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Bioactive peptides identified in pea and faba bean after *in vitro* digestion with human gastrointestinal enzymes

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ABSTRACT

We investigated the digestive peptide profiles of pea and faba bean proteins to evaluate possible bioactive peptides (BAPs), and to test immune-modulating properties of selected peptides using *in vitro* cell model. More than 400 unique peptides were released upon gastrointestinal digestion of pea and faba bean proteins and identified by high-resolution mass spectrometry (HPLC-ESI-MS/MS). Among these, 24 peptides were potentially bioactive as predicted by *in silico* analysis. Also, up to 275 recognized BAPs and eight known allergens were identified within the peptide sequences of the digests. Four selected peptides significantly decreased the IL-8 response in Caco-2 cells, with a specific pea peptide, DKPWWPK, reducing the IL-8 response up to 40 % during IL-1β induction. Combined with other nutritional benefits, the immune-modulatory properties of legume proteins point to a regular introduction of legumes in the diet, as they represent a still scarcely exploited food category, especially in the Nordic countries.

1. Introduction

The increasing world population, climate changes and the current geopolitical situation demands a higher food supply. Not only the fastest growing regions of the world, but all countries need to produce more food to minimize import and to increase their own self-sufficiency. Moreover, food production is responsible for up to 30 % of global greenhouse-gas emission (Willett et al., 2019), where red meat has on average 60 times higher emission per gram of protein produced compared to legumes (Tilman & Clark, 2014). A high intake of processed meat and low intake of whole grains are also dietary risk factors contributing to disease development (Stanaway et al., 2018). For these reasons, together with consumer awareness on climate effects of food production, new, high-quality, sustainable protein sources need to be exploited and developed. Legumes remain underexploited in the western diet, although they are considered a healthy and sustainable protein source, beneficial for the prevention of several non-communicable diseases. Moreover, the mechanisms activated in the human body when legume proteins are digested and release bioactive compounds, are not well known. Therefore, the exploration of health beneficial biological activities originating from a legume-rich diet is needed.

Peas and beans are regarded as a good source of proteins, and the exploitation of these legumes in future food will be necessary to comply with the shift towards a more sustainable and healthy diet. On average, peas contain 25 % protein, whereas faba bean seeds contain about 29 % proteins, including the major seed storage proteins legumin, vicilin and convicilin (Lu et al., 2020, Warsame et al., 2020). These proteins can account for more than 80 % of the total protein content in the beans (Warsame et al., 2020) and up to 65 % in peas (Lu et al., 2020). Both peas and faba beans are low in the sulphur-containing amino acids, but rich in the essential amino acid lysin, which make them excellent in combination with grain proteins (Warsame et al., 2020, 2022; Dahl, Foster, & Tyler, 2012). These legumes can be grown in a wide geographical area, including high-ground regions, with a short and low-demanding cultivation season. Faba beans are also superior in nitrogen fixation compared to other legumes (Neugschwandtner et al., 2015).

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Compared to proteins from animal sources such as milk, there is limited knowledge on the bioactive properties of peptides released during gastrointestinal digestion of proteins from pulses such as pea and faba bean. However, during the recent years increasing interest has been seen regarding BAPs from legume proteins and their use as pharmaceutical ingredients and in functional foods (Martineau-Cote et al., 2022a). In several studies presented in this review legume derived BAPs were obtained by enzymatic hydrolysis using either enzyme sources and/or conditions that do not mimic the environment of the human gastrointestinal tract.

Most recently Martineau-Côté et al. (2022b) compared the antioxidant, antidiabetic and antihypertensive activities of raw and cooked flours of faba bean, soy bean and pea after *in vitro* digestion using the standardized gastrointestinal INFOGEST protocol with gastrointestinal enzymes of animal origin and including ileal digestion. In total 11 peptides from faba beans were identified and the bioactivities of di- and tri-peptide fragments were predicted silico.

Due to the amount of information on protein structures and activity of peptides that has been accumulated over the last years and the increase of information coming from present studies, it is evident that *in silico* tools or machine learning methods are good approaches for increasing our knowledge on food bioactives. To this purpose, Corrochano et al. (2021) stated that computational approaches represent a significant promise to characterize bioactive elements within functional food, concerning both release and stability, as well as the efficacy of the activity.

For peptides to exert biological activities in humans they need to be absorbed through the gut epithelium and transported via the blood stream to their target receptors (León-Espinosa et al., 2016; Jakubczyk et al., 2019). Certain BPAs may also act locally in the human gut as ligands for nutrient receptors, e.g. iron chelators (Martineau-Cote et al., 2022a). These receptors relay signals affecting the functions of the intestinal epithelium and of different immune cells, both in the gut and the periphery (Corrochano et al., 2021). Bioactive compounds may function in many ways, for example as receptor agonists, partial agonists or antagonists, that influence intracellular signalling pathways that affect cell function (Hruby, 2002). Such effects can be traced using *in vitro* cell systems.

In general, there is a lack of in-dept research on the health effects of BAPs in legumes, especially concerning their immune-modulating properties. There is also a need to improve the knowledge of the allergenic potential of legumes when introducing more and new vegetable ingredients in our diet.

This study aimed to investigate the peptidomic profile of pea and faba bean protein after *in vitro* digestion using human gastrointestinal (GI) juices to simulate human digestion, where *in silico* approaches were applied to identify homology of released peptides to known allergenic epitopes, and to predict new possible bioactive peptides to be tested for immune-modulating properties using *in vitro* cell models.The workflow is shown in Fig. 1

2. Materials and methods

2.1. Plant materials

Faba bean (*Vicia faba* L. var. Vertigo) and pea (*Pisum* sativum L. var. Ingrid) varieties adapted to the Norwegian climate were supplied by Nofima (Aas, Norway). Protein rich fractions were obtained through dry milling and air-classification and their proximate composition was analysed as described by Saldanha do Carmo et al. (2020). Proximate composition of the fractions can be found in Table 1. Prior to *in vitro* model digestion the protein rich fractions were solubilized 1:10 w/v in water to obtain a paste-like consistency.

2.2. In vitro model digestion of protein rich fraction of peas and faba beans

Human gastric and duodenal juices were collected according to Ulleberg et al. (2011) by aspiration from healthy volunteers at Lovisenberg Diaconal Hospital (Oslo, Norway). The aspiration procedure was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (REK 2012/2230 and 2012/2210). The gastric and duodenal juices were aspirated simultaneously through three-lumen silicone tube, and the aspirates from 20 healthy subjects were pooled into one gastric and one duodenal juice batch, and stored at −20 °C. The pepsin and trypsin activities of the human gastric and duodenal juices were assayed according to Minekus et al. (2014) prior to the simulated model digestion.

