Optimising amino acid composition and protein digestion of pea-based foods.

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Abstract

As world population growth continues to pressure agricultural development, the need for developing sustainable, environmentally friendly food alternatives becomes increasingly important. In Asia, tempeh fermentation has long been used to improve the nutritional value of soybean and other legumes to create meat alternatives. By combining tempeh fermentation with locally grown substrates, new alternatives can be created, increasing nutritional quality while reducing environmental impact of intercontinental import. Here, a critical examination of the tempeh fermentation process and the factors governing the nutritional composition of field pea aims to assess the potential of field pea tempeh as a high-quality meat analogue. Field pea can contain up to 37.1% dw protein and a variety of beneficial micronutrients. Its indispensable amino acid profile is relatively well-balanced, limited by methionine, cysteine and tryptophan. Tempeh fermentation using *Rhizopus* spp. improves protein content, protein solubility and free amino acid content. In addition, tempeh fermentation produces several vitamins while reducing levels of anti-nutrients and flatulence-causing oligosaccharides. The nutritional value of field pea and field pea tempeh is strongly influenced by cultivar and environmental conditions, and can be improved through substrate mixing, co-inoculation and genetic modification. Combining tempeh fermentation with improved field pea protein quality has the potential to produce a high-quality meat alternative.

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Layman's Summary

As the world population keeps growing, more pressure is put on agriculture and the environment to produce food sustainably. Due to the big impact of the meat industry, finding alternatives to meat products is an important challenge. In Asia, people have been using tempeh fermentation to make meat substitutes from soybeans and other plants for a long time. By combining the tempeh fermentation process with locally grown crops, it may be possible to create new meat alternatives. In this review, the effect of tempeh fermentation and the nutritional content of field pea are discussed. Field pea is a great source of protein and other important nutrients. When fermented with one of several fungi from the *Rhizopus* genus, the protein content can be improved, and made to be digested more easily. Fermentation can also add a variety of vitamins and remove compounds that can make us feel bloated. The nutrition of field pea and the tempeh made from it depends strongly on the cultivated variety and the environment it grows in. By mixing field pea with other grains or seeds, combining different fungi and bacteria or using genetic modification, it can be possible to make a high-quality meat alternative that is healthy not only for us but also for the planet.

Introduction

In November 2022, the world's population surpassed 8 billion people. Prospects for the near future estimate it will have reached 9.7 billion by 2050 and over 10.4 billion by the turn of the century (UN Population Division, 2022)[1]. As the world's population continues to grow, so does the demand for food, which is expected to increase by 35% to 56% over the period of 2010 to 2050 (van Dijk *et al.*, 2021)[13]. Everything related to the production of food, from raw materials until it reaches the consumer, is part of the food supply chain. Globally, it is responsible for more than 26% of all anthropogenic greenhouse gas emissions (GHG), 32% of terrestrial acidification and 78% of eutrophication. The majority of these effects are directly caused by agriculture, which produces 81% of GHG emissions, including 20% caused by farming-related deforestation, 79% of acidification and 95% of eutrophication (Poore & Nemecek, 2018)[2]. Already this has resulted in significant and long-lasting changes to the planet's ecosystems, altering and diminishing biodiversity and ecological resilience (Bouwman *et al.*, 2002)[3]. In 2021, agriculture covered 4.8 billion ha, about 37% of the world's total land area, encompassing most available arable land with the remaining 63% being roughly equally divided between forests and barren land (including deserts, urban land and infrastructure). Agricultural land consists of 33% cropland and 67% meadows and pastures (FAO, 2023)[4]. Already the mounting pressure on our agricultural system is showing, as increase in arable land over the last few decades has come largely at the expense of tropical rainforests, and farming has intensified predominantly due to significantly increased use of fertiliser

and the reduction of fallow land. This is reflected in the fact that cropland per capita has decreased by about 18% over the last 20 years (Foley *et al.*, 2011; FAO, 2023)[4,5].

Alongside the rising demand for food due to population growth, developing countries show an increase in wealth, urbanisation and socioeconomic change. This shifts consumption patterns towards a more western diet, increasing the demand for commodities such as meat, fat and processed foods (Kearney, 2010)[6]. Unfortunately, this further exasperates the problems faced with feeding the world population, as animal products require large amounts of energy and water to produce. They are also highly inefficient in terms of energy and land-use, as 67% of agricultural land is used for pastures, and half of all cropland is used for animal feed, while animal products only contribute 18% of energy to our diet on average (FAO, 2023; Alexander *et al.*, 2015; Alexander *et al.*, 2016)[4,7,8].

While increasing arable land at the cost of forests and further intensification of our current agricultural practices is one possible solution for the projected increase in demand for food, it will enact a high cost on our planet's already pressured ecosystems. A more sustainable alternative would be to reduce the consumption of animal products as part of our diet. However, traditions and beliefs concerning meat-consumption are generally strong, and substitution of meat with vegetables or fungi of equal nutritional value is generally not well received (Leroy & Praet, 2015)[14]. Fortunately, meat analogues generally gain better reception, and as a result, scientific and commercial interest in them has greatly increased in recent years. Meat analogues consist predominantly of soy protein, owing to its high availability, low price and similar protein digestibility-corrected amino acid scores (PDCAAS) comparable to meat. Wheat, and more specifically gluten, is often present in small quantities due to its excellent ability to act as a binding agent. Other sources of protein found in meat analogues come from legumes, rice, potato, fungi and seaweed (Boukid, 2020; Chiang *et al.*, 2019)[9,10].

While fungal fermentation is well known for its use in the production of desirable flavours and alcohol, it can also offer a variety of beneficial effects to improve nutritional quality. An example of this is tempeh, a traditional Indonesian food which is made by fermenting soybeans with *Rhizopus* spp., which has been a part of the Indonesian diet for over 300 years (Shurtleff & Aoyagi, 2020)[11]. During the production of tempeh, fermentation can decrease levels of antinutrients, allergens and other undesirable compounds, increase protein quantity and quality, and promote the production of vitamins (Ahnan-Winarno *et al.*, 2021)[12]. Despite great scientific interest in soy products from 1990 onwards, tempeh has received relatively little attention until recently. In addition, alternatives to soy as the main ingredient for tempeh are being considered, with a variety of results (Ahnan-Winarno *et al.*, 2021)[12]. One such alternative is pea (*Pisum sativum*) which is grown worldwide and is well-suited for long-term storage like other legumes. Peas are a highly condensed source of protein and contain fibre, carbohydrates and a variety of minerals and vitamins (Frías *et al.*, 2010)[15]. While protein content is high, pea-protein is of limited quality compared to animal products due to its lower digestibility and

limited amino acid profile (FAO, 2011)[16]. Additionally, peas contain antinutrients and undigestible oligosaccharides, causing flatulence. Through preparation and tempeh fermentation, pea protein quality may be improved, and levels of antinutrients and undesirable compounds may be reduced. The objective of this paper is to combine the current state of tempeh research with pea production and processing literature. This review aims to provide an understanding of the potential of creating a high-quality meat analogue through pea tempeh fermentation in order to contribute to a more sustainable global food supply.

Soybean tempeh

Originally perceived as a lower-class food eaten as an affordable alternative to meat or fish, tempeh carries with it a long tradition and historical significance to the Indonesian people. Its popularity increased during the latter half of the 20th century, becoming a staple of the Indonesian diet regardless of socio-economic standing. Since the 1980's tempeh has also gained a following internationally, especially among vegetarians. An estimated 2.4 million tons of tempeh was produced in Indonesia in 2012, predominantly by small-scale producers using traditional methods. Comparatively, international tempeh production has modernised to accommodate large-scale production and increased consistency. The global tempeh market is estimated to be worth US$ 4.2 billion (GIA report, 2023)[37]. Indonesian cuisine includes a wide variety of tempeh products in terms of production methods and substrates. In addition to soybean, substrates such as soy pulp (a byproduct of tofu pressing), coconut press cake (a byproduct of coconut oil production) and peanuts are used (Romulo & Surya, 2021)[36]. Tempeh is also distinguished depending on fermentation time, resulting in products with different organoleptic properties. Examples are *tempe gembus* (fresh tempeh, 20 to 30 h fermentation), *tempe semangit* ('day-old tempeh', around 48 h) and *tempe bosok* (lit. 'rotten tempeh', up to 72 h), each with their own culinary uses. Despite this variety, tempeh outside of Indonesia is almost exclusively available as fresh soybean tempeh.

