NUTRITIONAL AND BIOACTIVE PROPERTIES OF CANADIAN FABA BEAN (*VICIA FABA* L.) PROTEINS

by

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ABSTRACT

Faba bean (*Vicia faba* L.) is emerging as a sustainable source of plant proteins. As such, a comprehensive portrait of faba bean protein quality is required to clearly seize the opportunities it has to offer as an alternative proteineous ingredient. In this context, the nutritional and bioactive properties of three processed Canadian faba bean varieties (Fabelle, Malik and Snowbird) were compared to two control legumes (pea and soy) following an *in vitro* human gastrointestinal digestion model.

Firstly, the nutritional value of faba bean proteins was assessed. The *in vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS) of raw faba bean flours were determined (13-38) and found in a similar range as pea (13-31) and soy (11-40). The IV-DIAAS significantly decreased ($p < 0.05$) in all boiled legumes, possibly as a result of increased protein aggregation. Since the primary obtained IV-DIAAS were underestimated compared to *in vivo* data, the digestion protocol was further improved to include a jejunal-ileal digestion phase. This addition led to a significant increase ($p < 0.05$) of amino acids digestibility (30% on average) and of IV-DIAAS (over 80% on average). Although the IV-DIAAS remained underestimated compared to *in vivo* data, a strong correlation was observed between *in vitro* and *in vivo* data ($r = 0.879$, $p = 0.009$), demonstrating the physiological relevance of the improved digestion protocol.

Secondly, the health benefits of faba bean flour was investigated through the screening of bioactive peptides resulting from *in vitro* gastrointestinal digestion. The *in vitro* antioxidant assays revealed that faba beans, depending on the variety, had either similar or better activities compared to the pea digestate, and similar or lower activities compared to the soy digestate. By using cellular models, however, the faba bean varieties Fabelle and Snowbird showed higher antioxidant activities than both pea and soy. The antihypertensive properties of Fabelle and Malik varieties were significantly higher ($p < 0.05$) than pea but lower than soy. The antidiabetic activity was higher for soy ($p < 0.05$), but no significant differences were found at the cellular level ($p > 0.05$). Eleven faba bean peptides with *in silico* predicted bioactivities were identified, confirming that the measured bioactive properties can be attributed to peptides.

Thirdly, the 11 peptides identified in the faba bean digestate were chemically synthesized to further investigate and ascertain their antioxidant and antihypertensive activities. Their mechanisms of actions were also investigated with a combination of *in vitro*, cellular and

computational analysis. Seven of the synthesized faba bean peptides showed potent antioxidant activities. These peptides were free radical scavengers through a dual mechanism of hydrogen atom transfer (HAT) and single electron transfer (SET). Four of the antioxidant peptides were also potent angiotensin-converting enzyme (ACE) inhibitors, making them multifunctional peptides. A kinetic study demonstrated that these peptides were noncompetitive ACE inhibitors. Their binding sites were located at the entrance of the active site cavity, as suggested by a molecular docking investigation.

Lastly, the *in vitro* bioavailability of the faba bean peptides released after gastrointestinal digestion was investigated through the assessment of their transport capacity across the intestinal barrier using a co-culture of Caco2 and HT29-MTX cells. Three and five faba bean potent antihypertensive and antioxidants peptides, respectively, were transported intact across the cell monolayer, while maintaining their bioactivities, thereby demonstrating their potential bioavailability.

This research demonstrated that Canadian faba beans have very good nutritional and bioactive properties indicating an excellent potential for use as a functional food ingredient in the development of high-quality protein products with health promoting attributes.

RÉSUMÉ

La gourgane (*Vicia faba* L.) est de plus en plus considérée et utilisée en tant que nouvelle source de protéine viable et durable. Cependant, pour être en mesure d'évaluer son plein potentiel en tant qu'ingrédient protéique alternatif dans l'industrie alimentaire, il est nécessaire d'avoir un portrait global de la qualité de ses protéines. Dans cette optique, les propriétés nutritionnelles et bioactives de trois nouvelles variétés canadiennes de gourganes (Fabelle, Malik et Snowbird) ont été comparées à deux sources de protéines végétales communément utilisées dans l'industrie alimentaire, soit les pois (*Pisum sativum*) et le soya (*Glycine max*) en utilisant un modèle *in vitro* de digestion gastrointestinal humaine.