The samples were digested according to (Brodkorb et al., 2019) with some modifications. In short, 500 mg pea and faba bean paste was mixed 1:1 with simulated salivary fluid (SSF) containing human alpha amylase (75 U/mL). Samples were incubated at 37 °C for 2 min. Gastric phase was performed by mixing one volume of oral bolus with an equal volume of human gastric juice that was adjusted with simulated gastric fluid up to pepsin activity of 2000 U/mL in the final digestion mixture. The pH was adjusted to 3 and the samples were incubated in a water bath with magnetic stirring at 37 °C for 120 min. The intestinal digestion phase was performed by mixing the gastric chyme (1:1, v:v) with human duodenal juice combined with simulated intestinal fluid to achieve a trypsin activity of 100 U/mL in the final mixture. The pH was adjusted to 7 and the samples were incubated as described above for 120 min. The enzyme activity was terminated at sampling by adding 5 mM Pefabloc® (Sigma Aldrich, St. Louis, MO, US). The digestion was performed in

Table 1
Composition of protein fractions obtained by air classification of peas and faba beans adapted to the Norwegian climate.

	Dry matter (dm)	Protein (% dm)	Starch (% dm)	Fat (% dm)
Pea protein	89.0 %	56.2	4.5	3.4
Faba bean protein	92.5 %	63.2	8.8	3.3

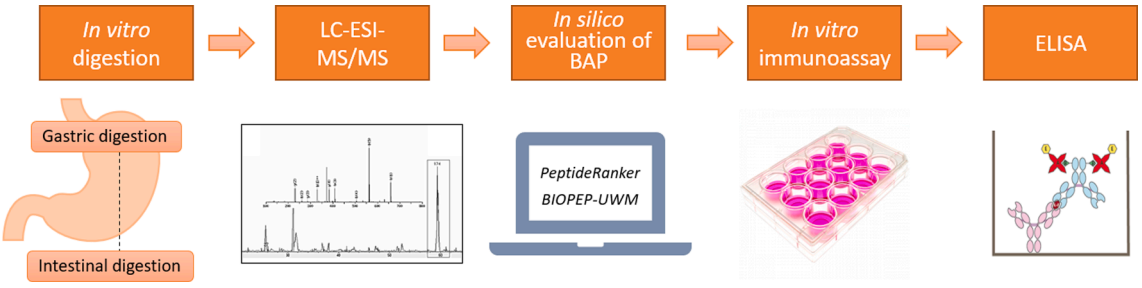

Fig. 1. Experimental overview: Pea and faba bean protein was digested with human GI juices. The digest was analyzed by LC-ESI-MS/MS, and the peptide profiles were further elaborated by *in silico* tools. Selected peptides were tested in Caco-2 cells *in vitro* to establish any immune-modulatory properties.

parallel, and all digested samples were immediately stored at −20 °C until further analysis.

2.3. Peptide profile by HPLC-ESI-MS/MS

Identification of peptide profiles of the digests was done according to Asledottir et al. (2020). In short, digests (100 μL) were desalted using a C18 spin column (Thermo Scientific, San Jose, CA, USA), according to the manufacturer's instructions, eluting with 70 % acetonitrile (v/v)/ 0.1 % trifluoroacetic acid (TFA). Mass spectrometry (MS) analysis was performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled with an Ultimate 3000 ultra-high-performance liquid chromatography instrument (Thermo Scientific, San Jose, CA, USA). Purified peptides were diluted in 50 μL of 0.1 % (v/v) formic acid solution, loaded through a 5 mm long, 300 mm internal diameter pre-column (LC Packings, San Jose, CA, USA) and separated by an EASY-Spray. PepM*****ap C18 column (2 μm, 15 cm–75 μm; 3 mm particles; 100 Å pore size (Thermo Scientific, San Jose, CA, USA)). Eluent A was 0.1 % formic acid (v/v) in Milli-Q water and eluent B was 0.1 % formic acid (v/v) in acetonitrile. The column was equilibrated with 5 % eluent B. Peptides were separated by a 4–40 % eluent B gradient over 60 min (300 nL/min). The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode by scanning the 1800–350 *m/z* range. A maximum of 10 of the most intense MS1 ions were fragmented in MS/MS mode. The resolving power was set at 70,000 full width at half maximum (FWHM), using automatic gain control (AGC) target of 1x 10^6 ions and 100 ms as a maximum ion injection time (IT) to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM and 10 s dynamic exclusion was used to prevent repeated fragmentation of the most abundant ions. Ions with one or more than six charges were excluded from fragmentation. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific, San Jose, CA, USA).

2.4. Spectra identification and database search

Peptides were identified from the MS/MS spectra using the Proteome Discoverer 2.1 software (Thermo Scientific, San Jose, CA, USA), based on the Sequest searching algorithm. Searches for pea and faba bean proteins were taxonomically restricted to *Pisum sativum* and *Fabaceae* database, respectively, extracted from UniProtKB (downloaded in February 2018). Due to limited database entries for Faba bean (*Vicia faba*) proteins, the search with wider taxonomic group was chosen to maximize the identification of peptides by sequence homology. Search parameters were: Met oxidation and pyroglutamic acid for *N*-terminus Gln as variable protein modifications; a mass tolerance value of 10 ppm for precursor ions and 0.01 Da for MS/MS fragments; no proteolytic enzyme selected. The false discovery rate and protein probabilities were calculated by a target decoy peptide spectrum match (PSM) validator working between 0.01 and 0.05 for strict and relaxed searches, respectively. Data from three replicate LC-MS/MS analyses were merged.

2.5. In silico analysis of bioactive peptides in digests

The potential bioactivity of all peptides identified after *in vitro* GI digestion was predicted and scored using PeptideRanker (https://distilldeep.ucd.ie/PeptideRanker/) (Mooney et al., 2012). Peptides with scores above 0.5 are considered possible bioactives. Two peptides from faba bean digests and two peptides from pea digests with PeptideRanker score greater than 0.89 were selected and purchased from Genosphere Biotechnologies for *in vitro* testing (see section 2.7). Only peptides with a known origin (parent protein) were considered for further study. All identified peptides in digests were also searched against the Bioactive peptide database BIOPEP-UWM (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/) (Minkiewicz et al., 2019), to identify encrypted BAPs not yet released from the digested protein. In addition, all peptide sequences were searched against the Immune Epitope Database (https://www.iedb.org/) restricted to Fabaceae family, to identify possible allergenic determinants within the digest. These peptides were matched by sequence homology with more than 90 % identity.

3. Cell culture

Human colon cancer cells (Caco-2 cells) and the selected test system was based on Quévrain et al. (2016). In brief, Caco-2 cells were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 20 % heat-inactivated fetal bovine serum, 1 % non-essential amino acids and 1 % penicillin–streptomycin in 75 cm^2 culture flasks. The cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. Cell culture medium was changed every 2–3 days. Subculturing was performed at approx. 70 % confluency by washing cells with phosphate buffered saline (PBS) and incubating with trypsin-EDTA (Biowest) for 5 min at 37 °C in a humidified atmosphere containing 5 % CO_2. The cell suspension was centrifuged at 1200*g* for 10 min, the supernatant discarded, and the cell pellet was resuspended in fresh growth medium before seeding at a density of 6x10^6 cells/mL. All cell assays were performed on cells in passage number 55–60 to ascertain reproducibility. The use of cells cultivated for the same number of passages is of utmost importance since cell lines tend to change properties upon prolonged culture due to various cultivating conditions (Lea, 2015a, 2015b).