Production process

While all soybean tempeh production involves the general processes of substrate preparation, inoculation, packaging and incubation, it does not necessarily follow a unified set of steps and varies between tempeh producers. The largest variation between producing methods can be found during the preparation of raw soybeans, where some methods soak and dehull the beans before boiling, while others boil them first, boil them twice, or omit the soaking step altogether. Inoculation, packaging and incubation are always the last three steps of the process, and vary depending on *Rhizopus* species and strain, co-inoculation, type of packaging material, aeration, duration and temperature (Ahnan-Winarno

et al., 2021)[12]. All beans must be dehulled before inoculation as hulls are considered to be a contaminant in the final product (FAO Codex, 2017)[17]. Dehulling can be performed mechanically on dry beans but is generally preceded by soaking, boiling or both. Soaking makes the beans tender and easier to peel and is done using tap water at 20 to 37° C for 6 to 36 h. While soaking facilitates dehulling, it also enables the growth of a variety of pathogens, predominantly *Lactobacillus casei*, *Streptococcus faecium*, *Streptococcus dysgalactiae* and *Staphylococcus epidermidis* (Mulyowidarso *et al.*, 1989)[18]. Soaking of raw soybeans causes natural acidification of the water, which slows or inhibits the growth of these pathogens, but if boiling is performed before soaking, acidification no longer occurs. To counteract this, some producers artificially lower the pH using food additives. Another method used is the addition of *Lactobacillus plantarum* to the soaking water, which consistently inhibits growth of pathogens and other microorganisms, and may act as a probiotic (Ashenafi & Busse, 1989; Ashenafi & Busse, 1992)[19,20]. Boiling is generally performed for 20 to 30 m and serves to both eliminate the undesirable raw soybean flavour and kill pathogens and other microorganisms. Next, the beans are drained to remove excess water which may adversely affect the fermentation process. Optimal relative humidity ranges from 60% to 90%, although it has been reported that values above 75% may cause undesirable sporulation during fermentation (Nout & Rombouts, 1990)[21]. Inoculation is performed by mixing the prepared beans with *Rhizopus* tempeh starter before being packaged. Traditionally, packaging involved the use of banana or teak leaves, but this practice has mostly been replaced in favour of using perforated polyethylene bags due to ease of use and worldwide availability. While convenient, this change in packaging does have an effect on the final product. Certain flavour-promoting aromatic compounds were found to be associated with the type of packaging used (Ahnan-Winarno *et al.*, 2021)[12].

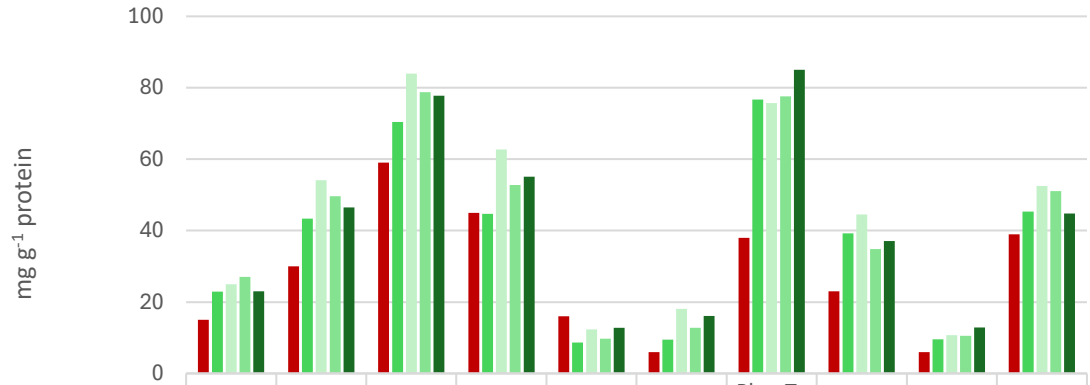
The creation of traditional tempeh starter cultures called *usar* involves the use of *waru* (*Hibiscus* spp.) leaves. The leaves are prepared by pressing a small amount of inoculated substrate between them for up to 24 h, allowing the growing mycelium to adhere to the roughly textured underside of the leaves. The substrate is then discarded and the leaves are dried and stored for up to 6 weeks at 25 to 30° C (Nout *et al.*, 1992)[26]. Modern starter cultures are prepared by growing *Rhizopus* spp. on rice powder, which is then desiccated and can be stored for over a year at room temperature (Ahnan-Winarno *et al.*, 2021)[12]. Starter cultures generally contain either *R. oligosporus*, *R. arrhizus* or *R. stolonifer*. Due to industrial upscaling and widespread commercialisation of standardised starter, *R. oligosporus* has become the predominant species for tempeh production worldwide. This resulted in a huge loss of starter diversity in Indonesia, where a few commercial brands replaced a multitude of cultivated species and strains (Sjamsuridzal *et al.*, 2021)[27]. *R. oligosporus*, alternatively named *R. microsporus* var. *oligosporus*, is considered to be a domesticated variant of *R. microsporus* which lacks the ability to produce several mycotoxins compared to its wild counterpart. *R.arrhizus*, also known as *R. oryzae*, is

currently debated to be two species, or variants, suggested to be named *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *delemar*. The distinction was only recently discovered through the use of rDNA Internal Transcribed Spacer (ITS) sequencing, and their effects on tempeh fermentation may differ despite the high genetic similarity (Dolatabadi *et al.*, 2014)[39].

Although the progression of tempeh fermentation is not strictly defined, it can be generally divided into three phases. The first phase lasts 30 to 36 h and is characterised by rapid mycelial growth, high lipase and protease activity, alkalisation, increased temperature, bacterial growth and a loss of total biomass (Sudarmadji & Markakis, 1978; Ruiz-Terán & Owens, 1996)[23,24]. Temperature and pH at the start of this phase are generally around 30° C and pH 4. Both increase during fermentation, with temperatures exceeding 42° C if not regulated. Growth of *R. oligosporus* on potato dextrose agar shows a correlation between temperature and pH, with optimal growth occurring around 37 °C at pH 3.5 compared to 42 °C or higher around pH 5.5 to 7.5. For optimal growth, water activity should be above 0.98 (Sparringa *et al.*, 2002; Sarette *et al.*, 1992)[22,38]. After 30 to 36 h, fermentation enters the maturation phase. Growth slows down markedly as fungal biomass approaches its peak, and temperature decreases. Protease and lipase activity continues at a lower rate, and there is no further loss of total biomass during this stage. Fermentation enters the third phase after 54 to 60 h, which is characterised by mycelial senescence, progressive discolouration and recommencement of bacterial growth. Protease activity increases sharply, and a further loss of total biomass is seen, primarily due to the consumption of lipids. Alkalisation continues, reaching pH 7 around 70 h (Sudarmadji & Markakis, 1978; Ruiz-Terán & Owens, 1996)[23,24]. Tempeh can be harvested at any time during fermentation depending on the desired organoleptic properties of the final product. Young tempeh has a mild taste, retaining some of the soybean flavour and has relatively little umami flavouring. Older tempeh tastes more fungal, and umami flavouring is stronger. Aged tempeh called *tempe bosok* may be fermented for as long as 70 h, has a slight bitterness to it, and contains high quantities of umami flavoured compounds, which reach their peak around 72 h (Utami *et al.*, 2016)[25].