En premier lieu, ce sont les propriétés nutritionnelles de la gourgane qui ont été investiguées. Les DIAAS (pour Digestible Indispensable Amino Acid Score) des farines de gourganes variaient de 13 à 16 (lorsque calculés en considérant la digestibilité des acides aminés libres) jusqu'à 32 à 38 (lorsque calculé selon la digestibilité des acides aminés totaux). Les DIAAS des farines de gourganes étaient dans un intervalle comparable à ceux du pois (13-31) et du soya (11-40). La variété de gourganes Malik avait au global une qualité nutritionnelle légèrement supérieure à celle de la variété Snowbird. Suite à la cuisson, les DIAAS des farines de l'ensemble des légumineuses étudiées a significativement diminué (p <0.05), probablement en raison de l'augmentation de l'agrégation des protéines. Puisque les premières valeurs de DIAAS obtenues *in vitro* ont révélé être inférieures aux valeurs habituellement rapportées dans les modèles *in vivo*, le protocole de digestion a été modifié pour inclure une phase de digestion jéjunale et iléale. L'ajout de cette dernière phase de digestion a causé une importante augmentation de la digestibilité des acides aminés (de 30% en moyenne) et des valeurs de DIAAS (de 80% en moyenne). Bien que les valeurs de DIAAS demeuraient tout de même sous-estimées par rapport au modèle *in vivo*, une forte corrélation a été observée entre les valeurs *in vitro* et *in vivo* (r=0.879 et p=0.009) ce qui démontrait la pertinence de ce modèle de digestion amélioré.

En deuxième lieu, les effets bénéfiques pour la santé des farines de gourganes ont été comparés à ceux du pois et du soya par le criblage des peptides bioactifs libérés lors de la digestion *in vitro*. Les divers tests antioxydants *in vitro* soit le DPPH (2.2-diphényl 1-pycrilhydrazyle), l'ABTS (acide 2,2'-azino-bis-(3-éthylbenzothiazoline-6-sulfonique), l'ORAC (Capacité d'absorption des radicaux dérivés de l'oxygène) et la chélation du fer ont révélé que les farines de gourgane, dépendamment des variétés, avaient un pouvoir antioxydant similaire ou supérieur à

celui du pois et similaire ou inférieur à celui du soya. Par contre, deux variétés de gourganes (Fabelle et Snowbird) avaient un pouvoir antioxydant supérieur à celui du pois et du soya au niveau cellulaire. L'effet hypotenseur des variétés de gourganes Fabelle et Malik était significativement supérieur à celui du pois, mais inférieur à celui du soya ($p < 0.05$). L'effet antidiabétique *in vitro* du soya était supérieur à celui des farines de gourgane ($p < 0.05$), mais il n'y avait pas de différence significative au niveau cellulaire ($p > 0.05$). Onze peptides provenant de la digestion *in vitro* de la gourgane ont été identifiés et une analyse *in silico* a confirmé que ceux-ci avaient un excellent potentiel bioactif.

En troisième lieu, les peptides identifiés dans les digestats de gourgane ont été synthétisés pour évaluer leurs activités antioxydantes et hypotensives. Leurs mécanismes d'actions ont aussi été étudiés avec une combinaison d'essais *in vitro*, *in silico* et cellulaires. Sept peptides avec une forte activité antioxydante ont été identifiés. Il a été démontré que ces peptides piégeaient les radicaux libres de deux manières différentes, soit par HAT (pour hydrogen atom transfer) et par SET (pour single electron transfer). Quatre de ces peptides avaient aussi un pouvoir inhibiteur contre l'enzyme de conversion de l'angiotensine (ECA), faisant d'eux des peptides multifonctionnels. Une étude cinétique et une étude d'amarrage moléculaire ont révélé que ces peptides étaient des inhibiteurs non compétitifs de l'ECA avec un site de liaison près de l'entrée de la cavité du site actif de l'enzyme.

En dernier lieu, la biodisponibilité *in vitro* des peptides provenant de la digestion gastrointestinal des farines de gourgane a été étudiée. Pour ce faire, une co-culture de cellules Caco2 et HT29-MTX cultivées en monocouche sur un support perméable a été utilisé pour simuler la barrière intestinale. Trois et cinq peptides avec des propriétés hypotensives et antioxydantes, respectivement, ont été transportés, démontrant leur potentiel d'être biodisponible *in vivo*.

Cette recherche a démontré que les protéines des nouvelles variétés de gourganes canadiennes ont d'excellentes valeurs nutritionnelles et bioactives, indiquant un excellent potentiel d'être utilisées en tant qu'ingrédients fonctionnels pour la formulation de produits alimentaires avec des propriétés bénéfiques pour la santé.