3.1. In vitro immunoassay of selected peptides in Caco-2 cells by induction of IL-1β

The *in vitro* assay was mainly carried out as described by Quévrain et al. (2016). In brief, Caco-2 cells were plated in Falcon 12-well flat bottom TC-Cell Culture plates with 1.8x10^5 cells per well and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 48 h. Then, growth medium (DMEM) was changed, and cells were incubated for another 48 h. For cell starvation, old growth medium was aspirated from culture plates following addition of serum-free growth medium for 24 h. For cell stimulation, serum-free growth medium was aspirated and discarded. Fresh serum-free growth medium containing the pro-inflammatory cytokine IL-1β (15 ng/mL, Sigma Aldrich, Germany) and synthesized legume peptides at concentrations ranging from 0.1 to 1000 μM was added (Genosphere Biotechnologies, France). A positive and negative control containing only IL-1β and no IL-1β, respectively, was included together with an IL-1 receptor antagonist (IL-1Ra, 30 ng/mL Sigma Aldrich) control, to demonstrate the inhibition of IL-1β by blocking the IL-1 receptor. Lack of test peptide cytotoxicity was verified by the trypan blue exclusion assay using a Countess Automated Cell Counter (ThermoFischer) according to the manufacturer's instructions. Cells were first exposed to the synthesized legume peptides for 30 min before the addition of IL-1β. Plates were gently shaken in a figure eight movement before another 24 h incubation at abovementioned conditions. After incubation, the supernatant was aspirated and immediately centrifuged at 15 000 rpm at 4 °C for 10 min, to remove any harvested cells, before being stored at −20 °C until further analysis. Stimulation of Caco-2 cells with peptides was performed in duplicate wells in every culture plate, and each plate was repeated three times (n = 6).

3.2. Quantification of IL-8 response in Caco-2 cells measured by ELISA

Supernatants collected from the *in vitro* immunoassay in Caco-2 cells were tested with enzyme-linked immunosorbent assay (ELISA) to quantify the IL-8 response following peptide exposure. Human IL-8 (CXCL8) Standard ABTS ELISA Development Kit was purchased from PeproTech and the assay performed according to the manufacturer's instructions. Absorbance was read by SpectraMax spectrophotometer (Molecular Devices) set at shaking for 5 *sec* prior to each plate reading.

All samples were measured in duplicates. The variability in the calculated IL-8 response was evaluated by box-plot analysis, and the significance set at $p < 0.05$ was evaluated by paired T-test. The IL-8 response was expressed as percentage of positive control.

4. Results and discussion

4.1. Identification of peptides released from pea and faba bean proteins

In vitro GI digestion of protein rich fractions of peas and faba beans was performed using the conditions of the consensus model developed through the INFOGEST network (Brodkorb et al., 2019), though with the use of human GI juices aspirated from healthy volunteers. A variety of peptides in the range of 7–24 amino acids released from several proteins within the Fabaceae family was identified by HPLC-ESI-MS/MS. Shorter peptides, which are likely released upon digestion, escaped the MS identification under the current conditions of analysis. Overall, peptides deriving from 48 proteins from different legume species, including peas, beans and lentils, were identified within the faba bean digest. In fact, Warsame et al. (2020) recently pointed out the great structural diversity in the faba bean proteome, resulting in high polymorphism and strict sequence homology between legume species. In the pea digest, peptides from 24 different proteins were identified; however, this search was more specific as the restriction was set to *Pisum sativum*, which reduced protein identification based on sequence homology with other species. All proteins identified are listed in Table 3. As illustrated in Fig. 2, the major peptide-producing proteins in faba bean were the storage proteins legumins and convicillin, which accounted for 75 % of the peptides in the digest. For pea, the major peptide-producing proteins were vicilin and legumins, accounting for close to 80 % of the peptides produced. The major peptide-producing proteins also reflect the most abundant proteins in peas and faba beans (Tzitzikas et al., 2006; Warsame et al., 2020). Overall, 1740 and 2054 peptides were identified within the faba bean and pea protein digests, respectively (Table 2). The peptide list was

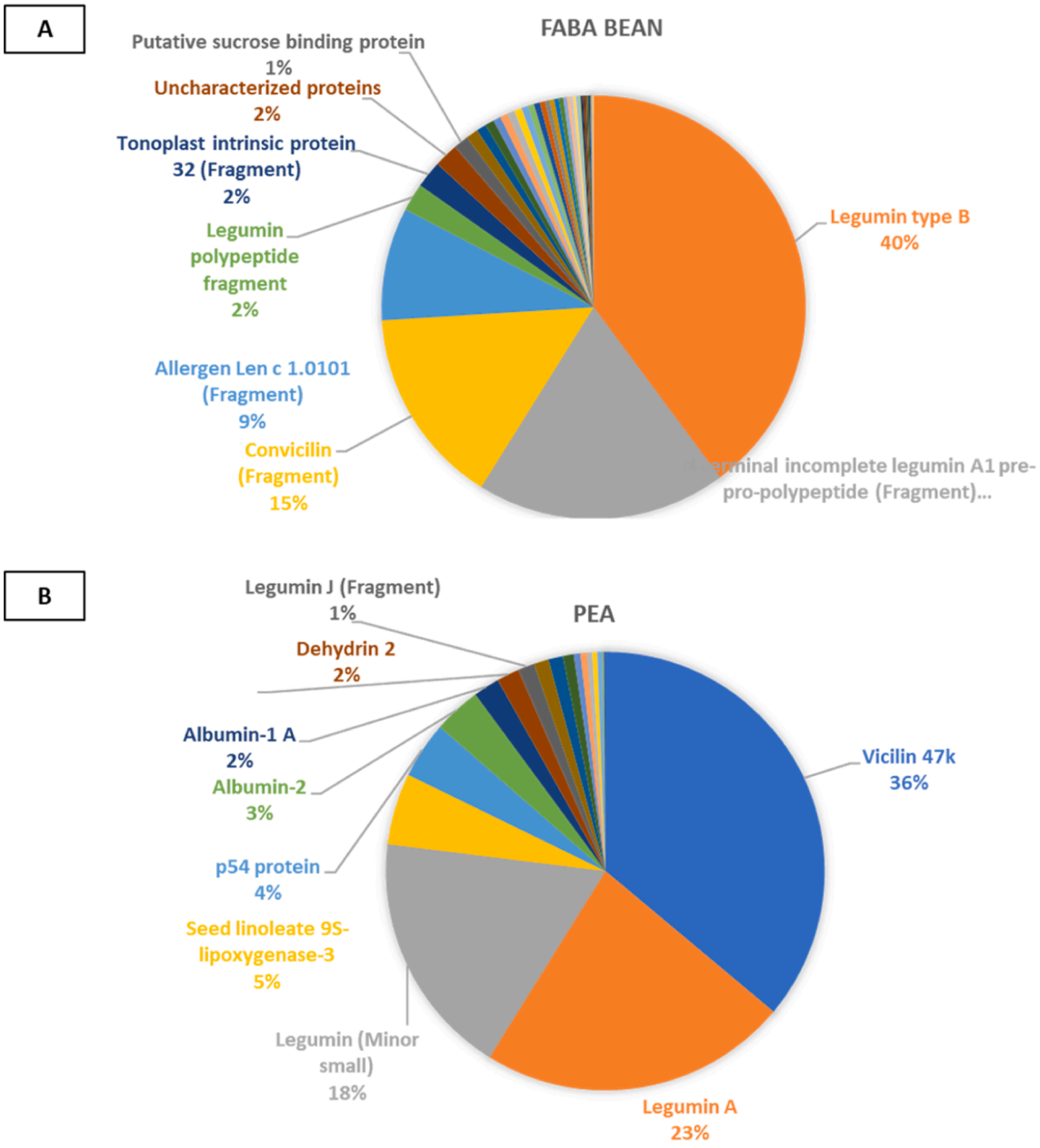

Fig. 2. Representation of parent protein giving rise to peptides in A) faba bean digest and B) pea digest. The peptides were searched against the Pisum sativum and Fabeacae database in UniProtKB.