Proteins, lipids and carbohydrates

During tempeh fermentation, soybean protein is hydrolysed and partially assimilated into fungal biomass. Despite relatively little change in total crude protein, soluble protein content increases significantly, up to 66.4% at 35 h (Ashenafi & Busse, 1991)[40]. At 46 h, an estimated 25% of the initial protein is hydrolysed, 25% of which is assimilated into fungal biomass, 10% is oxidized and 65% remains in the tempeh as amino acids and peptides (Nout & Kiers, 2005)[41]. The amino acid composition of soybean tempeh remains relatively unchanged during fermentation, although some minor changes can occur depending on production conditions or fungal strain. These changes are limited to at most a 20% increase or decrease to one or two amino acids (Stillings & Hackler, 1965; Murata *et al.*, 1967)[42,43].



	His	Ile	Leu	Lys	Met	Cys	Phe+Tyr	Thr	Trp	Val
recommended daily intake	15	30	59	45	16	6	38	23	6	39
USDA reference tempeh	23	43	70	45	9	10	77	39	10	45
Tempeh A	25	54	84	63	12	18	76	44	11	52
Tempeh B	27	50	79	53	10	13	78	35	11	51
Tempeh C	23	47	78	55	13	16	85	37	13	45

Figure 1. *Comparison of the WHO/FAO recommended daily intake of indispensable amino acids and the amino acid composition of standard reference soybean tempeh (USDA, 2018)[45], and three tempeh grown for research purposes (mg g^{-1} protein). Tempeh A (Indonesian, 48 h) and B (Japanese, 48 h) adapted from (Murata et al., 1967)[43]. Tempeh C (USA, 36 h) adapted from (Stillings & Hackler, 1965)[42].*

Looking at the indispensable amino acid requirement for human adults, the main limiting amino acid in soybean tempeh is methionine (**Fig.1**). Further limiting amino acids are lysine, valine and leucine. A notable difference can be seen in cysteine content between the USDA reference strain and the lab-grown tempeh, possibly due to analysis methods. Despite little change in total amino acid composition, the amount of free amino acids fluctuates markedly during fermentation, generally increasing up to 48 h, some by as much as 85-times compared to the unfermented substrate. As fermentation continues, some free amino acid concentrations decline (threonine, serine, glycine, alanine, arginine) while others continue to increase. With the exception of arginine, all free amino acid concentrations remain higher compared to the unfermented substrate (Murata *et al.*, 1967)[43]. While increased soluble protein and free amino acid concentrations improve nutrient bioavailability, protein digestibility does not necessarily increase concurrently. To quantify digestibility, the protein digestibility-corrected amino acid score (PDCAAS) is generally used. This method takes into account both the suggested required amounts and the human ability to digest each amino acid. More recently, limitations to this method in relation to its truncated scoring method and overcompensation has led to a proposal by the FAO to start using the digestible indispensable amino acid score (DIAAS) instead (Schaafsma, 2000; FAO, 2011)[16,61]. While PDCAAS scores for soybean (1.00) are touted to be equal to meat and dairy products, the more refined DIAAS suggest dairy products to contain protein of higher quality. Despite the

pervasive use of PDCAAS in the food industry, no PDCAAS values for soybean tempeh have been published. Lipase activity increases continuously during fermentation, most rapidly during the growth phase. Free fatty acid content also increases similarly during this phase, stagnating during maturation and eventually declining once senescence begins. Total crude lipid content decreases during the growth phase, remains constant during maturation, then decreases again once the third phase begins (Ruiz-Terán & Owens, 1996)[24]. Most carbohydrates are removed during substrate preparation and reduced further during fermentation. Of particular interest are stachyose and raffinose, two flatulence-causing oligosaccharides found in soybean and other legumes. Several *Rhizopus* spp. including *R. oligosporus*, *R. arrhizus* and *R. stolonifer* are known to consume both oligosaccharides, and a reduction of total saccharide content can be seen during fermentation. Sucrose and starch concentrations are reduced, while stachyose is almost completely eliminated after 72 h. Raffinose concentration increases slightly, which can be explained by the cleavage of stachyose by α-galactosidase into raffinose and a monosaccharide (Nout & Kiers, 2005; van der Riet *et al.*, 1987)[28,41]. Verbascose, a third flatulence-inducing oligosaccharide, is present in soybean at low concentrations (1% of dry weight) but most often omitted from publications (Nowak & Szebiotka, 1992)[47].

Vitamins and other compounds

In addition to increased nutrient bioavailability, tempeh provides several vitamins and other beneficial nutritional compounds. Increased concentrations of riboflavin (B2), nicotinic acid and nicotinamide (B3), pyridoxine, pyridoxal and pyridoxamine (B6), biotin (B7) and folate (B9) have been found depending on species and strain used (van der Riet *et al.*, 1987; Keuth & Bisping, 1993; Murata *et al.*, 1970; Ginting & Arcot, 2004)[28,30,32,33]. Tempeh is also often praised for being one of the few plant-based sources of vitamin B12. However, vitamin B12 is present in soybeans only at very low concentrations (<1 ng g^{-1}) and is not produced by *Rhizopus* spp. used for tempeh fermentation. The presence of vitamin B12 in tempeh is generally caused by contamination by bacteria such as *Klebsiella* spp. or *Citrobacter freundii*. Because of this, vitamin B12 concentrations tend to vary strongly, ranging from 0.7 to 150 ng g^{-1} in commercially available tempeh (Ahnan-Winarno *et al.*, 2021)[12]. *R. oligosporus* was also found to produce significant amounts of β-carotene and ergosterol during fermentation, unlike *R. arrhizus* which did not produce any β-carotene under similar conditions (Denter, Rehm & Bisping, 1998)[31]. Additionally, tempeh fermentation can increase antioxidant activity up to 12-fold compared to unfermented soybean due to the presence of polyphenols (Kuligowski *et al.*, 2017)[35]. Soybeans contain high concentrations of isoflavones, particularly daidzin and genistin, which have received a lot of scientific and medical interest in recent years due to indications of positive effects on disease prevention and health. Tempeh fermentation hydrolyses these isoflavones into their aglycone forms, which are considered to be the more biologically active (Ahnan-Winarno *et al.*, 2021; Yuan *et al.*, 2012)[12,34]. While

most micronutrient content increases or remains constant during fermentation, both thiamine (B1) and potassium concentrations are reduced significantly. After 24 h of fermentation thiamine is no longer detected, and potassium concentration is reduced by up to 43%. Significant amounts of potassium were detected in the condensation on the inside of the packaging (van der Riet, 1987)[28].

Anti-nutritional compounds

Soybean contains several undesirable anti-nutritional compounds which can reduce nutrient bioavailability or be harmful to human health. In several regions where soybean is a significant part of the diet, the presence of phytic acid exacerbates iron-deficiencies due to its high binding affinity to trace elements such as iron, zinc and calcium, resulting in an insoluble precipitate and strongly reducing their bioavailability. Fermentation using *Rhizopus* spp. reduces phytic acid contents of soybean by up to 22% due to the production of phytase, which hydrolyses phytic acid to inositol and phosphoric acid (Sutardi & Buckle, 1985)[44]. Soybean also contains significant levels of oxalate, an anti-nutrient which binds calcium to form insoluble calcium oxalate, a major contributor to the formation of kidney stones. While other known dietary sources such as spinach contain higher concentrations, soybean oxalate nevertheless exceeds recommended amounts for patients with a history of kidney stones (Massey *et al.*, 2001)[55]. Soaking, cooking and fermentation all contribute to the reduction of oxalate concentrations, resulting in tempeh with up to 84.5% reduced oxalate content compared to raw soybean (Haron & Raob, 2014)[56]. Raw soybean contains appreciable amounts of trypsin inhibitors, a group of proteins which reduce protein digestibility due to their inhibiting effect on both trypsin and chymotrypsin. Additionally, trypsin inhibitors are known to have various deleterious effects on human health, such as pancreatic enlargement and cancer. The cooking step during tempeh substrate preparation strongly reduces trypsin inhibitor activity (Vagadia *et al.*, 2017)[58]. Another heat-labile anti-nutrient present in soybean are lectins, a diverse group of proteins present in most plants. Soybean lectins have various anti-nutritional effects, reducing digestibility of proteins and polysaccharides due to their affinity for binding saccharide-groups. They are also linked to a wide range of negative health effects such as deterioration of the intestinal walls and degenerative organ damage (Liener, 1994)[52]. Cooking of soybean inhibits over 99.6% of lectin activity (Shi & Nickerson, 2018)[57]. Tannins, present in most legumes, are a group of large polyphenolic compounds considered to be anti-nutrients due to their ability to bind proteins which inhibits enzymatic digestion. While some legumes such as fava bean contain high concentrations of tannins (up to 20 mg g^{-1}), soybean contains negligible amounts (0.45 mg g^{-1}), similar to wheat (Liener, 1994; Rao & Prabhavathi, 1982)[52,53]. After dehulling, no detectible tannins remain (Egounlety & Aworh, 2003)[59].

