appreciated, particularly for the low cost of production and carbon footprint (34–36). The Alternatives to legumes are needed to cater to the increasing demand for plant based protein. The high protein content (15–50%) is reported in different oilseeds, making them ideal sources. Kjeldahl method is most commonly used for determining protein content in the food samples. As a standard practice, samples are ground before digestion. However, oilseeds often form cake while grinding due to high oil content; thus, cleaning grinding mills is challenging and increases the risk of sample cross contamination. The introduction of novel analytical approach in laboratory analysis requires validation of previous methods for assurance of

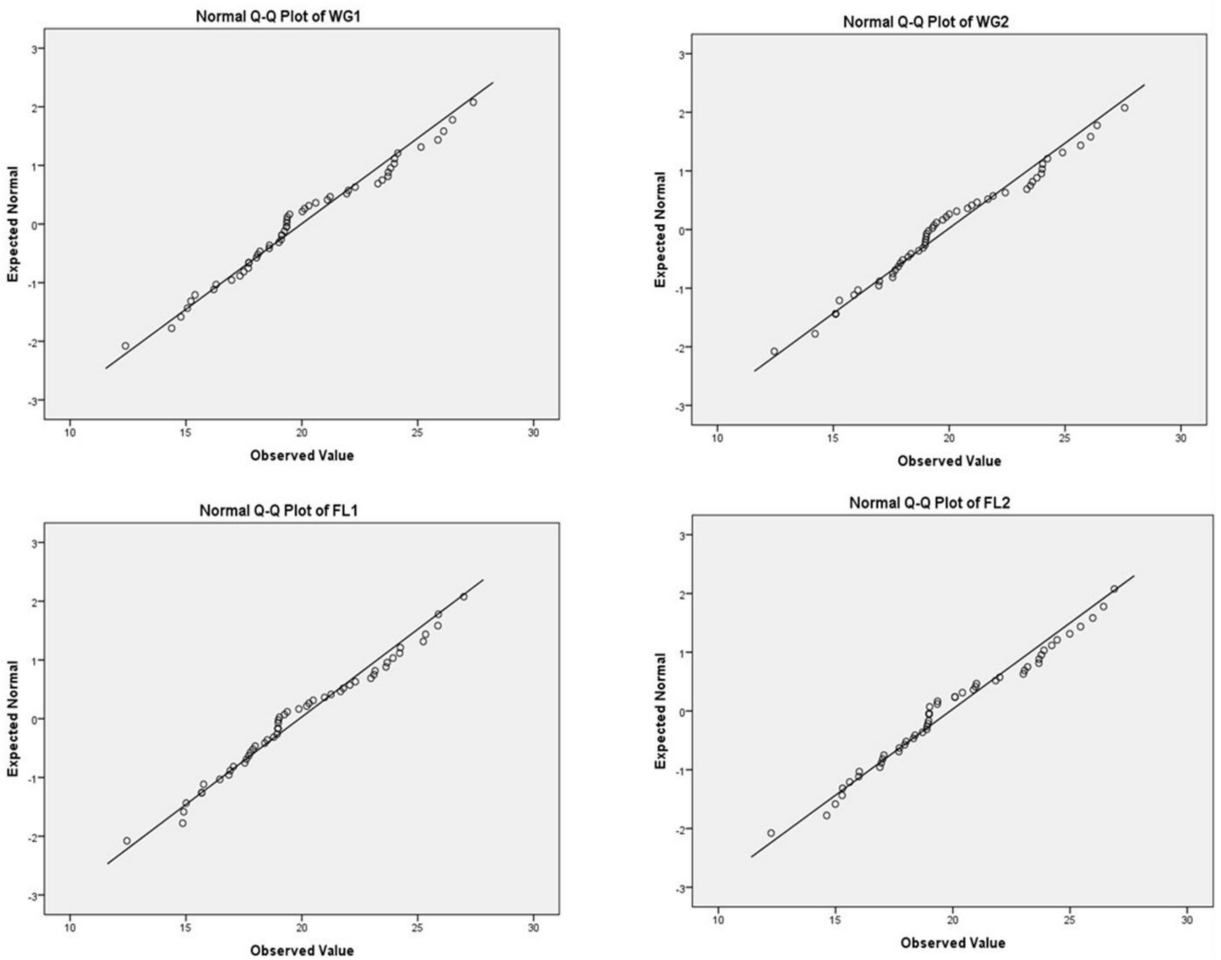

FIGURE 5 | Normal Q-Q plots of WG1, WG2, FL1, and FL2.

its quality. Some modifications in the standard Kjeldahl method have enhanced the versatility and decreased the procedural time for analyzing different samples (16, 18–20). Hydrogen peroxide is added to digest the samples with high oil content to prevent foaming and aid rapid digestion. However, proper sample handling, their sampling, and analytical procedure followed are essential for accurate and precise analysis. In our research, we directly digested the whole grain of five different oilseed crops using two different digestion methods to accomplish quick protein quantification. And with the modification in digestion mixture and catalysts the process of protein quantification speeds up. There were two hypotheses in this study. First, for small-seeded oilseeds, whole grain (WG) gives equally precise results as with flour samples (FL). Therefore, using WG will speed up the protein quantification method. Second, the modified digestion mixture and traditional digestion mixture are equally effective and have no influence on nitrogen recovery. The descriptive statistical analysis shows that no major difference in the values of mean, standard deviation, variance, and skewness for the flour as well as whole grain samples. With paired sample test, which is used for determination of mean difference between two datasets, it has been found that the difference in the mean, as well as standard deviation is near to zero for each pair, i.e., pair 1 (WG1 and FL1), pair 2 (WG2 and FL2), pair 3 (WG1 and WG2), and pair 4 (FL1 and FL2). The Sig (2-tailed) Pearson correlation describes 100% significant result in whole grain and flour samples. Reliability test based on the results from 52 accessions belonging to five different oilseeds, demonstrated consistency of the results. The value of chi-square test for all the 52 accessions of oilseed crops was found to be 38.278 with 11 degree of freedom at zero significance, which shows the relationship between two categorical variables. Hotelling's *T*-squared distribution (T2), a multivariate probability distribution, which is tightly related to the F-distribution and a generalization of 'Student's *t*-test, shows the significance of 0.186. Tukey's test of non-additivity is a test that shows an interaction between

TABLE 5A | Hotelling's *T*-squared test.

Hotelling's *T*-squared	*F*	*df*1	*df*2	Sig
5.206	1.667	3	49	0.186

TABLE 5B | ANOVA with Tukey's test for non-additivity.

			Sum of squares	*df*	Mean square	*F*	Sig
Between people			2361.887	51	46.312		
Within people	Between items		0.260	3	0.087	1.309	0.274
	Residual	Non-additivity	0.059[a]	1	0.059	0.884	0.349
		Balance	10.073	152	0.066		
		Total	10.131	153	0.066		
	Total		10.391	156	0.067		
Total			2372.279	207	11.460		

Grand mean = 19.9192.
[a]Tukey's estimate of power to which observations must be raised to achieve additivity = –1.806.

TABLE 5C | Intraclass correlation coefficient.

	Intraclass correlation[b]	95% confidence interval		*F* test with true value 0			
		Lower bound	Upper bound	Value	*df*1	*df*2	Sig
Single measures	0.994[a]	0.991	0.996	699.389	51	153	0.000
Average measures	0.999	0.998	0.999	699.389	51	153	0.000

Two-way random effects model where both people effects and measures effects are random.
[a]The estimator is the same, whether the interaction effect is present or not.
[b]Type A intraclass correlation coefficients using an absolute agreement definition.

TABLE 6A | Paired sample statistics and paired samples test showing mean difference and standard deviation.

Paired samples statistics

		Paired differences					*t*-score	*df*	Sig. (2-tailed)
		Mean	Std. dev	Std. error mean	95% confidence interval of the difference				
					Lower	Upper			
Pair 1	WG1-L1	0.08 ± 0.05	0.38	0.05	–0.02	0.18	1.52	51	0.13
Pair 2	WG2-L2	0.03 ± 0.04	0.30	0.04	–0.05	0.11	0.79	51	0.43
Pair 3	WG1-G2	0.05 ± 0.03	0.24	0.03	–0.00	0.12	1.73	51	0.09
Pair 4	FL1-FL2	0.00 ± 0.05	0.41	0.05	–0.10	0.12	0.15	51	0.87

TABLE 6B | Paired sample statistics and correlations showing mean, standard deviation, and correlations.

Paired samples statistics and correlations

		Mean	*N*	Std. deviation	Std. error mean	Correlation	Sig.
Pair 1	WG1	19.97	52	3.42	0.47	0.994	0.000
	FL1	19.89	52	3.35	0.46		
Pair 2	WG2	19.91	52	3.44	0.47	0.996	0.000
	FL2	19.88	52	3.41	0.47		
Pair 3	WG1	19.97	52	3.42	0.47	0.998	0.000
	WG2	19.91	52	3.44	0.47		
Pair 4	FL1	19.89	52	3.35	0.46	0.993	0.000
	FL2	19.88	52	3.41	0.47		

different factors with no replication. Thus the validation of this novel analysis/modified approach of using whole grain in small-seeded oilseeds demonstrated non-significant differences with their respective flour samples.

CONCLUSION

For the determination of "food"s protein quantity, it is very essential to standardized analytical methods and shortens the

overall analysis time. The correct quantification as well as determination of content of protein is important, and hence in the present study we analyze and compare the modification of Kjeldahl method for determination of protein content in five oilseed crops. Here, we directly digested whole grain of five oilseed crops instead of making flour to reduce processessing time and accomplish quick protein quantification. Further, to cope up with oil frothing during digestion time, we developed a modified digestion mixture. On the basis of validation parameters analyzed, it can be concluded that the protein content from whole grain of oilseed crops shows 100% non-significant results compared with the flour matrix indicating that there is no difference in the protein content if we digest the whole rain rather than that of the flour. Statistical analyses such as paired *T*-test, Pearson correlation, a range of reliability tests, and different types of plot analysis showed similar results in both flour and whole grain (reduce procedural time) for both the digestion mixtures. Hence, this method will help saving sample processing time, costs, and minimize the risk of cross-contamination with accurate results. This will enable a large data set to be finished in a very less duration of time.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SL conceived the idea and contributed to experimentation and manuscript drafting. RB standardized digestion methods and proofread the manuscript. RB and SL contributed to statistical analysis. RY and JR provided diverse accessions of oilseeds. AK and SK contributed to resources, editing, and formal analysis. All authors contributed to the article and approved the submitted version.

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Effects of Cotton–Peanut Intercropping Patterns on Cotton Yield Formation and Economic Benefits

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Intercropping has been widely adopted by farmers because it often enhances crop productivity and economic returns. However, to increase the comprehensive production benefits of agricultural cultivation and increase the economic benefits of cotton in Northwest Shandong Province, a set of green, ecological, and efficient intercropping mode suitable for Northwest Shandong Province was preliminarily formed. A 2-year intercropping experiment was conducted in Xiajin and Dongping counties in Shandong Province, with six alternative intercropping patterns proposed. After analyzing the experimental data, it was determined that the traditional cotton–peanut intercropping method is not mechanized and that a new intercropping mode has been proposed: four rows of cotton and six rows of peanut. We selected the appropriate intercropping mode for Xiajin and Dongping counties. The production efficiency of 4:4 cotton intercropping in Peanut Ridge was the best in Dongping and Xiajin counties, which was 28–123% higher than that of monoculture. This planting pattern is suitable for demonstration and promotion in the two counties, as well as in the traditional cotton area of the old Yellow River in Northwest Shandong.

Keywords: cotton, peanut, intercropping, economic benefit, yield

INTRODUCTION

Cotton is the main fiber crop, and peanut is a popular oil crop in China (Chi et al., 2019; Wang et al., 2021). The incompatibility with cotton and grain, oil, vegetables, melon, and fruit has become one of the major impediments to the development of cotton production (Abd El-Zaher Sh et al., 2009; Ahmed et al., 2015). The development of a multi-maturity, three-dimensional intercropping mode is essential to improving planting efficiency, resolving cropland conflict, and stabilizing cotton production. Peanuts have a good nitrogen-fixing ability, and they can improve the utilization rate of land and resources in time and space through the rational allocation of crop population (Chen et al., 2016). Peanut root properties and soil distribution are complementary to cotton, which may

increase soil fertility and allow for a combination of land and nutrition uses (Singh and Ahlawat, 2011; Singh et al., 2015). Numerous studies have shown that intercropping of Gramineae and legumes improve the ecological environment of farms through interspecific competition and spatial complementarity (Latati et al., 2014; Singh et al., 2017).

Planting two or more crop species in the same field and at the same time is known as intercropping (Li et al., 2001, 2003; Stomph et al., 2020). Intercropping has become popular in Asia and Africa because it allows farmers to make the most of available resources (Jordan et al., 2017; Chi et al., 2019). Intercropping patterns that are commonly used include legume/cereal, cotton/cereal, and legume/cotton. These intercropping arrangements allow not only the interception and utilization of sunlight energy and the absorption and utilization rate of water and fertilizer, but they also increase the biodiversity of farmland, effectively suppress weeds, and reduce the occurrence of diseases and insect pests, thus improving the system productivity and promoting the sustainable development of agriculture (Zhang et al., 2010; Gitari et al., 2017; Jordan et al., 2017). Cotton and peanuts are commonly grown in China, and cotton–peanut intercropping is widely utilized to harvest both crops simultaneously (Jordan et al., 2017; Zhao Y. et al., 2019; Salama et al., 2022). In addition, crop diversity in cotton–peanut intercropping systems increased soil environment and field stability (Zhang et al., 2016; Chen et al., 2018) found that in the maize/peanut intercropping system, intercropping enhanced the utilization capacity of maize to strong light, increased the net photosynthetic rate of functional leaves at the late growth stage of maize, and promoted the distribution of photosynthetic substances to grains, and produced in an obvious yield advantage (Singh and Ahlawat, 2013; Qian et al., 2018). At present, the commonly used intercropping patterns include two rows of cotton and peanut (2:2), two rows of cotton and three rows of peanut (2:3), two rows of cotton and four rows of peanut (2:4), and four rows of cotton and two rows of peanut (4:2). Farmers in the Yellow River Basin often harvest two crops in a year, believing it will increase their economic income. Cotton–peanut intercropping can meet these needs (Afrin et al., 2017; Gao et al., 2020; Gowton et al., 2021). However, because the traditional cotton–peanut intercropping method is not mechanized, a new intercropping mode has been proposed: four rows of cotton and six rows of peanut (Xu et al., 2013; Liang et al., 2020; Tang et al., 2020).

In Northwest Shandong Province, Xiajin and Dongping counties are both located on the alluvial plain of the historic Yellow River Channel. Monoculture cotton has been the main cultivation method in Xiajin and Dongping counties for many years. In recent years, the monoculture has led to low land yield, low cotton yield, and sparse comparative benefits. Monoculture cotton has resulted in the degradation of soil's physical and chemical structure, resulting in soil hardening, a decrease in soil fertility, and a decrease in ecological benefit due to its single planting structure. Furthermore, the market price of cotton has been low in recent years; the international trading environment for cotton has been severely hampered, and the cost of cotton planting has increased (Singh et al., 2015; Liu et al., 2019; Maitra et al., 2021). Cotton planting benefits are dwindling in both counties. Cotton planting regions are also gradually concentrated on saline-alkaline and sandy dry land. Innovation and development in the cotton-growing sector must find better planting models (Li et al., 2021; Li W. et al., 2022).

We have developed a series of green. Such an approach is needed to promote ecologically more diverse cropping systems that may be better suited to serve the multiple functions of northwest Shandong by conducting innovative experiments on cotton production and cultivation techniques to study the impact of cotton and peanut planting patterns on land resource use and changes to be further determined in soil structure and properties, crop yield, and field income. Our ultimate goal is to determine the feasibility and advantages of cotton/peanut intercropping, as well as the underlying mechanisms.

MATERIALS AND METHODS

Field Experimental Site and Cultivar

In 2018 and 2019, two field trials were conducted in Dongping County (116.48°E, 35.94°N), Tai'an City, Shandong Province of China, and Xiajin County (116.00°E, 36.95°N), and Dezhou City, Shandong Province of China. Luhua 8 was chosen as the peanut cultivar, and Lu6269 was chosen as the cotton cultivar in this experiment.

Experimental Design and Field Management

In 2018 and 2019, seven treatments were set up; monoculture of cotton (M), two rows of cotton and four rows of peanut intercropping (F2:4), four rows of cotton and four rows of peanut intercropping (F4:4), four rows of cotton and six rows of peanut intercropping (F4:6), two rows of cotton and four rows of peanut intercropping (R2:4), four rows of cotton and four rows of peanut intercropping (R4:4), and four rows of cotton and six rows of peanut intercropping (R4:6).

Monoculture cotton was planted on the flat land at a planting density of 67,500 plants ha^{-1}. Each plot measured 60.8 m^2 (6.08 m × 10 m) with a row spacing of 76 cm.

Cotton and peanut intercropping, cotton planting density of 67,500 plants ha^{-1}, and a row spacing of 76 cm. Peanut planting methods were classified as ridge planting and flat planting, with a row spacing of 30 cm, respectively, and a planting density of 300,000 plants ha^{-1}. The distance between cotton and peanut was 70 cm.

For 2 years, between mid- and late-February, chicken manure 20 thm^{-2} nitrogen, phosphorus, and potassium compound fertilizer 380 $kghm^{-2}$ was used as the base fertilizer on cultivated land. After sowing, the second true cotton leaf was coated, and the seedlings were planted. Peanut seeds were planted at the time of 2–3 pairs of true leaves.

Yield and Yield Components

Yield samples were collected 1 day before harvest. After drying for 14 days, the yield and yield composition of peanut and cotton were determined, respectively. At harvest, peanut pods from 10 randomly sampled plants were weighed after sun-drying for 14 days. Peanut pod yield and 100-pods weight were collected (Chi

et al., 2019). For cotton, all plants were collected in the sampling area to quantify cotton seed, cotton yield, boll density, and weight. After bolling, the seed cotton in the plot was gradually harvested. After drying, weight was used to calculate the yield of seed cotton in plots.

Benefit-Cost Measurement

Material inputs such as seeds, fertilizers, pesticides, and irrigation systems, as well as labor costs such as fertilization, irrigation, weeding, and harvesting, were recorded at each experimental station. Input costs were calculated according to the local material and labor daily prices in Shandong Province, while the production costs of peanut pods and seed cotton were calculated according to the average local market prices in Shandong Province in 2018 and 2019.

Data Collection

Data were collected for the cotton growth and development process, cotton yield and yield components, cotton plant biomass accumulation, cotton production cost, and economic benefits.

Statistical Analysis

Data were analyzed following analysis of variance 35, and means of crop management treatments were compared based on the least significant difference test (LSD) at the 0.05 probability.

RESULTS

Growth and Development Process

Different intercropping strategies for Xiajin cotton in 2018 had no effect on the sowing to squaring process; nevertheless, intercropping advanced flowering and boll opening, with F2:4 and R2:4 flowering 2 days earlier and bolls opening 4 days earlier than in monoculture cotton. The flowering time of other intercropping configurations was 1 day earlier, and the boll-setting time was 2 days earlier. In 2018, the process of cotton growth and development was delayed in Xiajin County, with seeding being 1–2 days slower than monoculture cotton, squaring being 2 days slower, flowering being 8 days slower, and boll opening being 3 days slower, while in 2019, only boll opening monoculture was 3 days slower than intercropping in Dongping County. Intercropping delayed the growth and development of cotton in Xiajin County. Intercropping seedlings were 1–2 days slower than monoculture cotton; seeding occurred 2 days later, flowering occurred 5 days later, and boll opening occurred 3 days later (**Table 1**).

Yield and Yield Components

The yield of cotton seed during 2018 in Dongping is shown in **Table 2**. This stable shows that the yield of cotton seed was high at M (258.97 kg ha^{-1}). Similarly, the estimated yield of cotton seed under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 shows a decline of 45.7, 27.8, 40.5, 3.0, 47.7, and 32.8%, respectively, from the yield of cotton seed at M. The results also show that the lint cotton yield was observed to be high at M (106.38 kg ha^{-1}) during 2018 in Dongping, while the lint cotton yield showed a decline of 44.2, 27.3, 39.6, 3.0, 47.0, and 31.0% under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6. The study also observed that the cottonseed yield during 2019 in Dongping was high at M (253.88 kg ha^{-1}), but at F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6, the cottonseed yield dropped by 46.2, 29.3, 41.7, 2.3, 48.9, and 34.3%. In 2019, in Dongping, the highest lint cotton yield was 102.36 kg ha^{-1} at M, but it showed a reduction of 43.8, 28.2, 39.8, 2.8, 46.6, and 31.1% lint cotton yield under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 flat.

The estimated result of Xiajin during 2018 shows that the seed cotton yield was low at M (307.27 kg ha^{-1}), but it showed a rise of 26.1, 30.0, 0.24, 5.9, 13.6, and 5.7% in cottonseed yield under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 flat. Similarly, the lint cotton yield was observed to be low at M (132.72 kg ha^{-1}), but it showed growth of 28.0, 30.6, 0.25, 4.9, 14.0, and 6.6% in lint cotton yield under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 flat. The study also observed that the cottonseed yield during 2019 in Xiajin was low at M (291.93 kg ha^{-1}), but it showed a growth of 26.3, 34.1, 2.4, 9.5, 15, and 5.7% in seed cotton yield under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 flat. Similarly, the lint cotton yield was observed to be low at M (127.5 kg ha^{-1}), but it was increased by 26.0, 33.3, 0.28, 6.0, 15.1, and 5.4% under F2:4, F4:4, F4:6, R2:4, R4:4, and R4:6 flat.

In 2018, in Dongping, the lint percentage of F2:4 was the highest, which was 2.7% higher than M. The boll number of F2:4 was the highest, which was 9.8% higher than M. Dongping in 2019; the lint percentage of F2:4 was the highest, which was 4.8% higher than M. The boll number of F2:4 was the highest, which was 5.2% higher than M. Xiajin in 2018; the lint percentage of F2:4 was the highest, which was 1.0% higher than M. The boll number of F2:4 was the highest, which was 19.7% higher than M. Xiajin in 2019; the lint percentage of F2:4 was the highest, which was 3.6% higher than M. The boll number of F2:4 was the highest, which was 19.5% higher than M (**Table 2**).

Cotton Biomass Accumulation

Between 2018 and 2019, in Dongping and Xiajin Counties, the ROB accumulation of monoculture cotton was the highest at squaring, first flowering, and boll opening and the lowest at full bolling. At initial and full flowering, ROB accumulation were 69.4–67.9%, and 35.4–38.3% that were lower than that of monoculture cotton, respectively. At the boll opening stage, F4:4 had the lowest ROB accumulation, which was 52.2–5.2% lower than that of monoculture cotton. F4:6 had the lowest ROB accumulation at squaring, which was 59.8–63.8% lower than monoculture cotton. R2:4, The ROB accumulation of R2:4 was the highest in all cropping-−178.0–202.1% higher than that in monoculture cotton. At full flowering, R4:6 had the highest ROB accumulation, which was 1.0–6.8% more than monoculture cotton (**Tables 3**, **4**).

In 2018, the largest VOB accumulation occurred in R2:4 monoculture, R4:4, F2:4, and R2:4 treatments at squaring, first and full flowering, full bolling, and boll opening were in R2:4. The minimum accumulative quantities were F4:4, F2:4, F2:4, monoculture, and F4:4. In Dongping County, the maximum accumulation of VOB was 17.2, 57.3, 43.6, 77.1, and 102.2% greater than the minimum accumulation, while in Xiajin County, the maximum accumulation was 19.5, 57.7, 40.9, 77.1, and 106.1% greater than the minimum accumulation (**Tables 3**, **4**).

TABLE 1 | Cotton growth and development process (days after sowing) in Dongping and Xiajin counties from 2018 to 2019.

Year	Location	Trt	Sowing	Seeding	Squaring	Flowering	Boll opening
2018	Dongping	M	4/28	5	51	72	117
		F2:4	4/28	5	51	70	113
		F4:4	4/28	5	51	71	115
		F4:6	4/28	5	51	71	115
		R2:4	4/28	5	51	70	113
		R4:4	4/28	5	51	71	115
		R4:6	4/28	5	51	71	115
	Xiajin	M	4/23	12	46	68	125
		F2:4	4/23	13	48	76	128
		F4:4	4/23	13	48	76	128
		F4:6	4/23	14	48	76	128
		R2:4	4/23	13	48	76	128
		R4:4	4/23	13	48	76	128
		R4:6	4/23	14	48	76	128
		LSD	**1**	**1**	**1**	**1**	**1**
2019	Dongping	M	4/28	7	55	74	120
		F2:4	4/28	7	55	74	117
		F4:4	4/28	7	55	74	117
		F4:6	4/28	7	55	74	117
		R2:4	4/28	7	55	74	117
		R4:4	4/28	7	55	74	117
		R4:6	4/28	7	55	74	117
	Xiajin	M	4/23	9	44	68	123
		F2:4	4/23	11	46	73	126
		F4:4	4/23	11	46	73	126
		F4:6	4/23	12	46	73	126
		R2:4	4/23	11	46	73	126
		R4:4	4/23	11	46	73	126
		R4:6	4/23	11	46	73	126
		LSD	**1**	**1**	**1**	**1**	**1**

Values followed by the same letters indicate non-significance difference among treatments within years at $P < 0.05$ (LSD test).

In Dongping County, the maximum accumulation of VOB from various treatments was in the R2:4, monoculture, R4:4, R2:4, and R2:4, respectively, in 2019. The minimum accumulation was F4:6, F2:4, R2:4, monoculture, and F4:4, respectively. The maximum accumulation of VOB in Dongping County was 13.3, 155.0, 43.0, 75.0, and 107.8% higher than the minimum accumulation of VOB. In 2019, the maximum VOB accumulation in different treatments of Xiajin County was in the treatments of R2:4, monoculture, R4:4, R2:4, and F4:4, respectively. The minimum accumulation was R4:4, F2:4, R2:4, monoculture, and F4:4. At various growth stages in Xiajin County, the maximum accumulation of VOB was 26.4, 147.7, 34.7, 64.5, and 111.1% greater than the minimum accumulation (**Table 4**).

Donping and Xiajin counties had the least organ biomass accumulation at squaring from 2018 and 2019 and the difference between treatments were modest. Monoculture had the largest accumulation of organ biomass at first flowering, while R2:4 and F2:4 had the lowest. The organ biomass accumulation of R4:6 was the most at full flowering, and that of R2:4 was the least. The organ biomass accumulation of R2:4 and F2:4 was greater at full bolling, whereas the organ biomass accumulation of monoculture was the least. At boll opening, monoculture had the largest biomass accumulation of organs, while F4:4 and R4:4 had the lowest (**Figure 1**).

Cost and Benefit

The net income of F4:4 and R4:4 was the highest in Dongping and Xiajin counties in 2018, while the net income of monoculture was the lowest. R4:4 and F4:4 had a net income of 123.3% higher than that of monoculture (**Table 5**). In 2019, Dongping and Xiajin Counties had the highest net income of R4:4 and the lowest net income from monoculture. Dongping County and Xiajin County increased by 127.8 and 125.6%, respectively (**Table 5**). Under the same treatments, the net incomes of Dongping and Xiajin counties were comparable. In Dongping County, the net income of each treatment is often lower than it is in Xiajin County. This is because Dongping County's lint and seed cotton yields were

TABLE 2 | From 2018 to 2019, cotton yield and yield components (Dongping and Xiajin).