Table 2
Overview of number of proteins and peptides identified in the digestas, including bioactive peptides and allergens detected through computational approaches.

Legume	Proteins identified	Peptides identified	Unique peptides identified	BAPs (BIOPEP-UWM)	BAPs (PeptideRanker)	Allergens (Immune Epitope DB)
Faba bean	48	1740	186	268	13	3
Pea	24	2054	227	275	11	6

manually sorted, and a non-redundant list consisted of 186 unique peptides from faba bean and 227 from pea protein digests.

4.2. In silico evaluation of bioactivity within digests

All unique peptides in the digests were tested *in silico* for possible bioactivity using PeptideRanker and searched against the bioactive peptide database BOIPEP-UWM to identify smaller peptides encrypted within the identified sequences. The BIOPEP search engine returned previously identified BAPs within the digests, all listed in Table 4. In total, 268 and 275 BAPs, mostly di- and tripeptides contained within the peptide sequences identified in faba bean and pea digest, respectively, were reported by BIOPEP. Most of the BAPs identified were recognized as inhibitors to angiotensin converting enzymes (ACE), dipeptidyl peptidase IV (DPPIV) inhibitors and some antioxidants. Although appearing to be the major bioactivities in legumes, this identification is rather explained by the database size, as there are over a thousand peptides listed as ACE inhibitors in BIOPEP, and several hundred as DPPIV-inhibitors and antioxidants, compared to, for instance, immune-modulatory peptides with only 78 entries (Minkiewicz et al., 2019). These numbers show the deficit of in-depth research on bioactivity found in food and the need to expand the knowledge to unravel new beneficial health effects, especially concerning immune-modulating properties.

Investigation of bioactives as di- and tripeptides within the peptide sequences of the *in vitro* digest is of importance as these peptides may be released during digestion by peptidases at the brush border membrane (BBM). The pre-absorptive phase of digestion occurring at the BBM is not included in the consensus digestion model used, however, based on previous research, we know that the activity at the BBM results in the release of small peptides and amino acids. In a recent study we observed that a heptapeptide from bovine milk was further degraded from both C-terminal and *N*-terminal end when subjected to *in vitro* digestion by porcine jejunal BBM vesicles (Asledottir et al., 2019). Jakubczyk et al. (2019) investigated a faba bean peptide fraction, obtained by fermentation and hydrolysis under GI conditions using porcine enzymes, with the highest potential for inhibition of metabolic syndrome. The authors identified by HPLC-ESI-MS/MS, six unique sequences in this fraction, 12–25 amino acid residues long, harboring a range of different smaller peptides identified as bioactive in the BIOPEP database.

All unique peptides identified in this study were also analyzed using the online tool PeptideRanker, to predict bioactive sequences released during *in vitro* digestion. The test returned 13 unique peptides as possible BAPs within the faba bean digest, and 11 unique peptides within the pea protein digest as shown in Table 5. The prediction of bioactivity returned peptides released from some of the less abundant proteins identified in the faba bean digest, such as glutamine amidotransferase type-2 domain-containing protein, previously identified in soybean, and phytocyanin domain-containing protein, previously identified in Phaseolus vulgaris. Two peptides released from these proteins, namely QQGPPPPPPPISL (PR score 0.93) and ATPPPPPPPPMSL (PR score 0.94), were selected for in vitro immune-modulatory assay. Both peptides are proline rich sequences which makes them considerably resistant to hydrolysis during GI digestion. The two peptides selected from pea digest were DKPWWPK (PR score 0.94) and NEPWWPK (PR score 0.89), released from the proteins seed linoleate 9S-lipoxygenase-2 and −3, respectively.

4.3. In silico evaluation of allergens within digest

The Immune Epitope Database was used to search for known allergens within the digests. All peptides were search against epitopes from the *Fabaceae* family. In total, 22 peptide sequences in pea and faba bean digests were matched with higher than 90 % homology with 8 different epitopes from *Arachis hypogaea* (peanut), *Glycine* max (soybean) and *Lens culinaris* (lentil). All sequences are listed in Table 6. Also, Santos-Hernandez et al. (2020) identified several resistant peptides from soybean, lentil, and pea after *in vitro* digestion using porcine GI enzymes, that exclusively matched epitopes from other species. This is in consistency with the described IgE cross-reactivity between peanut and soybean, which has been demonstrated both clinically and by epitope mapping. However, cross-reactivity between other species is also likely but has not yet been demonstrated in a similar way. EU regulations for allergen labeling of food contain 14 different allergens at present, including wheat, egg, milk, peanut, fish, crustaceans, soy, tree nuts, sesame, shellfish, mustard, celery and lupin (EFSA Panel on Dietetic Products & Allergies, 2014). Three of these established allergens, namely peanut, soy and lupin belong to the *Fabaceae* family together with many other common legumes, such as beans, peas, and chickpeas to name a few. Considered the high sequence homology with established allergens it is important to investigate the potential allergenicity of pea and faba proteins, as species within the same family can share the same motif introducing cross-reactivity.

4.4. In vitro immune-modulatory response of selected peptides

Immune-modulatory properties of selected legume peptides were evaluated by challenging Caco-2 cells with the inflammatory cytokine IL-1β in the presence of selected peptides followed by measurement of the IL-8 response. No cytotoxic effects of the test peptides were observed either when tested alone or in combination. As illustrated in Fig. 3, peptide exposure during IL-1β induction resulted in a significant decrease in IL-8 release, compared to the positive control (no legume peptide present). The peptides QQGPPPPPPPISL and ATPPPPPPPPMSL found in faba bean digests showed immune-modulating properties at higher concentrations of the peptides (greater than 100 μM), and even stronger modulation, i.e., 40 % inhibition, when cells were co-exposed to both selected peptides (Fig. 3A). Interestingly, Corrochano et al. (2021) also discovered a peptide, TIKIPAGT, from faba beans with anti-inflammatory activity using a machine-learning approach. This peptide was identified through a combination of targeted and untargeted approaches, before validation *in vitro* by measuring the decrease in LPS-induced TNF-α response in human macrophages, showing promising immune-modulating properties.