Suggested health benefits

There are several suggested health benefits attributed to the consumption of tempeh based predominantly on limited *in vitro*, animal and population studies. Most notably as a cancer preventative, through isoflavone and antioxidant activity, and the promotion of cardiovascular health (Nout & Kiers, 2005; Messina, 1995; Ahnan-winarno *et al.*, 2021)[12,41,60]. Tempeh consumption may improve gut health and reduce severity of diarrhoea, with consistent reports demonstrating anti-microbial activity against gram-positive bacteria, in which tempeh extracts limit the ability of pathogenic *E. coli* to adhere to intestinal membranes (Nout & Kiers, 2005)[41]. Other suggested benefits include prevention and treatment of anaemia, improvement of liver, lung and bone health, positive effects on type 2 diabetes, obesity, skeletal muscle recovery and recovery from malnutrition (Ahnan-winarno *et al.*, 2021)[12]. Several suggested benefits of tempeh consumption merit additional research, as current studies are often exploratory, and should be approached with a modicum of scepticism due to experimental limitations, low sample size and ambiguous results. Interestingly, soybean contains several compounds that are considered both health-promoting and anti-nutrients. Examples of this are genistein and daidzein, which are attributed various health-promoting effects such as anti-oxidative activity and angiogenesis inhibition, but are also considered anti-nutrients by some due to their anti-thyroidal properties implicating them as a cause of diet-induced goitre (Divi *et al.*, 1997)[65]. Other ambiguous compounds include saponins and lipoxygenases.

Table 1. Effects of tempeh production. Changes in macro-, micro- and anti-nutrient contents during soybean preparation and fermentation.

macronutrients	**micronutrients**	**anti-nutrients**
Increased total crude and soluble protein	Increased vitamin B2, B3, B6, B7, B9 and B12	Decreased flatulence-causing oligosaccharides
Increased free amino acids	Increased β-carotene	Decreased phytic acid
Increased small peptides	Increased ergosterol content	Decreased oxalate
Decreased total crude fats	Increased anti-oxidative capacity through production of polyphenols	Decreased trypsin inhibitor activity
Increased free fatty acids	Activation of isoflavones through hydrolysation	Decreased lectins
Decreased carbohydrates	Decreased vitamin B1	
	Decreased potassium	

Tempeh fermentation is a complex process based on tradition and a wide variety of adjustable parameters. There is no singular final product but rather a whole scala of products based on the subjective taste of producers and consumers. No optimal process exists, and optimisation should instead be focussed on parameters that may add or improve desired traits. Substrate preparation and fermentation have a wide range of effects on the final product (**Tbl.1**). Nutrient bioavailability is

improved by increasing protein solubility, free amino acid content and free fatty acid content. Several health-promoting compounds such as vitamins and antioxidants are increased during fermentation, although vitamin B1 and potassium contents are decreased. Anti-nutrients and flatulence-causing oligosaccharides naturally present in raw soybean are strongly reduced. Despite these laudable qualities, the mechanisms behind their production are not completely understood, and desired results are often a process of trial and error. Most tempeh research limits itself to one or a few strains of the same *Rhizopus* species and may benefit from the potential wealth of attributes present in the wide variety of available inoculates. The range of tempeh products and their health benefits may further be enhanced by exploring substrate variety and the use of co-inoculation.

Field pea

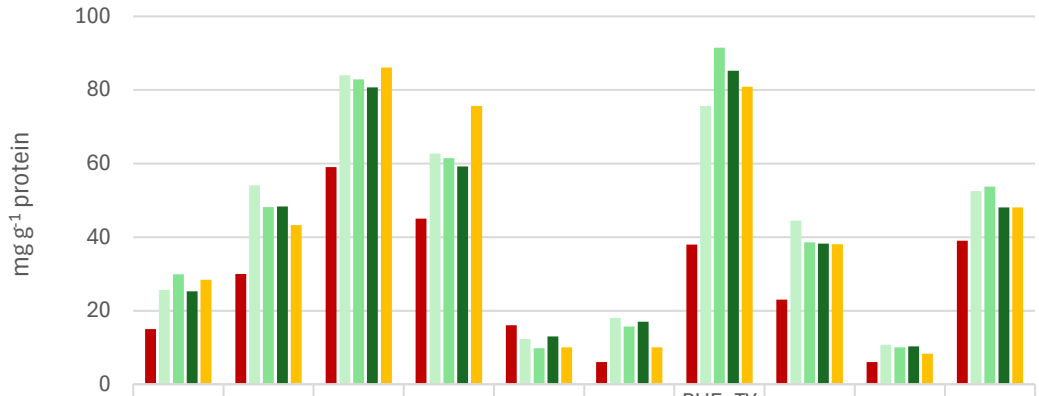
While the Indonesian cuisine uses several different substrates for tempeh production, international interest in substrates other than soybean has remained largely scientific. The use of alternative substrates results in tempeh with different nutritional compositions and organoleptic properties. While fermentation using *Rhizopus* spp. generally results in similar effects such as increased protein content, free amino acids, vitamin content and reduced anti-nutrients, changes are not necessarily uniform. An example of this is thiamine production during tempeh fermentation of wheat, which is instead reduced during soybean fermentation (Wang & Hesseltine, 1966)[77].

When considering alternative substrates for European tempeh production, localized availability of substrate crops is highly relevant in light of environmental impact and sustainability. While an annual average of 2.7 Mt of soybean was produced in the European Union between 2014 and 2018, a further 14 Mt of soybean was imported to meet demands. In total, less than 7% of soy imports came from the European continent (Karges *et al.*, 2022)[49]. The second most abundant legume grown in the European Union is field pea, with an estimated 2.1 Mt produced annually (Kezeya Sepngang *et al.*, 2020)[46]. Field pea, also known as dry pea or split pea, is part of the highly diverse pea species (*Pisum sativum*) and is specifically used to describe the dry grain varieties of the species. Its fresh counterpart is called garden pea, fresh pea or green pea, although there are several cases where terms are used interchangeably. The term split pea specifically refers to processed field pea, which is dried, dehulled and split along its cotyledons after harvesting, and includes both green and yellow varieties. Scientific literature is very often unclear on whether experiments involve fresh or dried pea, their colour, and whether they are split or not. Unlike soybean, field pea is a cool season crop, well suited for cultivation in central and northern Europe, preferring mean seasonal temperatures of 10 – 18° C (Devi *et al.*, 2023)[50]. Both are valued by the agricultural industry for their symbiotic relationship with *Rhizobium* species, which allows them to fixate nitrogen from the atmosphere, reducing fertilizer requirements. Field pea nitrogen

derived from atmosphere (NDFA) was measured at 69-71%, slightly higher than average soybean NDFA at 55%, although this is likely to be an overestimation due to limited data. NDFA strongly fluctuates depending on plant cultivar, soil type and additional nitrogen fertilization (Kumar & Goh, 2000; Ciampitti & Salvagiotti, 2018)[62,63]. Some attempts at producing pea tempeh have been made, describing generally positive results and palatability (Ashenafi & Busse, 1991; Nowak & Szebiotka, 1992; Reiss, 1993)[40,47,48].