Year	Location	Trt	Boll number (bolls plant $^{-1}$)	Boll weight (g boll^{-1})	Seed cotton yield (kg ha^{-1})	Lint cotton yield (kg ha^{-1})	Lint percentage (%)
2018	Dongping	M	13.3	4.96	258.97	106.38	41
		F2:4	14.6	4.78	140.58	59.35	42.1
		F4:4	14.2	4.57	187.02	77.36	41.3
		F4:6	13.7	4.54	154.02	64.22	41.6
		R2:4	12.5	4.75	251.22	103.14	39.8
		R4:4	13.9	4.52	135.36	56.36	39.2
		R4:6	13.2	4.21	173.94	73.43	39.3
	Xiajin	M	15.7	4.33	307.27	132.72	43.2
		F2:4	18.8	4.52	387.42	169.88	43.6
		F4:4	18.5	4.7	399.36	173.34	43.4
		F4:6	15.4	4.39	308.01	133.05	43.2
		R2:4	16.3	4.39	325.51	139.22	42.7
		R4:4	16.5	4.62	348.98	151.26	43.3
		R4:6	15.8	4.52	324.87	141.43	43.5
		LSD	**2.2**	**0.81**	**19.30**	**15.25**	**1.7**
2019	Dongping	M	13.5	4.88	253.88	102.36	39.4
		F2:4	14.2	4.69	136.49	57.55	41.3
		F4:4	13.8	4.45	179.55	73.49	40.5
		F4:6	13.3	4.36	147.97	61.65	39.6
		R2:4	11.9	4.54	246.26	99.52	37.8
		R4:4	13.2	4.32	129.61	54.66	37.2
		R4:6	12.9	4.13	166.92	70.52	37.7
	Xiajin	M	15.4	4.12	291.93	127.5	41.3
		F2:4	18.4	4.35	368.85	160.64	42.8
		F4:4	17.8	4.62	391.46	169.95	42.1
		F4:6	14.6	4.26	298.89	127.86	41.5
		R2:4	15.6	4.33	319.58	135.1	41.1
		R4:4	16.2	4.54	335.67	146.73	42.5
		R4:6	15.2	4.38	308.65	134.36	42.7
		LSD	**2.0**	**0.76**	**18.52**	**13.91**	**1.4**

Values followed by the same letters indicate non-significance difference among treatments within years at $P < 0.05$ (LSD test).

lower than those in Xiajin County, resulting in lower sales and thus poorer net income in Xiajin County. Cotton and peanut intercropping will raise production costs. Seed, planting, and artificial inputs are the key cost increases. Intertillage, fertilizer, and pesticide input remain unchanged. Its net income was higher than the final net income, demonstrating that these inputs were worthwhile. R4:4 offers the best production benefits in both places, based on net income in 2018 and 2019 (**Table 5**).

DISCUSSION

In Dongping and Xiajin counties, the impact of cotton monoculture and various cotton–peanut intercropping patterns on cotton growth and development, yield components, biomass accumulation of multiple organs, costs, and benefits of cotton production were investigated. The F4:4 model had the highest yield in Xiajin County, with a 30 and 34.1% increase in yield over 2 years compared with monoculture seed cotton. Among six intercropping patterns, the 2-year yield of R2:4 was the highest in Dongping County, which was 27.8 and 3.0% lower than that of monoculture cotton. The net profit of cotton and peanut intercropping is 127.8% higher than that of cotton monoculture. This research offers a new perspective on cotton growing in Shandong Province. The cotton and peanut intercropping option increases field utilization efficiency. Even though it only increases a modest amount of investment, it has a significant impact on economic benefit (Yang et al., 2017; Zhao W. et al., 2019; Zhi et al., 2019). It is a critical innovation that will help boost the economic benefits of cotton production.

The process of cotton production and development is essential for yield formation (Dong et al., 2012; Ai and Ma, 2020; Zhang et al., 2020; Wang et al., 2021). In several intercropping patterns, different intercropping patterns have little effect on the early growth and development of cotton (Sun et al., 2014). Intercropping will reach the flowering stage 0–2 days ahead of schedule, and boll opening 2–4 days ahead of schedule in Dongping County. In Xiajin, intercropping modes delay reaching the sowing stage by 1–3 days, squaring by 2 days, flowering by 5–8 days, and seed pod opening by 3 days. The whole growing cycle of cotton in Dongping County is 7–25 days shorter than in Xiajin

TABLE 3 | Cotton reproductive organs biomass (ROB) and vegetative organs biomass (VOB) accumulation in 2018.

Location	Trt	Squaring		First flowering		Full flowering		Full bolling		Boll opening	
		ROB	VOB	ROB	VOB	ROB	VOB	ROB	VOB	ROB	VOB
Dongping	M	8.23	188.79	140.39	557.45	197.97	473.96	99.76	333.91	602.88	352.94
	F2:4	6.41	206.74	43.00	237.86	122.24	375.89	271.07	574.06	306.50	414.97
	F4:4	4.86	183.59	66.15	385.68	168.13	518.78	166.58	480.91	280.65	213.42
	F4:6	3.10	186.22	103.20	526.27	201.34	503.79	175.96	503.32	332.22	242.58
	R2:4	6.75	215.11	44.28	247.44	124.73	394.86	284.77	591.47	315.82	431.71
	R4:4	5.13	189.13	68.85	405.18	176.64	539.77	171.64	495.49	294.83	219.90
	R4:6	3.17	191.89	106.31	542.20	207.42	524.18	181.30	518.58	338.90	252.37
Xiajin	**LSD**	**1.11**	**19.32**	**16.52**	**23.26**	**21.92**	**25.62**	**23.56**	**30.23**	**27.36**	**24.88**
	M	8.71	196.42	146.06	602.47	206.00	493.13	105.83	347.41	651.61	381.49
	F2:4	6.88	223.48	44.75	254.66	130.88	402.48	284.77	620.43	328.17	435.96
	F4:4	5.20	194.73	70.13	412.94	180.01	560.69	173.33	500.35	303.33	224.22
	F4:6	3.24	197.50	110.49	568.79	217.61	544.50	190.21	544.02	359.08	252.37
	R2:4	7.02	232.53	46.98	267.42	132.29	410.78	302.05	615.37	341.33	462.22
	R4:4	5.40	200.60	72.36	421.58	185.55	567.04	182.04	530.52	306.70	228.81
	R4:6	3.44	203.50	113.80	564.14	219.97	561.23	192.30	539.50	352.60	262.56
	LSD	**1.32**	**18.65**	**17.25**	**24.11**	**26.82**	**19.45**	**35.61**	**22.64**	**25.45**	**18.99**

Values followed by the same letters indicate non-significance difference among treatments within years at $P < 0.05$ (LSD test).

TABLE 4 | Cotton reproductive organs biomass (ROB) and vegetative organs biomass (VOB) accumulation in 2019.

Location	Trt	Squaring		First flowering		Full flowering		Full bolling		Boll opening	
		ROB	VOB	ROB	VOB	ROB	ROB	VOB	ROB	VOB	ROB
Dongping	M	8.65	213.58	151.74	625.02	213.96	488.32	103.79	371.01	669.87	370.77
	F2:4	7.32	213.01	48.64	245.07	138.29	421.45	282.02	649.44	352.94	456.89
	F4:4	5.50	211.41	68.82	424.63	174.93	550.22	174.99	505.20	320.34	243.61
	F4:6	3.48	195.63	116.75	558.17	209.48	519.06	183.07	528.74	359.06	259.74
	R2:4	7.02	221.63	49.20	272.44	133.55	414.80	313.53	609.40	360.48	475.32
	R4:4	5.39	200.59	77.89	454.30	190.91	583.39	195.92	540.54	303.76	228.79
	R4:6	3.62	211.28	121.34	558.63	226.27	593.01	188.62	570.96	359.44	262.57
	LSD	**1.20**	**18.45**	**16.52**	**22.75**	**23.50**	**19.03**	**27.55**	**26.70**	**26.13**	**23.09**
Xiajin	M	9.94	204.35	154.91	675.50	230.97	552.90	121.87	393.02	704.27	396.90
	F2:4	7.86	230.25	48.37	272.67	144.10	463.46	304.90	645.50	338.11	502.02
	F4:4	5.62	224.23	80.75	450.48	205.47	589.01	189.09	530.68	315.59	237.81
	F4:6	3.60	215.45	122.77	603.27	237.39	615.99	201.73	593.48	377.22	265.12
	R2:4	7.87	258.36	53.15	302.54	138.97	448.13	338.66	646.45	379.26	480.89
	R4:4	5.73	206.68	83.32	451.39	198.67	624.32	209.62	578.75	325.29	249.61
	R4:6	3.89	228.17	129.89	649.61	233.30	600.92	219.49	610.34	366.85	286.43
	LSD	**1.09**	**22.3**	**18.64**	**21.78**	**29.12**	**23.85**	**26.47**	**28.04**	**27.91**	**24.65**

The unit of biomass accumulation is g m^{-2}.

County (**Table 1**), so the biomass accumulation in Dongping County was lower than that in Xiajin County, and the yield was increased by 25.92–56.8% lower than in Xiajin County (**Table 2**).

In previous studies, several cotton–peanut intercropping methods were found to have a greater impact on cotton yield than others (Singh and Ahlawat, 2014; Singh et al., 2015). Cotton–peanut intercropping under conventional fertilization enhanced seed cotton production by 16.9% when compared to monoculture (Meso et al., 2007; Singh et al., 2015). Cotton and peanut are complementary in terms of time and space, as well as in terms of growth and development. Peanuts grow quickly and have a brief growth phase, but cotton grows slowly in the early stages (Sharma and Mathur, 2006). Peanut had podded and formed a yield before cotton reached

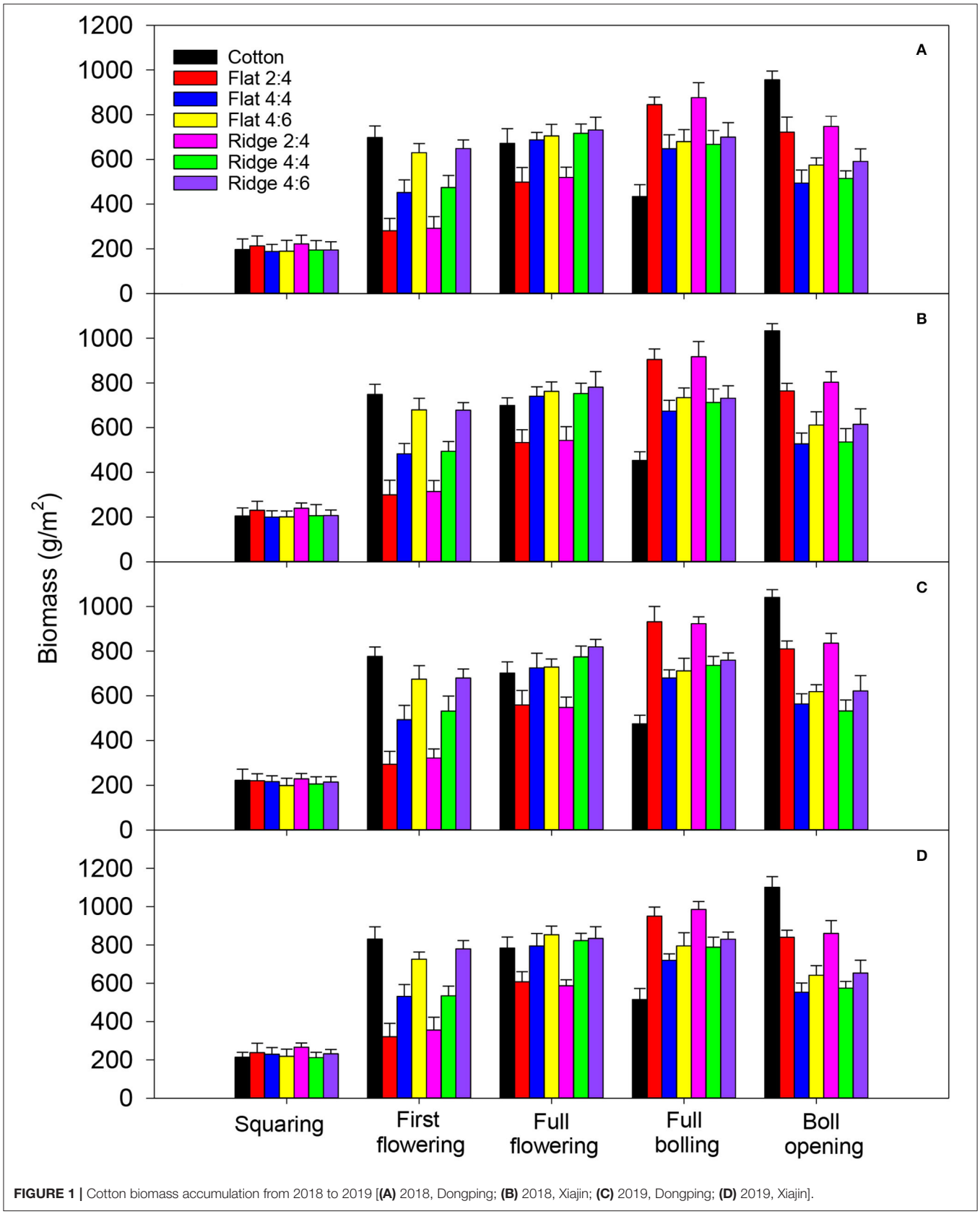

FIGURE 1 | Cotton biomass accumulation from 2018 to 2019 [**(A)** 2018, Dongping; **(B)** 2018, Xiajin; **(C)** 2019, Dongping; **(D)** 2019, Xiajin].

TABLE 5 | Cost and benefit of cotton production in 2018 and 2019.

Location	Trt	Seed	Sow	Intertillage	Fertilizer	Pesticide	Worker	Labor cost (yuan/worker)	Net income (yuan/mu)
		yuan/mu							
2018 Dongping	M	26.5	20	100	130	20	9	100	409.6
	F2:4	191.2	111.8	100	130	20	9	100	694.5
	F4:4	142.6	84.8	100	130	20	10	100	914.5
	F4:6	171.8	101	100	130	20	10	100	726.6
	R2:4	191.2	111.8	100	130	20	9	100	694.6
	R4:4	142.6	84.8	100	130	20	10	100	914.5
	R4:6	171.8	101	100	130	20	10	100	726.6
2018 Xiajin	M	27	20.8	100	130	20	10	100	422.0
	F2:4	197	116.3	100	130	20	10	100	708.0
	F4:4	148.4	89.1	100	130	20	11	100	942.2
	F4:6	178.7	103	100	130	20	11	100	763.3
	R2:4	198.9	117.4	100	130	20	10	100	715.0
	R4:4	145.5	86.5	100	130	20	11	100	942.2
	R4:6	177	106.1	100	130	20	11	100	763.3
2019 Dongping	M	26.5	20.5	100	130	20	9	100	409.6
	F2:4	191.2	111.8	100	130	20	9	100	694.7
	F4:4	142.6	84.8	100	130	20	10	100	914.5
	F4:6	171.8	101.6	100	130	20	10	100	726.6
	R2:4	198.9	116.3	100	130	20	9	100	722
	R4:4	149.8	86.5	100	130	20	10	100	933
	R4:6	177	106.1	100	130	20	10	100	741.3
2019 Xiajin	M	27.3	20.6	100	130	20	10	100	426.1
	F2:4	197	116.3	100	130	20	10	100	729.1
	F4:4	146.9	89.1	100	130	20	11	100	933
	F4:6	175.3	103	100	130	20	11	100	748.6
	R2:4	206.9	121	100	130	20	10	100	736.6
	R4:4	155.9	90	100	130	20	11	100	961.3
	R4:6	180.6	109.3	100	130	20	11	100	778.7

the phase of rapid accumulation, which had little effect on cotton yield, allowing cotton to make maximum use of light and heat resources. In addition, peanut nitrogen fixation can increase soil nitrogen, which is conducive to cotton growth (Singh and Ahlawat, 2015; Kumar et al., 2019; Xie et al., 2022).

The yield of lint cotton and seed cotton in Xiajin County in the past 2 years was the highest in F4:4, which was 30.0–34.1% higher than monoculture. Although the monoculture cotton yields were the highest in 2 years in Dongping County, the R2:4 lint yields were just 2.8–3.0% lower than the monoculture yield, indicating that the appropriate intercropping method had no effect on yield but was conducive to yield increase (Tang et al., 2021; Li L. et al., 2022). The improvement in the yield of intercropping was reflected in the influence of boll weight, lint percentage, and boll number per plant. At the full bolling stage, intercropping boosted biomass accumulation in reproductive organs by 178.0–202.1%, which aided in yield increase (**Figure 2**). Cotton–peanut intercropping can help to speed up the accumulation of nutrients in reproductive organs, as well as the growth and development of flowers and bolls and their settings (Wang et al., 2007; Liu et al., 2019; Maitra et al., 2020; Zhao et al., 2020). However, due to the longer growing period and larger biomass accumulation, Xiajin County's cotton production is higher than Dongping County's.

In field production, the purpose of intercropping is not only to increase the yield of crops but also to boost the economic benefits so as to obtain better benefits for cotton farmers (Dai et al., 2015; Wang et al., 2021). Cotton–peanut intercropping can boost cotton yield, improve field utilization rate, and provide greater production benefits, according to the results of a 2-year experiment. When compared to the net profit of monoculture, Dongping and Xiajin counties had the most economic benefit from adopting the R4:4 cotton method, which increased from 123.3 to 127.8% (**Table 5**).

CONCLUSIONS

In Dongping and Xiajin counties, different cotton intercropping patterns had substantial effects on cotton growth and development, cotton yield, yield components, biomass accumulation of different organs, and the cost and benefit of cotton production. The yield of lint cotton and seed cotton in Xiajin County in the last 2 years was the highest under F4:4,

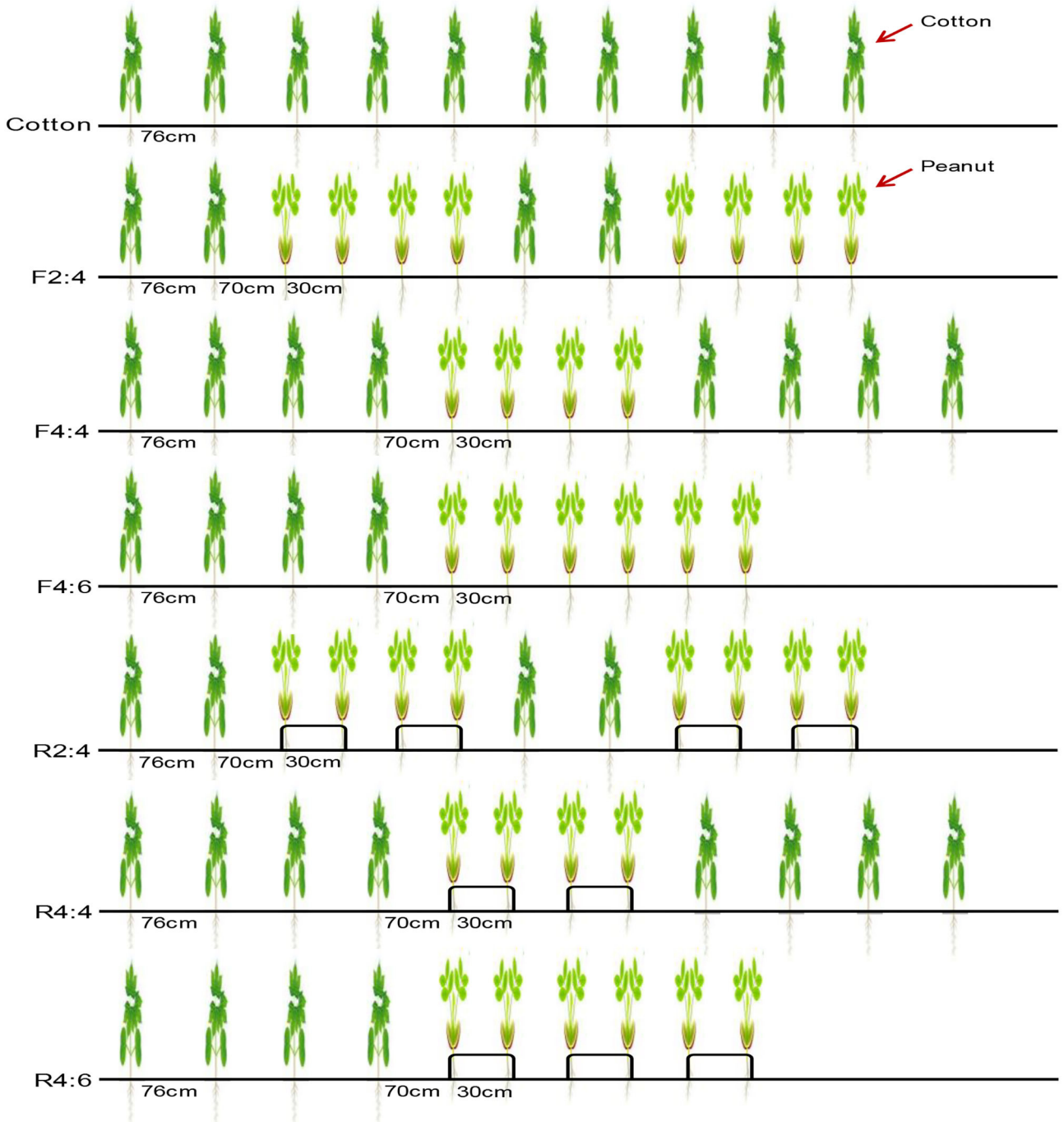

FIGURE 2 | Sketch of field experiments in cropping patterns.

while the yield of monoculture cotton in Dongping County was the highest. In Dongping County and Xiajin County, the economic benefits of F4:4 and R4:4 under various treatments were the highest in 2018, while the economic benefits of monoculture were the lowest. The economic benefit of R4:4 was the highest in Dongping and Xiajin counties in 2019, while the economic benefit of monoculture was the lowest. Based on the experiments between 2018 and 2019, we can select the best intercropping mode R4:4 that offers the highest production benefits in Dongping and Xiajin counties, which increased from 123.3 to 127.8% compared with the net profit of monoculture. The planting pattern is suitable for promotion in two counties.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GW, DW, and SS: conceptualization. XZ, LW, SF, GW, SS, and DW: collecting data. MA, SS, RS, and XZ: methodology. SS, DW, and SF: writing—original draft. LW and SF: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Ultra-Performance Liquid Chromatography-Mass Spectrometry-Based Untargeted Metabolomics Reveals the Key Potential Biomarkers for Castor Meal-Induced Enteritis in Juvenile Hybrid Grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂)

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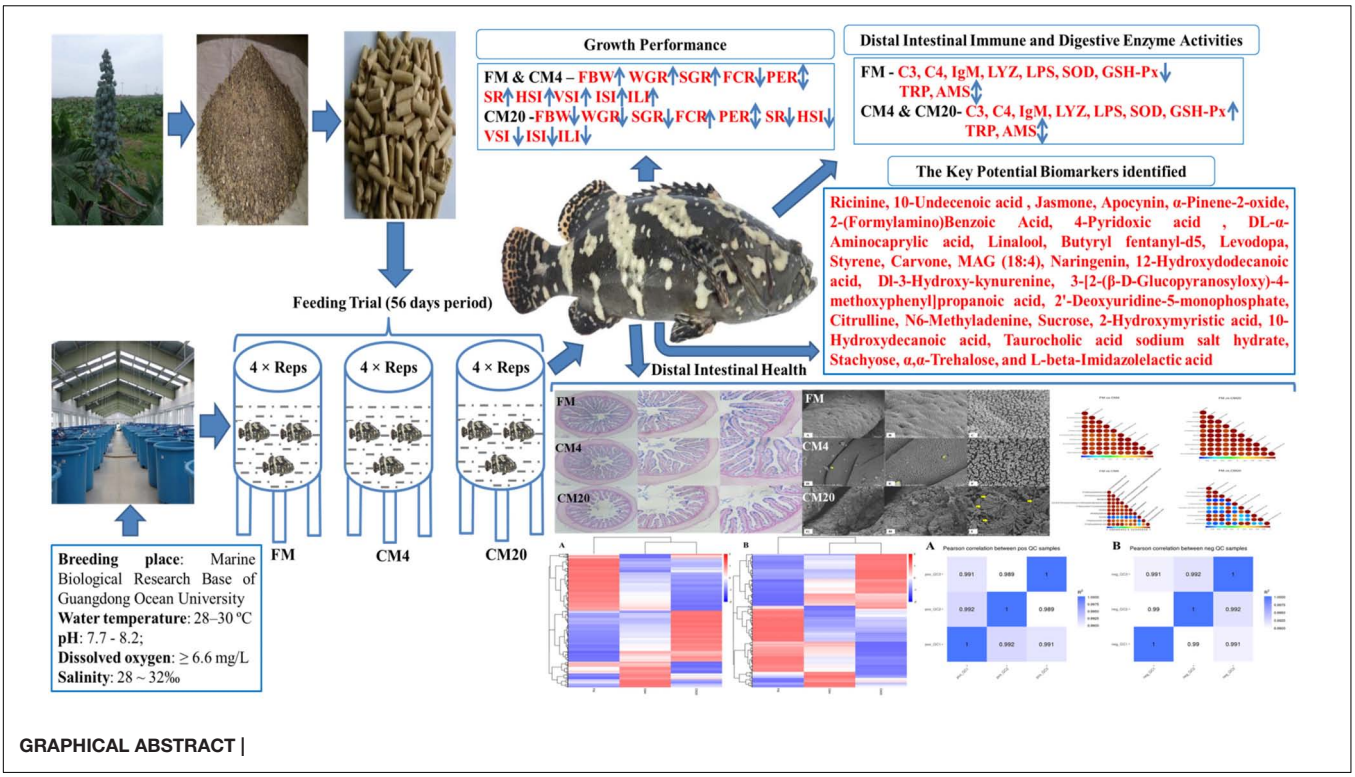
The intensification of aquaculture to help kerb global food security issues has led to the quest for more economical new protein-rich ingredients for the feed-based aquaculture since fishmeal (FM, the ingredient with the finest protein and lipid profile) is losing its acceptability due to high cost and demand. Although very high in protein, castor meal (CM), a by-product after oil-extraction, is disposed-off due to the high presence of toxins. Concurrently, the agro-industrial wastes' consistent production and disposal are of utmost concern; however, having better nutritional profiles of these wastes can lead to their adoption. This study was conducted to identify potential biomarkers of CM-induced enteritis in juvenile hybrid-grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) alongside their growth and distal intestinal (DI) health evaluation. A total of 360 fish (initial weight = 9.13 ± 0.01g) were randomly assigned into three groups, namely, fish-meal (FM) (control), 4% CM (CM4), and 20% CM (CM20). After the 56-days feeding-trial, the DI tissues of FM, CM4, and CM20 groups were collected for metabolomics analysis. Principal components analysis and partial least-squares discriminant-analysis (PLS-DA, used to differentiate the CM20 and CM4, from the FM group with satisfactory explanation and predictive ability) were used to analyze the UPLC-MS data. The results revealed a significant improvement in the growth, DI immune responses and digestive enzyme activities, and DI histological examinations in the CM4 group than the others. Nonetheless, CM20



GRAPHICAL ABSTRACT |

replacement caused DI physiological damage and enteritis in grouper as shown by AB-PAS staining and scanning electron microscopy examinations, respectively. The most influential metabolites in DI contents identified as the potential biomarkers in the positive and negative modes using the metabolomics UPLC-MS profiles were 28 which included five organoheterocyclic compounds, seven lipids, and lipid-like molecules, seven organic oxygen compounds, two benzenoids, five organic acids and derivatives, one phenylpropanoids and polyketides, and one from nucleosides, nucleotides, and analogues superclass. The present study identified a broad array of DI tissue metabolites that differed between FM and CM diets, which provides a valuable reference for further managing fish intestinal health issues. A replacement level of 4% is recommended based on the growth and immunity of fish.