Exposing Caco-2 cells to peptides deriving from pea protein digests resulted in an even stronger reduction in the IL-8 response compared to faba bean peptides. When cells were exposed to the peptide DKPWWPK at concentration from 0.1 to 100 μM, the IL-8 response was reduced by approx. 40 % for all concentrations, showing no particular correlation between concentration of the peptide and the IL-8 response (Fig. 3B). The other pea peptide, NEPWWPK, showed a dose-dependent response ($R^2 = 0.89$), with significant reduction of the IL-8 response at peptide concentration of 10 and 100 μM. Similar to the faba bean peptides, the pea peptides showed the highest reduction of the IL-8 response when tested in combination, at concentration 10 and 100 μM. To our best knowledge this assay has not been applied to the study of food derived peptides. Quévrain et al. (2016) used the same system to study the effect

Table 3
Parent proteins of peptides identified within pea and faba bean digest obtained by HPLC-ESI-MS/MS.

Legume digested	Accession number	Protein Name	Coverage (%)	Protein MW	Species Identified
Pea	P30164	Actin-1	2.4	41726.2	PEA
	P46258	Actin-3	4.8	41635.1	PEA
	P62926	Albumin-1 A	6.9	13912.1	PEA
	P08688	Albumin-2	15.6	26238.6	PEA
	P12886	Alcohol dehydrogenase 1	5.8	41155.8	PEA
	B0BCJ0	Convicilin (Fragment)	8.4	19239.8	PEA
	O04117	Dehydrin 2	7.5	27088.1	PEA
	P12227	DNA-directed RNA polymerase subunit beta'' (Fragment)	1.2	133600.0	PEA
	P46257	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	2.8	38491.1	PEA
	O24648	Gibberellin 3-beta-dioxygenase 1	2.7	41726.0	PEA
	Q40980	Heat shock protein hsp70	1.9	71167.3	PEA
	Q5NJL5	Late embryogenesis abundant protein	4.7	38828.3	PEA
	O24294	Legumin (Minor small)	22.3	64872.9	PEA
	P02857	Legumin A	36.8	58805.8	PEA
	Q41032	Legumin J (Fragment)	47.2	4187.5	PEA
	Q7M1N3	Legumin L1 beta chain (Fragment)	15.7	7849.1	PEA
	A8I354	Mitochondrial pyruvate dehydrogenase kinase isoform 1	2.4	41705.4	PEA
	Q41068	P.sativum vicilin (Fragment)	14.3	7283.4	PEA
	O49927	p54 protein	9.5	54662.8	PEA
	Q6WG36	RPA 70 kDa subunit	1.7	71510.9	PEA
	P14856	Seed linoleate 9S-lipoxygenase-2	5.6	97134.3	PEA
	P09918	Seed linoleate 9S-lipoxygenase-3	10.3	97629.8	PEA
	Q5W915	UDP-sugar pyrophospharylase	1.7	66177.3	PEA
	D3VNE0	Vicilin 47 k	45.8	49895.5	PEA
Faba bean	Q6E2Z6	1-Cys peroxiredoxin	6.9	24414.2	MEDTR
	P02581	Actin-1	2.4	41362.9	SOYBN
	Q43015	Alcohol dehydrogenase-1CN	2.4	40909.3	PHAAT
	Q84UI1	Allergen Len c 1.0101 (Fragment)	11.5	47826.7	LENCU
	C0HJB3	Alpha-mannosidase	1.4	111065.2	CANEN
	A0A151TKC9	Auxin efflux carrier component	1.6	61,386	CAJCA
	A0A0L9VFP1	Auxin response factor	1.6	80479.2	PHAAN
	A0A1S3UMS3	beta-conglycinin, beta chain-like	2.1	49055.9	VIGRR
	A0A151SLM9	Blue copper protein	7.1	20721.2	CAJCA
	B0BCL8	Convicilin (Fragment)	29.4	57501.5	VICFA
	A0A1S2XSR4	Cytokinin riboside 5′-monophosphate phosphoribohydrolase	6.1	27251.4	CICAR
	A0A0B2QHG3	DEAD-box ATP-dependent RNA helicase 24	2.4	85,544	GLYSO
	O04117	Dehydrin 2	3.5	27088.1	PEA
	P21226	Endochitinase A2	4.3	34678.3	PEA
	A0A1S3TV88	FHA domain-containing protein PS1 isoform X1	1.1	131486.4	VIGRR
	A0A0R0IBI6	Formin-like protein	1.2	131062.7	SOYBN
	G7IHC9	Formin-like protein	1.3	95005.4	MEDTR
	A0A151SFW7	Formin-like protein	1.2	110348.8	CAJCA
	A0A0R0EX18	Glutamine amidotransferase type-2 domain-containing protein	7.6	19068.8	SOYBN
	A0A151TUL0	Glycinin G3	2.2	50177.5	CAJCA
	P26413	Heat shock 70 kDa protein	1.9	70880.2	SOYBN
	A0A072UP10	Hydroxyproline-rich glycoprotein family protein, putative	1.6	80,803	MEDTR
	G7LAF4	Inner membrane protein	1.2	84592.4	MEDTR
	G7IKW1	KRI1-like protein	2.6	76,263	MEDTR
	O82464	Late embryogenic abundant protein	11.6	12207.5	VIGRR
	P05190	Legumin type B	26	54448.1	VICFA
	Q43672	Legumin; legumin-related high molecular weight polypeptide (Fragment)	32.4	15316.9	VICFA
	A0A072VLM9	Major intrinsic protein (MIP) family transporter	3.9	27568.4	MEDTR
	Q2HTL8	NAD(P)-binding rossmannfold protein	7.2	31804.5	MEDTR
	Q03971	*N*-terminal incomplete legumin A1 pre-pro-polypeptide (Fragment)	23.5	56793.2	VICFA
	A0A151TN42	Phototropin-1	1.7	109184.8	CAJCA
	V7AQC3	Phytocyanin domain-containing protein	7.2	19565.4	PHAVU
	P02854	Provicilin (Fragment)	2.7	46385.3	PEA
	A0A1S3VMC3	pumilio homolog 12-like	2.1	47710.7	VIGRR
	A0A151UAJ0	Putative gibberellin receptor GID1L3	4.7	34763.8	CAJCA
	Q9AVP7	Putative sucrose binding protein	12.2	54614.9	VICFA
	A0A072VM90	Seed biotin containing protein SBP65, putative	1.3	80195.8	MEDTR
	P09918	Seed linoleate 9S-lipoxygenase-3	3.1	97629.8	PEA
	A0A024NRI7	Tonoplast intrinsic protein 32 (Fragment)	8.1	18253.7	VICFA
	A0A0L9TSE1	TPR_REGION domain-containing protein	1.9	73770.7	PHAAN
	C6TK03	TPR_REGION domain-containing protein	4	33079.4	SOYBN
	G7JHP7	Transmembrane protein, putative	3.1	55321.2	MEDTR
	A0A0L9UWJ7	Uncharacterized protein	4	26151.5	PHAAN
	A0A1J7IDJ4	Uncharacterized protein	1.2	109000.1	LUPAN
	V7CZ66	Uncharacterized protein	9.5	23705.2	PHAVU
	A0A1J7I6K9	Uncharacterized protein	1.3	90780.3	LUPAN
	V7B4Z3	Uncharacterized protein	1.3	89470.7	PHAVU
	A0A1S2Z249	zinc finger protein WIP2-like	4.2	41083.2	CICAR

Table 4
Bioactive peptides identified within the digests of pea and faba bean protein, search through BIOPEP database.