Nutritional comparison

On a macronutrient level, raw field pea contains less protein (22.6% of dry weight) and fat (1.6% dw) but more carbohydrates (70.4% dw) compared to soybean (Ashenafi & Busse, 1991)[40]. In addition, pea contains less than half the fibre content, although removal of fibre due to dehulling should be taken into consideration. Field pea contains less total nitrogen compared to soybean (65.2% dw) but the relative amount of both trichloroacetic acid soluble nitrogen and amino nitrogen is higher in pea, indicating higher concentrations of soluble protein and free amino acids compared to soybean (Nowak & Szebiotka, 1992)[47]. Pea carbohydrates are comprised largely of starch (33-48% dw), non-starch polysaccharides, sucrose (2% dw), raffinose, stachyose and verbascose (5.8-7.3% dw combined) (Nikolopoulou *et al.*, 2007)[64]. Other findings indicate sucrose to be the most abundant low-molecular weight sugar in both soybean and pea (6.3% and 5.23% dw, respectively), and lower values of flatulence-causing oligosaccharides in pea compared to soybean (1.7% and 4.3% dw, respectively) (Nowak & Szebiotka, 1992)[47]. A comparison of 6 field pea varieties found 2.6-5.4% dw sucrose and between 5.0-6.1% dw stachyose, raffinose and verbascose in varying ratios (Wang *et al.*, 2008)[67]. A comparison of indispensable amino acid compositions between soybean and yellow split pea after soaking and cooking shows a largely similar distribution, with high levels of leucine, lysine, phenylalanine and tyrosine (**Fig.2**). Notably, yellow split pea contains higher concentrations of lysine compared to soybean, while cysteine and tryptophan concentrations are slightly lower (Murata *et al.*, 1967; Stillings & Hackler, 1965; Nosworthy *et al.*, 2017)[42,43,51]. Like soybean, split pea faces the challenge of overcoming low concentrations of methionine, cysteine and tryptophan when attempting to improve protein quality. The protein quality of yellow split pea and soybean is relatively similar, with PDCAAS scores of 69% for cooked yellow split pea, and 73% for cooked soybean (Nosworthy *et al.*, 2017; Nosworthy *et al.*, 2022)[51,76]. However, it should be noted that data on yellow split pea



	HIS	ILE	LEU	LYS	MET	CYS	PHE+TYR	THR	TRP	VAL
recommended daily intake	15	30	59	45	16	6	38	23	6	39
Soybean A	26	54	84	63	12	18	76	44	11	52
Soybean B	30	48	83	61	10	16	92	39	10	54
Soybean C	25	48	81	59	13	17	85	38	10	48
Yellow split pea	28	43	86	76	10	10	81	38	8	48

Figure 2. Comparison of indispensable amino acid compositions of prepared soybean and yellow split pea substrates (mg g^{-1} protein). *Preparation includes dehulling, soaking and cooking. Soybean substrate A (Indonesian) and B (Japanese) adapted from (Murata et al., 1967)[43], C (USA) adapted from (Stillings & Hackler, 1965)[42] and yellow split pea substrate adapted from (Nosworthy et al., 2017)[51]. Recommended daily intake as indicated by the WHO/FAO (FAO; 2011)[16]. No USDA standard reference is available for yellow split pea.*

PDCAAS is very limited compared to soybean, and soybean PDCAAS may be as high as 85% ±17.2 (van den Berg *et al.*, 2022)[69]. Like soybean, field pea contains a variety of vitamins and other micronutrients before processing, including vitamins B1, B2, B3, B5, B6, B7, B9, ascorbic acid (C), tocopherol (E), inositol, vitamin K and β-carotene, as well as appreciable levels of iron, zinc and magnesium (Savage & Deo, 1989; Amarakoon *et al.*, 2012)[74,116].

Anti-nutritional compounds

Similar to other legumes, field pea contains several anti-nutrients such as phytic acid, oxalate, trypsin inhibiting compounds, lectins and tannins. Concentrations of anti-nutrients are relatively low when compared to soybean. Phytic acid content of yellow split pea is less than most other beans, and nearly half that of soybean (Shi & Nickerson, 2018)[57]. Cooking reduces phytic acid content of yellow split pea by up to 23%, and fermentation could potentially reduce this even further. Total oxalate contents are about 20% lower compared to soybean, although soaking and cooking has a less pronounced effect on yellow split pea oxalate (-41%) compared to soybean oxalate (-66%) (Shi & Nickerson, 2018)[57]. When looking at soluble oxalate only, yellow split pea concentrations are less than half that soybean. This may indicate a less pronounced detrimental effect as insoluble oxalate is not readily absorbed and thus

does not contribute to the formation of kidney stones. Trypsin inhibitor concentrations in field pea are generally low, and are unlikely to have much impact even when consumed uncooked (Wang *et al.*, 1998b)[66]. As trypsin inhibiting compounds found in legumes are not heat-stable, cooking or autoclaving is likely to further reduce their effect (Vagadia *et al.*, 2017)[58]. Lectins are not considered anti-nutrients in field pea as concentrations are less than 1% compared to soybean (Shi & Nickerson, 2018)[57]. Field pea varieties meant for human consumption contain negligible levels of tannins, similar to soybean (Wang *et al.*, 1998a)[54]. Other potentially anti-nutritional compounds found in field pea are saponins and lipoxygenases.

Effects of processing

Most raw legumes have a strong undesirable flavour, and their consumption causes gastrointestinal distress, vomiting and diarrhoea. Long term consumption causes growth inhibition and organ damage. Because of this, cooking is considered non-optional for human consumption of legumes, as it improves palatability and neutralises toxins and anti-nutrients. When considering yellow split pea as a substrate for tempeh fermentation, several options for processing are available. Soaking is generally considered the first step, although dry processing is possible. The primary purpose of soaking is to improve the effects of heat treatment by softening the cotyledons, but it also affects macro- and micronutrient concentrations and leaches sucrose and oligosaccharides from the peas. Soaking also improves substrate susceptibility to fungal colonisation. As heat treatment, pea can be boiled, steamed, baked, autoclaved, extruded or treated with microwave or infrared radiation. While the general concept of each treatment is clearly defined, parameters such as time and temperature can vary widely, and are often arbitrarily chosen. An example of this is extrusion processing, which can be performed at 135° C with an entry speed of 500 kg/h and a screw speed of 60 rpm in one experiment, while another uses a multi-temperature barrel extruder at 30 to 120° C with a screw speed of 650 rpm and unknown residence time (Nosworthy *et al.*, 2017; Frias *et al.*, 2010)[15,51]. As differences of less than 10° C can have a significant impact on the nutritional contents of the extruded substrate, this strongly limits the ability to draw comparisons between experiments.

Table 2. Comparison of amino acid composition after heat treatment in yellow split pea. Values in mg g^{-1} protein. Adapted from (Nosworthy *et al.*, 2017)[51].

amino acid	HIS	ILE	LEU	LYS	MET	CYS	PHE+TYR	THR	TRP	VAL
untreated	27	37	74	71	9	11	70	36	9	43
cooked	28	43	86	76	10	10	81	38	8	48
baked	29	41	80	69	9	11	76	38	9	48
extruded	28	40	77	71	9	11	76	37	8	46

Boiling, baking and extrusion processing of yellow split pea shows minor changes to relative crude protein, crude fat, and amino acid content, although extrusion results in slightly lower indispensable amino acid contents compared to boiling and baking (**Tbl.2**) (Nosworthy *et al.*, 2017)[51]. A comparison of 5 heat treatments shows improved amino acid composition and protein quality compared to raw pea, with amino acid compositions changing markedly depending on the treatment (Khattab *et al.*, 2009)[72]. Considering methionine, cysteine and tryptophan as the first limiting indispensable amino acids in yellow split pea, boiling provides the highest levels of methionine and tryptophan, while microwave and infrared treatments result in the highest cysteine content. While infrared treatment shows a positive impact on the amino acid composition of pea, it also reduces protein solubility and flavour (McCurdy, 1992)[68].