Keywords: *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂, fishmeal, metabolomics, ricinine, castor meal, scanning electron microscopy, ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), partial least-squares discriminant-analysis (PLS-DA)

INTRODUCTION

With reference to the highlights of the global food security and nutrition (1), there are about 11% (over 820 million people) of the world's population (majority from south Asia and sub-Saharan Africa) that remain undernourished, a little up from the 10.6% in 2015. The direct causes emanate from increasing population, income inequalities, conflicts, instability, poverty, and ineffective nutrition policies. Over the last two decades, the aquaculture industry has expanded rapidly to produce highly nutritious food at a relatively lower price (2). The FAO, in their 2020 report, revealed that, despite the decline in aquaculture production, the sector played a major role in the 179 million tonnes of the global fish produced, of which 156 million tonnes were used for human consumption (3). However, the aquaculture industry in increasing production to meet the global demands has been faced with lots of challenges including the over-exploitation of natural stocks, perishability, and food-borne diseases. Market globalisation and recurring food-safety alerts have resulted in increasing consumer awareness. In the last decade, the seafood sector has incentivised the application of relevant novel molecular techniques for the monitoring and assessment of food safety, traceability, and quality due to the perishable nature of seafood and the key role they play as a

protein source for the global population (4, 5). Thus, regarding the development of the global economy and the upgrading of living standards, humans are calling for higher quality seafood, and the problem of fish health deterioration and quality instigated by feed has become one of the central problems that cannot be disregarded in the aquaculture industry today. The increasing demand for fish products further intensifies the supply pressure of feed materials, especially fishmeal (FM) which is mainly sourced from marine capture fisheries. FM is the preferred source of protein in aquaculture because of its high protein content, balanced essential amino acids (EAA), high palatability, and digestibility (6). Among the other animal food-producing sectors, the aquaculture industry is noted as the largest consumer of FM because the industry consumes about 68–78% (7, 8). The current shortfall of marine capture fisheries has drastically increased the cost of FM as demand exceeds the supply (3). Exploring alternative conventional feeds such as plant-based protein (PBP) diets is a necessity as they are less costly and readily accessible (9, 10). However, the extensive research and documentation of most of the PBP ingredients, including soybean meal (SBM), cottonseed meal, peanut meal, corn gluten meal, wheat gluten meal, and others used in substituting FM partially or fully are gradually making them costly and not accessible enough as farmers' acceptance rates and demands keep on increasing (9–13). There is therefore an urgent need for the assessment of other PBPs that are readily available in larger quantities and can substitute FM.

Castor meal (CM) is a by-product generated after the extraction of oil from the castor plant (*Ricinus communis* L.) seed (14). Hitherto, the consistent production and safe discarding of agro-industrial wastes have been a matter of concern since their natural oxidation directly fortifies the quantum of greenhouse gases creating awful effects on human and animal health. CM has the potential of being used as a protein supplement due to its high protein (35–55.8% dry matter depending on the seed characteristics) and energy (32–49% dry matter) levels as compared to other PBP ingredients (15, 16). A comparative study of the amino acid compositions of CM revealed an almost similar pattern for all EAAs in SBM (mostly substituted PBP for FM) except lysine and sulphur amino acids (17). Castor meal-induced enteritis (CMIE) like other PBP-induced enteritis (18, 19) refers to the non-infectious subacute enteritis, and histological characteristics such as shortened mucosal folds, lamina propria and submucosa swelling, infiltration of various inflammatory cells, and reduced absorption of intestinal epithelial cells after dietary supplementation of CM. The intestine is an important organ that suffers lots of pathogenic microorganism effects and toxic damages (20, 21). Generally, the available evidence postulates that the effects of CM on intestinal health of animals are related to the imbalanced amino acids and the anti-nutritional factors (ANFs) such as ricin, ricinine, allergen, agglutinins, tannins, lectin, oxalate, and phytases contained in CM (22–25). Thus, CM is being precluded from being used as an animal feed supplement. Previously conducted studies show that CM raw material can usually be added to feed at a relatively low level since excessive addition to diets may not only reduce feed intake and growth but also affect the intestinal structure and cause enteritis in the gut of animals (16, 26–30). Although there are very limited studies on the supplementation effects of CM in fish, the studies conducted on hybrid catfish (*Hetero clarias*) exceeding 12.5% inclusion (31) and juvenile grass carp (*Ctenopharyngodon idella*) exceeding 5% inclusion (32) all show a reduction in growth performance, feed utilisation, and body composition, unless CM raw material is reduced or detoxified. However, there are lack of systematic studies on the effect of effects of CM on intestinal enteritis in fish.

In understanding the effects of aquaculture feeds on the physiology of fish, there must be a shift in the methods used to discover diet and intestinal health relations from different standpoints. The use of new omics technologies is noted to show enormous potential to aid in the understanding of the complex interplay between the nutrition and immunity of fish (33). As one of the newest "omics" sciences, metabolomics deals with the supplementation of data from transcriptomics, genomics, and proteomics to promote the understanding of biological systems (34). Metabolomic studies provide great potential insight into biomarker identification, diagnosis of diseases, and toxicological mechanism (35, 36) since they can reveal the changes and laws of endogenous metabolites in an organism after external interference (37). Currently, metabolomics has been extensively used in drug discovery and food safety fields, thus, becoming an effective tool to investigate the biochemical effects of toxic substances (38), which warrants more research.

The hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂), is one of the most sought after fish in China and the world and is currently used for intensive and super-intensive aquaculture as a result of their enormous attributes such as faster growth, efficient food utilisation, higher resistive capacity to disease infection, being able to withstand higher population density (39, 40), higher nutritional and market value (41, 42), aside from their ability to adapt to high salinity conditions (43). As a typical carnivore marine new species, the hybrid grouper's dietary protein requirement ranges between 50 and 55% (44). FM is the primary protein source for grouper, but this fish is less adapted to PBP diets due to utilisation and enteritis problems (45).

This study aimed at identifying differential metabolites linked with dietary CM by conducting untargeted metabolomics using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Notably, there is non-existence of studies correlating dietary CM supplementation with their metabolite profiling in fish. For the first time, this study demonstrates the effects of replacing FM with CM on growth, feed utilisation, immune response, digestive enzyme activities, and histological examination in hybrid grouper. Furthermore, our study's main attraction and novelty are premised on the fact that we have been able to identify candidate biomarkers of the overall CM pattern, which is a more comprehensive perspective given that nutrients do not act in isolation.

MATERIALS AND METHODS

Experimental Diets

All of the procedures were performed following the relevant policies of Animal Welfare in China. The Animal Research and the Ethics Review Board of Guangdong Ocean University approved the animal protocol used in the present study. The FM used in the current study was supplied by China National Township Enterprise, whereas the CM was purchased from the Shangdong Weifang Supply and Marketing Industrial Co. Ltd. (Shangdong, China). Three iso-nitrogenous (approximately 50% crude protein), and iso-lipidic (approximately 10% of total lipid) experimental diets were formulated to contain 0, 4.76, and 23.79% of CM by replacing 0% (FM, control), 4% (CM4), and 20% (CM20) of FM protein. The **Supplementary Table 1** illustrates the amino acid profiles of the FM and CM ingredients used in this study. The ingredient formulation composition and the proximate chemical analysis of the experimental diets are presented in **Table 1**. Crystalline amino acid (AA) methionine, lysine, threonine, and leucine were added to the diets to achieve the required amino acid for grouper feed. The balancing of AA profiles during the formulation was done in strict accordance with previously reported work (46).

The proximate chemical analysis of the experimental diets followed standardised methods of AOAC (47). Briefly, the moisture content was determined by drying feed in an oven at 105°C until a constant weight was obtained. The crude lipid was determined by the Soxhlet method using ether extraction. Also, while the crude protein (N × 6.25) was determined by the method of Kjeldahl, which involves the use of an Auto Kjeldahl System (8400-Autoanalyzer, FOSS), the ash content was analysed by muffle furnace combustion involving oven incineration at 550°C for 5 h. In preparing the diets, all the ingredients were ground and sieved (60 mesh size sieve). Afterward, they were gradually mixed with fish oil, corn oil, and soybean lecithin, and finally, purified water was added to make dough. The dough was then pelleted through a double helix extrusion machine (F-75 pelletizer, South China University of Technology, China). The pellets made (2 and 2.5 mm diameter) were air-dried, and kept in sealed Ziploc bags, which were subsequently stored at –20°C till the commencement of the feeding trial. The EAA and non-essential amino acid (NEAA) contents in the various experimental diets are shown in the **Supplementary Table 2**.

TABLE 1 | Formulation and proximate composition of experimental diets (% dry matter).

Ingredients	FM	CM4	CM20
Red fish meal[a]	50	46	30
Castor meal[b]	0	4.76	23.79
Wheat gluten meal[c]	9	9	9
Soy protein concentrate[d]	7	7	7
Wheat Flour[c]	16	16	16
Casein[e]	2	2	2
Corn oil[c]	2	2	2
Fish oil[c]	2.5	2.8	4
Soy lecithin[c]	1.5	1.5	1.5
Vitamin premix[f]	0.5	0.5	0.5
Mineral premix[g]	0.5	0.5	0.5
Choline chloride[h]	0.5	0.5	0.5
Vitamin C[a]	0.05	0.05	0.05
$Ca(H_2PO_4)_2$[h]	1	1	1
Attractant[a]	0.1	0.1	0.1
Ethoxyquin[a]	0.05	0.05	0.05
Microcrystaline cellulose[i]	6.8	5.53	0.47
Carboxymethyl cellulose[i]	0.5	0.5	0.5
Methionine[j]	0	0.04	0.19
Lysine[j]	0	0.12	0.6
Threonine[j]	0	0.02	0.08
Leucine[j]	0	0.03	0.16
Total	100	100	100
Proximate composition			
Crude protein	50.00	49.98	49.43
Crude lipid	10.10	10.57	10.03
Moisture	7.10	7.13	7.88
Ash	12.58	12.18	11.71

[a]*Red fish meal: crude protein, 70.03%, and crude lipid 8.24% (supplied by China National Township Enterprises Corporation).*
[b]*Castor meal: crude protein, 58.87% and crude lipid, 0.42% (purchased from Shandong Weifang Supply and Marketing Industrial Co., Ltd., Shandong, China).*
[c]*Wheat gluten meal: crude protein, 81.22%, and crude lipid, 0.11%; Wheat flour: crude protein, 10.52%, and crude lipid, 0.36%; corn oil; fish oil; soy lecithin; vitamin C; (purchased from Zhanjiang Haibao Feed Co. Ltd., Guangdong, China).*
[d]*Soy protein concentrate: crude protein, 67.87% and crude lipid, 0.46% (supplied by Shandong Changrun Biology Co., Ltd).*
[e]*Casein: crude protein, 92.43% and crude lipid, 0.11 (purchased from Sigma Chemical Co., Ltd., Shanghai, China).*
[f]*Vitamin premix (g kg^{-1} mixture): vitamin B_1, 17.00 g; vitamin B_2, 16.67 g; vitamin B_6, 33.33 g; vitamin B_{12}, 0.07 g; vitamin E, 66.00 g; vitamin K, 3.33 g; vitamin D, 33.33 g, retinyl acetate, 6.67 g;* D*-calcium pantothenate, 40.67 g; nicotinic acid, 67.33 g; folic acid, 4.17 g; biotin, 16.67; inositol, cellulose, 592.72 g; and 102.04 g (obtained from Zhanjiang Yuehua Feed Co. Ltd., Zhanjiang, China).*
[g]*Mineral premix (g kg^{-1} premix): $ZnSO_4.H_2O$, 32.0991 g; $FeSO_4{\cdot}7H_2O$, 18.785 g; $MgSO_4.H_2O$, 65.19927g, $CoCl_2.6H_2O$ (10%), 5.5555g; $CuSO_5.5H_2O$, 11.0721 g; KIO_3, 0.0213 g; Na_2SeO_3 (10%), 0.5555 g, KCl, 22.7411 g; zeolite powder, 843.9777 g (Obtained from Zhanjiang Yuehua Feed Co. Ltd., Zhanjiang, China).*
[h]*Purchased from Shanghai Macklin Biochemical Co. Ltd., Shanghai, China.*
[i]*Purchased from Shantou Xilong Chemical Factory, Guangdong, China.*
[j]*These amino acids were added to balance the amino acid content in the FM (control) diet. Purchased from Shanghai Doublewin Bio-Tech. Co., Ltd.*

Experimental Fish and Culture Condition

Healthy hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) were obtained from a commercial farm (Zhanjiang, Guangdong Province, China). Upon arrival, all fish were acclimatised in aerated cement pools (4.5 m [L] × 3.45 m [W] × 1.8 m [H]) for 2 weeks. Fish were hand-fed twice daily with a commercial diet during the acclimatisation period. Afterward, 360 juvenile fish of uniform size were starved for 24 h. They were then weighed and randomly distributed into 12 cylindrical fibreglass tanks (0.5 m^3) at 30 fish densities per tank. They were hand-fed during the 8-weeks experiment period to apparent satiation twice daily (08:00 and 16:30) with the three experimental diets (FM, CM4, and CM20). The experiment was conducted at an indoor facility of the Marine Biological Research Base of Guangdong Ocean University (China) for 56 days. Single-airstones provided water aeration.

During the feeding period, the water quality parameters were maintained daily by renewing 30% of the filtered seawater for the first 2 weeks, which shifted to 50% renewal to keep the temperature, pH, dissolved oxygen, and salinity within the ranges 28–30°C, 7.7–8.2, ≥6.6 mg L^{-1}, and 28–32‰, respectively (YSI 556 multiprobe system, YSI Inc., United States). The photoperiod was 12 h L: 12 h D, with the light period from 7:30 am to 7:30 pm.

Sampling of Fish

The Performance of Growth

At the termination of the experiment, all fish after 24 h starvation period were anaesthetised with eugenol (1:10,000) before harvest. The total numbers of fish left per individual tank were counted, and their mean body weights were taken. Three fish were randomly sampled to record their body and intestinal lengths, liver, and intestinal weights. Based on the obtained records, the growth performance parameters, including the survival rate (SR), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), hepatosomatic index (his), viscerosomatic index (VSI), intestinal somatic index (ISI), and intestinal length index (ILI) were calculated as described below;

$$SR, \% = 100 \times \frac{Final\ fish\ number}{Initial\ fish\ number}; \quad (1)$$

$$WGR, \% = 100 \times \frac{Final\ fish\ body\ weight\ (g) - Initial\ fish\ body\ weight\ (g)}{Initial\ fish\ body\ weight\ (g)}; \quad (2)$$

$$SGR, \% = 100 \times \frac{\ln[Final\ fish\ body\ weight\ (g)] - \ln[Initial\ fish\ body\ weight\ (g)]}{Days\ of\ experiment}; \quad (3)$$

$$FCR = \frac{Total\ dry\ feed\ intake\ (g)}{Final\ fish\ body\ weight\ (g) - Initial\ fish\ body\ weight\ (g)}; \quad (4)$$

$$HSI, \% = 100 \times \frac{Fish\ liver\ weight\ (g)}{Fish\ body\ weight\ (g)}; \quad (5)$$

$$VSI, \% = 100 \times \frac{Fish\ viscera\ weight\ (g)}{Fish\ body\ weight\ (g)}; \quad (6)$$

$$ISI, \% = 100 \times \frac{Final\ fish\ intestine\ weight\ (g)}{Final\ fish\ body\ weight\ (\mathrm{g})};\ and \quad (7)$$

$$ILI, \% = 100 \times \frac{Final\ fish\ intestine\ length\ (cm)}{Final\ fish\ body\ length\ (\mathrm{cm})}. \quad (8)$$

Distal Intestinal Sampling for Histological Examination and Metabolomics Analysis

To aid in the analysis of intestinal enzyme activity, two fish from each tank were randomly selected and their intestines removed. The intestines were cleared of any mesenteric adipose tissue and rinsed with deionised water. The cleaned distal intestines (DI) were cut, placed in Eppendorf tubes, and immediately frozen in liquid nitrogen. Samples were later stored at –80°C for subsequent enzyme activity analysis.

Eight fish from each treatment group (two fish per replicate tank) were randomly selected and their intestines removed for the scanning electron microscopy (SEM) analysis. The removal of the DI of the fish was done within 1–3 min, and the tissue mass was not more than 3 mm. The bloodstains and other tissues on the sample surface were removed by gently washing with PBS (pH 7.4) for the 30 s followed by fixing in a 2.5% glutaraldehyde (purchased from Wuhan Servicebio Technology Co. Ltd., Wuhan, China). The samples were kept for 24 h (at 4°C) until further processing. For Alcian Blue-Periodic Acid-Shiff (AB-PAS) staining analysis, a fish per replicate tank was randomly selected to remove the DI tissue samples, which were immediately placed in a 4% formaldehyde solution and stored for subsequent analysis.

For metabolomics analysis, six fish were randomly sampled from each treatment group to get the DI samples. The samples were instantaneously frozen in liquid nitrogen and stored at –80°C for subsequent analysis.

Determination of Enzyme Activities

After removing the frozen intestinal samples, they were thawed, weighed, and homogenised in 0.9% sterilised saline at 1:9 (*tissue*:*volume*) with the help of a bead homogeniser in ice for 10 min. The homogenate was later centrifuged (3000 × *g* for 15 min at 4°C). The supernatant was collected in 1.5 mL tubes and used the determination of enzyme activities. The immune and antioxidant titres of the intestine, including lysozyme (LYZ), Immunoglobulin M (IgM), complement 3 (C3) and 4 (C4), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities, were determined using the fish LYZ Elisa detection kit, fish IgM Elisa detection kit, fish C3 and C4 Elisa detection kit, fish SOD Elisa detection kit, and fish GSH-Px Elisa detection kit, respectively. The kits were all procured from the Shanghai Jianglai Biotechnology Co. Ltd. (Shanghai, China).

Following the manufacturer's protocol, to a flat-bottomed 96-well plate pre-coated with fish LYZ antibody, 50 μl each of the standard or intestinal sample (sample final dilution is fivefolds) solution was added to the appropriate wells and mixed gently without touching the good wall. Subsequently, 100 μl of horseradish Peroxide (HRP)-conjugate LYZ reagent was added to the sample and standard wells with the exception of blank wells. Plates were covered with closure plates and incubated for 60 min at 37°C. Afterward, liquid in wells was discarded, dried by gently swinging plates, and washed five times (solutions were kept still in wells for 30 s before drying by pat) with wash buffer solution (diluted 20-fold with double distilled water). After plate drying,

the 50 μl each of Chromogen Solution A and Solution B were added to all wells and incubated for 15 min at 37°C, and the reaction stopped with 50 μl of stop solution. The optical density (OD) of the LYZ level in each well was read using a Rayto RT-6100 microplate reader (Shenzhen, China) set to 450 nm wavelength. The IgM, C3, and C4 enzymes followed the same procedure as the LYZ, however, fish IgM antibody, fish C3 antibody, and fish C4 antibody were used instead of fish LYZ antibody. The trypsin (TRP) activity was determined according to the methods of Erlanger et al. (48). Lipase (LPS) and amylase (AMS) activities were measured following the methods of Gjellesvik et al. (49) and Yaghoubi et al. (50), respectively. The kits used for the TRP, LPS, and AMS activities were as well purchased from Shanghai Jianglai Biotechnology Co. Ltd. (Shanghai, China). The intestinal enzyme activities were expressed per mg protein concentration (bicinchoninic acid, BCA) (51).

Distal Intestinal Alcian Blue-Periodic Acid Schiff Section and Scanning Electron Microscopy Analysis

Samples were washed with PBS after removing from the fixation solution and post-fixed by washing tissue blocks with 0.1 M PBS (pH 7.4) three times (15 min per time). The tissue blocks were transferred into 1% osmium tetroxide (OsO_4) in 0.1 M PBS (pH 7.4) for 1–2 h at room temperature and washed again in 0.1 M PBS (pH 7.4) three times (15 min per each time). Samples were dehydrated in graded ethanol doses (30, 50, 70, 80, 90, 95, and 100% with two baths in 100% ethanol). Isoamyl acetate was used for the final dehydration stage, during which the dehydration time lasted 15 min at each step. Samples were then submitted to critical point drying (Quorum K850, Quorum Tech. Ltd., United Kingdom) and attached to metallic stubs using carbon stickers. Later, they were sputter-coated with gold using MC1000 sputter coater (Hitachi Ltd., Tokyo, Japan) for 30 s. The prepared SEM samples were examined and photographed using an MSIP-REM-htn-SU8100 scanning electron microscope (Hitachi High-Technologies Corporation Corporate Manufacturing Strategy Group, Japan).

For the AB-PAS histological examinations, the tissues removed from the 4% formalin buffer were paraffin-embedded (JB-P5, Wuhan Junjie Electronics Co., Ltd., Wuhan, China), cut into 4μm sections using a microtome (Leica Instruments, Shanghai, China, RM2016). Samples were dewaxed by Xylene I and Xylene II for 20 min each, followed by 100% ethanol I and ethanol II (Servicebio, G1049, Sinaopharm Group Chemical Reagent Co., Ltd., Shanghai, China) for 5 min each, then 75% ethanol for 5 min. Samples were later rinsed with running tap water. Subsequently, the sections were stained with Alcian blue dyes for 15 min. They were rinsed with running tap water till it was colourless and then stained with periodic acid dye for 15 min. Afterward, they were rinsed with running tap water and rinsed twice again with distilled water. They were then placed in Schiff's reagent and stained again at room temperature for 30 min in the dark followed by rinsing for 5 min. The sections were dehydrated by 100% ethanol I (5 min), 100% ethanol II (5 min), 100% ethanol III (5 min), Xylene I (5 min), Xylene II (5 min), and later sealed with the neutral gum. The images were then captured as previously described (52, 53) with Olympus model BX51 (Serial number: 9K18395, Tokyo, Japan). The villi height (VH), villi width (VW), crypt depth (CD), lamina propria (LP), and intestinal epithelial muscle thickness (MT) were measured using the software Image-Pro Plus 6.3 (Media Cybernetics, Inc., Rockville, MD, United States). The goblet cell (GC) counts were measured using cellSens Standard 1.8 software.

Analysis of Metabolomics

Metabolites Extraction and Ultra-Performance Liquid Chromatography-Mass Spectrometry Analysis

The individual DI samples (100 mg) were ground with liquid nitrogen, and their homogenate was re-suspended using prechilled 80 and 0.1% formic acid by the good vortex. Subsequently, the samples were incubated on ice (5 min), centrifuged (15,000 × *g* for 20 min at 4°C), and aliquots of supernatant samples were diluted to a final concentration containing 53% methanol by LC-MS grade water. The samples were consequently transferred into fresh Eppendorf tubes (with 0.22 μm), centrifuged (15,000 × *g* for 20 min at 4°C), and finally, the filtrate was injected into the LC-MS/MS system analysis (54). UPLC-MS/MS analyses were performed using a Vanquish UPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive^{TM} HF-X mass spectrometer (Thermo Fisher, Germany) at Novogene Co., Ltd. (Beijing, China). Samples were injected onto a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) using a 17 min linear gradient at 0.2 ml/min flow rate. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 12 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 17 min. Q Exactive^{TM} HF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 40 arb, and aux gas flow rate of 10 arb.

Quality Control

As metabolomics is easily disturbed by external factors and changes rapidly, data quality control (QC), which can detect anomalies in time, is necessary to obtain stable and accurate metabolome results. In controlling the quality of the experiment conducted while sample processing, QC samples were prepared. The QC samples are the equal mixing samples of treatment samples used to balance the chromatographic-mass spectrometry system, monitor the LC-MS system performance state, and evaluate the system's stability during the whole experiment process. Based on the relative quantitative value of metabolites, the Pearson correlation coefficient between QC samples was calculated. The higher the correlation of QC samples (the closer to 1), the better the stability of the whole method. Again, the distribution of QC samples in the PCA analysis diagram can be termed as having better stability of the whole method and that there is a higher data quality when QC samples are reflected as being smaller and are clustered together (55). At the same time, blank samples are set up to aid in the removal of background ions.

Processing of Data and Metabolite Identification

The UPLC-MS/MS raw data generated were processed using Compound Discoverer 3.1 (CD 3.1, Thermo Fisher) to perform a peak alignment, peak picking, and quantitation of each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100,000. Subsequently, peak intensities were normalised to the total spectral intensity. The normalised data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. The peaks were then matched with the *mz*Cloud[1], mzVault, ChemSpider[2], and MassList databases to obtain accurate qualitative and relative quantitative results. Statistical analyses were then performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using the area normalisation method. The metabolites were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[3], Human Metabolome Database (HMDB)[4] and LIPID MAPS® database[5]. Later on, the principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) was performed using a flexible and comprehensive software for metabolomics processing known as meta X software. An application of univariate analysis (*t*-test) was made to calculate the statistical significance (*P*-value). The Variable Importance in the Projection (VIP) value reflects the contribution of each variable to the model. Larger VIP values were considered as the major potential biomarkers for differentiating the control and the experimental groups. The metabolites with VIP $>$ 1 and $P < 0.05$ and fold change ≥ 2 or FC ≤ 0.5 were considered as differential metabolites (56).

Differential Metabolites' Filtering

The Venn plot of differential metabolites between the DI tissues was made to search for the co-contained differential metabolite, that is, the overlapping part in the Venn plot. The Log2FC values of the differential metabolites in the coinciding part were calculated, and the co-contained differential metabolites changed with contrary trends and were considered to be significantly affected after ingestion by fish. The left of the co-contained metabolites with the same trend and differential times of Log2FC < 2 were considered no-significantly changed and removed in the whole differential metabolites of the DI contents. Volcano plots were used to filter metabolites of interest-based on log2FC and –log10 (*p*-value) of metabolites. For heatmap clustering, the data were normalised using *z*-Scores of the intensity areas of differential metabolites and were plotted by Pheatmap package in R language. The correlation between differential metabolites was analysed by cor.mtest function in the R-package (method = Pearson). The statistically significant correlation between differential metabolites was calculated by cor.mtest in the R-package where the threshold level of significant correlation was $P < 0.05$. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was performed. When the ratio was satisfied by x/n $>$ y/N, metabolic pathways were considered enriched, and when the *P*-value of the metabolic pathway was < 0.05, metabolic pathways were regarded as statistically significantly enriched.

Statistical Analysis

The statistical analyses were done using the Statistical Package for Social Sciences (SPSS) for Windows software (IBM SPSS version 20, Inc., 2010, Chicago, IL, United States). To examine the differences between groups, a one-way analysis of variance (ANOVA) was conducted when the data variance was homogenous. Differences were considered statistically significant at $P < 0.05$ between treatment groups using Tukey's Honest Significant Difference (HSD) tests. Receiver operator characteristic (ROC) curve analysis was conducted for metabolites to determine the Area Under Curve (AUC), which compares the predictive ability of metabolites.

RESULTS

Growth Performance

As illustrated in **Table 2**, the CM4 group used in replacing the FM group experienced significantly high ($P < 0.05$) final body weight (FBW), WGR, SGR, HSI, VSI, ISI, and ILI. In contrast, the FCR was significantly higher ($P < 0.05$) in the CM20 group than in the CM4 and FM groups. No significant difference ($P > 0.05$) was observed in the PER and SR between all groups.

Immune, Antioxidant, and Digestive Enzyme Indices

The results of the DI immune and digestive enzyme indices are shown in **Table 2**. The C3 activity was significantly higher in the CM4 group than in the other groups. On the other hand, CM's replacement levels in fish diets significantly increased ($P < 0.05$) the C4, IgM, LYZ, SOD, and GSH-Px immune and antioxidant enzymes concentrations, with the CM20 groups witnessing the highest value. No significant differences ($P > 0.05$) were observed among all groups concerning the TRP and AMS digestive enzymes. Nonetheless, a significantly elevated ($P < 0.05$) LPS activity was observed in the CM4 and CM20 groups than in the FM group, with the CM4 revealing the highest activity.

Distal Histological Examination

Castor-meal substitution in the diet caused a significant change in the histological analysis of the DI. The results obtained regarding the DI histology examined by AB-PAS staining are presented in **Figure 1** and **Table 3**. It was revealed after the 56-day feeding trial that the 20% CM replacement level led to a significant reduction ($P < 0.05$) in the villi height, villi width, crypt depth, and goblet cells. On the other hand, a significant increase ($P < 0.05$) in

[1]https://www.mzcloud.org/

[2]http://www.chemspider.com

[3]https://www.genome.jp/kegg/pathway.html

[4]https://hmdb.ca/metabolites

[5]http://www.lipidmaps.org/

TABLE 2 | Effects of different levels of castor meal substitute for fish meal protein on the growth performance, immune and digestive enzyme activities of juvenile hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂).