Legume	Activity	Sequence
Faba bean	Antiamnestic	PGP, PG, GP
	ACE inhibitor	VLP, LHLP, RL, IR, AAP, IY, VF, LPP, TAP, VY, FP, IPA, VAP, VPP, GY, PR, LSP, LNP, YL, LF, YG, AY, YP, LLP, GLP, GP, PL, VK, PSY, IA, GW, IP, RP, AF, AP, LA, VP, RA, AA, GF, IF, VG, IG, GI, GA, GL, AG, GH, HL, GR, KG, FG, DA, GS, GV, GQ, GK, GT, GE, GG, QG, AI, SG, LG, GD, TG, EG, EA, NG, PG, PAP, VAV, YGG, QK, DG, NY, SY, KY, KF, KL, NK, RR, AR, KP, GPP, EI, IE, EV, VE, TE, LQ, LN, PT, TQ, AH, PP, PQ, EW, EK, KE, HP, PH, ALEP, LVY, VNP, AV, LEE, GQP, AGP, EAP, LEK, VGP, TP, DF, YV, YE, WL, GAGP, QGP, PPP, RG, GTG, ST, YN, DFG, LR, QP, KLP, EF, ER, FF, DR, LP
	Celiac toxic	QQQP
	Antithrombotic	GP, PGP, PG, DEE
	Immunomodulating	YG, YGG, EAE
	Stimulating vasoactive substance release	EEE, SSS, LLL, EE, SE
	Glucose uptake stimulating peptide	VL, LV, IV, LI, II, LL
	Neuropeptide	GQ, YL
	Peptide regulating the stomach mucosal membrane activity	PGP, PG, GP
	Regulator of phosphoglycerate kinase activity	SL
	Anticancer (Dvl protein binding)	VVV
	Antioxidative	LH, LLPH, HL, AY, IY, AH, EL, HAH, LHL, PHA, PHF, PHT, IKL, GGE, K_D, PEL, IR, KP, TY, VY, TW, ALEPDHR, TETWNPNHPEL, GPP, NEN, KKY
	Bacterial permease ligand	KK
	Anti-inflammatory	VPP, PY, GGW
	Inhibitor of insulin secretion	PGP
	Dipeptidyl carboxypeptidase inhibitor	PPPE, PPPG
	Chemotactic	PGP, GVAPG
	Hypotensive	AA
	Activating ubiquitin-mediated proteolysis	RA, LA
	Dipeptidyl peptidase IV inhibitor	PPPP, GP, PP, MP, VA, LA, AP, PA, LP, VP, LL, VV, HA, IPA, APG, IP, TP, WP, SP, FP, RP, KP, HP, YP, GA, IA, RA, EP, NP, TA, QP, FL, HL, EK, AL, SL, GL, AA, PL, PPG, WL, WN, YT, AD, AE, AF, AG, AH, AS, AT, AV, AY, DN, DP, DQ, DR, EG, EH, EI, ES, ET, EV, EW, GE, GF, GG, GH, GI, GV, GW, GY, HE, HF, HI, HR, HS, HT, HV, II, IN, IQ, IR, KE, KF, KG, KH, KI, KK, KT, KY, LH, LI, LN, LT, LV, MK, MV, NA, ND, NE, NG, NH, NL, NN, NQ, NR, NT, NY, PG, PH, PI, PK, PM, PN, PQ, PS, PT, PV, PY, QA, QD, QE, QG, QH, QL, QN, QQ, QS, QT, QV, QY, RG, RI
	Alpha-glucosidase inhibitor	YP, EA, PP, VE, PE, AD
	Dipeptidyl peptidase III inhibitor	LR, YL, RR, GE, GF, PR, RV, DA, HL, HF, HP, LA, FL, PE, YG, VY
	Campde inhibitor	IR, KF, EF
	Renin inhibitor	LR, IR, KF, EF, NR
	Hypolipidemic	EF
	Tyrosinase inhibitor	FPY
Pea	Antiamnestic	PGP, PG, GP
	ACE inhibitor	RL, IR, GPA, AVP, PYP, LY, IY, VF, MF, LPP, HY, FP, IPA, VAP, PR, LSP, LNP, YL, LF, YG, FY, AY, AFP, AIP, YP, LLP, GPL, GP, PL, LKP, VK, PSY, IA, IP, RP, AF, AP, LA, VP, RA, YA, AA, GF, FR, IF, VG, IG, GI, GA, GL, AG, GH, HL, GR, KG, FG, DA, GS, GV, MG, GQ, GK, GT, HG, GE, GG, QG, AI, SG, LG, GD, TG, EG, EA, NG, PG, YVP, DG, NY, NF, SY, SF, KF, KL, NK, RR, AR, EY, KP, EI, IE, EV, VE, TE, LQ, LN, PT, TQ, PP, PQ, EW, EK, KE, HP, PH, AGSS, ALEP, FVP, AV, LEE, GLY, LVQ, VLY, MPP, EAP, LEK, VGP, DY, TP, DF, DM, YV, YE, SGP, RG, ST, YN, AGS, LR, LDY, QP, KLP, NLR, EF, ER, LPL, YNL, FF, DR, LP
	Antithrombotic	GP, PGP, PG, DEE, RGD
	Immunomodulating	YG
	Stimulating vasoactive substance release	EEE, SSS, LLL, EE, SE
	Glucose uptake stimulating peptide	VL, LV, IV, LI, II, LL
	Neuropeptide	GQ, YL, YLG
	Peptide regulating phosphoinositol metabolism	GLY
	Peptide regulating ion flow	DY
	Peptide regulating the stomach mucosal membrane activity	PGP, PG, GP
	Regulator of phosphoglycerate kinase activity	SL
	Antioxidative	LH, HL, HH, AY, LY, IY, EL, DHH, LHE, PHY, PWW, IKL, GGE, K_D, PW, IR, LKP, LK, KP, TY, LDY, TW, DHG, ALEPDHR, FVPH, SAEHGSLH, NEN, LPL, YNL, YLG
	Bacterial permease ligand	KK
	Anti-inflammatory peptide	PY, HY
	Inhibitor of insulin secretion	PGP
	Chemotactic peptide	PGP
	Hypotensive peptide	AA
	Embryotoxic	RGD
	Activating ubiquitin-mediated proteolysis	RA, LA
	Dipeptidyl peptidase IV inhibitor	GP, PP, MP, VA, LA, FA, AP, PA, LP, VP, LL, VV, HA, IPA, APG, IP, TP, WP, SP, FP, RP, KP, HP, YP, GPA, GA, IA, RA, EP, NP, TA, QP, FL, HL, EK, AL, SL, GL, LPL, AA, PL, WN, WW, AE, AF, AG, AT, AV, AY, DN, DP, DQ, DR, EG, EH, EI, ES, ET, EV, EW, EY, FN, FR, GE, GF, GG, GH, GI, GV, HD, HE, HF, HH, HI, HR, HS, HT, HY, IH, II, IM, IN, IR, KE, KF, KG, KI, KK, KS, KT, KV, LH, LI, LN, LT, LV, MF, MG, NA, ND, NE, NF, NG, NH, NL, NN, NQ, NR, NT, NV, NY, PF, PG, PH, PI, PK, PM, PN, PQ, PS, PT, PV, PW, PY, QA, QD, QE, QG, QH, QI, QL, QN, QQ, QS, QV, RG, RI, RL, RN, RR, SF, SH, SI, SK, SV, SY, TD, TE, TG, TH, TI, TK, TL, TN, TQ, TR, TS, TV, TW, TY, VD, VE, VF, VG, VH, VI, VK, VL, VN, VQ, vS VT, WD, YA, YD, YE, YG, YL, YN, YS, YV, GPAE, FF
	HMG-coa reductase inhibitor	GGV
	Alpha-glucosidase inhibitor	YP, EA, PP, VE, PE
	DPP-III inhibitor	LR, YL, RR, GE, GF, PR, RV, DA, HL, HF, HP, IH, LA, FA, FR, FL, PE, PF, YG
	Campde inhibitor	IR, KF, EF
	Renin inhibitor	FT, LR, IR, KF, EF, NR, SF, YA, LY, LPL, YNL
	Hypolipidemic	EF
	Tyrosinase inhibitor	FPY