The substrate with the highest protein quality after processing depends on the method of measurement. When using the protein efficiency ratio (PER), based on weight gain, autoclaving results in the highest protein quality. Alternatively, *in vitro* protein digestibility (IVPD), based on a simulated digestive environment, shows boiling to be best (Khattab *et al.*, 2009)[72]. PDCAAS values of cooked yellow split pea are highest (69.2%), followed by baking (68.9%) and extrusion (65.4%).

Interestingly, when protein quality is calculated using DIAAS, extrusion scores highest (67%) compared to cooking (66%) and baking (64%) (Nosworthy *et al.*, 2017)[51]. There are no PDCAAS or DIAAS scores available for other heat treatments. Soaking, cooking and autoclaving improve starch digestibility, and a combination of soaking and autoclaving results in the most pronounced effect (Bishnoi & Khetarpaul, 1993)[71]. Heat treatment also reduces flatulence-causing oligosaccharide content, with high temperatures having a more pronounced effect. Extrusion processing shows lower concentrations of stachyose, raffinose and verbascose at 135° C compared to 129° C by up to 25% (Frias *et al.*, 2010)[15].

Processing causes a reduction in vitamin content depending on the type of food and type of treatment. Some vitamins are more affected by heat treatment than others (Lešková *et al.*, 2006)[75]. The effects of different processing methods on field pea vitamin concentrations have received relatively little interest. Both boiling and autoclaving cause a reduction of folate (B9) content in pea, with lowest total folate content seen after cooking (Dang *et al.*, 2000)[70]. Extrusion processing reduces thiamine (B1) and riboflavin (B2) content by up to 50% and 10%, respectively (Frias *et al.*, 2010)[15].

Other than soaking and cooking, mechanical processing may be required if a certain substrate consistency is desired. While mild mechanical processing such as maceration up to 24,000 rpm has no significant effect on the protein profile of pea, high-pressure industrial processing can cause denaturation and aggregation (Sirtori *et al.*, 2012)[73].

Effects of variety and environment

There are many varieties of field pea, and attributes such as seed weight, nutrient composition and anti-nutrients can vary widely depending on cultivar, location and environmental conditions (**Tbl.3**). A large-scale quantitative characterisation of field pea seeds shows protein content can vary between 13.7 and 30.7% dw. Starch content is also highly variable and negatively correlated to protein content, ranging from 27.6% to 56.3% dw (Tzitzikas *et al.*, 2006)[79]. Contrary to this, Hood-Niefer *et al.* (2012) reported only minimal effects of cultivar and no significant effect of location on protein or starch contents, ascribing their findings to improved agricultural management practices (Hood-Niefer *et al.*, 2012)[82]. The effect of variety appears less pronounced for fat content (Wang *et al.*, 2010)[78]. Interestingly, indispensable amino acid profiles also change depending on cultivar, although only arginine and cysteine contents were significantly correlated, possibly due to limited sampling (Wang & Daun, 2004)[80]. Both mineral and anti-nutrient content is affected by cultivar, although significant correlation was only found for calcium, copper, potassium, manganese and phosphorus. In the case of anti-nutrients and flatulence-causing oligosaccharides, a significant correlation was found between cultivars for raffinose and phytic acid, but not for stachyose or verbascose (Wang & Daun, 2004)[80].

Table 3. Effects of variety and environment on field pea composition. All correlations are statistically significant ($p < 0.05$). Adapted from (Tzitzikas *et al.*, 2006; Wang *et al.*, 2010; Nikolopoulou *et al.*, 2007; Wang & Daun, 2004; Wang *et al.*, 1998b)[64,66,78,79,80].

Composition	Range	Influenced by
Protein content (% dw)	13.7 – 30.7	Cultivar, location, year
Starch content (% dw)	27.6 – 56.3	Cultivar, location, year
Fat content (% dw)	12.1 – 15.4	Cultivar, location, year
Amino acid content (mg g^{-1} protein)		Cultivar
Arginine	74 – 85	
Cysteine	NA	
Mineral content (mg 100 g^{-1} dw)		Cultivar
Calcium	70.4 – 89.5	
Copper	0.6 – 0.8	
Potassium	1012.3 – 1330.4	
Manganese	1.1 – 1.5	
Phosphor	401.9 – 605.2	
Sucrose (mg g^{-1} dw)	24.8 – 32.8	Cultivar, location, year
Raffinose (mg g^{-1} dw)	5.6 – 6.4	Cultivar
Phytic acid (mg g^{-1} dw)	6.2 – 11.0	Cultivar, location, year
Trypsin inhibitor activity (mg g^{-1} dw)	1.9 – 2.1	Cultivar, location
Tannins (% dw)	0.45 – 0.92	Cultivar, location, year

Trypsin inhibitor activity can be very high in some cultivars, and is additionally correlated to location (Wang *et al.*, 1998b)[66]. A comparison of 6 field pea cultivars shows protein, starch, fibre, fat, ash and phytic acid content to be significantly different depending on year and location, in addition to cultivar (Wang *et al.*, 2010)[78]. Additional conditions that may affect nutrient and anti-nutrient composition include climate and the mechanical and chemical composition of the soil (Nikolopoulou *et al.*, 2007)[64]. Low-tannin field pea cultivars generally contain less than 0.9% dw tannins, depending on cultivar, location and year (Wang *et al.*, 1998a; Nikolopoulou *et al.*, 2007)[54,64]. High-tannin cultivars are used as animal feed, where balanced levels of tannins can help optimise nutritional value for ruminants (Frutos *et al.*, 2004)[81].

Altogether, field pea is a logical alternative to soybean when considering more localized tempeh production in the European Union. Although it contains less protein and more starch compared to soybean, indispensable amino acid profiles are similar, and protein solubility is higher. Additionally, field pea contains lower concentrations of anti-nutrients than soybean. Field pea nutritional composition and anti-nutrient content depends heavily on variety and environment and should always be considered when using field pea as a tempeh substrate.

Improving tempeh quality

The selection of an appropriate substrate, *Rhizopus* species and production parameters all help to define the characteristics of the resulting tempeh. Additionally, several options such as co-inoculation, substrate mixing and genetic modification can help to improve the final product. Co-inoculation combines the beneficial effects of multiple microorganisms, increasing micronutrients while decreasing anti-nutrients, flatulence-causing oligosaccharides and immunoreactivity. Bacterial co-inoculation can also act as a safeguard against pathogenic contamination during production. The addition of secondary substrates can help balance nutritional content and improve protein quality, and genetic modification can improve both substrate crops and microorganisms in a variety of ways.