Parameters	FM	CM4	CM20
Growth			
IBW (g)	9.12 ± 0.01	9.13 ± 0.00	9.13 ± 0.00
FBW (g)	80.44 ± 1.31^{b}	85.07 ± 0.87^{b}	56.21 ± 3.10^{a}
WGR (%)	781.99 ± 14.67^{b}	832.02 ± 9.53^{b}	515.59 ± 33.76^{a}
SGR (% day^{-1})	3.89 ± 0.03^{b}	3.92 ± 0.07^{b}	3.24 ± 0.10^{a}
FCR	0.78 ± 0.03^{a}	0.74 ± 0.01^{a}	0.93 ± 0.02^{b}
PER	2.90 ± 0.10	2.86 ± 0.10	2.59 ± 0.14
SR (%)	98.33 ± 1.68	95.00 ± 2.16	95.57 ± 4.43
Morphological			
HSI (%)	2.12 ± 0.16^{ab}	2.42 ± 0.13^{b}	1.71 ± 0.09^{a}
VSI (%)	9.06 ± 0.15^{b}	9.55 ± 0.16^{b}	7.63 ± 0.49^{a}
ISI (%)	0.63 ± 0.02^{a}	0.78 ± 0.03^{b}	0.59 ± 0.04^{a}
ILI (%)	144.44 ± 2.45^{b}	147.45 ± 2.62^{b}	126.48 ± 3.06^{a}
Immune			
C3 (μg mgprot^{-1})	103.67 ± 2.83^{a}	148.72 ± 14.81^{b}	120.08 ± 8.27^{ab}
C4 (μg mgprot^{-1})	222.22 ± 5.05^{a}	265.04 ± 13.54^{ab}	292.14 ± 12.16^{b}
IgM (μg mgprot^{-1})	94.47 ± 1.11^{a}	101.27 ± 1.14^{b}	106.34 ± 1.29^{c}
LYZ (U gprot^{-1})	6.18 ± 0.50^{a}	13.71 ± 0.93^{b}	17.90 ± 1.29^{c}
SOD (ng mg.prot^{-1})	9.61 ± 0.34^{a}	13.37 ± 1.25^{b}	17.65 ± 0.94^{c}
GSH-Px (ng mg.prot^{-1})	27.21 ± 1.78^{a}	40.58 ± 1.98^{b}	47.84 ± 2.38^{b}
Digestive enzymes			
TRP (U mgprot^{-1})	1868.71 ± 110.84	2317.29 ± 171.15	2156.58 ± 107.61
LPS (U mgprot^{-1}	120.56 ± 10.75^{a}	474.57 ± 38.91^{c}	279.67 ± 25.23^{b}
AMS (U mgprot^{-1})	299.25 ± 13.47	382.55 ± 35.30	380.11 ± 18.10

Data are mean values of four replicates ± SE. The means in the same line with no superscript letters do not differ significantly among groups (P > 0:05) based on Tukey's HSD test. Where: IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; SR, survival rate; HSI, hepatosomatic index; VSI, viscerosomatic index; ISI, intestinal somatic index; ILI, intestinal length index; C3, complement 3; C4, complement 4; IgM, Immunoglobulin M; LYZ, lysozyme; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; TRP, trypsin; LPS, lipase; and AMS, amylase; FM, fish meal (control group); CM4, 4% CM protein replacement to FM protein; CM20, 20% CM protein replacement to FM protein.

muscle thickness and lamina propria width was observed in the CM20 group in comparison to the FM and CM4 groups (**Figure 1** and **Table 3**).

As illustrated in **Figure 2**, the SEM results show significant change among groups. Compared with the other groups, the highest CM replacement group (CM20) was observed to have few and weak mucosal villi density, alongside its irregular villi orientation (**Figures 2G–I**). Again, there was villi atrophy (flattening or blunting) which in a way led to some of the villi disappearing in contrast to what was observed in the CM4 (**Figures 2D–F**) and the control group (FM; **Figures 2A–C**).

Metabolic Profiling by Ultra-Performance Liquid Chromatography-Mass Spectrometry Analysis

Figure 3 and **Supplementary Figure 1** show DI content's representative spectra. It must be noted that the samples of the DI tissue were analysed with UPLC-MS in positive and negative modes. **Figure 4A** illustrates the score plots of the PCA in the two modes. The QC samples were observed in the centre and were clustered tightly, depicting the better stability of the whole detection process and the higher quality of the data; thus, its sample correlation is shown in **Figure 4B**.

In characterising the profiles of the metabolites, the Partial Least Squares Discrimination Analysis (PLS-DA) was used, and **Figure 5** shows the results of the DI contents in the positive (**Figure 5A**) and negative modes (**Figure 5B**). There were clear separations between all groups, and all samples in the respective groups were fundamentally observed to be in the 95% confidence ellipses (**Supplementary Figure 2**). Consequently, in preventing model over-fitting, permutation tests were conducted in positive and negative modes (**Figure 6**). The permutation test parameters R2Y and Q2Y in the DI samples' positive modes were 0.97 and 0.59 between groups of FM and CM4, and 0.98 and 0.85 between groups of FM and CM20. In the negative modes, the permutation test parameters R2Y and Q2Y were 0.96 and 0.49 between groups of FM and CM4, and 0.98 and 0.82 between groups of FM and CM4 of which they were $\geq$ 0.5. To judge the quality of the model, it is sorted and verified to check whether the model is over-fitting. Model over-fitting reflects the accuracy of the model construction. If the model is not over-fitted, the model can describe the sample well and be used as the premise of searching for the model biomarker group. But if the model is over-fitted, then the model is not suitable for describing the sample and cannot be used for later data analysis. The modelling and prediction are conducted after randomly shuffling the grouping markers of each sample where each of the modellings corresponds to a group of values R2 and Q2 and their regression lines are obtained according to the values of Q2 and R2 after 200 shuffling and modelling. When the R2 data is larger than the Q2 data and the intercept between the Q2 regression line and Y-axis is less than '0,' the model is described as not over-fitted. Thus, the results of the red line (Q2) and the blue line (R2) on the left indicate a low risk of over-fitting the models (**Figure 6**).

Differential Metabolites' Filtering

Venn diagram is used to display multiple comparative combinations of differential metabolites, which can intuitively compare common and unique differential metabolites between different groups to display the relationship between multiple groups of differential metabolites. From the analysis of the Venn plots, there were 567 differential metabolites in the DI tissue contents, of which 34 were co-contained in positive modes between the various groups. Nonetheless, in the negative modes, 368 differential metabolites were observed in the DI tissue contents, of which 17 were co-contained metabolites between the FM, CM4, and CM20 groups (**Figure 7**). The Venn plots revealed that most of the differential metabolites observed in the DI tissues for hybrid grouper occurred due to the physiological response of the intestine to diet metabolism. The **Supplementary Table 3** illustrates the co-contained differential metabolites in the DI intestine in positive and negative modes.

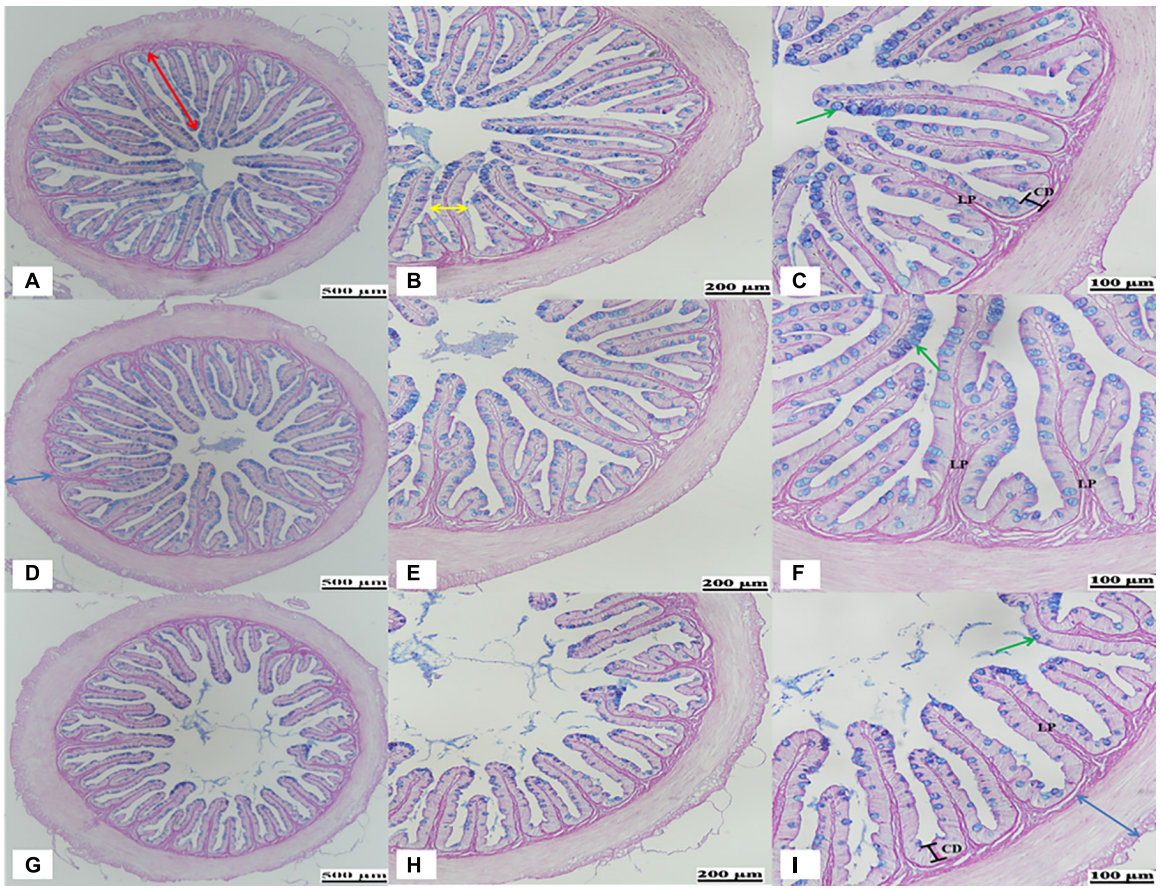


FIGURE 1 | Representative histological evaluation of the distal intestine in hybrid grouper fed the FM **(A–C)**, CM4 **(D–F)**, and CM20 **(G–I)** diets based on Alcian Blue-Periodic Acid-Schiff (AB-PAS) staining. **(A,D,E)** Shows a decreasing villi height (red arrows) and increased mucosal folds (blue arrows) as replacement levels increases. **(B,C,E,F,H,I)** Shows the changes in the villi width (yellow arrow), lamina propria (LP), crypt depth (CD), and goblet cells (green arrows). FM, fish meal (control group); CM4, 4% castor meal (CM) protein replacement to FM protein; CM20, 20% CM protein replacement to FM protein.

TABLE 3 | The distal intestinal tissue morphology of hybrid grouper fed with experiment diets.

Treatment	Villi height (μm)	Villi width (μm)	Muscle thickness (μm)	Crypt depth (μm)	Lamina propria width (μm)	Goblet cells/μm villi height
FM	797.55 ± 9.59^{c}	128.64 ± 3.76^{b}	191.79 ± 1.33^{a}	75.08 ± 1.81^{b}	14.02 ± 0.69^{a}	0.06 ± 0.00^{b}
CM4	730.56 ± 7.94^{b}	112.40 ± 4.20^{a}	221.41 ± 4.26^{b}	80.69 ± 2.64^{b}	15.06 ± 0.97^{a}	0.07 ± 0.00^{b}
CM20	481.55 ± 6.91^{a}	97.51 ± 3.52^{a}	309.04 ± 5.70^{c}	64.91 ± 1.66^{a}	28.35 ± 1.14^{b}	0.04 ± 0.00^{a}

Data are mean values ± SE. The means in the same column with no superscript letters do not differ significantly among groups (P > 0:05) based on Tukey's HSD test. Where: FM, fish meal (control group); CM4, 4% castor meal (CM) protein replacement to FM protein; CM20, 20% CM protein replacement to FM protein.

Analysis of Differential Metabolite

As illustrated in **Figures 7B1,B2**, the volcano plot in the positive mode showed significantly that 127 metabolites were up-regulated and 126 metabolites were down-regulated between the FM and CM4 groups (**Supplementary Table 4**). On the other hand, 213 metabolites were up-regulated and 199 metabolites down-regulated between the FM and CM20 groups (**Supplementary Table 5**). Again in **Figures 7B3,B4**, the volcano plot in the negative mode showed significantly that 83 metabolites were up-regulated and 106 metabolites down-regulated between the FM and CM4 groups (**Supplementary Table 6**). However, 128 metabolites were up-regulated, and 126 metabolites were down-regulated between the FM and CM20 groups in the negative mode (**Supplementary Table 7**). Hierarchical Clustering Analysis (HCA) was conducted for all the differential metabolites obtained between comparison pairs (57). In the end, the relative quantitative values of differential metabolites were normalised and clustered as presented in **Figure 8A** (positive mode) and **Figure 8B** (negative mode) with its details provided in **Supplementary Figure 3**.

The 567 differential metabolites in the positive modes were enriched into 52 pathways (**Supplementary Table 8**) between the FM and CM4 groups of which the top 10 pathways

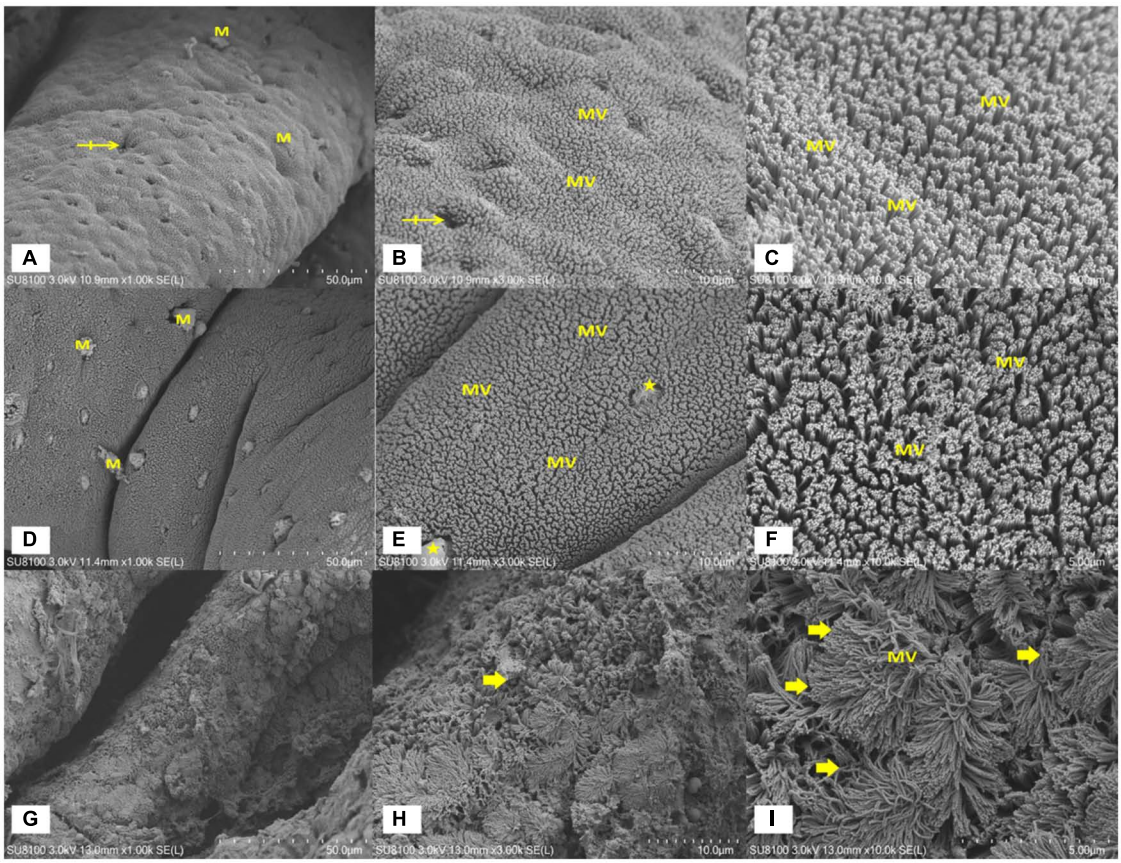


FIGURE 2 | A scanning electron microscopy (SEM) of the distal intestinal mucosal surface of juvenile hybrid grouper fed with FM **(A–C)**, CM4 **(D–F)**, and CM20 **(G–I)**. Bar markers represent 50 μm **(A,D,G)**, 10 μm **(B,E,H)**, and 5 μm **(C,F,I)**. **(A–C)** Shows more and pebbled mucosal surface or villi (MV) density which contains orifices of several goblet cells that may be seen protruding mucus (M) or not (crossed arrow); **(D–F)** shows more and pebbled mucosal surface or villi (MV) density which contains orifices of several goblet cells that may be seen protruding mucus (M) in addition to very few noticeable villi detachments of from the epithelial layer (stars); **(G–I)** shows few and weak mucosal surface or villi (MV) density, irregular orientation of villi, villi atrophy or blunting (flattening) causing some villi to disappear (arrows). Abbreviations are as defined in **Figure 1**.

were "Purine metabolism," "cGMP-PKG signalling pathways," "Starch and sucrose metabolism," "Antifolate resistance," "Vascular smooth muscle contraction," "Olfactory transduction," "Phototransduction," "Parkinson's disease," "Alcoholism," and "Metabolic pathways." On the other hand, there were 64 pathways enriched in positive modes between the FM and CM20 groups (**Supplementary Table 9**), with the top 10 being "Galactose metabolism," "beta-Alanine metabolism," "Vitamin digestion and absorption," "Folate biosynthesis," "Fc epsilon RI signalling pathway," "Dopaminergic synapse," "Parkinson's disease," "Alcoholism," "Tryptophan metabolism," and "Caffeine metabolism." The KEGG-enrichment scatterplot showed that there was only one distinct pathway, "Purine metabolism," which was significantly enriched ($P < 0.05$) when the FM to CM4 were compared together. In contrast, two distinct pathways, "Galactose metabolism" and "beta-Alanine metabolism," were significantly enriched ($P < 0.05$) when comparing the FM to CM20 (**Figure 8C**) in the positive modes.

However, in the negative modes, the 368 differential metabolites were enriched into 60 pathways (**Supplementary Table 10**) between the FM and CM4 groups of which the top 10 pathways were "Taste transduction," "Pyrimidine metabolism," "Metabolic pathways," "Pentose and glucuronate interconversions," "Inositol phosphate metabolism," "FoxO signalling pathway," "AMPK signalling pathway," "Thyroid hormone synthesis," "Renin secretion," and "Cortisol synthesis and secretion." On the other hand, there were 64 pathways enriched in negative modes between the FM and CM20 groups (**Supplementary Table 11**), with the top 10 beings "Galactose metabolism," "Starch and sucrose metabolism," "Fc epsilon RI signalling pathway," "Insulin resistance," "Central carbon metabolism in cancer," "Fructose and mannose metabolism," "Valine, leucine, and isoleucine degradation," "Neomycin, kanamycin, and gentamicin biosynthesis," "Inflammatory mediator regulation of TRP channels," and "Thyroid hormone synthesis." The KEGG-enrichment scatterplot disclosed that there was only one distinguishing pathway, "Taste transduction," which was significantly enriched ($P < 0.05$) when the FM to CM4 were compared together, whereas five distinct pathways, "Starch and sucrose metabolism," "Insulin

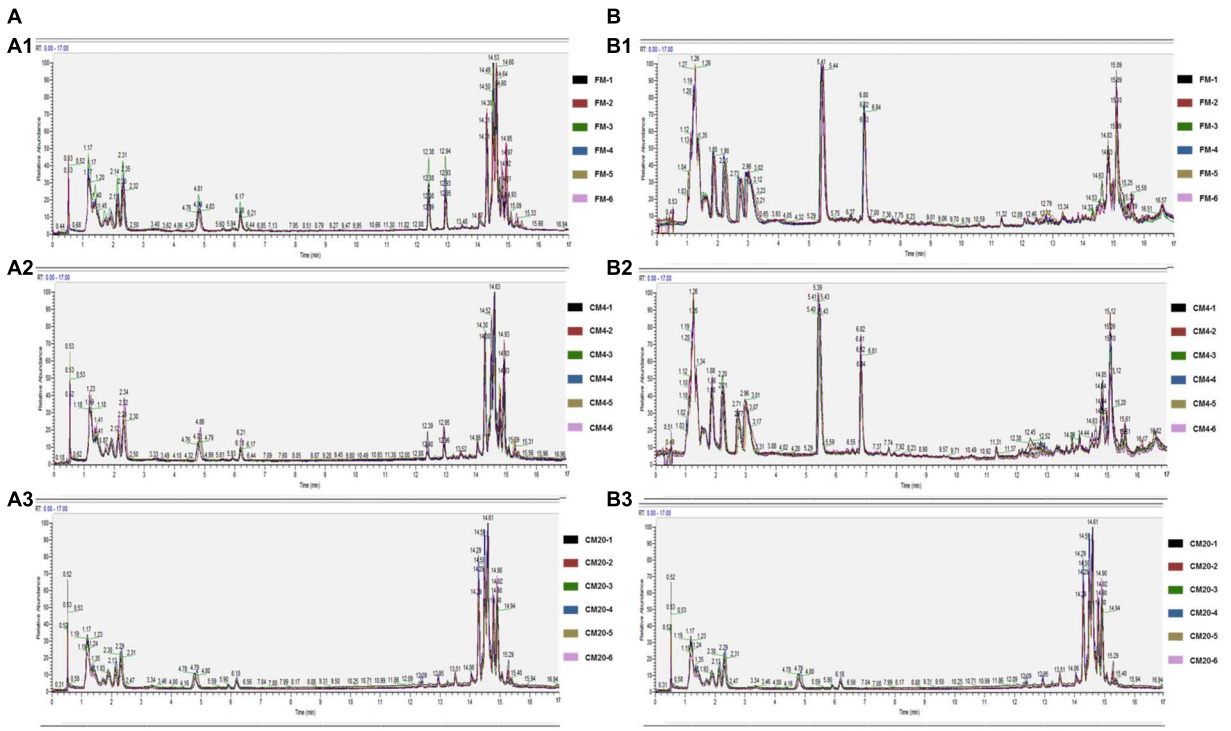


FIGURE 3 | The representative UPLC-MC spectra of the distal intestine in positive **(A)** and negative **(B)** modes. **(A1,B1)** Respectively represent the individual sample repeats of FM in positive and negative modes, **(A2,B2)** respectively represent the individual sample repeats of CM4 in positive and negative modes, and **(A3,B3)** respectively represent the individual sample repeats of CM20 in positive and negative modes. Abbreviations are as defined in **Figure 1**.

resistance," "Galactose metabolism," "Fc epsilon RI signalling pathway," and "Central carbon metabolism in cancer," were significantly enriched ($P < 0.05$) comparing FM to CM20 (**Figure 8C**) in the negative modes.

Based on the VIP > 1 score, the absolute value of log2(fold change) > 1, and $P < 0.05$, the 10 most influential metabolites differentiating the CM4 from the FM group in the positive mode (**Supplementary Table 12**) were: "Ricinine ($C_8H_8N_2O_2$)," "10-Undecenoic acid ($C_{11}H_{20}O_2$)," "Jasmone ($C_{11}H_{16}O$)," "Apocynin ($C_9H_{10}O_3$)," "α-Pinene-2-oxide ($C_{10}H_{16}O$)," "2-(Formylamino)Benzoic Acid ($C_8H_7NO_3$)," "4-Pyridoxic acid ($C_8H_9NO_4$)," "DL-α-Aminocaprylic acid ($C_8H_{17}NO_2$)," "Linalool ($C_{10}H_{18}O$)," and "Butyryl fentanyl-d5 ($C_{23}H_{25}[2]H_5N_2O$)." Nevertheless, those differentiating the CM20 from the FM group (**Supplementary Table 13**) were: "Ricinine ($C_8H_8N_2O_2$)," "Jasmone ($C_{11}H_{16}O$)," "α-Pinene-2-oxide ($C_{10}H_{16}O$)," "Levodopa ($C_9H_{11}NO_4$)," "10-Undecenoic acid ($C_{11}H_{20}O_2$)," "Styrene (C_8H_8)," "Carvone ($C_{10}H_{14}O$)," "Butyryl fentanyl-d5 ($C_{23}H_{25}[2]H_5N_2O$)," "MAG (18:4) ($C_{21}H_{34}O_4$)," and "DL-α-Aminocaprylic acid ($C_8H_{17}NO_2$)" in the positive mode. In the negative mode, the 10 most influential metabolites differentiating the CM4 from the FM group (**Supplementary Table 14**) were: "12-Hydroxydodecanoic acid ($C_{12}H_{24}O_3$)," "Dl-3-Hydroxy-kynurenine ($C_{10}H_{12}N_2O_4$)," "Naringenin ($C_{15}H_{12}O_5$)," "3-[2-(β-D-Glucopyranosyloxy)-4-methoxyphenyl]propanoic acid ($C_{16}H_{22}O_9$)," "2′-Deoxyuridine-5-monophosphate ($C_9H_{13}N_2O_8P$)," "Citrulline ($C_6H_{13}N_3O_3$)," "*N6*-Methyladenine ($C_6H_7N_5$)," "Sucrose ($C_{12}H_{22}O_{11}$)," "2-Hydroxymyristic acid ($C_{14}H_{28}O_3$)," and "10-Hydroxydecanoic acid ($C_{10}H_{20}O_3$)." However, those differentiating the CM20 from the FM group (**Supplementary Table 15**) were: "Naringenin ($C_{15}H_{12}O_5$)," "Dl-3-Hydroxy-kynurenine ($C_{10}H_{12}N_2O_4$)," "12-Hydroxydodecanoic acid ($C_{12}H_{24}O_3$)," "Sucrose ($C_{12}H_{22}O_{11}$)," "2-Hydroxymyristic acid ($C_{14}H_{28}O_3$)," "Taurocholic acid sodium salt hydrate ($C_{26}H_{45}NNaO_7S$)," "Stachyose ($C_{24}H_{42}O_{21}$)," "10-Hydroxydecanoic acid ($C_{10}H_{20}O_3$)," "α,α-Trehalose ($C_{12}H_{22}O_{11}$)," and "L-beta-Imidazolelactic acid ($C_6H_8N_2O_3$)" in the negative mode. Given this, a total of 20 metabolites were observed as the key metabolites in the FM and CM4 group (lower replacement level), and 20 key metabolites were also observed in the FM and CM20 group (higher replacement level). However, in analysing the key metabolites, 12 of them were observed in both replacement groups. As a result, a total of 28 metabolites were identified and selected as the key potential biomarkers.