STATEMENT FROM THE THESIS OFFICE

According to the regulation of the Faculty of Graduate Studies and Research of McGill University, guidelines for thesis preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guideline for Thesis Preparation" in addition to the manuscripts.

As manuscripts for publication are frequently very concise documents, where appropriate additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reports in the thesis.

In general when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contribution of Authors" as a preface of the thesis.

When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

CONTRIBUTION OF AUTHORS

The thesis consists of nine chapters. **Chapter I** provides a short introduction and describes the research rational as well as the main and specific objectives of the study.

Chapter II gives a literature overview of the current body of knowledge on the nutritional and bioactive values of faba beans in comparison to pea and soy with a particular focus on proteins. The amino acid composition, protein digestibility, impact of food processing on protein digestibility and quality of faba beans are discussed. Then, the potential of faba beans as a health-promoting food ingredients is discussed in presenting the different bioactive properties of faba bean protein hydrolysates reported in literature. The contribution of other faba bean nutrients to its nutritional and bioactive value (starch, fibre, minerals, lipids, GABA, L-Dopa) are also discussed. Finally, the potential challenges to the use of faba bean proteins are presented (favism, lectins and allergenicity).

Chapter III to **Chapter VII** are presented in the form of manuscripts and have been either published, or submitted for publication. The connecting statements provide the rationale linking the different parts of this study.

Chapter III provides a comprehensive investigation of the nutritional quality of three raw and processed Canadian faba bean varieties in comparison to pea and soy using a standardized *in vitro* human digestion protocol. The nutritional value of the different legumes is compared in terms of proximate composition, electrophoretic profile, amino acid profile, amino acid score, protein digestibility, antinutritional factors and *in vitro* digestible indispensable amino acid score (IV-DIAAS).

Chapter IV presents an original improvement of the *in vitro* gastrointestinal digestion protocol to enable a more physiologically relevant assessment of the nutritional quality of food proteins *in vitro*. The digestion protocol was modified through the addition of a jejunal-ileal digestion phase to mimic brush border digestion and was applied to the different legume variety to assess the impact on protein digestibility and the *in vitro* DIAAS.

Chapter V is a comprehensive investigation of the bioactive value (antioxidant, antihypertensive and antidiabetic activities) of faba bean flours after *in vitro* gastrointestinal digestion in comparison to pea and soy. A combination of *in silico*, *in vitro* and cellular models were used to measure these bioactive activities. The peptides responsible for the bioactive effects were purified and identified by mass spectrometry.

Chapter VI presents an investigation of the mechanisms of actions of antioxidant and antihypertensive faba bean-derived peptides identified after *in vitro* gastrointestinal digestion through the use of computational, *in vitro* and cellular models.

Chapter VII is an assessment of the *in vitro* bioavailability of faba bean peptides after *in vitro* gastrointestinal digestion in comparison to pea and soy through the use of a cellular model mimicking the intestinal barrier.

Chapter VIII provides a general conclusion to the thesis with a summary of major findings.

Chapter IX outlines the contribution of this study to the field and provides recommendations regarding future research.

Delphine Martineau-Côté, the author, was responsible for the experimental work, writing of the thesis and preparation of the manuscripts for publication.

Dr. Salwa Karboune, the PhD student's supervisor, guided the research and critically revised the thesis.

Dr. Lamia L'Hocine, the PhD student's co-supervisor, was responsible for funding acquisition, project administration, provided the resources, provide scientific guidance to all the research and critically revised and edited the thesis.

Dr. Allaoua Achouri, the co-author of manuscripts #1-6 (Chapter II-VII) provided scientific guidance, laboratory assistance for the HPLC analysis and critically revised the manuscripts.

Dr. Janitha Wanasundara, the co-author of manuscripts #2 and #4 (Chapter III and Chapter V) was responsible for funding acquisition, project administration and contributed to the revision and editing of the manuscripts.

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PUBLICATIONS

1. Martineau-Côté, D., Achouri, A., Karboune, S., & L'Hocine, L. (2022). Faba Bean: An Untapped Source of Quality Plant Proteins and Bioactives. *Nutrients, 14*(8). https://doi.org/10.3390/nu14081541.

2. Martineau-Côté, D., Achouri, A., Pitre, M., Wanasundara, J., Karboune, S. & L'Hocine, L. (2023). Investigation of the Nutritional Quality of Raw and Processed Canadian Faba Bean (Vicia faba L.) Flours in Comparison to Pea And Soy Using a Human *in vitro* Gastrointestinal Digestion Model. *Food Research International*, 113264. doi:https://doi.org/10.1016/j.foodres.2023.113264.