Co-inoculation

A mixed inoculum consisting of multiple *Rhizopus* species, other fungal species or bacteria can augment the result of tempeh fermentation by increasing desirable compounds, decreasing anti-nutrients and reducing contamination risk. Co-inoculation of grass pea with *Rhizopus microsporus* var. *chinensis* and *Aspergillus oryzae* results in tempeh with increased protein content, increased free amino acid content and higher *in-vitro* protein bioavailability compared to grass pea tempeh fermented with *R. microsporus* var. *chinensis* alone (Starzynska-Janiszewska *et al.*, 2015)[88]. Soybean tempeh produced using a combination of *R. oligosporus* and *Actinomucor elegans* shows greater reduction of flatulence-

causing oligosaccharides compared to fermentation using *R. oligosporus* alone, and the addition of *A. elegans* also increases the reduction of IgE immunoreactivity against soy proteins, which may be invaluable for the production of hypoallergenic tempeh (Huang *et al.*, 2019)[89]. The ergosterol content of barley tempeh increases by 12 to 31% compared to the control when *R. oligosporus* is co-inoculated with *Saccharomyces cerevisiae* and several other yeast species. Co-inoculation with *S. cerevisiae* also affects vitamin content, increasing vitamin B3 and B6, while reducing vitamin B1 and B7 (Feng *et al.*, 2007)[90]. A mixed inoculation of *R. oligosporus* and *Yarrowia lipolytica* in soy pulp results in increased concentrations of methionine and tryptophan, at the cost of cysteine, which *Y. lipolytica* can catabolise (Vong *et al.*, 2018)[113]. Surprisingly, there is very little mention of mixed starters containing multiple *Rhizopus* species in literature, while they are readily available commercially. Co-inoculation experiments using *R. oligosporus* and *R. arrhizus* show that, while *R. oligosporus* alone does not significantly decrease flatulence-causing oligosaccharide content, inoculum containing as little as 10% *R. arrhizus* reduces stachyose and raffinose concentrations by up to 73%. Additionally, several micronutrient concentrations appear to be strongly affected by the inoculation ratio, including vitamin B2, B3, B6, B8 and ergosterol. Based on these findings, a co-inoculation of 80% *R. oligosporus* and 20% *R. arrhizus* will result in the most nutritionally balanced tempeh (Wiesel *et al.*, 1997)[112].

In addition to fungi, bacterial co-inoculation can also confer several beneficial effects. The addition of *Lactobacillus plantarum* to the soaking water during soybean preparation can partially or completely inhibit the growth of several bacterial pathogens, including *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella infantis*, *Escherichia coli*, *Enterobacter aerogenes* and *Staphylococcus aureus* (Ashenafi, 1991; Ashenafi & Busse, 1989, 1991b, 1991c, 1992)[19,20,83,84,85]. Similar inhibition is observed when *L. plantarum* is added during tempeh fermentation of pea, chickpea and faba bean, although the effect is somewhat less pronounced in pea and only partially effective in faba bean. In most cases, the combination of acidification and *L. plantarum* yields the best results. While attempts at producing soybean tempeh with reliable vitamin B12 content using *Citrobacter freundii* have met with limited success (up to 59 ng g^{-1} dw), the co-inoculation of lupin seed (*Lupinus* spp.) with *R. oligosporus, R. arrhizus and Propionibacterium freudenreichii* yields tempeh with very high levels of vitamin B12 (up to 1230 ng g^{-1} dw) (Denter & Bisping, 1994; Signorini *et al.*, 2018)[29.86]. This is especially relevant as vitamin B12 deficiency is one of the most prevalent issues among vegetarians and vegans, affecting up to 76 and 90% of the adult population, respectively (Pawlak *et al.*, 2013)[87]. Co-inoculation experiments of soybean tempeh with *R. oligosporus* and several bacteria show an increase in vitamin B6 and B12 content, while vitamin B3 content decreased. While some of the bacterial species tested are not suitable for human consumption, it does show that vitamin content of tempeh can be augmented through bacterial co-inoculation (Keuth & Bisping, 1993)[30].

Mixed substrates

While tempeh fermentation generally increases the total protein content and digestibility of the substrate, amino acid contents tend to remain relatively unchanged. By balancing the amino acid profile of the substrate, the protein quality of the resulting tempeh can be improved. Importantly, a secondary substrate may cause a change in organoleptic properties, nutrient composition or introduce additional anti-nutrients. An exploratory study combining soybean with peanut or sunflower seed shows both combinations yield tempeh with positive organoleptic results. The roughly 1:1 ratio of substrates is reflected in the composition and indispensable amino acid profiles of the resulting tempeh. For both soybean-peanut and soybean-sunflower tempeh, the macronutrient composition is a rough average between tempehs created from their unmixed constituents, with reduced protein and increased fat content compared to soybean tempeh. The addition of peanut as a secondary substrate lowers the total concentration of indispensable amino acids compared to pure soybean tempeh, with the notable exception of tryptophan, which is increased relative to both soybean and peanut tempeh. The addition of sunflower seed increases methionine + cysteine and tryptophan concentrations compared to pure soybean tempeh, resulting in a more balanced amino acid profile (Vaidehi et al 1985)[96]. Fermentation of a 1:1 mixture of soybean and wheat results in tempeh with high protein and carbohydrate content. Amino acid profiling shows relatively high levels of sulfur-containing amino acids, and PER measurements result in a higher protein quality score compared to both pure soybean and wheat tempeh (Wang *et al.*, 1968)[91]. A mixed soybean and oat groats (*Avenia sativa*) substrate also produces a high protein tempeh with markedly increased protein solubility compared to the unfermented control (Nowak, 1992)[94].

Taking into consideration that the first limiting indispensable amino acids in field pea are methionine, cysteine and tryptophan, potential complementary substrates can be suggested based on high contents of those amino acids. Because of this, other legumes are generally unsuited as secondary substrates, as their amino acid compositions are generally similar (Hall *et al.*, 2017)[100]. Cereals are potential candidates due to their relatively high protein content, with oat groats being of particular interest because of their high protein quality, containing significantly more methionine, cysteine and tryptophan than wheat, corn and rice (Gulvady *et al.*, 2013)[92]. Of various pseudocereals, buckwheat (*fagopyrum esculentum*) may be a potential candidate, as it contains respectable concentrations of methionine, cysteine and tryptophan, although protein content is relatively low (12.3% dw) (Mota *et al.*, 2016; Wijngaard & Arendt, 2006)[98,99]. Amaranth (*Amaranthus caudatus*) another pseudocereal originating from South America, also contains methionine, cysteine and tryptophan concentrations that could complement the amino acid profile of field pea (Pedersen *et al.*, 1987)[101]. Protein quality assessments in various edible nuts show concentrations of sulfur-containing amino acids and tryptophan to be relatively similar or lower compared to field pea, with the exception of Brazil nut

(*Bertholletia excelsa*) and cashew nut (*Anacardium occidentale*). Brazil nut contains surprisingly high levels of methionine (up to 89.8 mg g^{-1} protein) while containing similar cysteine and lower tryptophan concentrations than field pea, and a total protein content of 14.4% dw. Cashew nut contains all three first-limiting amino acids at concentrations above field pea, and a reasonable protein content (21.3% dw) (Venkatachalam & Sathe, 2006; Rico *et al.*, 2015)[95,97].

Genetic modification

Another approach to improve protein quality and obtain further desirable attributes can be through genetic modification. While several *Rhizopus* spp. have been successfully modified to improve production of compounds for the bioindustry, there appears to be no mention in literature of *Rhizopus* spp. modification for the purpose of nutritional improvement. Genetic modification of the substrate can be considered instead, due to its strong influence on the nutritional composition of the final product. While different depending on species, most legumes are known to be relatively resistant to genetic engineering. Transformation rates tend to be low for many legume species, and regeneration after attempted transformation can be difficult and highly genotype specific (Somers *et al.*, 2003)[102]. Most legumes, including field pea, rely on *Agrobacterium tumefaciens* mediated transformation, although biolistic methods are also used. While genetic modification experiments have produced several promising results in field pea, there has thus far been no commercialisation either due to the lack of field experiments, or because modified strains displayed unintended negative characteristics (Ludvikova & Griga, 2022)[103]. In recent years, CRISPR/Cas9 gene-editing has received an incredible amount of attention, and its use for the transformation of legume species has started to pick up, including for pea (Li *et al.*, 2023)[104]. The emergence of new techniques such as CRISPR/Cas9 and speed breeding, in combination with developments in genomics, proteomics, transcriptomics and resource databases, is paving the way for the creation of new and improved field pea varieties (Pandey *et al.*, 2021)[105].