Subsequently, *z*-score plots of the metabolites were analysed to define the potential biomarkers further (**Figure 9**). **Figure 10** and **Supplementary Tables 16, 17** illustrate the intensities of the potential biomarkers identified in the positive and negative modes. Compared with the FM group, increasing the replacement levels of CM caused a significant increasing trend ($P < 0.05$) in the intensities of Ricinine, 10-Undecenoic acid, Jasmone, Apocynin, α-Pinene-2-oxide, 2-(Formylamino)Benzoic Acid, 4-Pyridoxic acid, DL-α-Aminocaprylic acid, Linalool,

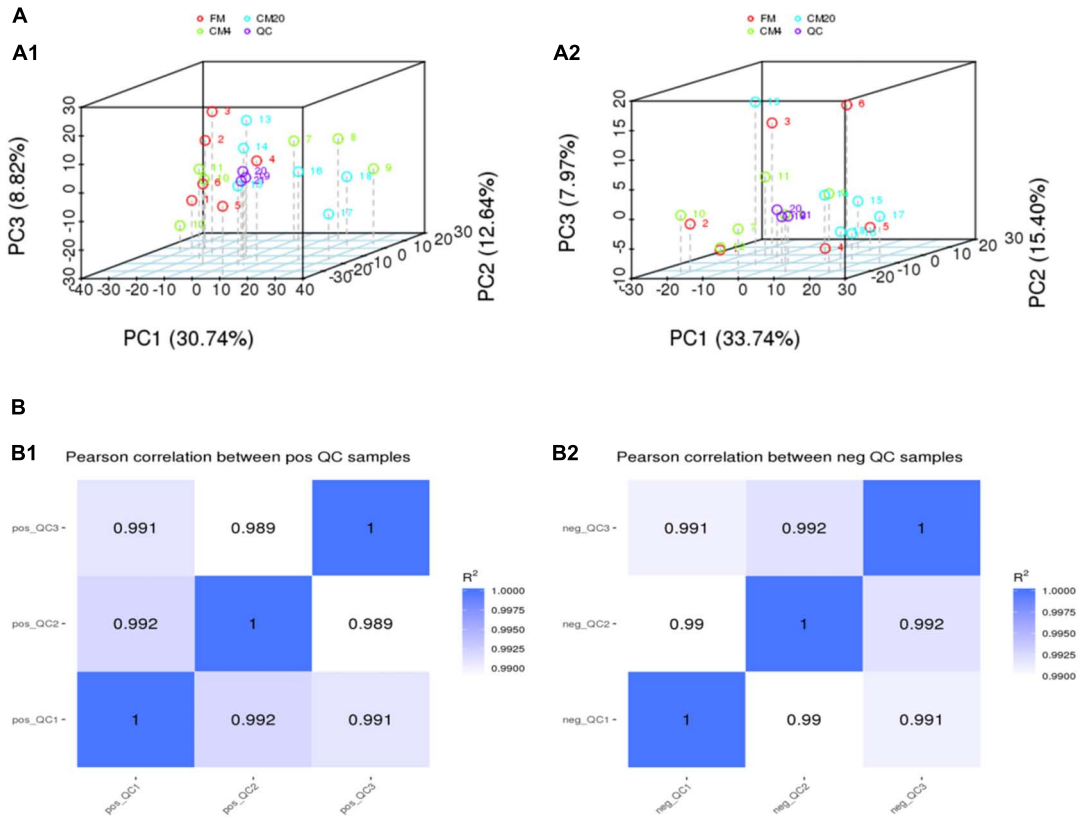


FIGURE 4 | (A) The results of the total sample PCA score plots from UPLC-MS spectra of the distal intestinal tissue in the positive **(A1)** and negative **(A2)** modes. Abscissa PC1 and ordinate PC2 represent the scores of the first and second principal components. Scattered dots in different colours represent samples from different experimental groups as illustrated in **Figure 3**. QC represents the quality control group. **(B)** The QC samples of the distal intestinal contents' correlation in the positive **(B1)** and negative **(B2)** modes. The abscissa is log10 (Peak. Area + 1), the ordinate (Peak. Area + 1), and the R^2 is the square of the Pearson correlation coefficient. $n = 6$.

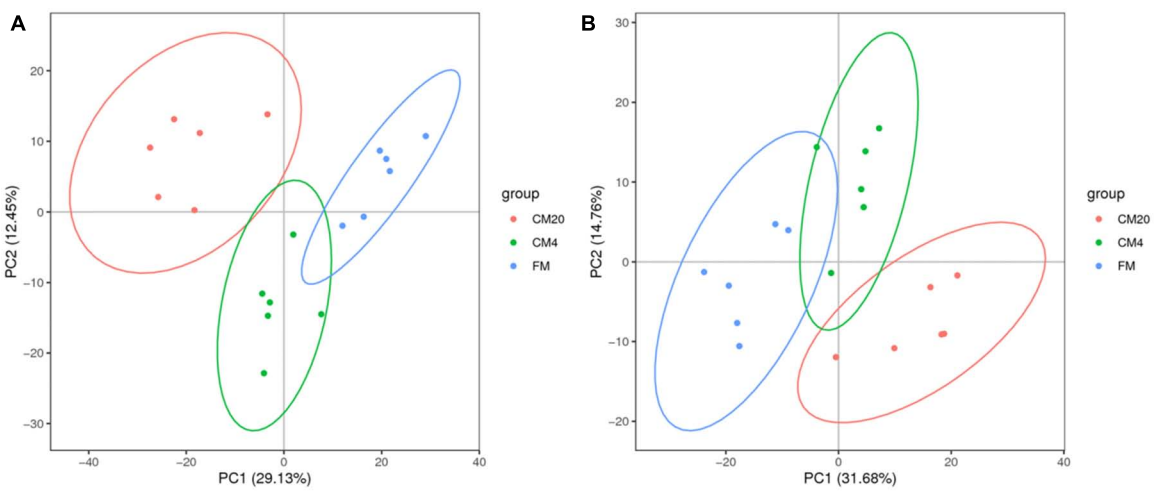


FIGURE 5 | The results of the total PLS-DA score plots from the UPLC-MS spectra of the distal intestinal contents in positive **(A)** and negative **(B)** modes. Blue dots represent the FM group (fishmeal group-control); green dots represent the CM4 groups [4% CM (castor meal) protein replacement to FM protein]; red dots represent CM20 groups (20% CM protein replacement to FM protein). $n = 6$.

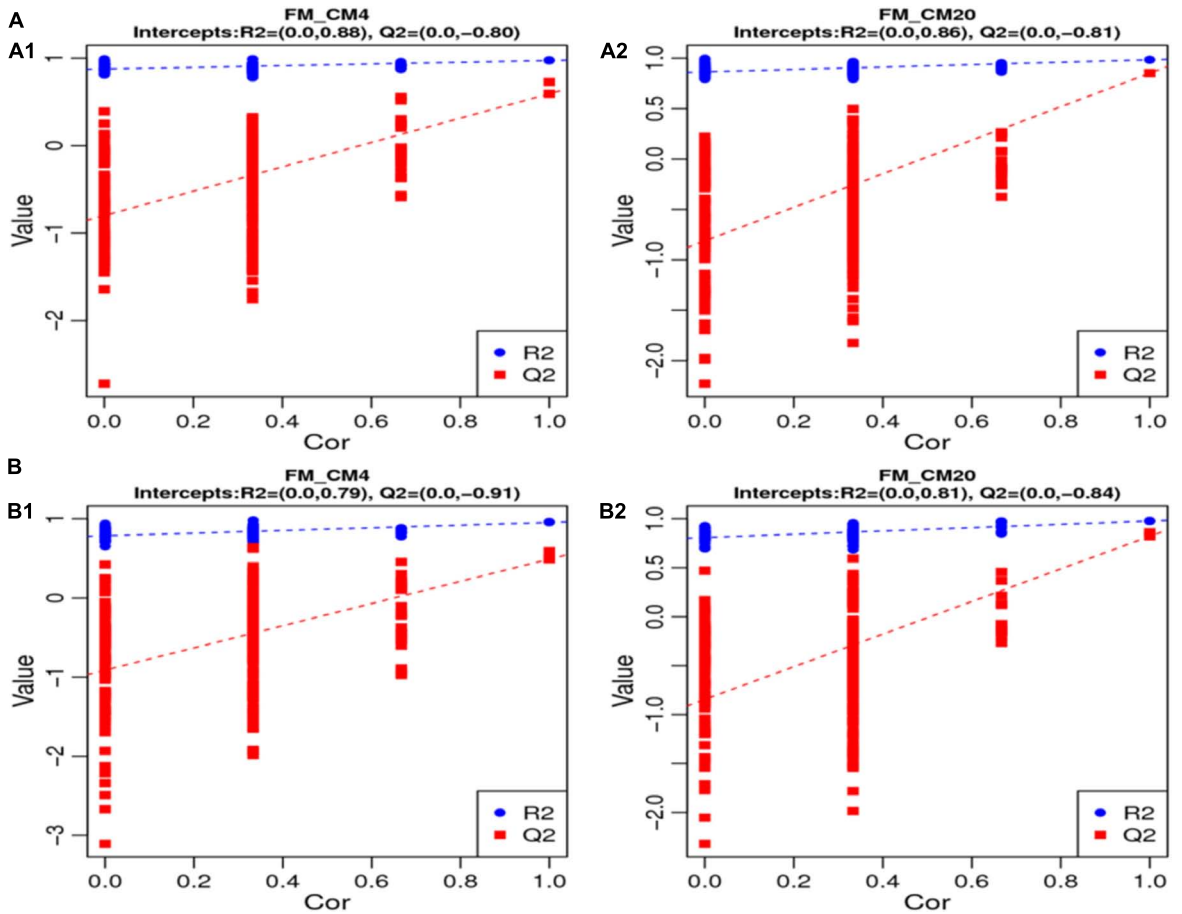


FIGURE 6 | The results of the permutation test of the PLS-DA models of the distal intestinal contents between FM, CM4, and CM20 groups in the positive **(A1,A2)** and negative **(B1,B2)** modes. The R2Y value represents the model's goodness of fit, whereas the Q2 value represents the predictability of the models. Abbreviations are as defined in **Figure 1**. $n = 6$.

Butyryl fentanyl-d5, Levodopa, Styrene, MAG (18:4), 12-Hydroxydodecanoic acid, Dl-3-Hydroxy-kynurenine, 3-[2-(β-D-Glucopyranosyloxy)-4-methoxyphenyl]propanoic acid, 2′-Deoxyuridine-5-monophosphate, Citrulline, *N6*-Methyladenine, 2-Hydroxymyristic acid, and 10-Hydroxydecanoic acid, contrast to as observed in the intensities of Sucrose, Taurocholic acid sodium salt hydrate, Stachyose, and L-beta-Imidazolelactic acid. Nonetheless, no significant differences ($P > 0.05$) were observed between the FM and CM4 groups concerning the intensities of 2-(Formylamino)Benzoic Acid, DL-α-Aminocaprylic acid, Linalool, Levodopa, 12-Hydroxydodecanoic acid, 3-[2-(β-D-Glucopyranosyloxy)-4-methoxyphenyl]propanoic acid, and 10-Hydroxydecanoic acid. Again, for the intensities of Naringenin, no significant difference ($P > 0.05$) was observed among all groups. Interestingly, there were zero intensities of Carvone, Stachyose, and α,α-Trehalose biomarkers observed in the CM4 group. **Supplementary Table 18** shows the classification of the 28 differential metabolites identified in the DI tissues of FM vs. CM4 and CM20 groups (HMDB).

The area under the ROC curve is recognised as the area under curve (AUC), which is used to assess the sensitivity and specificity of biomarkers for predicting the occurrence of events (58). The sensitivity and specificity of each metabolite are determined by the optimal threshold of the ROC Curve. Thus, (i) when AUC = 0.5, the biomarker has no predictive value for predicting the occurrence of events, and (ii) when AUC value is > 0.5, the closer it is to 1 depicts the higher the accuracy of prediction. The prediction accuracy is generally low when the AUC value is between 0.5 and 0.7. The prediction accuracy is certain when the AUC value is between 0.7 and 0.9, and the prediction accuracy becomes high when the AUC value is above 0.9. The results of the ROC analysis, as shown in **Figure 11**, resulted in the AUC of the metabolites exceeding 0.8 at a 95% CI, depicting that there were good predictive abilities for the screened potential metabolite biomarker. The correlation analysis conducted revealed Sucrose, Taurocholic acid sodium salt hydrate, Stachyose, and L-beta-Imidazolelactic acid to have a negative correlation with the other potential metabolite biomarkers, whereas the other potential metabolite biomarkers actively correlated positively with each other (**Figure 12**).

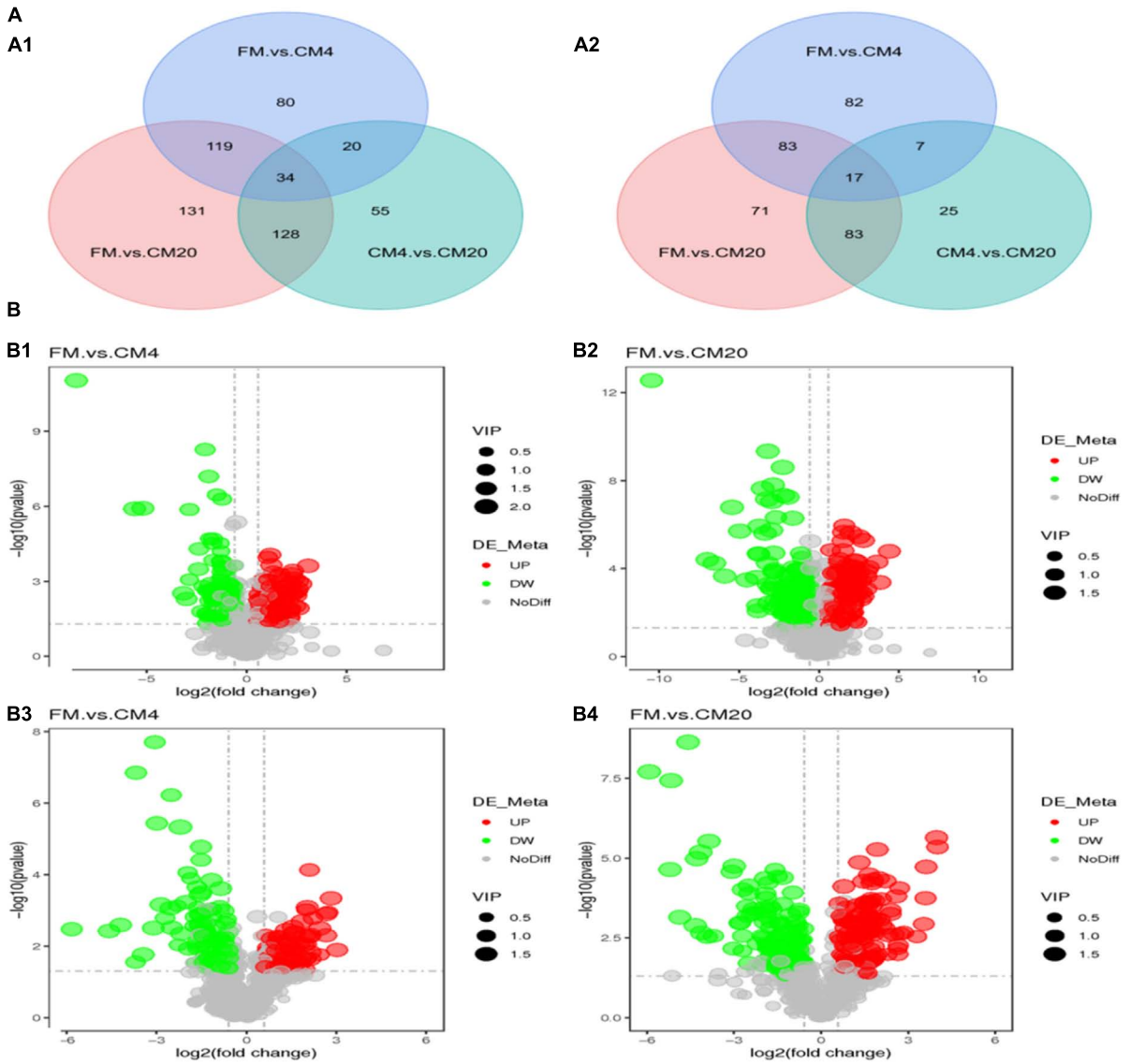


FIGURE 7 | (A) The Venn plot displays the differential metabolites of distal intestinal contents of hybrid grouper between experimental groups in positive **(A1)** and negative **(A2)** modes. **(B)** Volcano plots of *p*-values between the FM, CM4, and CM20 groups in the positive **(B1,B2)** and negative **(B3,B4)** modes. The horizontal coordinate represents the change of expression of multiple metabolites in different groups (log2FC), and the vertical coordinate represents the significance level of difference [–log10(*p*-value)]. Each dot in the figure represents a metabolite, and the size of the dot represents the VIP value. The significantly up-regulated metabolites are represented by red dots, the significantly down-regulated metabolites are represented by green dots, and gray dots represent no significantly different metabolites. Abbreviations are as defined in **Figure 1**. $n = 6$.

DISCUSSION

The present study revealed that the growth performance, intestinal histology, digestion, immune responses, and metabolic profiles identified were significantly changed by dietary replacement of CM. Generally, plant-based proteins (PBPs) are reported to have imbalanced EAA profiles. The inclusion of PBPs in fish diets exposes them to various phytochemicals, including antinutritional factors (ANFs), which interfere with nutrient digestibility, absorption, and utilisation and ultimately affect the growth performance and health status (9, 10, 17). CM also contains ANFs such as ricin, ricinine, allergen, agglutinins, tannins, lectin, oxalate, and phytases, as well as low levels of EAA, including lysine and methionine (22–25, 59). As a carnivorous fish, the demand for protein and amino acids for grouper must be of high quality in their right proportion (45). While it is promising in making good use of alternative PBP sources such as CM in a particular range via nutrient balancing, previously conducted research has illustrated that the supplementation of EAA like lysine, methionine, and tryptophan to balance the AA profiles in CM enhances growth as well as improvement in the whole body composition (17, 22, 26, 59). Correspondingly, the

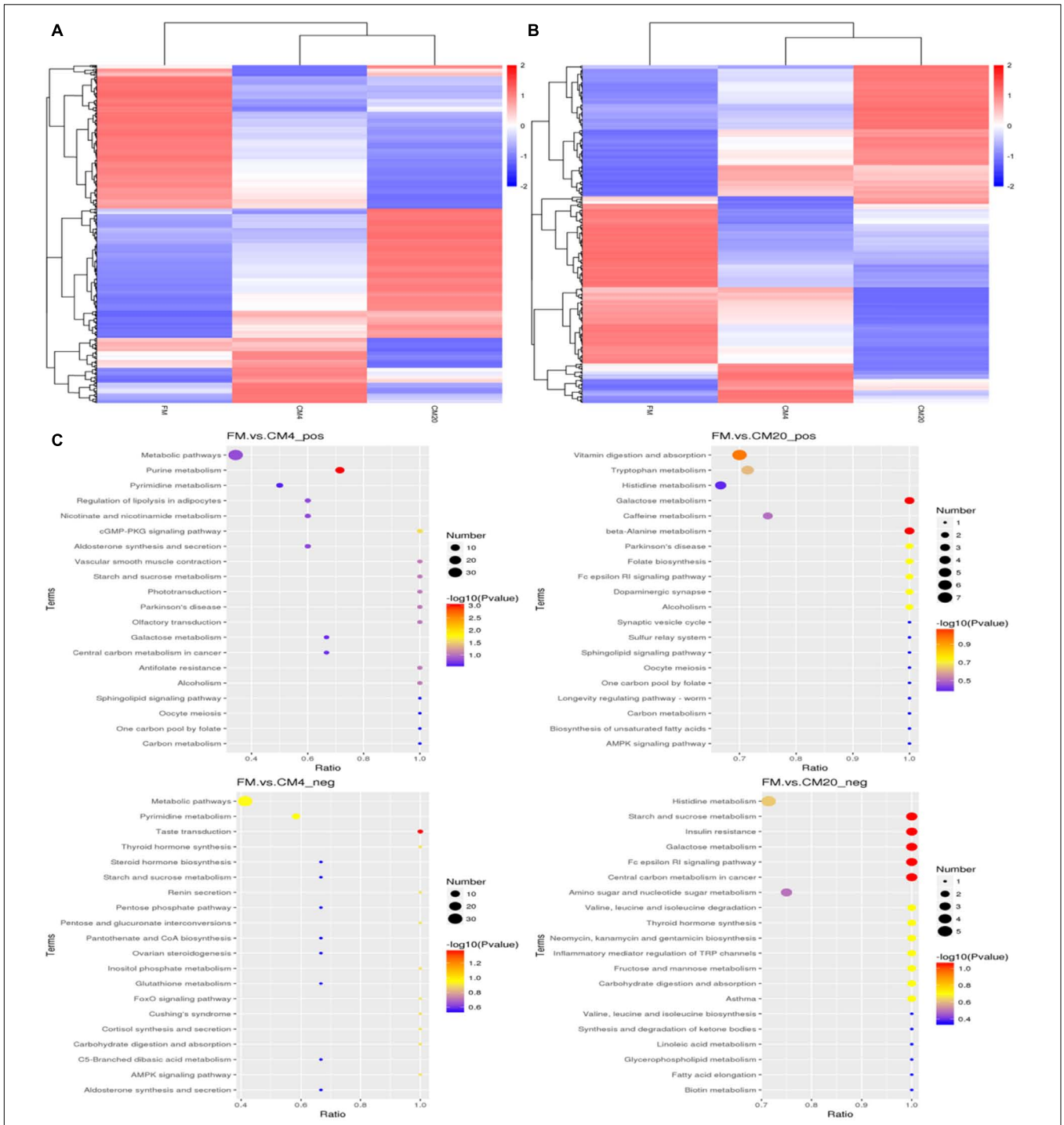


FIGURE 8 | Clustering heatmap of total differential metabolites in the positive **(A)** and negative **(B)** modes after fishmeal replacement with castor meal. Individual groups are clustered in the vertical part, whereas those of the metabolites are clustered in the horizontal part. Colour intensity indicates the intensity of the metabolite. The relationship of metabolite content clustering between groups can be seen horizontally. The shorter the cluster branch is, the higher the similarity is. **(C)** Scatter plot of the top 20 KEGG pathway enrichment for differential metabolites in the FM, CM4, and CM20 groups in positive (pos) and negative (neg) modes. Abbreviations are as defined in **Figure 1**. *n* = 6.

plethora of research available on FM replacement with partial or full PBP supports the supplementation of EAA (mainly lysine, methionine, and threonine) to aid in achieving favourable AA profiles and improve palatability (59, 60). In the present study, we supplemented lysine, methionine, threonine, and leucine EAA to balance CM's AA profiles.

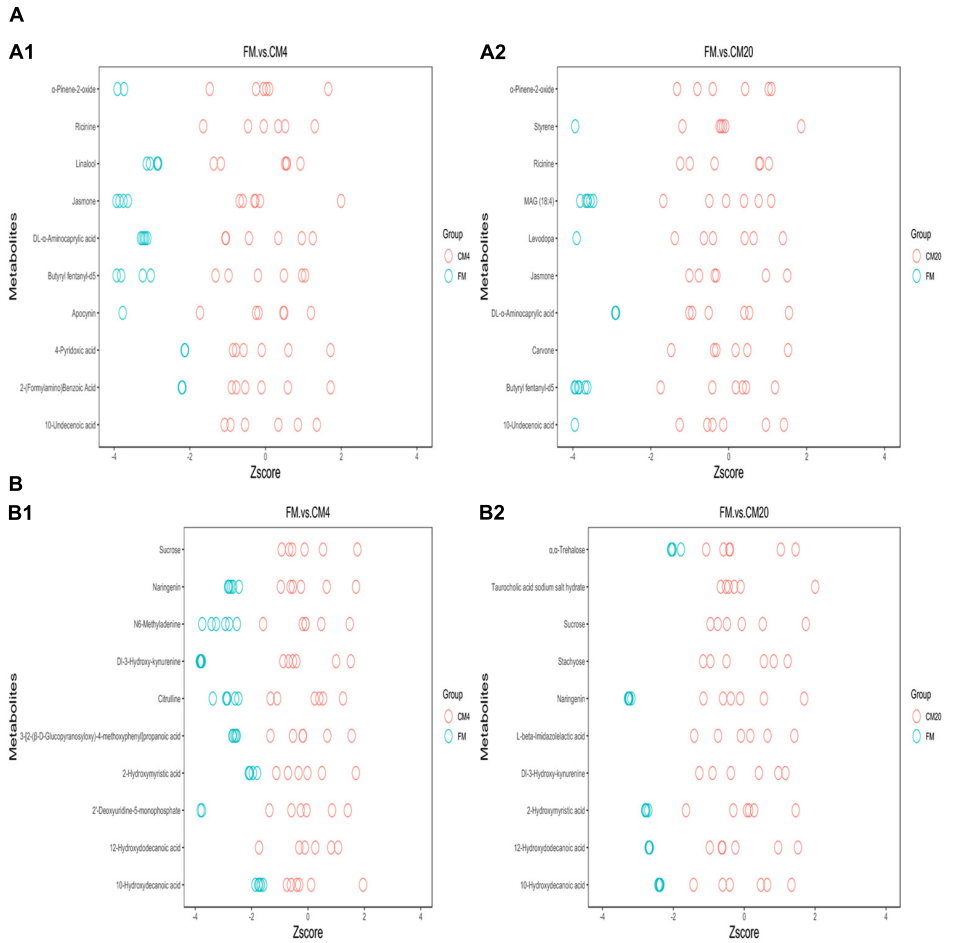


FIGURE 9 | The *z*-Score plot of the potential biomarkers for the comparison between FM, CM4, and CM20 groups in the positive **(A1,A2)** and negative **(B1,B2)** modes. Abbreviations are as defined in **Figure 1**. $n = 6$.

At the end of the 56-day feeding trial, a significant improvement in the growth performance (FW, WGR, SGR, FCR HSI, VSI, ISI, and ILI) was witnessed in fish fed at the 4% replacement level in comparison with the other groups. Nonetheless, the fish fed with the highest replacement level (CM20 group) showed the worst growth performance. Similarly, dietary supplementation of CM above 8% (27) or 10% (26) reveals a reduction in feed intake, which causes a decrease in growth. Increasing the inclusion of CM in diets resulted in an increase in ricinine contents which led to a reduction in feed intake and growth performance in rainbow trout (*Oncorhynchus mykiss*) (61) and grass carp (*Ctenopharyngodon idellus*) as against lower CM replacement [40 g/kg feed (4%)] which had lesser ricinine content (< 20 mg/kg) (32). The supplementation of CM in broiler diets led to lower feed intake, poor FCR, and PER (62, 63), and even 83% mortality in growing chicks (64). The reduction in growth could be attributed to the higher contents of ANFs such as ricin, lectins, allergen, and ricinine in the diets. Although the contents of ANFs such as ricinine in feed were not analysed in the current study, the reduction in the growth performance could be attributed to the higher levels of ricinine content. More to this explanation is the metabolomics analysis which revealed the intensity of ricinine metabolite as being significantly high in the CM20 group than in the others.

Digestive enzyme activities, including amylase (AMS), trypsin (TRP), and lipase (LPS), aid in the breaking down of food for nutrient absorption (65, 66). The current study revealed a significantly high LPS activity in the CM groups compared to the other groups. No significant differences in the TRP and AMS were observed after FM's dietary replacement with CM. A previous report shows a significant decrease in the activities of intestinal TRP and chymotrypsin (C-TRP) and liver TRP, C-TRP, AMS, and LPS after replacing FM with soybean meal in red seabream (*Pagrus major*) which was not in agreement with our findings (67). The digestive enzyme contents produced are undoubtedly regulated by the fish type, age, and diet (68, 69). Thus, the inconsistency can be attributed to the dietary CM supplement used in the current study since there is limited information on the subject matter.