3. Martineau-Côté, D., Achouri, A., Pitre, M., Karboune, S., & L'Hocine, L. (2024). Improved in vitro gastrointestinal digestion protocol mimicking brush border digestion for the determination of the Digestible Indispensable Amino Acid Score (DIAAS) of different food matrices. *Food Research International*, 178, 113932. https://doi.org/https://doi.org/10.1016/j.foodres.2024.113932.

4. Martineau-Côté, D., Achouri, A., Wanasundara, J., Karboune, S. & L'Hocine, L. (2022). Health Beneficial Bioactivities of Faba Bean Gastrointestinal (*in vitro*) Digestate in Comparison to Soybean and Pea. *International Journal of Molecular Sciences, 23*(16). doi:10.3390/ijms23169210.

5. Martineau-Côté, D., Achouri, A., Karboune, S., & L'Hocine, L. (2024). Antioxidant and Angiotensin-Converting Enzyme Inhibitory Activity of Faba Bean-Derived Peptides After In Vitro Gastrointestinal Digestion: Insight into Their Mechanism of Action. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.4c00829.

6. Martineau-Côté, D., Achouri, A., Pitre, M., Karboune, S. & L'Hocine, L. (2024). Transepithelial Transport Across a Caco-2 and HT29-MTX-E12 Co-Culture Monolayer of Faba Bean Bioactive Peptides, in Comparison to Pea and Soy, After *In Vitro* Gastrointestinal Digestion and Assessment of their Antioxidant Activity (submitted).

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NOMENCLATURE/LIST OF ABBREVIATIONS

4PL	4 parameter logistic curve
AA	Amino Acids
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
AAS	Amino Acid Score
ABS	Absorbance
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)
ACE	Angiotensin-converting enzyme
A_E	Frequency of the release of peptide fragments with a given activity by selected enzymes
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
ALP	Alkaline Phosphatase
ANOVA	Analysis of variance
AP	Apical
ARE	Antioxidant response element
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	area under the curve
BAPNA	Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride
BBM	Brush Border Membrane
BCA	Bicinchoninic acid
BIOPEP-UWM	Bioactive Peptide Database of University of Warmia and Mazury
BL	Basolateral
BPPb	Bradykinin-potentiating peptide b
CAA	Cellular Antioxidant Assay
CCK	Cholecystokinin
CI	Combination Index
D	The dose of peptides required to scavenge free radicals at x% level when used in combination
DCFH	dichloro-dihydro-fluorescein
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DH	Degree of Hydrolysis
DIAAS	Digestible indispensable amino acid score
Dm	half-maximal effective concentration
DMEM	Dulbecco's Modified Eagle Medium
D-PBS	Dulbecco's Phosphate-Buffered-Saline
DPPH	2,2′-diphenyl-1-picrylhydrazyl
DPP-III	dipeptidyl peptidase III
DPP-IV	dipeptidyl peptidase IV
DRI	Dose Reduction Index
D_x	The dose of peptide required to scavenge free radicals x% level when used alone

EC_{50}	half-maximal effective concentration
ECACC	European Collection of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
F_a	Fraction affected
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
G6PD	glucose-6-phosphate dehydrogenase
GABA	γ-Aminobutyric acid
GAE	Gallic Acid Equivalent
GLP-I	Glucagon-like peptide-1
Gly-Pro-AMC	Gly-Pro-7-amido-4-methylcoumarin hydrobromide
GMO	Genetically Modified Organism
GR	Glutathione Reductase
HAT	hydrogen atom transfer
HBSS	Hanks' Balanced Salt Solution
HDL	High-Density Lipoprotein
HHL	N-Hippuryl—His—Leu hydrate
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPEPDOCK	Hierarchical flexible Peptide Docking
HPLC	High-performance liquid chromatography
HSD	honestly significant difference
IC_{50}	half-maximal inhibitory concentration
IDF	Insoluble dietary fibres
INFOGEST	International network of excellence on the fate of food in the gastrointestinal tract
IV-DIAAS	*in vitro* Digestible indispensable amino acid score
IV-PDCAAS	*in vitro* Protein Digestibility Corrected Amino Acid Score
Keap1	Kelch-like ECH-associated protein 1
K_i	Inhibition constant
K_m	Michaelis constant
L	Luminescence
LAB	Lactic Acid Bacteria
LDL	low-density lipoprotein
L-Dopa	L-3,4-dihydroxyphenylalanine
LOX	Lipoxygenase
LY	Lucifer Yellow
MEM	Minimum Essential Medium
mOsm	milliosmole
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry

MTX	Methotrexate
MW	Molecular Weight
MWCO	Molecular weight cut-off
Nrf2/ARE	Nuclear Factor Erythroid 2-Related Factor 2/Antioxidant Response Element Cellular Pathway
OPA	ortho-phthalaldehyde
ORAC	oxygen radical absorbance capacity
P_a	Peak area
PBS	Phosphate-Buffered-Saline
PBS+	Phosphate-Buffered-Saline with Ca2+ and Mg2+
PDB	Protein Data Bank
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PepT1	Peptide transporter 1
PER	Protein Efficiency Ratio
pH	potential hydrogen
PHA	phytohemagglutinin
pI	Isoelectric Point
p-NP	2-nitrophenol
p-NPP	p-nitrophenyl phosphate
Ppm	parts per million
R	pearson coefficient
RAAS	renin-angiotensin-aldosterone system
RCSB	Research Collaboratory for Structural Bioinformatics
RFU	Relative Fluorescence Unit
RIPA	Radioimmunoprecipitation assay buffer
RMSD	root mean square deviation
ROS	Reactive oxygen species
RP	Reverse Phase
SD	Standard Deviation
SDF	Soluble dietary fibres
SDS	sodium dodecyl sulfate
SDS-Page	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SET	single electron transfer
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOD1	Superoxide Dismutase
SPT	skin prick test
SSF	Simulated Salivary Fluid
TBHQ	Tert-Butylhydroquinone
TCH	Total carbohydrate
TDF	Total dietary fibres

TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
TIA	Trypsin inhibitor activity
TID	True Ileal Digestibility
TIU	Trypsin inhibitor unit
TNBS	2,4,6-trinitrobenzene sulfonic acid
TPC	Total Phenolic Content
Tris	tris(hydroxymethyl)aminomethane
Trx1	Thioredoxin 1
U	enzymatic unit
V_{max}	maximum rate
WHO	World Health Organization
WPI	Whey Protein Isolate

List of Amino Acids

Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Cys	C	Cysteine
Glu	E	Glutamic Acid
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Ala	A	Alanine

CHAPTER I. GENERAL INTRODUCTION

1.1 General Introduction

With climate change, population growth and diet-related diseases, there is an increasing need for high-quality protein sources with reduced environmental footprint. Pulses are advantageous alternatives due to their well established nutritive, economic and environmental benefits (Erbersdobler et al., 2017). Faba bean is a modestly produced pulse that has recently attracted the attention of both the scientific community and the food industry due to its various environmental, agronomic and nutritional desirable characteristics (Multari et al., 2015). Faba bean is well adapted to the Canadian climate and is increasingly cultivated in the western provinces of Alberta, Saskatchewan and Manitoba (Penner, 2017). Faba bean has the highest nitrogen fixation capacity among cultivated pulses, thereby, limiting the need for nitrogen fertilization when used in crop rotation. Faba bean is also nutritionally dense with a high content of proteins (30% on average) (Raikos et al., 2014), fibres, starch, minerals (Millar et al., 2019) and health-beneficial bioactive compounds, including GABA (Coda et al., 2017), L-DOPA (Purves et al., 2017) and phenolics (Amarowicz & Shahidi, 2017). Besides, new faba bean varieties with low antinutritional factors content (vicine, convicine and tannins) were developed (Hou et al., 2018; Khazaei et al., 2019), which further improves the nutritional value of faba beans. Based on that, faba bean is a promising pulse to assist in the transition toward a healthier diet and sustainable food system.

Canada is a world-leading producer and exporter of pulses, in particular lentils and peas (FAOStat, 2021). There is, therefore, a high potential to establish faba bean production, exportation and processing in Canada to diversify the Canadian pulse protein offer. New Canadian faba bean varieties have been developed and implemented over the last years (Khazaei et al., 2021). Those possess different quality traits to respond to different markets and consumer expectations. The Malik variety is a large seeded variety with a high-tannin content that is preferred for the human consumption market (Khazaei et al., 2021). The Snowbird variety is a small seeded variety with low-tannin content, which is preferred for the feed industry (Khazaei et al., 2021). The Fabelle variety is a medium seeded variety, with a high-tannin and low-vicine and convicine content. Vicine and convicine are two antinutrients that are specifically found in faba bean and are responsible for favism in a sensitive population. The Fabelle variety possesses therefore an appreciable advantage in a food safety and consumer acceptance perspective.