There are several examples of genetic modification to improve protein quality in other legumes. In soybean, glycinin and β-conglycinin storage proteins make up over 70% of total protein content. These storage proteins differ in their amino acid composition, with β-conglycinin containing substantially lower amounts of sulphur-containing amino acids. As β-conglycinin accumulates, relative concentrations of methionine and cysteine decrease, resulting in reduced protein quality (Krishnan, 2005)[106]. When the storage protein ratio is shifted by a combination of conglycinin overexpression and RNA interference to reduce β-conglycinin, the sulphur-containing amino acid content of soybean significantly increases (Wang *et al*, 2022)[111]. Comparable to soybean, field pea protein consists predominantly of legumins, vicilins and albumins, with methionine content in albumins being substantially higher compared to legumins (185%) and vicilins (259%) (Rubio *et al.*, 2013)[107].

Overexpression of albumins and knock-down or knock-out transformations of legumins and vicilins may similarly improve sulphur-containing amino acid content in field pea.

Another approach could be the heterologous expression of complementary proteins from other sources. An example of this is Brazil nut albumin, which contains high levels of methionine and has successfully been expressed in soybean, resulting in up to 40% higher methionine content compared to regular soybean (Rubio *et al.*, 2013)[107]. Unfortunately, Brazil nut albumin has since been identified as the major allergen in Brazil nut, limiting its potential. Another candidate is sunflower albumin, which is shown to increase methionine content by up to 94% when expressed in lupin compared to the non-transgenic parent line. While there was a small decrease in cysteine content, the amino acid profile appeared to be otherwise unaffected (Molvig *et al.*, 1997)[108]. A potential target to improve tryptophan concentrations in field pea is anthranilate synthase, a critical enzyme for the biosynthesis of tryptophan in plants, regulated through tryptophan feedback inhibition. When a modified, feedback-resistant rice homologue of anthranilate synthase (OASA1D) is expressed in azuki bean (*Vigna angularis*) the concentration of accumulated tryptophan is increased (Hanafy *et al.*, 2006)[109]. Moreover, the expression of OASA1D in glycinin and β-conglycinin deficient soybean lines shows an additive effect, resulting in significantly improved tryptophan content (Kita *et al.*, 2010)[11-].

Discussion

The primary dietary role of meat is to provide a rich source of high-quality protein. Commonly consumed types of meat contain an average of 19.4% wet weight (ww) protein when raw, and ideally, meat alternatives should aim for similar or higher content (Bohrer, 2017)[114]. Soybean is one of the richest plant-based sources of protein (26.5-47.6% dw), and soybean tempeh contains 20.3% ww protein on average (Vollmann *et al.*, 2000; USDA, 2018)[45,115]. While field pea protein content is lower (13.7-37.7% dw), tempeh fermentation shows an increased effect on protein content compared to soybean (Tzitzikas *et al.*, 2006; Ashenafi & Busse, 1991a)[40,79]. In addition, field pea retains less water after soaking, resulting in tempeh with higher relative protein content. Assuming these effects are consistent, field pea tempeh can be estimated to have a protein content of 15.6% ww on average. By selecting only high-protein field pea cultivars, the protein content of field pea tempeh could be improved to equal that of soybean and meat. Although tempeh fermentation is shown to increase relative protein content in a variety of substrates, some findings instead indicate a decrease (Ahnan-Winarno *et al.*, 2021; Nowak & Szebiotka, 1992; Reiss, 1993)[12,47,48]. These contrasting results emphasise the need for increased consistency in both production and analytical methods.

Protein quality is influenced by the composition, bioavailability and digestibility of proteins, and can be quantified as a score using several methods. Quality scores can vary significantly depending on the chosen method, which is often subject to researcher preference. Even when FAO/WHO recommended methods PDCAAS or DIAAS are used, comparisons may be of limited value as choice of scoring pattern and digestibility assay can significantly impact results (FAO, 2011)[16]. Amino acid profile comparisons between field pea and soybean show a highly similar pattern, with methionine, cysteine and tryptophan as the first three limiting amino acids in both substrates. PDCAAS assessment of cooked yellow split pea (69.2) and soybean (73.2) using the same methodology resulted in similar scores, suggesting comparable quality (Nosworthy *et al.*, 2017; Nosworthy *et al.*, 2022)[51,76]. While there is relatively little change in amino acid composition during tempeh fermentation, protein solubility and free amino acid content increases significantly, potentially increasing protein bioavailability and digestibility (Nowak & Szebiotka, 1992)[47]. Relative soluble protein and amino acid content in field pea tempeh is higher compared to soybean tempeh. Compounds such as trypsin inhibitors, lectins and tannins could potentially impact protein quality, but concentrations of these anti-nutrients in field pea are too low to be of significant influence (Wang *et al.*, 1998a, 1998b; Shi & Nickerson, 2018)[54,57,66]. While true protein digestibility of field pea tempeh cannot be determined without *in vivo* assessment, protein quality is already on par with its main alternative and can plausibly be expected to increase further through tempeh fermentation, being limited predominantly by the methionine, cysteine and tryptophan content of the substrate.

Genetic variation and environmental conditions during growth are the most important factors influencing the nutritional content of field pea (Tzitzikas *et al.*, 2006)[79]. As such, appropriate selection of cultivar and location will be crucial for the production of high-protein field pea tempeh. Substrate processing methods influence the nutritional composition in several ways, affecting minor changes in amino acid profile and protein solubility (Nosworthy *et al.*, 2017; McCurdy, 1992)[51,68]. As optimal growth of *Rhizopus* spp. is affected by water content, dry processing methods such as baking or infrared radiation treatment are best avoided (Sarette *et al.*, 1992)[38]. Autoclaving yields similar results to boiling in terms of amino acid content but may be preferable due to increased reduction of flatulence-causing oligosaccharides at higher temperatures (Khattab *et al.*, 2009; Frias *et al.*, 2010)[15,72]. However, as high temperature treatments also have increased impact on the vitamin content of field pea, boiling could be considered instead (Lešková *et al.*, 2006)[75]. As concentrations of heat-labile anti-nutrients in field pea are already low, the duration of heat-treatments should be kept to a minimum to avoid unnecessary negative impact (Shi & Nickerson, 2018; Wang *et al.*, 1998a; Wang *et al.*, 1998b)[54,57,66].

The nutritional quality of field pea tempeh can be improved in several ways. Co-inoculation of *R. oligosporus* and *R. arrhizus* can combine the vitamin producing qualities of both strains and the ability of *R. arrhizus* to metabolise stachyose (Wiesel *et al.*, 1997)[112]. Co-inoculation of *P. freudenreichii* can be used to add desirable concentrations of vitamin B12 to tempeh, which is normally only found in animal products (Signorini *et al.*, 2018)[29]. Addition of *L. plantarum* to the soaking water can partially or completely inhibit growth of pathogenic bacteria and should be considered depending on production environment (Ashenafi, 1991; Ashenafi & Busse, 1989, 1991b, 1991c, 1992)[19,20,83,84,85]. Other than co-inoculation, the protein quality of field pea tempeh may be improved through the addition of complementary substrates. A promising European candidate would be sunflower seed, which contains high methionine, cysteine and tryptophan content (Vaidehi et al 1985)[96]. In Canada, a leading producer of field pea, oat groats can be considered as a locally produced alternative. Finally, genetic modification can be used to improve amino acid composition by shifting the balance of storage proteins, the heterologous expression of desirable proteins, or the expression of modified proteins such as OASA1D (Kita *et al.*, 2010)[110]. A possible avenue of future research could be the attempted inhibition of cysteine catabolism in *Y. lipolitica*, which may then significantly improve amino acid concentrations when co-inoculated (Vong *et al.*, 2018)[113]. While genetic modification can offer a variety of benefits, consumer opinion is generally negative and may severely impact the viability of the product.

Conclusion

Altogether, the nutritional profile of field pea makes it a promising candidate for the creation of a high-quality meat alternative through tempeh fermentation. The nutritional quality of field pea tempeh will depend greatly on the genetic variation and growth environment of the substrate and can be further improved by co-inoculation, the introduction of secondary substrates and genetic modification.

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