of bacterial peptides and found a 40 % reduction which is in the same order of magnitude as observed in our study.

Although this study has not described the cellular mechanism behind the inhibition of IL-8, it is known that IL-1β-induced IL-8 production is facilitated through a series of intracellular cascade reactions. The signal

Table 5
Peptides identified in faba bean and pea protein digest as possible bioactives. Peptides were search through the online tool PeptideRanker. Alle peptides with scores above 0.5 were considered possible bioactives, and bolded ones were selected for immune-modulatory assay.

Legume Digested	Peptide Sequence	PR Score	Protein MW	Accession number	Protein Name	Species
Pea	**DKPWWPK**	0.938	97134.3	P14856	Seed linoleate 9S-lipoxygenase-2	PEA
	NEPWWPK	0.893	97629.8	P09918	Seed linoleate 9S-lipoxygenase-3	PEA
	SNNPFKFLVPA	0.802	58805.8	P02857	Legumin A	PEA
	LPPSSRVGFT	0.695	66177.3	Q5W915	UDP-sugar pyrophospharylase	PEA
	SRSDPQNPF	0.669	49895.5	D3VNE0	Vicilin 47 k	PEA
	ALEPDNRIESEGGLIETWNPNNK	0.620	58805.8	P02857	Legumin A	PEA
	DLAIPVNKPGQLQSFL	0.583	49895.5	D3VNE0	Vicilin 47 k	PEA
	ALEPDNRIESEGGLIETWNPNNKQ	0.551	58805.8	P02857	Legumin A	PEA
	HFEWDDDMGIPGA	0.543	97629.8	P09918	Seed linoleate 9S-lipoxygenase-3	PEA
	NAMFVPHYNL	0.543	58805.8	P02857	Legumin A	PEA
	LSPGDVFVIPA	0.540	49895.5	D3VNE0	Vicilin 47 k	PEA
Faba bean	GAGPPPPPPGA*	0.949	90780.3	A0A1J7I6K9	Uncharacterized protein	LUPAN
	QGPPPPPPPISL*	0.948	19068.8	A0A0R0EX18	Glutamine amidotransferase type-2 domain-containing protein	SOYBN
	ATPPPPPPPPMSL	0.944	19565.4	V7AQC3	Phytocyanin domain-containing protein	PHAVU
	PPPPPPGAKPG*	0.940	95005.4	G7IHC9	Formin-like protein	MEDTR
	QQGPPPPPPPISL	0.931	19068.8	A0A0R0EX18	Glutamine amidotransferase type-2 domain-containing protein	SOYBN
	TAAPPPPPPPMK	0.872	80803.0	A0A072UP10	Hydroxyproline-rich glycoprotein family protein, putative	MEDTR
	QTGSAPPPPPPPG	0.817	131062.7	A0A0R0IBI6	Formin-like protein	SOYBN
	PNAPPPPQP	0.804	61386.0	A0A151TKC9	Auxin efflux carrier component	CAJCA
	GSTIVPPPPPPP	0.698	110348.8	A0A151SFW7	Formin-like protein	CAJCA
	PPPPPEIKH	0.698	47710.7	A0A1S3VMC3	pumilio homolog 12-like	VIGRR
	VQTGSAPPPPPPPG	0.684	131062.7	A0A0R0IBI6	Formin-like protein	SOYBN
	ALEPDNRIESEGGLIETWNPNN	0.586	56793.2	Q03971	*N*-terminal incomplete legumin A1 pre-pro-polypeptide (Fragment)	VICFA
	VTSGLPPPPPPP	0.566	109000.1	A0A1J7IDJ4	Uncharacterized protein	LUPAN

Footnote to Table 5: * At the time when the activities were evaluated by PeptideRanker, the origin of these peptides were unknown.

Table 6
Identified peptides sequences within the digesta matched with known epitopes. The search was performed using the Immune Epitope Database (IEDB) restricted to Fabaceae family and matched against homology with 90% identity. The highest scoring sequence per matched epitope is listed and the matching is bolded in the epitope sequence.

Food	Peptide sequence identified in digest	Epitope sequence	Epitope ID	Parent protein	Species
Pea	QEQENEGNNIFSG	**EQENEGSNIFSG**FAQ	837,156	Ara h 3	Arachis hypogaea
Faba bean	FEITPEKNPQ	DPIYSNKLGKF**FEITPEKNPQ**LRDLD	181,292	Gly m 5	Glycine max
Faba bean	FEITPEKNPQLQ	DPIYSNKLGKF**FEITPEKNPQ**LRDLD	181,292	Gly m 5	Glycine max
Faba bean	FFEITPEKNPQ	DPIYSNKLGK**FFEITPEKNPQ**LRDLD	181,292	Gly m 5	Glycine max
Faba bean	FFEITPEKNPQLQ	DPIYSNKLGK**FFEITPEKNPQ**LRDLD	181,292	Gly m 5	Glycine max
Faba bean	EITPEKNPQL	GKFF**EITPEKNPQL**RD	227,769	Gly m 5	Glycine max
Faba bean	EITPEKNPQLQ	GKFF**EITPEKNPQL**RD	227,769	Gly m 5	Glycine max
Faba bean	FEITPEKNPQLQD	GKF**FEITPEKNPQLRD**	227,769	Gly m 5	Glycine max
Pea	NAMFVPHYNL	SLRK**NAMFVPHYT**LN	59,438	Gly m 6	Glycine max
Faba bean	ALEPDNRIESEGGLIETWNPNN	**IESEGGLIETWNPNN**K	187,845	Gly m 6	Glycine max
Pea	ALEPDNRIESEGGLIETWNPNNK	**IESEGGLIETWNPNNK**	187,845	Gly m 6	Glycine max
Pea	ALEPDNRIESEGGLIETWNPNNKQ	**IESEGGLIETWNPNNK**	187,845	Gly m 6	Glycine max
Pea, Faba bean	IESEGGLIETWNPNN	**IESEGGLIETWNPNNK**	187,845	Gly m 6	Glycine max
Pea	IESEGGLIETWNPNNK	**IESEGGLIETWNPNNK**	187,845	Gly m 6	Glycine max
Pea	IESEGGLIETWNPNNKQ	**IESEGGLIETWNPNNK**	187,845	Gly m 6	Glycine max
Pea	SEGGLIETWNPNN	IE**SEGGLIETWNPNN**K	187,845	Gly m 6	Glycine max
Pea	ELAFPGSSHEVD	EEDNVISQIQRPVK**ELAFPGSS**R**EVD**R	141,628	Len c 1	Lens culinaris
Pea	FVIPAGHPVAI	EEGQEEETTKQVQRYRARLSPGDVL**VIPAGHPVAI**N	141,631	Len c 1	Lens culinaris
Pea	LSPGDVFVIPA	EEGQEEETTKQVQRYRAR**LSPGDV**L**VIPA**GHPVAIN	141,631	Len c 1	Lens culinaris
Pea	FLAGEEDNVIS	RN**FLAGEEDNVIS**QI	141,741	Len c 1	Lens culinaris
Pea	LAGEEDNVISQ	RNF**LAGEEDNVISQ**I	141,741	Len c 1	Lens culinaris
Pea	NFLAGEEDNVIS	R**NFLAGEEDNVIS**QI	141,741	Len c 1	Lens culinaris