Fish rely mainly on their non-specific and innate immunity in dealing with pathogenic invasion or the presence of toxins. Immune and antioxidant enzymes including C3, C4, IgM, LYZ,

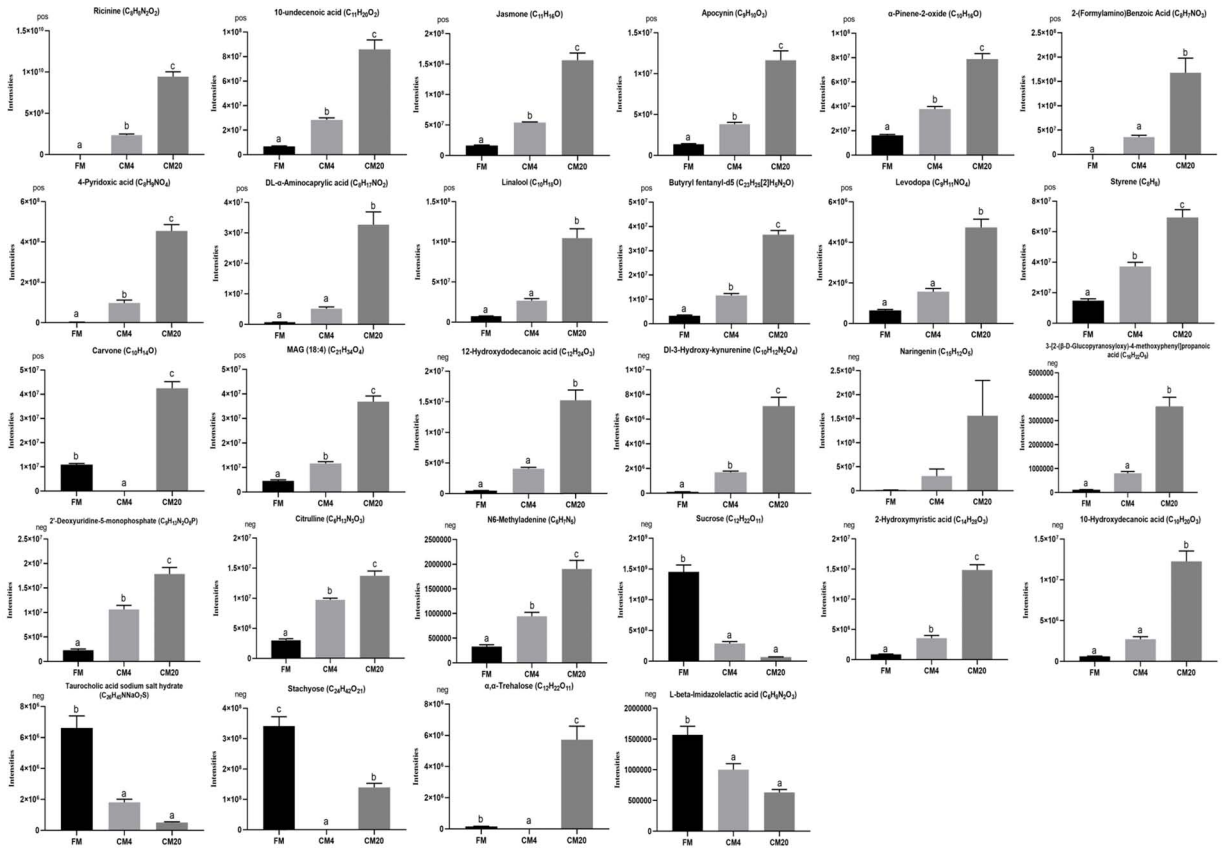


FIGURE 10 | Intensities of the metabolites identified in the positive and negative modes. Data represented the mean ± SE (Turkey's HSD, $P < 0.05$; $n = 6$). Vertical bars marked with no letters do not differ significantly ($P > 0.05$) among groups. In the bar plots, FM, fishmeal; CM4, 4% replacement of fishmeal with castor meal; and CM20, 20% replacement of fishmeal with castor meal.

SOD, and GSH-Px help in host defence functions for growth and development as they can reflect the stress responses of fish due to infectious agents or toxins (70, 71). Complement is mainly responsible for the destruction and elimination of toxins. C3 and C4 are primarily produced by hepatocytes which can be activated to participate in immune response (72). IgM plays a vital role in bacterial opsonisation, toxin, and virus neutralisation, making them liable to phagocyte destruction in the host organism (73, 74). As part of the innate immune system, the LYZ functions by attacking, hydrolysing, and breaking glycosidic bonds in the peptidoglycan (75). In this study, FM replacement with CM significantly increased the C3, C4, IgM, and LYZ in the DI of juvenile hybrid groupers, indicating that CM substitution in diet could improve immunity, consistent with other reported studies (12, 76, 77). Contrarily, Zhang et al. (78), after substituting FM with soybean meal, showed significantly lower ($P < 0.05$) IgM, C3, and C4 levels in the intestine in comparison to the control. However, no significant differences were found in the LYZ enzyme activity among the treated groups (78). Optimum threonine (79) and leucine (80) in grass carp (*Ctenopharyngodon idella*) and black carp (*Mylopharyngodon piceus*), respectively, has been shown to significantly increase LYZ, C3, C4, and IgM immune parameters. Also, acute toxicity testing using chlorpyrifos in common carp (*Cyprinus carpio*) showed an LYZ increase in serum, hepatopancreas, and kidney, as well as an increase in serum and kidney IgM (81). Although this is the first report to reveal the effects of replacing FM with CM, the reason for the discrepancy can be ascribed to the supplementation of threonine and leucine AA in our study to achieve an optimum AA profile. Again, the changes could be attributed to the clearance and contributing effect of inflammatory reactions such that phagocytic cells are attracted to injury sites which can ultimately lyse pathogenic cells (75, 82, 83). We entreat further studies to explain the effects of CM on the non-specific immune systems of fish. For the antioxidant enzymes, while SOD is reported to support catalysing reactive O^{-2} to H_2O_2 partitioning (84), GSH-Px primarily shows the detoxification of hydrogen peroxides, as well as other peroxides such as lipid hydroperoxides (85). An increase in antioxidant enzyme implies that the antioxidant defence against reactive oxygen and free radical reaction is high; hence, the SOD and GSH-Px increase in the hybrid grouper's intestine after replacing FM with CM supplementation suggests an improvement in the antioxidant status. The CM20 group exhibited the highest SOD and GSH-Px which was significantly higher than the results obtained for the control group. This phenomenon may be because of the high

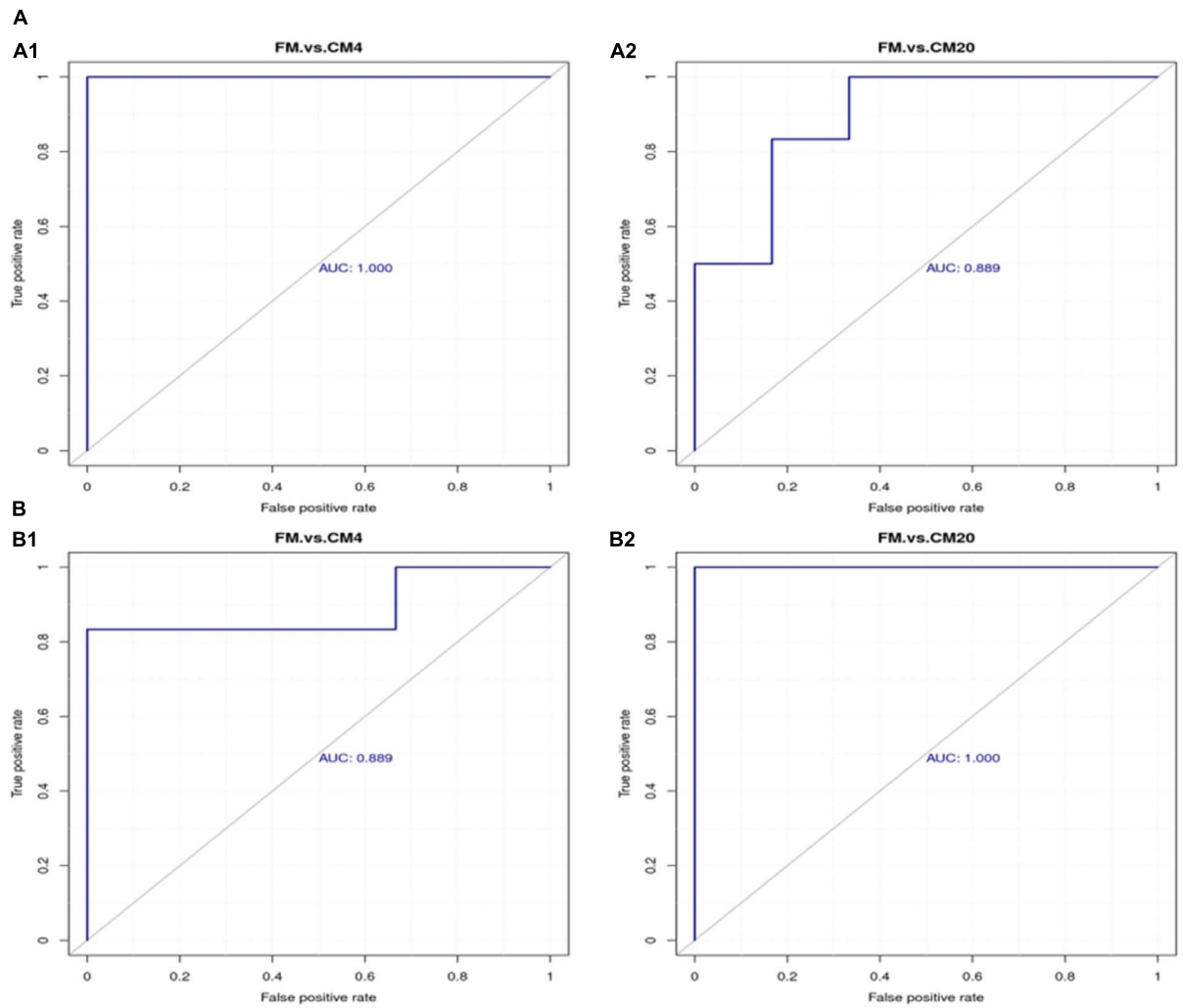


FIGURE 11 | The ROC curve analysis for differentiating among groups with respect to the potential biomarker metabolites in the positive **(A1,A2)** and negative **(B1,B2)** modes. Abbreviations are as defined in **Figure 1**. *n* = 6.

presence of monoterpene metabolites identified in the current study, such as the α-Pinene-2-oxide whose intensity was very high in the CM groups (CM20 obtaining the highest) than in the control. Pre-administration with α-Pinene-2-oxide sheltered U373-MG cells from stimulated oxidative damage of H_2O_2 via blocking the loss of cell viability (IC50: 79.70 mM to α-Pinene-2-oxide), which in turn prevented the formation of ROS and lipid peroxidation. As a result, there was a gross enhancement of endogenous antioxidant status via enhancing glutathione, SOD, CAT, GR, GSH-Px activities, HO-1 properties, and protein expression as reported by Porres-Martinez et al. (86). Thus the increase in the intensities of α-Pinene-2-oxide in the gut might have triggered the increase in the levels of SOD and GSH-Px observed in the present study.

The intestine is an important organ for digestion and absorption of nutrients. It plays an ardent role in regulating immunity, mucosa barrier, signal recognition, and the production of endogenous active molecules (87). Inducing enteritis has been one of the fascinating areas of studies that are now commonly used as the benchmark for the study of intestinal inflammation in fish, especially after FM is replaced with PBPs, including CM (18, 88). Fish physiology has been reported to improve along with the changes in the intestinal structure. Due to the high presence of ANFs in most PBP diets, the histopathological changes it comes with have been extensively researched. There is usually a swelling of the lamina propria (making their width bigger) and subepithelial mucosa, reduction in epithelial villi height, villi width, loss of normal enterocyte supranuclear absorptive vacuolisation, and infiltration of inflammatory cells. This, in a way, decreases the DI capacity to break down food into smaller particles, digest them and absorb the nutrients (18, 45, 88–91). Research accentuates that broader or taller epithelial villi and wider crypt depth are indications of higher absorption of nutrients in the gut (92). Although very little, other studies have also highlighted the histopathological changes of animals after CM supplementation,

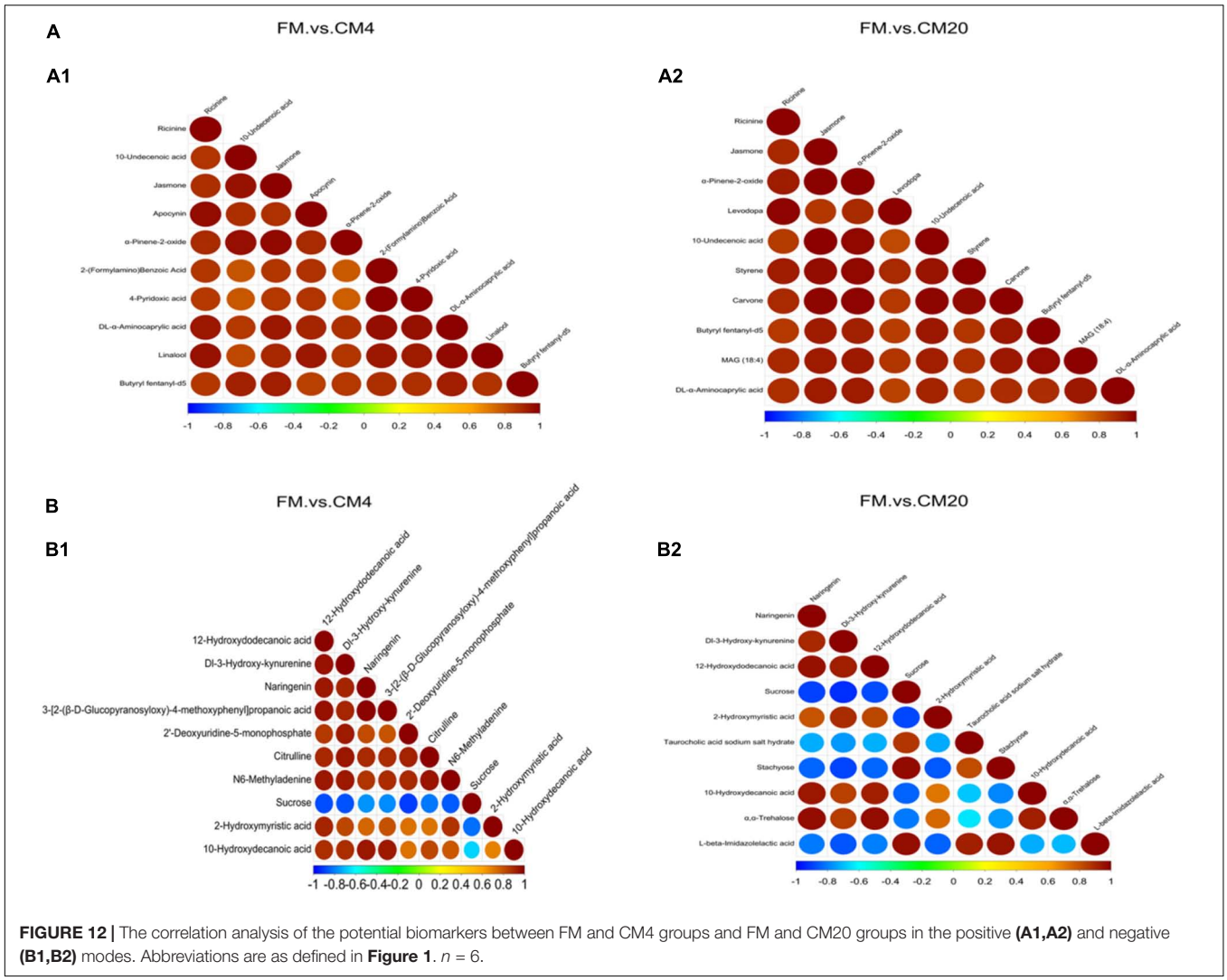


FIGURE 12 | The correlation analysis of the potential biomarkers between FM and CM4 groups and FM and CM20 groups in the positive **(A1,A2)** and negative **(B1,B2)** modes. Abbreviations are as defined in **Figure 1**. $n = 6$.

of which its severity is premised on the inclusion level. Diniz et al. (26), after 10% dietary CM supplementation, revealed an intense inflammation of the abomasums and intestines with corrosion of mucous membranes in cattle. Aslani et al. (27) observed hepatic necrosis, kidney acute tubular necrosis gastroenteritis, cardiac haemorrhage, and necrosis in sheep. Nagalakshm and Dhanalaksh (16) also revealed pathological lesions in the kidney, liver, intestines, and lungs in lambs after 10% CM as a result of the ANFs. On the contrary, an 8% supplementation level was purported to show no significant differences in the histopathological changes (28). There are some discrepancies looking at the results of previous studies. In the present study, the higher substitution of CM (CM20) in the diet caused CM-enteritis in the DI histological examination conducted by the AB-PAS staining and the SEM analysis. There was a significant decrease in the VH, VW, CD, and GC counts, as well as an increase in the MT and LP width in the CM20 group as compared to the other groups similar to the observation made previously in soybean enteritis (78). For the SEM results, there were fewer and weaker mucosal villi density, as well as villi atrophy (flattening or blunting), and this together might have caused the reduction in growth performance as the intestines were not tall and wider enough to absorb the needed nutrients for growth development. To the best of our knowledge, this is the first report to be conducted using a fish model (hybrid grouper) to assess the histopathological changes in the DI tissue by the AB-PAS staining and SEM after dietary CM supplementation, thus, we entreat that further studies be conducted.

Metabolomics has been effectively used to identify key potential metabolic biomarkers in the DI tissue of grouper after replacing FM with soybean meal (78). The application of LC-MS/MS analysis on four different types of castor bean revealed 60 key metabolites of high commercial value, which were all associated with primary and secondary metabolism, including fatty acids, amino acids, flavones, flavonol, flavanones, dopamine, and phenylpropanoids (93). Moreover, a recent report (94) identified *R. communis* to contain eighty-three metabolites, including alkaloids, flavonoids, terpenoids, derivatives of benzoic acid, tocopherols, coumarins, and fatty acids. However, it is vital to identify the key metabolites after their dietary supplement

and know their intensities after digestion. It must be stated that, for the first time, this study labels the DI tissue's metabolic profiling changes in juvenile grouper after being fed 4 and 20% CM protein replacement to FM using UPLC-MS, and the literature regarding UPLC-MS-based metabolomics on fish is rare. Compared with the current study, there were only 28 identified potential biomarkers.

Again, the present study discusses the biological relationships between the key potential biomarkers and their roles in intestinal health. Among the 28 potential biomarkers identified; 5 of them were organoheterocyclic compounds [Ricinine (sub-class: pyridinecarbonitriles), 4-Pyridoxic acid (sub-class: pyridinecarboxylic acids and derivatives), Butyryl fentanyl-d5 (sub-class: Fentanyl), L-beta-Imidazolelactic acid (sub-class: Imidazoles), and *N6*-Methyladenine (sub-class: Purines and purine derivatives)]; 7 belonged to Lipids and lipid-like molecules [10-Undecenoic acid (sub-class: Fatty acids and conjugates), α-Pinene-2-oxide (sub-class: Monoterpenoids), Linalool (sub-class: Monoterpenoids), Carvone (sub-class: Monoterpenoids), MAG (18:4) (sub-class: Lineolic acids and derivatives), 2-Hydroxymyristic acid (sub-class: Fatty acids and conjugates), and Taurocholic acid sodium salt hydrate (sub-class: Bile acids, alcohols and derivatives)]; 7 were in the super class of Organic Oxygen compounds (Jasmone (sub-class: Carbonyl compounds), Apocynin (sub-class: Carbonyl compounds), Dl-3-Hydroxy-kynurenine (sub-class: Carbonyl compounds), 3-[2-(β-D-Glucopyranosyloxy)-4-methoxyphenyl]propanoic acid (sub-class: Carbohydrates and carbohydrate conjugates), Sucrose (sub-class: Carbohydrates and carbohydrate conjugates), Stachyose (sub-class: Carbohydrates and carbohydrate conjugates), and α,α-Trehalose (sub-class: Carbohydrate and carbohydrate conjugates); 2 were in the super class of Benzenoids [2-(Formylamino)Benzoic Acid (sub-class: Benzoic acids and derivatives) and Styrene (sub-class: styrenes)]; 5 were in the super class Organic acids and derivatives [DL-α-Aminocaprylic acid (sub-class: Amino acids, peptides, and analogues), Levodopa (sub-class: Amino acids, peptides, and analogues), 12-Hydroxydodecanoic acid (sub-class: Medium-chain hydroxyl acids and derivatives), Citrulline (sub-class: Amino acids, peptides and analogues), and 10-Hydroxydecanoic acid (sub-class: Medium-chain hydroxyl acids, and derivatives)]; 1 was from super class Phenylpropanoids and polyketides [Naringenin (sub-class: Flavans)]; and 1 was from the super class Nucleosides, nucleotides, and analogues [2′-Deoxyuridine-5-monophosphate (sub-class: Pyrimidine deoxyribonucleotides)] which corresponds to previously identified metabolites in castor (*R. communis*) (94–96).

Ricinine, a metabolite of the class pyridines, and derivatives are considered a ricin toxin marker. Ricinine dosage varies but is found to be 2.3–32.9 g/kg in leaves, 3 g/kg in roots, 2.4 g/kg in stems, and 0.43–7.0 g.kg in its seeds (97, 98). Chickens are reported to be poisoned to death after consuming diets of 0.1 g/kg ricinine (99). 4-Pyridoxic acid (sub-class: pyridine carboxylic acids and derivatives) is the primary product of vitamin B_6 in animals formed by the pyridoxal oxidation by a non-specific flavin adenine dinucleotide (FAD)-dependent aldehyde oxidase. Having a higher 4-Pyridoxic acid/pyridoxine ratio is linked with a deficiency in vitamin B_6 (100, 101) which can affect intestinal morphology (decrease villi height and width) and absorption and metabolism of protein in animals (102). Butyryl fentanyl-d5 belongs to the class Piperidines (sub-class: Fentanyl), and Butyrates as dose-dependent is reported to promote intestinal barrier function at lower concentrations ($\leq$2 mM) (103) but may disrupt intestinal barrier function at high concentrations (5 or 8 mM) by inducing apoptosis (104). Although a previous study reveals a higher concentration of L-beta-Imidazolelactic acid to be associated with an increase in antioxidant enzyme activities such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (105) which are noted to play a vital role in the health of animals, the association of its relation with intestinal health is still unknown. Thus further research is warranted to explain such an association. This present study revealed significantly high intensities of Ricinine, 4-Pyridoxic acid, and Butyryl fentanyl-d5, and significantly low intensity of L-beta-Imidazolelactic acid in the higher replacement level as compared to the FM and the CM4 groups. Therefore the plausible reason for the witnessed disruption in the distal intestine can be attributed to the increase of Ricinine, 4-Pyridoxic acid, and Butyryl fentanyl-d5 intensities and decrease of L-beta-Imidazolelactic acid, which can also illustrate why higher replacement of FM with CM led to poorer growth performance.

The intensities of α-Pinene-2-oxide, Linalool, and Carvone were significantly increased with increasing replacement levels of CM. Monoterpenoids such as α-Pinene-2-oxide, Linalool, and Carvone are components of volatile essential oils from several plant products that are potent suppressors of plant growth (could be the reason for poor growth in the CM20 groups), but also cause a significant increase in antioxidant enzymes (106). Pre-treatment with α-Pinene-2-oxide inhibited ethanol-induced gastric lesions, reduced gastric juice volume and acidity, and increased gastric wall mucus in Swiss mice (107). α-Pinene-2-oxide is reported to stimulate oxidative damage of H_2O_2 through blocking the loss of cell viability, and prevents the formation of ROS and lipid peroxidation. Thus, gross enhancement of endogenous antioxidant status via enhancing glutathione, SOD, CAT, GR, GSH-Px activities, HO-1 properties, and protein expression exist (86). Various studies have described α-Pinene-2-oxide to display antimicrobial, anticancer, antitumor, anticancer, anti-inflammatory, and antiallergic properties. Nevertheless, most of these studies lack information concerning their relation to the intestinal health of animals (106); thus, more research is warranted in such areas.

The intensities of carbonyl compounds, including Jasmone, Apocynin, and Dl-3-Hydroxy-kynurenine, significantly increased with increasing replacement levels of CM to FM. In humans, intestinal ischemia usually occurs from impaired blood perfusion to the bowel due to various causes such as sepsis, cardiac insufficiency, vaso- and cardio-depressant drugs, and lifelong surgery, which in the long run progressively damages cell structures producing lesions that are further exacerbated (108). However, the treatment with Apocynin prevented such intestinal damage (109). Trehalose, a non-reducing disaccharide

of glucose, is formed by stress conditions such as heat, oxidative stress, and other toxins. Based on previous findings, cytoplasmic trehalose accumulation protects the proteins and membranes from denaturation due to stress (110).

The main differential benzenoid metabolites belonged to Benzoic acids and derivatives and the Styrenes subclass. Benzoic acid irritates the human mucous membrane (111). Styrene is connected to several human diseases such as ulcerative colitis, non-alcoholic fatty liver disease, and the hereditary metabolic disorder celiac disease (112, 113). Reiko et al. (114), in an *in utero* exposure with Styrene, revealed a reduction in pub body growth and induced alteration in behaviour and neurotransmitter levels in brains. The significant increase in the intensities of 2-(Formylamino)Benzoic Acid and Styrene metabolites could be linked to the poor growth attained for grouper fish fed 20% CM replacement. Since studies relating to the association of 2-(Formylamino)Benzoic Acid and Styrene metabolites with the intestine health are limited, we recommend further studies to ascertain such relations.

The organic acid and derivatives are very large super-class with numerous pathways. Only the "Amino acids, peptides and analogues" and "Hydroxy acids and derivatives" sub-classes were discussed herein. Various amino acids, peptides, and analogues sub-class compounds have been known to down-regulate tyrosinase (TYR) gene expression or inhibit TYR catalytic activity. An example is Levodopa which is made via the biosynthesis from amino acid L-tyrosine by tyrosine hydroxylase enzyme (115). Also, L-arginine is an essential amino acid that is a precursor in synthesising Citrulline, proteins, urea, ornithine, creatinine, and agmatine, supporting the glutamate, proline, and polyamine metabolism at the whole organism level or cellular level in mammals. L-arginine's availability and metabolisation can modulate inflammation, regulate the immune response to infections, and recover the physiological steady-state (116). Again, it has been long-established that hydroxy acids in crude extracts from plants have been used to treat diseases. The novel fatty acid, 12-Hydroxydodecanoic acid, was recently recognised as a metabolite with antifungal properties (117, 118). Citrulline has been involved in numerous regulatory roles, such as gut modulation, anti-inflammatory and antioxidative effects, protein synthesis, blood pressure regulation, nitrogen homeostasis, renal function, skeletal muscle function, cardiac function, and vascular health as well. The available information regarding the use of citrulline in animals is very limited; nevertheless, it is slightly gaining research interest as a result of its unique metabolism. Citrulline not only serve as functional marker for gut barrier dysfunction, but it has been associated also with several intestinal diseases, including short bowel syndrome, necrotizing enterocolitis and gastric ulcers (119). The observed increase in Citrulline metabolite intensity might have been due to the increased arginine content analysed (**Supplementary Table 1**) in the CM ingredient, which was higher than observed in the FM. Thus, there was modulation of inflammation and regulation of immune response, which sort of affirms why the immune enzyme activities analysed were higher than as obtained in the control group.

CONCLUSION

In summary, the results in this study for the first time demonstrated the effects of replacing FM with CM on the growth, feed utilisation, immune response, digestive enzyme activities, and intestinal health of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂). A significant enhancement in terms of the parameters mentioned was achieved after replacing FM with 4% of CM. With the application of metabolomics, there were 28 identified metabolites as biomarkers of adherence to the CM diet. We propose further research to be conducted to validate the identified metabolites. In this study, the universal metabolomics platform (untargeted metabolomics) was used which only obtained relative estimates of the metabolites. Thus we recommend further studies which will deal with the use of targetted assays to obtain quantitative results for the identified potential biomarkers. Furthermore, it is necessary to conduct metabolomics profiling to determine how stable these potential biomarkers are when dealing with multiple time points or over an extended period and with a wider range of adherence to dietary CM patterns. The current study is limited to the dietary patterns of the CM trial, and it will be prudent to investigate the specificity of the potential biomarkers for the CM diets in comparison to other dietary patterns that vary in terms of macronutrients, such as lipids, proteins, and carbohydrates. The methods of using the integrated analysis of multi-omic technologies concerning different organs will be considered in our future work to further reveal the CM induced-enteritis mechanism.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research and the Ethics Review Board of Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

KA conceived and designed the experiment in consultation with X-HD, wrote the first draft of the manuscript which was later reviewed and criticized by X-HD and B-PT, and performed the animal experiment. S-YC, Q-HY, H-YL, and X-BY helped to collect the samples. KA, S-YC, Q-HY, H-YL, and X-BY analysed the data. X-HD and SZ aided in the purchasing of the re-agents. KA, X-HD, H-YL, Y-ZY, and HZ interpreted the statistical outcome. All authors consented to the submission of the manuscript for publication.