In this context, it becomes crucial to assess the protein quality of the different faba bean varieties, to inform consumers and the food industry on how optimized consumption and utilization can be achieved. Protein quality is dependent on the essential amino acid composition, but also and more importantly, on their bioavailability after gastrointestinal digestion. It's the bioavailability of amino acids that indicate whether the protein source has the capacity to adequately fulfill human nutritional and metabolic requirements. Besides, the FAO Committee of Experts on Protein Quality Evaluation for human nutrition (FAO, 2013) has emphasized that beyond physiological and metabolic responses, the role of dietary proteins in health improvement and prevention of nutrition-related chronic diseases (ex. cardiovascular diseases, cancer, hypertension, oxidative damage, etc.) also need to be considered in protein quality assessment. Therefore, considering these health outcomes may be especially pertinent in the evaluation of the protein quality of Canadian faba bean varieties.

1.2 Objectives

Since Canadian faba bean varieties have been cultivated on a larger scale only for only a few years (Statistics Canada, 2020), they remain underconsumed and underutilised by the food industry. Information on their nutritional and health-promoting properties are scarce and not well studied in comparison to other legumes, like pea and soy. A better understanding of these properties will be greatly contributive to develop new markets for this highly valuable crop. Thereby, the overall objective of this work was to assess the nutritional and bioactive properties of Canadian faba bean proteins in comparison to two control legumes (pea and soy),widely used in the food industry. This main objective was divided in the following specific objectives:

1) Determine protein nutritional quality (amino acid profile, amino acid digestibility and the Digestible Indispensable Amino Acid Score (DIAAS)) of three processed Canadian faba bean varieties (Fabelle, Malik and Snowbird) comparatively to pea (Amarillo) and soy (AAC-26-15) using a harmonized static *in vitro* gastrointestinal digestion procedure adapted to mimic brush border digestion.
2) Investigate the health-promoting activities of three Canadian faba bean varieties (Fabelle, Malik and Snowbird) through bioactive peptides characterization and identification in the gastrointestinal protein digestate in comparison to pea (Amarillo) and soy (AAC-26-25).

3) Elucidate the underlying mechanism of action of synthesized faba bean bioactive peptides.
4) Assess the *in vitro* bioavailability of faba bean bioactive peptides derived from *in vitro* gastrointestinal digestion using a cellular model of intestinal absorption.

1.3 Hypothesis

- It is hypothesized that faba bean proteins have promising nutritional and bioactive properties, because of protein similarities between faba bean, pea and soy.
- It is hypothesized that the three Canadian faba bean varieties have different nutritional and bioactive properties, as a result of their particular quality traits:
    - Malik (high-tannin and high-vicine and convicine)
    - Snowbird (low-tannin and-high vicine and convicine)
    - Fabelle (high-tannin, low-vicine and convicine)

CHAPTER II. LITERATURE REVIEW

2.1 Abstract

Faba beans are emerging as sustainable quality plant protein sources, with the potential to help meet the growing global demand for more nutritious and healthy foods. The faba bean, in addition to its high protein content and well-balanced amino acid profile, contains bioactive constituents with health-enhancing properties, including bioactive peptides, phenolic compounds, GABA, and L-DOPA. Faba bean peptides released after gastrointestinal digestion have shown antioxidant, antidiabetic, antihypertensive, cholesterol-lowering, and anti-inflammatory effects, indicating a strong potential for this legume crop to be used as a functional food to help face the increasing incidences of non-communicable diseases. This paper provides a comprehensive review of the current body of knowledge on the nutritional and biofunctional qualities of faba beans, with a particular focus on protein-derived bioactive peptides and how they are affected by food processing. It further covers the adverse health effects of faba beans associated with the presence of anti-nutrients and potential allergens, and it outlines research gaps and needs.

2.2 Introduction

Agricultural practices and dietary habits are subject to changes due to the global population increase and climate change. Current predictions suggest that the world population will reach 10 billion people by 2050 and that agriculture will be responsible for up to 30% of greenhouse gas emissions (Willett et al., 2019). Livestock are significant contributors to these emissions by consuming substantial amounts of water and feed and by occupying large land surfaces (Tilman & Clark, 2014). Furthermore, excessive animal-based protein consumption is scientifically proven to be associated with various non-communicable diseases and metabolic disorders, such as obesity (Wang & Beydoun, 2009), type II diabetes (Chen et al., 2020), heart diseases (Abete, Romaguera, Vieira, Lopez de Munain, & Norat, 2014), and cancers (International Agency for Research on Cancer, 2015; Sinha, Cross, Graubard, Leitzmann, & Schatzkin, 2009). For the above reasons, along with increased consumer awareness about carbon emission reductions, there is a need to develop new, high-quality, and more sustainable protein sources. In this regard, legumes are well-acknowledged as valuable alternatives to animal-based proteins due to their economical, environmental, and medicinal properties (Erbersdobler, Barth, & Jahreis, 2017). The legume-based protein market is mainly composed of soy (*Glycine max* L.) and pea (*Pisum sativum* L.)