transduction of IL-1β occurs upon binding to IL-1 receptor 1 following an accelerated recruitment of IL-1 receptor accessory protein (Lin et al., 2018). Therefore, it is possible that the transduction process has been inhibited by antagonist activity either blocking the site of IL-1β binding or to the accessory protein, both actions terminating the transduction in an antagonistic manner. Alternatively, the BAPs could bind to or interfere with other receptor systems influencing IL-1β-receptor signaling.

5. Conclusion

Pea and faba bean proteins released a large number of peptides with possible bioactive properties during *in vitro* model digestion with human gastrointestinal juices. *In silico* evaluation of the peptide profiles elucidated the large potential of bioactivity within these protein food matrices, including both pro-bioactivity and allergenicity, however, the computational search revealed a deficit of in-depth research on bioactivity concerning immune-modulating properties. The prediction of

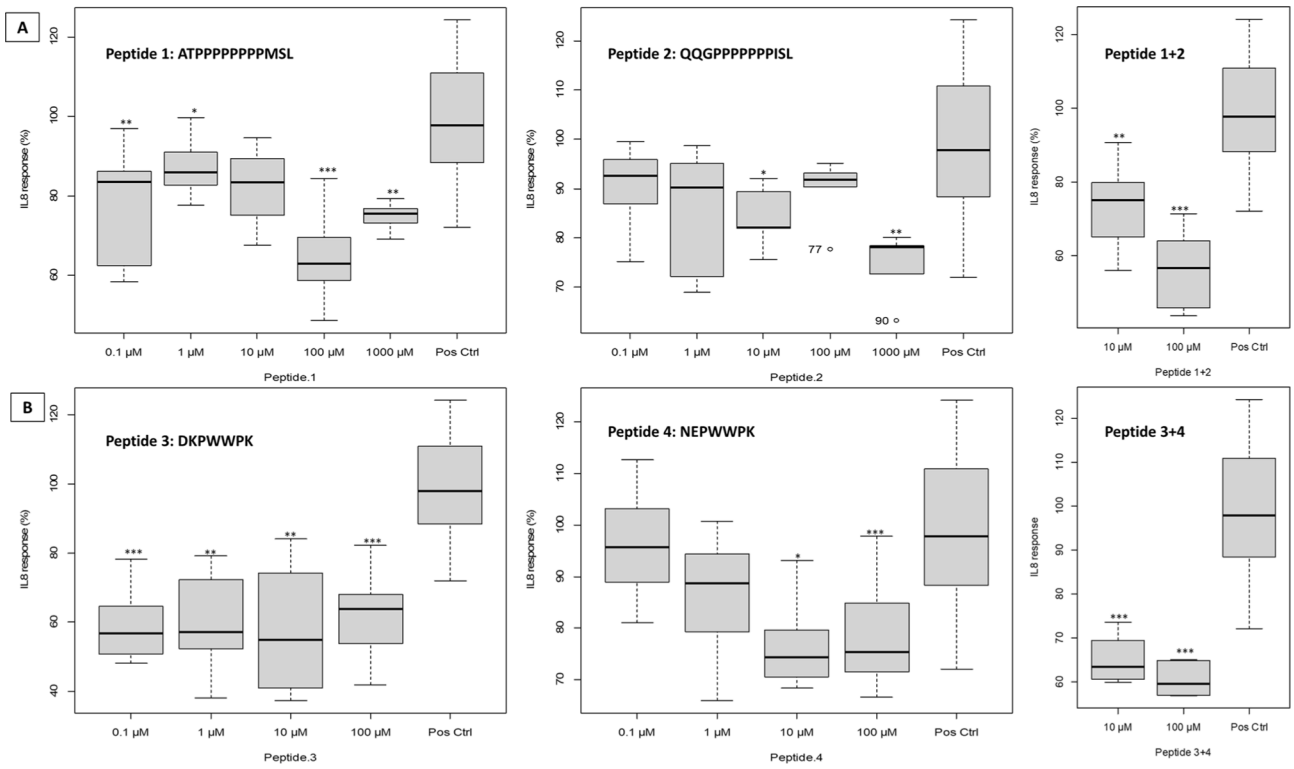

Fig. 3. Relative IL-8 response in Caco-2 cells after exposure to peptides from faba bean (**A**) and pea (**B**). Cells were exposed to IL1-β and test compounds at different concentrations (0.1–1000 µM). IL-8 response was measured by sandwich ELISA and expressed as a percentage response of positive control containing no test compound. Statistically significant IL-8 reduction was tested by paired t-test, and asterix indicates significance at $p < 0.05$(*), $p < 0.01$(**) and $p < 0.001$(***).

bioactivity also enabled the selection of four peptides for *in vitro* cell assay, which actually exhibited an immune-modulating effect by reducing the IL-1β induced IL-8 response in Caco-2 cells. This study illustrates the importance of combining peptidomic approaches and *in silico* tools for screening food digests and profiling new bioactives. The results obtained are useful for expanding the knowledge on bioactivity, in particular immune-modulatory potential of legume proteins, not only to be exploited as singular compounds, but also to unravel new beneficial health effects and to promote benefits of legumes in the common western diet, as this is still a heavily underutilized food category, especially in the Nordic countries.

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CRediT authorship contribution statement

Tora Asledottir: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Gerd Elisabeth Vegarud:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Gianluca Picariello:** Investigation, Writing – review & editing, Resources. **Gianfranco Mamone:** Investigation, Writing – review & editing, Resources. **Tor Erling Lea:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Arne Røseth:** Methodology, Resources. **Pasquale Ferranti:** . **Tove Gulbrandsen Devold:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Ethical Statement

Ethical approval for aspiration of gastric and duodenal juices from human subjects in this study was granted by Norwegian Regional Committees for Medical and Health Research Ethics (REK 2012/2230 118 and 2012/2210).

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