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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.2022.847425/full#supplementary-material

Supplementary Figure 1 | The individual sample repeats representative UPLC-MC spectra of the distal intestine in the **(A)** positive, and **(B)** negative modes. FM-1 to FM-6 represent the individual sample repeats of FM; CM4-1 to CM4-6 represent the individual sample repeats of CM4; CM20-1 to CM20-6 represent the individual sample repeats of CM20 FM, fish meal (control group); CM4, 4% Castor meal (CM) protein replacement to FM protein; CM20, 20% CM protein replacement to FM protein.

Supplementary Figure 2 | The results of the total PLS-DA score plots from the UPLC-MS spectra of the distal intestinal contents in positive **(A)** and negative **(B)** modes. A1 and A2 are, respectively the positive modes between FM and CM4, and FM and CM20 groups; B1 and B2 are respectively the negative modes between FM and CM4, and FM and CM20 groups. N = 6.

Supplementary Figure 3 | The detailed clustering heatmap of total differential metabolites in the positive **(A)** and negative **(B)** modes after fishmeal replacement with castor meal. Individual groups are clustered in the vertical part, whereas those of the metabolites are clustered in the horizontal part. Colour intensity indicates the intensity of the metabolite. The relationship of metabolite content clustering between groups can be seen horizontally. The shorter the cluster branch is, the higher the similarity is. FM, fish meal (control group); CM4, 4% castor meal (CM) protein replacement to FM protein; CM20, 20% CM protein replacement to FM protein.

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The Impact of Dehulling and Germination on the Physiochemical, Protein Solubility and Water and Oil Holding Capacities of Yellow Eye Bean (*Phaseolus vulgaris L.*) Protein Concentrates

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Pulse varieties including Yellow Eye (YE) beans (*Phaseolus vulgaris* L.) are a rich source of protein (~26.5%) that can be utilized to create value-added protein concentrates. Pre-treatments including dehulling and germination have been shown to be effective at improving the nutritional and functional properties of extracted protein concentrates. However, the composition and functionality of these protein concentrates can vary depending on the pre-treatments and the method of extraction used (salt vs. alkaline). Furthermore, little is known about the impact of combining these different processing methods on the properties of YE bean protein concentrates. The objective of this study was to evaluate how germination and dehulling pre-treatments individually and when combined influence protein extraction efficiency, physiochemical properties (surface hydrophobicity and intrinsic fluorescence), and the functionality (solubility, oil and water holding capacities) of salt and alkaline extracted protein concentrates. Compared to the salt extracted concentrates, the alkaline protein concentrates exhibited higher protein recovery yields (16–23% vs. 43–56%) respectively. Conversely, the salt extracted protein concentrates exhibited superior functional properties as observed by improved water holding capacities and less variation in their solubilities at different pH values (4 to 10). When the pre-treatments were combined, the salt extracted concentrates exhibited improved extraction efficiencies and improved hydrophobicity and intrinsic fluorescence, whereas the opposite trend was observed in the alkaline protein concentrates. These observations were attributed to differences in the protein content and composition of the salt vs. alkaline protein concentrates. Overall, these findings suggest that dehulling and germination are potential processing methods that may be used to improve the physiochemical characteristics of salt extracted protein concentrates from yellow eye beans. Future research may investigate the potential application of these ingredients in different food formulations.

Keywords: plant protein, germination, dehulling, protein extraction, protein concentrate

INTRODUCTION

Over the past decade there has been increased efforts to reduce animal-based proteins and replace them with plant-based proteins to form healthier, more affordable, and more sustainable food products (Vainio et al., 2016). Underutilized pulse varieties, such as Nova Scotian Yellow Eye (YE) beans (*Phaseolus vulgaris* L.), have a high protein content (26.5%) and can serve as alternative protein ingredients in food production, however, their efficacy must be shown (English et al., 2019a).

Proteins in the form of concentrates (65–90% protein dry basis) or isolates (>90% protein dry basis) can be separated from different plant or animal sources and used as food ingredients (Ma et al., 2022). Traditionally, the preferred protein sources have been from soy, wheat, or dairy products (such as whey), however, the presence of allergens and certain dietary restrictions are making pulse proteins a more attractive alternative for food formulations (Soller et al., 2015; Nwachukwu and Aluko, 2021). Although there are different ways to define protein functionality, this terms most often refers to the ability of proteins to form and stabilize networks in food systems such as gels, foams, and gels, and includes physiochemical properties such as solubility, and water and fat binding abilities (Foegeding and Davis, 2011; Vogelsang-O'Dwyer et al., 2021). Indeed, pulse proteins have shown similar functional properties compared to soy proteins (Burger and Zhang, 2019). For example, common bean proteins have shown promising emulsifying and gelling properties (Shevkani et al., 2015; Rahmati et al., 2018), whereas pea proteins have exhibited high solubility and foaming properties. Knowledge of the functional properties of proteins is therefore important in selecting the most ideal ingredients for successful food formulations (Vogelsang-O'Dwyer et al., 2021). However, in terms of their functional properties not all proteins are equal, differences in the functional properties can be attributed to inherent variations between cultivars or differences in protein content, composition, and conformation (Rui et al., 2011). The type of protein extraction methodology used can also contribute to differences in functional properties because of: (1) changes in the protein fraction extraction affinity, and (2) conformational changes achieved during the extraction process (Karaca et al., 2011).

Currently, the techniques used to extract plant proteins may be grouped into dry and wet processes (Fernando, 2021; Yang et al., 2021). Dry processes achieve protein separation based on differences in density whereas in wet processes, proteins are extracted based on their solubilities in alkaline or salt solutions, followed by isoelectric or micellar precipitation, respectively (Stone et al., 2015; Tanger et al., 2020). The work in the present study has focused on wet processes since these methods are commonly used with pulses (Cui et al., 2020). Among the wet extraction methods, the alkaline extraction process is more commonly used because of its simple and cost-effective nature (Zhang et al., 2014; Momen et al., 2021). Although this approach primarily targets globulins and produces higher protein concentrate yields, the alkaline and acidic environments necessary with this approach can result in partial denaturation of the proteins which in turn reduces protein quality and functionality (Yang et al., 2021). Conversely, protein extraction techniques using salt solutions have been shown to produce a more complete protein profile (Karaca et al., 2011). However, compared to alkaline methods, salt extraction procedures can be time-consuming because an additional desalting step is required (Hadnadev et al., 2017). Determining how these two extraction techniques impact the functionality of YE bean protein concentrates is critical to the successful development and application of these ingredients.

Apart from the challenges related to protein extractions, the presence of undesirable components in pulses can impact the functionality and quality of pulse proteins. Antinutrients such as tannins have been linked with decreased protein bioavailability which in turn limits protein functionality (Samtiya et al., 2020). Various pre-treatments including germination and the subsequent enzymatic hydrolysis of indigestible proteins have helped to improve protein digestibility (Ali and Elozeiri, 2017). Dehulling (removal of the seed coat) and soaking have also been used to reduce anti-nutrients that impact protein adsorption (Wang, 2008). Many of the studies evaluating the effectiveness of pre-treatments have focused on flour samples with little emphasis on the protein isolates or concentrates. For example, Liu et al. (2018), demonstrated an increase in protein content and water absorption index in germinated mung bean flour over a 72-h period. In addition, Ghavidel and Prakash (2007) used a combination of pre-treatments (dehulling and germination) on lentils, chickpeas, green gram, and cowpeas and observed an increase in protein and protein digestibility. Indeed, our own observations with other food model systems containing yellow eye beans have shown that blending pre-treatments (germination and soaking) were more effective strategies for improving the functional properties and the aroma profiles of these pulse ingredients (English et al., 2019b). Although the use of combined pre-treatments is not a new approach the improvement in protein functionality suggests that there may be merit in further investigating this strategy.

Accordingly, the main objective of the present study was to evaluate the impact of dehulling and germination individually and a combination of these pre-treatments on Nova Scotia Yellow-eye bean protein concentrates. Protein extractions were conducted using alkaline and salt solutions, and the physiochemical (hydrophobicity and intrinsic fluorescence) and functional properties (solubility and oil and water binding capacity) of the different concentrates were evaluated. It was hypothesized that improved protein functionality and physiochemical properties may be observed in protein concentrates exposed to combined pre-treatments, since blending pre-treatments has been shown to have a greater impact on protein functionality (English et al., 2019b). Moreover, because salt extraction techniques have been demonstrated to reduce conformational changes in the extracted protein isolates (Grover and Ryall, 2005), it was further hypothesized that the salt extraction protein concentrates would have improved physiochemical properties compared to their alkaline extracted counterparts. Understanding the impact of these processes on the physiochemical and functional properties of yellow eye bean protein concentrates is important for the production and development of new pulse ingredients of high quality.

METHODS

Materials

Yellow Eye beans (*Phaseolus vulgaris* L.) grown in Cambridge, Hants County, Nova Scotia, were purchased from a grocery store, Superstore in Antigonish, Nova Scotia and stored in a cool, dark cupboard until use. Bradford reagent, tris, and mini-PROTEAN electrophoresis gels were obtained from Bio-Rad (Mississauga, ON). Ethanol and sodium hypochlorite were purchased from VWR (Mississauga, ON). Sodium hydroxide, β-mercaptoethanol, and methanol were obtained from Fisher Scientific (Ottawa, ON). Sodium phosphate dibasic, sodium phosphate monobasic, and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Oakville, ON). Hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific (Ottawa Canada). All chemicals were reagent grade and did not require any additional purification.

Preparation of Bean Flours for Protein Extraction

A total of 5 bean variations were prepared for this study (**Figure 1**). Prior to flour preparation, beans were sorted and sanitized in a 0.07% (w/v) sodium hypochlorite solution for 30 min, then washed with distilled water until a pH of 7.0 was reached. Beans were soaked at a ratio of 1:4 of beans to distilled water at room temperature for 20 h. Once soaking was finished the remaining distilled water was decanted and excess water was removed from the beans. Whole/soaked (WS) beans were freeze dried and ground using a Blendtec 51-601-BHM Kitchen Mill. Untreated, raw beans (RB) were used as a control for this study and were milled using a Blendtec 51-601-BHM Kitchen Mill. The dehulled/soaked (DS) beans were first manually dehulled, then dried, ground, and stored as previously described.

Germination

Variations described as whole/germinated (WG) and dehulled/germinated (DG) were germinated using a method adapted from Ma et al. (2018) and Xu et al. (2019). Beans were first sanitized and soaked following the method outlined in Section Preparation of Bean Flours for Protein Extraction. Twenty-two beans were placed into a sterile petri dish lined with Grade 1 Whatman® filter paper moistened with 1 mL of distilled water. The beans were covered with another piece of filter paper moistened with 500 μL of distilled water. Beans were germinated in a Memmert Humidity Chamber HCP (Büchenbach, Germany) at 20 °C and 95% relative humidity for a maximum of 72 h, with germinated beans being collected every 24 h. Beans were considered germinated when their radicles reached a length of over 2 mm. Beans that failed to germinate after the 72 h were discarded. Variation WG was then freeze dried and ground into flour, whereas variation DG was dehulled prior to freeze drying and grinding. All flours were stored in airtight containers at 4 °C until they were used.

Protein Content and Ash Content Analysis

Protein and ash analyses, and crude fat determination of all flours were determined at the Department of Agriculture and Food Operations Laboratory (Truro, Nova Scotia, Canada) using similar methods outlined in English et al. (2019a). Ash content was determined following the Association of Official Agricultural Chemists (AOAC) method 923.03 (AOAC, 2005). Protein content was determined using combustion analysis (Laboratory Services Analytical Laboratory, LSAL, Method 410) using a LECO CN828 macro combustion instrument (St. Joseph, KS, USA) using a protein to nitrogen conversion rate of 6.25 (Yang et al., 2021). Crude fat was calculated using a solvent extraction method. Carbohydrate content was determined by subtraction of the other chemical components from 100.

Protein Isolation

Alkaline Extraction Method

A total of five protein concentrates were generated using an alkaline extraction protocol (**Figure 1**). A modified alkaline extraction procedure was developed using a combination of reports from de Evangelho et al. (2017), Du et al. (2018), and Karaca et al. (2011). Briefly, bean flours were suspended in distilled water (1:10 w/v) and the pH of the solutions were adjusted to 9.5 with a 4N NaOH solution, after which the solutions were left to stir for 1 h. The slurries were centrifuged at 5,000 x g for 40 min and the supernatants collected. The remaining pellets were resuspended in distilled water (1:5 w/v) and centrifuged at 5,000 x g for 30 min. The supernatants were pooled together, and the pH was adjusted to 4.5 using 1M HCl to precipitate the protein. The samples were then centrifuged at 3,500 x g for 15 min and the pellets of precipitated protein was kept and adjusted to a pH of 7.0.

Salt Extraction Method

Five protein concentrates were prepared using a salt extraction protocol as shown in **Figure 1**. The salt extraction method described by Mundi and Aluko (2012) was followed with some modifications. Bean flours were suspended in 0.1 M Phosphate buffer (pH 8.0, 1:10 w/v) and stirred constantly for 2 h at 4 °C. The slurries were then centrifuged at 4,260 x g for 45 min, after which the supernatants were collected. Ammonium sulfate was added to the supernatants until 40% saturation had been achieved. The mixtures were stirred for an additional 2 h at 4 °C and centrifuged following the previously described conditions. The supernatants were collected once again, and ammonium sulfate added until 80% saturation was reached. The slurries were centrifuged one more time, after which the pellets were collected. The pH values of the extracted pellets were adjusted to 7.0 and diafiltrated with deionized water through a Vivaflow 200 polysulfone membrane with a molecular weight cut-off of 10 kDa at a volume concentration ratio (VCR) = 10.

Ultrafiltration

Both protein extracts were ultrafiltered using a Vivaflow 200 polysulfone membrane with a molecular weight cut-off of 10 kDa. The ultrafiltration was repeated twice at VCR = 5. The collected protein solutions were freeze-dried and weighed to calculate the protein yield, and the protein recovery yields were calculated as a percentage of the isolate weight of the protein content found in the associated flour (Equations 1 and 2). Protein purity of each

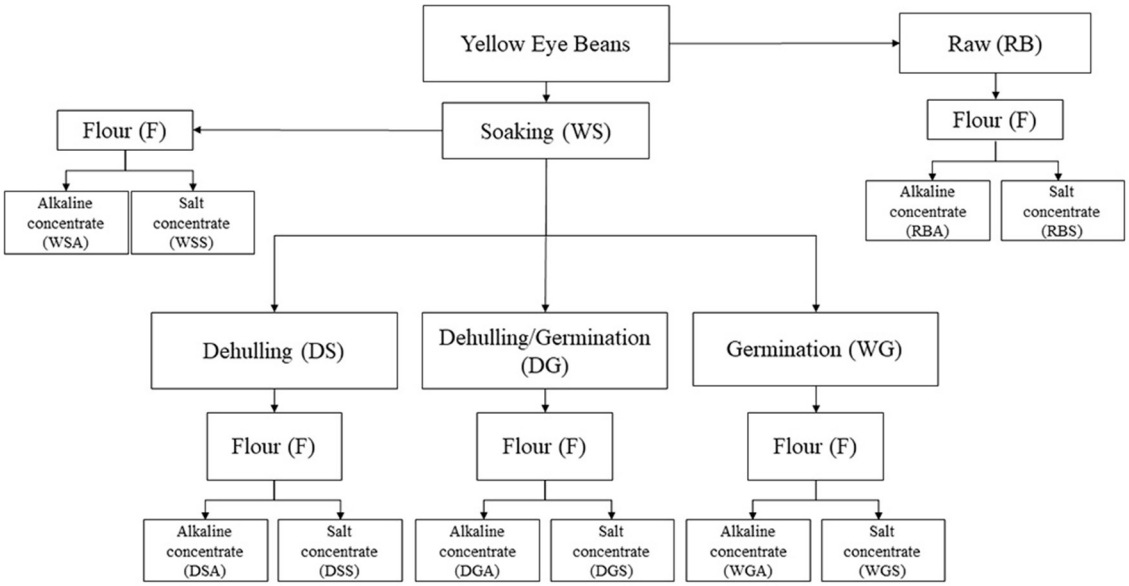

FIGURE 1 | Sample preparation of pre-treated (soaked, dehulled, germinated, and dehulled and germinated), Yellow Eye beans and the subsequent protein concentrates generated using salt and alkaline protein extraction solutions.

extracted protein concentrate (Equation 3) was determined by the Bradford protein assay (Bradford, 1976).

$$Extraction\ yield\ (\%) = \frac{weight\ of\ protein\ concentrate\ (g)}{total\ weight\ of\ flour\ (g)}\ x\ 100 \quad (1)$$

$$Protein\ recovery\ yield\ (\%) = \frac{weight\ of\ protein\ concetrate\ (g)}{Protein\ composition\ of\ flour\ (g)}\ x\ 100 \quad (2)$$

$$Protein\ purity\ (\%) = \frac{protein\ concetration\ (mg)}{Total\ weight\ of\ freeze-dried\ concentrate\ (mg)}\ x\ 100 \quad (3)$$

Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) samples were prepared follow a method adapted from Aluko and McIntosh (2004) and English et al. (2019b). Concentrations of 10 mg/mL of each PI were prepared in Tris/HCl buffer (pH 8.0). Three point five microlitres of filtered protein extract was mixed with 10 μL of distilled water and 5 μL of protein sample buffer (pH 6.8, 1 M Tris-HCl, 5% SDS, 50% glycerol, 0.5% Bromphenol blue, and 10% β-mercaptoethanol) and heated in a boiling water bath for 10 min.

Fifteen microlitres of each sample were loaded onto 12% Mini-PROTEAN® TGXTM gels obtained from BioRad (Mississauga, ON). A pre-stained protein ladder (New England BioLabs, P77066; 10-250 kDa) was loaded in a 5 μL aliquot to estimate the molecular weights of proteins in the samples. Gels were loaded into a Mini-PROTEAN® Tetra Cell (Bio-Rad, Mississauga, ON) and the system was run for 1 hour at 170 V and 30 mA. Gels were stained for 30 min with a 0.1% Coomassie Brilliant Blue (R-250) staining solution and then de-stained overnight. A pre-stained protein marker (New England BioLabs, P77066, 10-250 kDa) was used to estimate the molecular weights of proteins. Images of the gels were captured using a BioRad Chemic DocTM MP imaging system.

Intrinsic Fluorescence Measurement

The intrinsic fluorescence spectrums of the protein concentrates were measured following a modified method from He et al. (2020) and Yang et al. (2021). Concentrations of 0.2 mg/mL of protein solutions were made in 10 mM phosphate buffer (pH 7.2). The intrinsic fluorescence spectra of the protein solutions were measured spectrophotometrically using a QuantaMasterTM spectrofluorometer from Photon Technology International Inc. (London, ON). The excitation wavelength was set to 280 nm, and the emission spectra was recorded from 290–400 nm at a rate of 1 nm/s and a slit width of 2 nm.

Surface Hydrophobicity

A modified method by He et al. (2020) and Joshi et al. (2012), using 8 mM ANS prepared in 10 mM phosphate buffer (pH 7.2) as a probe, was followed. A concentration curve of 0.0–0.05% (w/v) and protein solutions were prepared using 10 mM phosphate buffer (pH 7.2). Briefly, 50 μL of ANS probe was added to 4 mL of each protein concentration, and then incubated in the dark for 5 min. The relative fluorescence intensity (RFI) of each dilution was then measured using a QuantaMaster^{TM} spectrofluorometer from Photon Technology International Inc.

(London, ON) with extraction/emission slits set to 5 nm, and excitation and emission wavelength set to 380 and 480 nm respectively. The net RFI was calculated using Equation 4:

$$Net\ RFI = \frac{RFI\ (ANS + Protein) - RFI(ANS + Blank)}{RFI\ (ANS + Blank)} \quad (4)$$

The slope of the net RFI vs. protein concentration was calculated using linear regression analysis and used as an index of surface hydrophobicity (H_0-ANS).

Protein Solubility

Protein solubility was determined using a combination of the methods described by Yin et al. (2010) and Ortiz and Wagner (2002). Protein solutions with concentrations of 10 mg/mL were prepared using 0.1 M phosphate buffers with pH values of 4.0, 6.0, 8.0, and 10.0. The samples were stirred for 45 min at room temperature and then centrifuged at 5,000 *x g* for 20 min. The protein concentrations of the supernatants were determined using the Bradford assay (1976) and the protein solubilities were determined using Equation 5:

$$Protein\ Solubility\ (\%) = \frac{Supernatent\ protein\ concetration\ (\frac{mg}{mL})}{Total\ protein\ concentration\ (\frac{mg}{mL})} \quad (5)$$

Water Holding Capacity and Oil Holding Capacity

Water holding capacity (WHC) and oil holding capacity (OHC) were determined following a modified version of the methods by Mohan and Mellem (2020). Briefly, 2.5 g of corn oil or distilled water were added to 0.25 g of sample to determine OHC and WHC, respectively. The samples were vortexed every 5 min for 10 sec for a total of 30 min. Afterwards, the samples were centrifuged for 15 min at 1800 *x g*. Finally, the supernatant was carefully pipetted off, and the remaining pellets were weighed. The following equation was used to calculate the OHC and WHC:

$$OHC\ or\ WHC\ \left(\frac{g}{g}\right) = \frac{Final\ sample\ wt\ (g) - Inital\ sample\ wt\ (g)}{Initial\ sample\ wt\ (g)} \quad (6)$$

Statistical Analysis

A minimum of three trials in triplicate for each sample were conducted for all protein concentrate experiments except for the bean flour samples (protein concentration determination and the ash analyses). The averaged means from the trials were calculated and reported as means ± standard deviation. Statistical differences were calculated using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test at $p < 0.05$ in XLSTAT® software version 2020.2 (New York, NY). In addition, Pearson correlation (r) analysis was used to determine significant correlations between physicochemical properties and functional properties.

RESULTS AND DISCUSSION

Protein and Ash Content of Flour Samples From Treated and Untreated YE Beans

Protein content in the raw YE bean flour was 26.5%, and the highest value was observed in the flour from the dehulled/soaked beans (27.9%). Similar protein yields (20–30%) have been reported in the literature for other beans (Mundi and Aluko, 2012). The flour from the raw and soaked samples registered the highest ash content, 4.1 and 4.2%, respectively, whereas the lowest ash value of 3.6% was registered in flour samples from dehulled/soaked and dehulled/germinated seeds (**Table 1**).

Characterization of Alkaline and Salt Extracted Bean Protein Concentrates

Protein Extraction Efficiency

Extraction yields and protein recovery yields for alkaline extracted protein concentrates (APCs) ranged from 12.2 ± 0.5%—14.9 ± 0.28% and 43–56%, respectively (**Table 2**). Concentrate yields and protein recovery for salt extracted protein concentrates (SPCs) were much lower, ranging from 4.2–6.4% and 16–23% respectively. Ammonium sulfate because of its high solubility in water and negligible effects on protein has been the preferred salt used to extract protein fractions from other pulses including mungbeans, (Mendoza et al., 2001), and cowpea (Rangel et al., 2003). However, compared to alkaline extraction methods, protein yields from salt concentrates are often lower. Karaca et al. (2011) reported lower protein yields in SPCs for chickpeas and lentils. In addition, Yang et al. (2021) also reported lower protein yields in extracts from yellow peas, and these observations were attributed to greater carbohydrate solubility in salt solutions. Sathe (2002) emphasized that carbohydrates are the major non-protein components in beans; and their complete removal is required for adequate protein separation. Other factors including high salt concentrations as well as the presence of lipids have been shown to impact protein solubilization which in turn impacts the protein yield (Aluko, 2004; Deak et al., 2006). **Table 2** shows the carbohydrate (59.3–64%) and lipid (0.8 to 1.4%) values reported in the present study which are similar to published results (Karaca et al., 2011). The low lipid contents in the flour samples, suggests that in this instance, lipids may not have a significant impact on the protein extraction yields, however, the interfering effect of carbohydrates cannot be ruled out.

Both germination and dehulling pre-treatments impacted the yields of the protein concentrates generated from the alkaline and salt extractions. Compared to the raw beans used for alkaline extractions (RBA), germination decreased the yields in concentrates from beans that were germinated then alkaline extracted (WGA). Yields were also decreased in samples that were dehulled/germinated before undergoing a similar extraction process (DGA) (**Table 2**). The concentrate yield and recovery yield of the whole germinated seeds that underwent salt extractions (WGS) were also significantly decreased when compared to concentrates obtained from beans that were soaked followed by protein extraction using salts (WSS). These observations are opposite to the results of Sofi et al. (2020),

TABLE 1 | Ash and protein content of flour samples generated from treated and untreated yellow eye (YE) beans.

Flour samples	Raw beans	Soaked	Whole germinated	Dehulled/ soaked	Dehulled/ germinated
Protein content (%)	26.5	26.8	25.7	27.9	27.7
Ash content (%)	4.1	4.2	3.7	3.6	3.6
Carbohydrate (%)	59.3	62.8	65.1	63.1	64
Total Lipids (%)	0.8	1.2	1.4	1.31	1.4

All analyses were carried out at the Department of Agriculture and Food Operations Laboratory (Truro, NS). Replicate data were not obtained therefore, no additional data analysis could be determined.

TABLE 2 | Protein extraction efficiency of untreated and pre-treated Yellow Eye bean protein concentrates prepared by alkaline and salt-extraction methods.

Sample	Extraction yield (%)	Protein recovery yield (%)	Protein purity (% protein)
(i) Alkaline extracted protein concentrates			
Raw bean	14.9 ± 0.30^{a}	56.1 ± 1.10^{a}	93.3 ± 1.50^{a}
Whole soaked	13.4 ± 0.70^{ab}	50.1 ± 2.61^{b}	98.6 ± 0.94^{b}
Whole germinated	12.2 ± 0.50^{b}	47.5 ± 1.90^{b}	98.7 ± 0.41^{b}
Dehulled soaked	13.4 ± 0.71^{ab}	48.0 ± 2.54^{b}	98.0 ± 0.60^{b}
Dehulled germinated	12.2 ± 0.71^{b}	43.8 ± 2.60^{b}	91.3 ± 2.91^{a}
(ii) Salt extracted protein concentrates			
Raw bean	4.26 ± 0.31^{c}	16.1 ± 1.20^{c}	76.8 ± 0.41^{c}
Whole soaked	6.19 ± 0.22^{d}	23.1 ± 0.82^{d}	75.5 ± 0.94^{c}
Whole germinated	4.31 ± 0.40^{c}	16.8 ± 1.20^{c}	86.2 ± 0.90^{c}
Dehulled soaked	5.87 ± 0.52^{d}	21.1 ± 1.90^{cd}	89.3 ± 0.91^{cd}
Dehulled germinated	6.34 ± 0.63^{d}	22.9 ± 2.30^{d}	90.0 ± 1.50^{d}

[1]*Extraction yield, recovery yield and protein content values shown are the mean ± the standard deviation (n = 3). Values with different letters in each column represent significant differences ($p < 0.05$) by Tukey's test.* [2]*The extraction yields (%) represent the weight of protein concentrate relative to the total weight of bean flour used for the extraction.* [3]*The protein recovery yields (%) represents the weight of protein concentrated relative to the protein composition of the beans.* [4]*The protein purity (%) represents the concentration of protein (determined by the Bradford assay) relative to the total weight of freeze-dried protein concentrate.*

who found a slight increase in yields from alkaline extracted protein concentrates generated from germinated chickpeas. This is potentially due to differences in germination time, as Sofi et al. (2020) used a shorter time of 48 h, whereas the beans in the present study were left to germinate for up to 72 h. Indeed, a study by Rumiyati and Jayasena (2012) highlighted a decrease in protein concentrate yield in dehulled/germination Australian sweet lupin APIs starting at 72 h of germination, and a continued decrease in concentrate yield from 17.6 ± 3.8% on day 3, to 4.7 ± 0.4% on day 9 of germination. Thus, it is likely that by 72 h, protein was being used as an energy source of germination in common beans (Ali and Elozeiri, 2017), which might offer a possible explanation for the lower extraction yields reported in the present study. For example, using the alkali method, the extraction yield registered for the germinated samples was 12.2 ± 0.49 % compared to 14.9± 0.28% for the raw YE bean (**Table 2**). Lower yields were observed in the salt extracted concentrates, 4.26 ± 0.31% for the raw beans vs. 4.31 ± 0.38% for the germinated samples. It is well known that beans are composed of different protein fractions, however, differences in the solubility of these fractions may have contributed to the lower yields obtained in the composite protein concentrates. Boyle et al. (2018) also highlighted that extraction procedures that combine more than one extraction solvent, can maximize solubilization of different protein fractions, which in turn improves extraction yields.