(Technavio, 2017). However, as the demand for these products continues to increase, it is necessary to diversify and put forward new sources, such as from other pulse crops.

Pulses, such as lentils, chickpeas, and beans, are of high interest due to their well-established nutritional, economical, and environmental benefits (Erbersdobler et al., 2017). Among pulses, the faba bean (*Vicia faba* L.), with its high protein content and agronomic advantages, represents an excellent, yet untapped, source of sustainable and quality dietary proteins. The faba bean is an annual dicotyledonous pulse (*Fabaceae* or *Leguminosae*) that has been grown for millennia in Asia, Africa, and in the Mediterranean region (Witcombe, 1982), but little is known about it/it remains underutilized in western countries (FAOSTAT, 2019). It is well adapted to various climates, including boreal-types (Lizarazo, Lampi, Jingwei, Sontag-Strohm, Piironen, & Stoddard, 2015), and can, therefore, easily grow in colder regions, including in Canada (Oomah, Luc, Leprelle, Drover, Harrison, & Olson, 2011). Worldwide production of faba beans (5.7 million tonnes) is currently modest when compared to soy (353 million tonnes) and pea (14.6 million tonnes) (FAOSTAT, 2019). The faba bean has agronomical, nutritional, and health benefits that may incite production growth in the future. Indeed, the faba bean is recognized as a pulse with a high ability to fix atmospheric nitrogen (Herridge, Peoples, & Boddey, 2008). This characteristic can be wisely used to reduce nitrogen fertilizer applications that lead to detrimental ecological effects, such as eutrophication (Köpke & Nemecek, 2010). Therefore, the faba bean can be used in land rotation (Aschi et al., 2017), in intercropping (to enrich soil) (Dubova, Alsiņa, Ruža, & Šenberga, 2018), and to increase the yield of other plants, such as barley (Mouradi, Farissi, Makoudi, Bouizgaren, & Ghoulam, 2018) and wheat (Xiao, Yin, Ren, Zhang, Tang, & Zheng, 2018; Xu, Qiu, Sun, Müller, & Lei, 2018). Faba bean intercropping also increases genetic diversity, which has a protective effect against the spread of diseases, as stated by a recent meta-analysis (Zhang et al., 2019).

The faba bean, in addition to being a highly nutritive pulse, is not genetically modified (non-GMO) and not a regulated allergen. These are competitive advantages when compared to soy (Calabrò et al., 2014; Health Canada, 2018). Although GMOs are safe to eat, some consumers are still reticent to include them in their diets (Wunderlich & Gatto, 2015). The dry seed is mainly composed of starch, proteins, and dietary fibres (Table 2-1). The faba bean has a higher protein content than most pulses, including peas, chickpeas, lentils, and beans (Estefania, Ivan, Maria, Rosa-Millan, & Othona, 2018; Raikos, Neacsu, Russell, & Duthie, 2014). It is a rich source of vitamins and

minerals and is low in fat. Similar to other pulses, it contains anti-nutritional factors, such as tannins, phytic acid, digestive enzyme inhibitors, oxalate, and lectins that can decrease the bioavailability and uptake of proteins and minerals during digestion and induce toxic effects (Mattila et al., 2018). One particularity of the faba bean involves the presence of vicine and convicine (Shi, Arntfield, & Nickerson, 2018; Shi, Mu, Arntfield, & Nickerson, 2017), which are associated with favism and are significant obstacles to faba bean utilization (Belsey, 1973; Chiremba, Vandenberg, Smits, Samaranayaka, Lam, & Hood-Niefer, 2018). However, those undesirable compounds can be considerably reduced through food processing (Shi et al., 2018; Shi et al., 2017) and breeding strategies (Khazaei et al., 2019). Newly improved faba bean varieties have been developed in recent years to further increase the nutritional qualities of the faba bean. Low tannins (Hou et al., 2018), as well as low-vicine and convicine (Khazaei et al., 2019) cultivars, are now available.