Dehulling did not improve the protein yields in the alkaline extracted concentrates, however, the dehulled salt concentrates showed a significant increase in yield compared to the concentrates generated from raw beans whose proteins were extracted with salt solutions (RBS). Seed coats primarily consist of carbohydrates and only contained 2–8% protein (Zhong et al., 2018). Therefore, removing the seed coat can increase the amount of protein to non-protein components, thus increasing concentrate yield.

Combined pre-treatments of dehulling and germination also improved protein extraction efficiency in the salt extracted samples (DGS). However, this was not observed for similar pre-treated beans that underwent alkaline extractions (DGA), in these samples, concentrate and protein recovery yields both decreased compared to concentrates from untreated beans (RBA) as well as those from beans that were soaked prior to alkali extractions, the WSA concentrates. These differences may be due to the presence of condensed tannins and protein-tannin interactions, which can form tannin-protein complexes and reduce protein solubility under alkaline conditions (Tajoddin et al., 2014; Shen et al., 2020). Because the seed coats were removed after germination in the present study, it is possible that these condensed tannins diffused into the cotyledon during the germination process (Chagas and Santoro, 1997). Condensed tannins can be extracted through alkaline methods and can form tannin-protein interactions which have been found to limit extraction yields at pH 8.0 (Brouwer et al., 2019). Moreover, the addition of salt solutions to proteins has been linked with decreased protein-tannin affinity (Kilmister et al., 2016).

Physiochemical Characteristics of Protein Concentrates

Significant variations were observed in the physiochemical properties of the untreated and pre-treated concentrates obtained from salt and alkaline extractions. As hypothesized, the measured physiochemical properties of the salt extracted concentrates exhibited reduced conformational changes when compared to

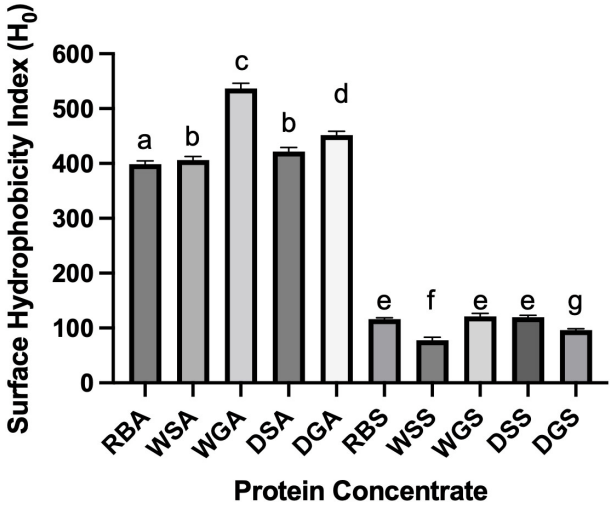

FIGURE 2 | Surface hydrophobicity index (H_0) of alkaline- and salt-extracted Yellow Eye bean protein concentrates ($n = 3$). Different letters (a to g) represent significant differences by Tukey's multiple comparison test ($p < 0.05$).

the alkaline extracted concentrates, which showed signs of protein denaturation. In addition, the pre-treatments resulted in changes to the tertiary structure of the protein concentrates and the exposure of more hydrophobic surface regions in the alkaline extracted concentrates, as indicated by the higher surface hydrophobicity values ranging from 398 ± 0.7–537 ± 9.6, (**Figure 2**). The opposite was observed in concentrates from salt extraction solutions where hydrophobicity values ranged from 77.6 ± 5.7 to 121.4 ± 5.3. Similar results were also reported by Yang et al. (2021) who recently showed that pea protein isolates extracted by an alkaline method exhibited a greater effect on protein conformation compared to protein extracted using salt solutions; higher surface hydrophobicity values (847.9 ± 32.9) were also reported for the alkaline extracted pea concentrates compared to the salt extracted counterparts (732.2 ± 42.8).

Variations in the physiochemical characteristics of the concentrates may be attributed to: (1) the types of proteins extracted and isolated during each extraction process and, (2) the conformation and structural changes to the proteins that occurred during the extraction process (Stone et al., 2015; Yang et al., 2021). Differences in the physiochemical properties may also occur because of the types of proteins extracted using salt vs. alkaline techniques. For example, salt extraction procedures typically isolate both globulins and albumins, whereas alkaline extraction methods primarily extract and precipitate the globulin fractions due to the pH of the precipitation step (Karaca et al., 2011; Tanger et al., 2020). Differences in protein fractions were also observed in the SDS-PAGE data, as the alkaline extracted concentrates had thicker bands because of the globulin subunits (40 and 45 kDa) whereas a smaller albumin band (27 kDa) was observed in the salt extracted concentrates (**Figure 3**). This alteration of the globulin/albumin ratio can influence the physiochemical characteristics of the protein. Importantly, since globulins tend to be more hydrophobic than albumins (Mundi and Aluko, 2012), this property can explain the higher surface hydrophobicity (H_0-ANS) observed in alkaline concentrates compared to those from the salt extraction (**Figure 3**). In addition, globulins can also dissociate into their 11S legumin and 7S vicilin subunits under acidic and alkaline conditions, thus further exposing more hydrophobic side chains which in turn increases surface hydrophobicity (Papalamprou et al., 2010).

The fluorescence spectra of the aromatic amino acids, phenylalanine, tryptophan, and tyrosine can be used as a tool to observe changes in the tertiary structure of proteins caused by solvents used for protein extraction as well as pre-treatments used before extraction (Johnson, 2006). Since bean protein isolates have been reported to contain ∼ 6.0% phenylalanine, and 3.4% tyrosine, (and tryptophan not determined) (Fernández-Quintela et al., 1997; Boye et al., 2010), this intrinsic fluorescence variability may be used to detect differences in the treated and untreated protein concentrates. Similar approaches have been used by other researchers to evaluate structural changes in pea protein isolates (Yang et al., 2021) and black turtle bean protein isolates (He et al., 2020). The maximum fluorescence emission registered for alkaline extracted concentrates in the present study was much lower than that observed for the salt extracted concentrates (**Figures 4A,B**), indicating a decrease of exposed aromatic amino acid residues (Yang et al., 2021). Indeed, a negative correlation was observed between hydrophobicity and intrinsic fluorescence ($r = -0.8725$). This negative correlation may result from the formation of less compact structures which are induced by partial protein unfolding that is promoted by the alkaline conditions (Jiang et al., 2009; Yang et al., 2021). Decreased maximum fluorescence may also be a result of inter-intermolecular hydrophobic interactions of previously exposed tryptophan chromophores, thus promoting aggregation (Shen and Tang, 2012).

In addition, a shift toward higher wavelengths (red shift) was observed in the pre-treated concentrates obtained by alkaline extraction, and the maximum emission wavelength of the soaked, WSA (328 nm), germinated, WGA (329 nm), and dehulled concentrates, DGA (328 nm) increased compared to the alkaline concentrates from raw beans, RBA (327 nm). Conversely, the maximum emission wavelength (327 nm) of the alkaline protein concentrates from dehulled beans, DSA, remained the same (**Figure 4A**). The fluorescence intensity of the germinated alkaline protein concentrates also increased compared to the alkaline protein concentrates from raw beans, RBA. This observation indicates that germination and the required soaking process had created a more hydrophobic environment for the tryptophan residues, thereby increasing the surface availability of hydrophobic amino acids (Ghavidel and Prakash, 2007). Indeed, the surface hydrophobicity of all the pre-treated samples also increased compared to the alkaline protein concentrates from raw beans, RBA (**Figure 2**). All these measurements can be linked to the conformational changes of the extracted proteins (He et al., 2020). Specifically, alkaline protein concentrates from germinated beans, WGA as well as alkaline protein concentrates from dehulled and germinated beans, DGA, had significantly higher surface hydrophobicity

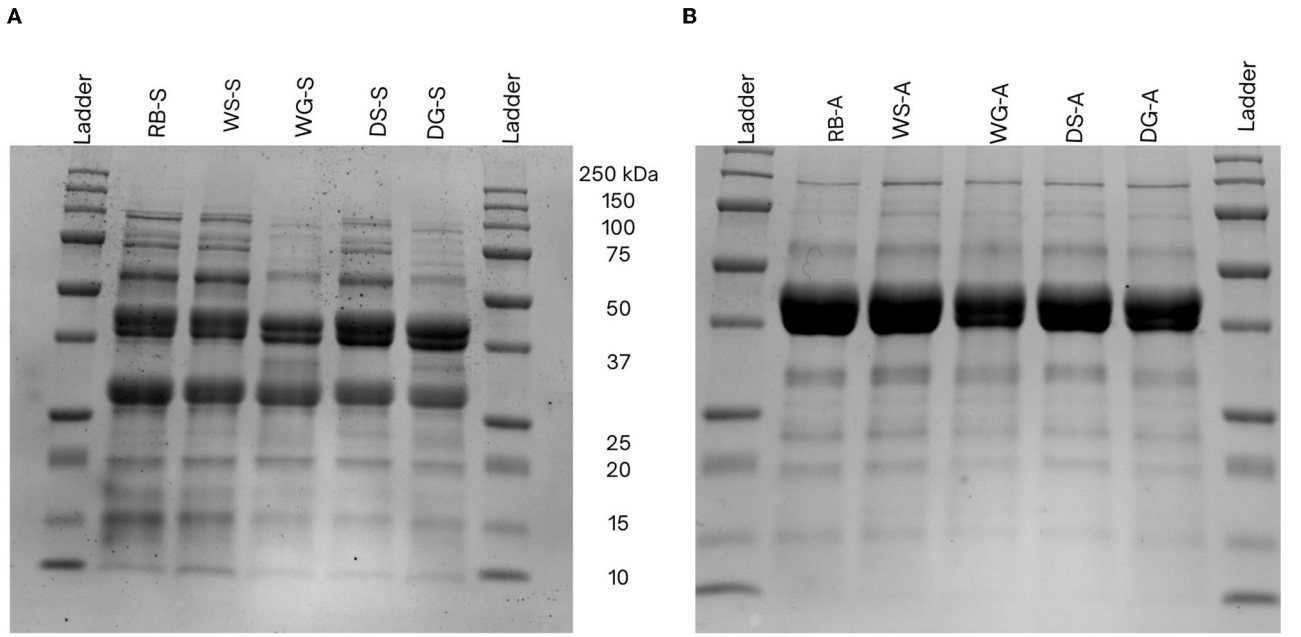

FIGURE 3 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of Yellow Eye bean proteins extracted using salt- **(A)** and alkaline-extraction **(B)** techniques. Molecular distribution was compared to a New England BioLabs Precision Plus pre-stained protein marker (P77066, 10–250 kDa) in lane 1. The samples were separated using a 12% mini-Protean precast gel stained with Coomassie Brilliant Blue (0.1%).

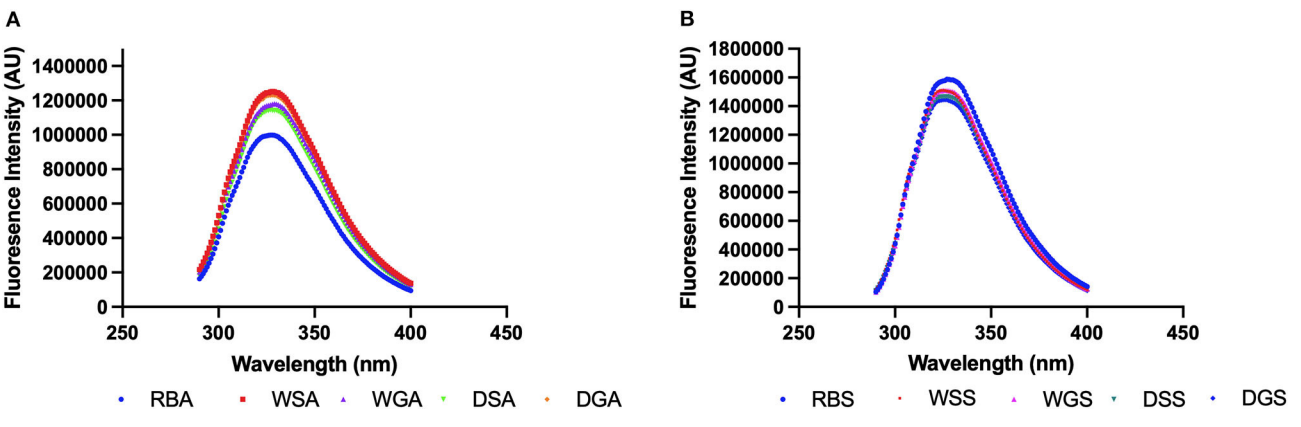

FIGURE 4 | (A) Intrinsic fluorescence measurement of alkaline-extracted protein concentrates and **(B)** salt-extracted protein concentrates prepared from untreated and pre-treated Yellow Eye beans (0.2 mg/mL in 10 mM phosphate buffer, pH 7.2) ($n = 3$).

compared to their ungerminated counterparts. This indicates an increase in exposed hydrophobic sites of amino acids. Enzymatic protein degradation is also one of the mechanisms initiated during germination which can alter protein conformation and/or denaturation and expose hydrophobic areas of the protein, thus increasing surface hydrophobicity (Ghavidel and Prakash, 2007; Zahir et al., 2021).

Figure 4B shows the impact of the various pre-treatments on the fluorescence patterns observed for the protein concentrates generated from salt extractions. A spectral shift toward lower wavelength (blue shift) was observed in the pre-treated, salt extracted protein concentrates, with decreased maximum emission wavelengths for the soaked, WSS (324 nm), germinated, WGS (325 nm), dehulled and germinated, DGS (325 nm) protein concentrates compared to those of the raw, RBS (327 nm) and dehulled salt extracted protein concentrates, DSS (327 nm) (**Figure 5**). In addition, the fluorescence intensity of the pre-treated salt extracted protein concentrates decreased compared to fluorescence intensity from the salt extracted concentrates from raw beans, RBS. This indicated an increase of tryptophan residues exposed to a hydrophilic environment, thus decreasing the surface availability of hydrophobic amino acids (He et al., 2020).

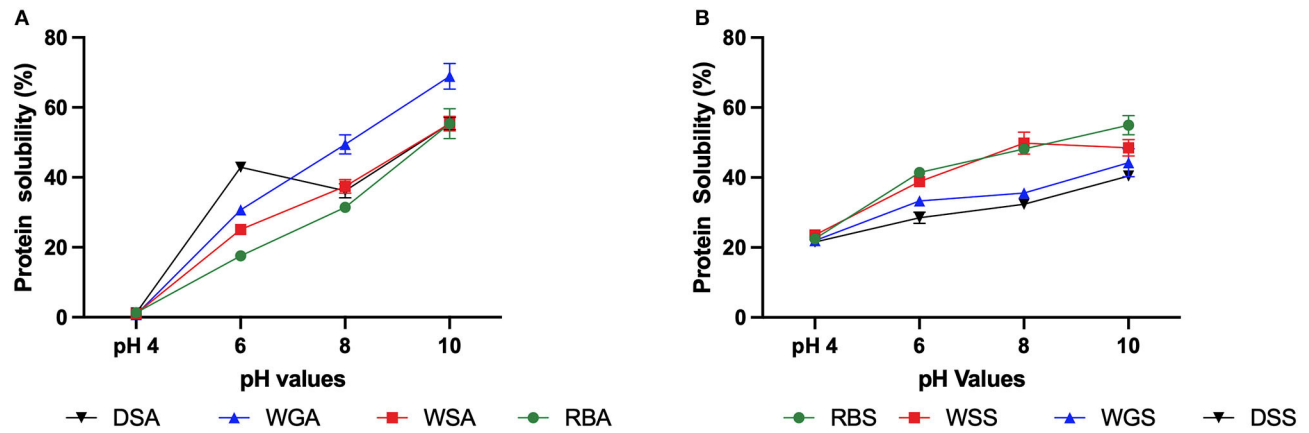

FIGURE 5 | (A) Protein solubility (% protein dissolved, 10 mg/mL in 0.1 M Phosphate buffer, pH values 4 to 10) of untreated and pre-treated Yellow Eye bean protein concentrates prepared using alkaline extraction; **(B)** and salt solutions ($n = 3$).

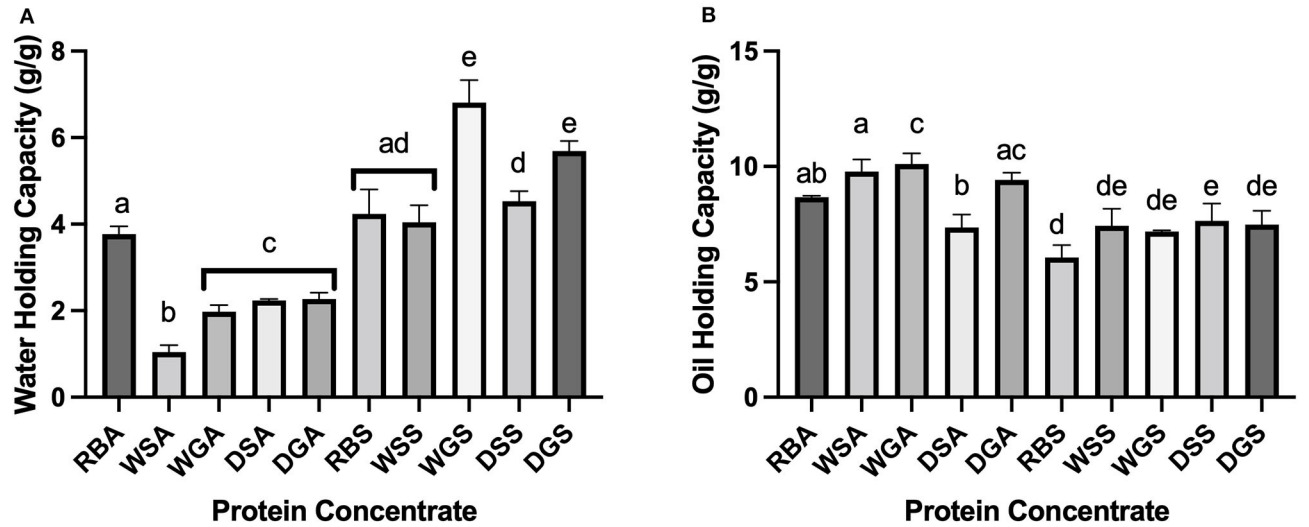

FIGURE 6 | (A) Water holding capacity of alkaline- and salt-extracted Yellow Eye bean protein; **(B)** Oil holding capacity of alkaline- and salt-extracted Yellow Eye bean protein concentrates. Different letters (a to e) represent significant differences by Tukey's multiple comparison test ($p < 0.05$), ($n = 3$).

In particular, the surface hydrophobicity of the soaked (WSS) and dehulled, (DSS) salt extracted protein concentrates decreased compared to the hydrophobicity of the salt extracted protein concentrates from raw beans, RBS, which is likely due to the leaching of insoluble proteins into the soaking water (Barak et al., 2014). However, it should be noted that the surface hydrophobicity registered for the salt extracted protein concentrates from the germinated, beans (WGS) was significantly higher when compared to the soaked samples (WSS). These changes can be linked to the degradation of nutrients used for energy during germination and the increased exposure of hydrophobic amino acids (Xu et al., 2020; Zahir et al., 2021).

Protein Functionality

The conformation of a protein has long been established as an important property that can impact protein functionality (Boye et al., 2010). Contrary to the initial hypothesis, salt extracted protein concentrates exhibited decreased functionality when compared to the alkaline extracted concentrates, particularly relating to protein solubility. The solubilities of the alkaline extracted protein concentrates were significantly impacted by pH, as the concentrates precipitated at pH 4.0, and then solubility steadily increased as the pH of the concentrates also increased (**Figure 5A**). Interestingly, the impact of pH on salt extracted concentrates was less severe (**Figure 5B**). This can once again be attributed to the type of protein fractions extracted.

The isoelectric point of globulins is pH 4.5, which explains the insolubility of alkaline extracted protein concentrates at pH 4.0 (Tanger et al., 2020). The combination of albumin and globulin protein fractions extracted in the salt extracted protein concentrates would explain the lack of precipitation at pH 4.0, as albumin has been found to be more soluble than globulin at

pH 4.0 (Makeri et al., 2017). It should be noted that the solubility of both alkaline and salt extracted protein concentrates in this study are typically lower than other pulse protein concentrates, particularly at more alkaline pH values (Mundi and Aluko, 2012; Shevkani et al., 2015; Ge et al., 2021). Paraman et al. (2007) attributed high insolubility to be a result of higher rates of interpeptide hydrophobic interactions or sulfhydryl-disulfide interactions. These changes can prevent protein-water interactions and thus reduce solubility. A better understanding of the bonds and linkages present in these bean protein concentrates would further elucidate these interactions.

Although the salt extracted concentrates had lower surface hydrophobicity values, these samples demonstrated lower solubility curves compared to raw beans extracted in salt solutions (RBS). Several factors including protein structure, surface charge, and the degree of aggregation can influence protein solubility (Hayakawa and Nakai, 1985). The lower solubility of the pre-treated salt extracted protein concentrates may be a result of intermolecular aggregation of its ß-sheets (Yang et al., 2021). On the other hand, the pre-treated alkaline extracted protein concentrates, particularly the germinated (WGA) and dehulled/germinated (DGA) samples, had increased solubility curves compared to the alkaline extracted protein concentrates from raw beans (RBA). This can be attributed once again to protein hydrolysis of globulins and the exposure of hydrophilic proteins for protein-water interactions (Cao et al., 2010).

Due to an increase in hydrophobic surface regions, the alkaline protein concentrates exhibited more hydrophobic functional characteristics, including increased OHC and decreased WHC when compared to the salt extracted concentrates. The higher OHC and lower WHC of alkaline protein concentrates compared to salt extracted concentrates can be explained by variations in protein conformation (**Figures 6A,B**). The exposure of hydrophobic side chains produced through the alkaline extraction process may be responsible for the higher OHC and the relatively low solubility of alkaline proteins (**Figures 5A**, **6A**) compared to the literature (Karaca et al., 2011). In theory, an increase in hydrophobicity would result in a decrease of hydrophilic groups on the surface of the protein, thus, limiting the potential for protein-water interactions and decreasing WHC (Stone et al., 2015; Mohan and Mellem, 2020). Indeed, our own observations showed that surface hydrophobicity was positively correlated with OHC ($r = 0.877$) and negatively correlated with WHC ($r = -0.618$).

Variation in pre-treatment effects on OHC, WHC, and protein solubility were also observed. In general, pre-treated alkaline extracted protein concentrates had decreased WHC compared to similar concentrates from raw beans, RBA. This can be attributed to the increase in surface hydrophobicity properties. In addition, the removal of water-binding matrix components including starch and fiber have been found to decrease WHC in yellow field pea alkaline extracted protein concentrates (Agboola et al., 2010). It is also possible that the extracted protein was denatured during the extraction process, which can also correlate with decreased WHC (Mohan and Mellem, 2020). These phenomena are most likely responsible for the decreased WHC of all other pre-treated alkaline extracted protein concentrates compared to similar extracted concentrates from raw beans (RBA). Conversely, the germinated salt extracted protein concentrates (WGS) and the germinated/ dehulled salt extracted concentrates (DGS) had significantly higher WHCs compared to salt extracted concentrates from raw (RBS) and soaked (WSS) beans. This can be explained by the increase in soluble proteins generated during proteolysis (Sofi et al., 2020).

Despite increases in hydrophobic properties in the alkaline extracted protein concentrates, no significant differences in OHC were established between the concentrates from raw beans (RBA) and other alkaline extracted concentrates. However, the OHC of dehulled, alkaline extracted concentrates DSA was significantly lower than that of the soaked, WSA, germinated, WGA and dehulled DGA, alkaline extracted protein concentrates. Indeed, the surface hydrophobicity of the dehulled concentrates (DSA) was significantly lower than the dehulled/germinated (DGA) alkaline extracted concentrates, and there was no red shift in maximum emission wavelength. These changes can be attributed to a decrease in exposed hydrophobic amino acids, potentially due to tannin-protein interactions (Pal et al., 2017). Interestingly, despite the lower surface hydrophobicity of the dehulled sample (DSS), its OHC was significantly higher than that of the raw beans (RBS). The solubility curve of the salt extracted dehulled concentrate (DSS) was also decreased compared to concentrates from raw beans (RBS). Conformational changes that occurred during the extraction process may have attributed to these changes (Yang et al., 2021).

Although the salt extracted concentrates had lower surface hydrophobicity values, these samples demonstrated lower solubility curves compared to raw beans extracted in salt solutions (RBS). Several factors including protein structure, surface charge, and the degree of aggregation can influence protein solubility (Hayakawa and Nakai, 1985). The lower solubility of the pre-treated salt extracted protein concentrates may be a result of intermolecular aggregation of its ß-sheets (Yang et al., 2021). On the other hand, the pre-treated alkaline extracted protein concentrates, particularly the germinated (WGA) and dehulled/germinated (DGA) samples, had increased solubility curves compared to the alkaline extracted protein concentrates from raw beans (RBA). This can be attributed once again to protein hydrolysis of globulins and the exposure of hydrophilic proteins for protein-water interactions (Cao et al., 2010).

CONCLUSIONS

This study assessed the impact of dehulling and germination and a combination of these pre-treatments on the protein extraction efficiency, physiochemical properties, protein solubility and the water/oil holding capacities of alkaline, and salt extracted protein concentrates from YE beans. In general, alkaline extracted protein concentrates had a higher protein extraction efficiency compared to salt extraction samples, however the former was more susceptible to conformational changes as indicated by the increase in surface hydrophobicity. This increase in hydrophobic surface regions, observed in the alkaline extracted protein concentrates resulted in an increase in OHC and a decrease in the WHC when compared to the salt extracted concentrates. When applied individually, dehulling of beans resulted in protein concentrates with increased yields in the salt extraction solutions

compared to the protein concentrates generated from raw beans when proteins were extracted in salt solutions. Conversely, beans that were germinated prior to protein extraction registered greater surface hydrophobicity when compared to the soaked samples. Moreover, the combination of dehulling and germination pre-treatment decreased protein extraction efficiency and functionality when compared to the untreated and soaked alkaline extracted protein concentrates. The combined pre-treatments also resulted in improved protein extraction yields, physiochemical properties, and the functionality of the salt extracted concentrates. The beneficial effect observed from the combined pre-treatment (dehulling/ germination) on the salt extracted protein concentrates suggests that these protein samples may be good potential candidates for further studies that evaluate their application in different food formulations.

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

LV co-designed the study, collected all data, interpreted results, drafted the manuscript, and approved the submission version. ME co-designed the study, supervision, and resources, edited, and proofread the manuscript, and approved the submission version. All authors contributed to the article and approved the submitted version.

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