Table 2-1. Proximate compositions of the faba bean compared to pea and soy (g/100 g dry base) compiled from various studies (Abdel-Aal, Ragaee, Rabalski, Warkentin, & Vandenberg, 2018; Adamidou, Nengas, Grigorakis, Nikolopoulou, & Jauncey, 2011; Agume, Njintang, & Mbofung, 2017; Salem S. Alghamdi, Khan, El-Harty, Ammar, Farooq, & Migdadi, 2018; Bhatty, 1974; Boye, Zare, & Pletch, 2010; Cipollone & Tironi, 2020; De Angelis et al., 2021; Frias et al., 2011; E. R. Grela & Günter, 1995; Eugeniusz R. Grela, Kiczorowska, et al., 2017; C. M. Grieshop & Fahey, 2001; Christine M. Grieshop et al., 2003; Hood-Niefer, Warkentin, Chibbar, Vandenberg, & Tyler, 2012; Ivarsson & Neil, 2018; Jiménez-Escrig, Serra, & Rupérez, 2010; Kahlon & Shao, 2004; Kotlarz, Sujak, Strobel, & Grzesiak, 2011; S. R. Kumar, Sadiq, & Anal, 2022; Maeta, Katsukawa, Hayase, & Takahashi, 2022; Martín-Cabrejas, Ariza, Esteban, Mollá, Waldron, & López-Andréu, 2003; P. Mattila et al., 2018; Mayer Labba, Frøkiær, & Sandberg, 2021; Moussou, 2019; Njoumi, Josephe Amiot, Rochette, Bellagha, & Mouquet-Rivier, 2019; Nti, Plahar, & Annan, 2016; Pisarikova & Zraly, 2010; Raikos et al., 2014; Redondo-Cuenca, Villanueva-Suárez, Rodríguez-Sevilla, & Mateos-Aparicio, 2007; Setia, Dai, Nickerson, Sopiwnyk, Malcolmson, & Ai, 2019; Singh, Bohra, & Sharma, 2019; Stoughton-Ens, Hatcher, Wang, & Warkentin, 2010; Ukwuru, 2003; Wilson, Birmingham, Moon, & Snyder, 1978; M. Xu, Jin, Simsek, Hall, Rao, & Chen, 2019).

Legume		Proteins	Carbohydrates						Ash	Fat
			TCH [1]	Starch	Amylose [2]	TDF [3]	IDF [4]	SDF [5]		
Faba bean	Mean	27.6	66.0	40.0	34.0	12.9	15.1	1.4	3.4	1.4
	SD	3.0	5.1	3.4	6.4	9.0	4.6	1.8	0.4	0.4
	Min	22.7	55.2	28.1	18.6	6.4	10.7	0.6	2.6	0.7
	Max	34.7	71.4	47.5	44.4	34.9	30.3	7.6	4.4	3.2
	n1 [6]	106	57	46	24	17	18	18	94	80
	n2 [7]	13	6	7	3	6	4	4	11	11
Pea	Mean	23.4	63.5	44.9	29.6	14.7	11.0	2.5	3.0	1.6
	SD	2.4	7.1	1.2	3.5	2.6	0.9	1.4	0.3	0.5
	Min	18.1	52.8	42.2	19.1	12.2	9.7	1.7	2.4	1.0
	Max	27.5	70.0	46.6	31.6	19.4	12.9	5.6	3.7	2.9
	n1	34	5	18	12	11	8	8	23	23
	n2	12	4	6	3	6	3	3	10	10
Soy	Mean	40.0	28.6	2.7	-	21.9	24.8	2.6	5.2	19.7
	SD	3.0	3.0	2.7	-	8.3	8.6	2.3	0.6	2.2
	Min	31.5	19.7	0.2	-	13.7	15.4	0.6	3.0	14.0
	Max	46.8	33.2	6.7	-	35.5	32.6	6.1	6.3	23.6
	n1	48	31	19	-	9	5	5	40	60
	n2	12	5	2	-	5	4	4	8	12

[1] TCH: total carbohydrate; [2] percentage of total starch; [3] TDF: total dietary fibres; [4] IDF: insoluble dietary fibres; [5] SDF: soluble dietary fibres; [6] n1: number of cultivars; [7] n2: number of references.

The faba bean, beyond its nutritional value, is also a rich source of bioactive compounds that have reported health-enhancing properties. These include phenolic compounds (Amarowicz & Shahidi, 2017), resistant starch (Li, Yuan, Setia, Raja, Zhang, & Ai, 2019), dietary fibres (Çalışkantürk Karataş, Günay, & Sayar, 2017), non-protein amino acids (Purves, Zhang, Khazaei, & Vandenberg, 2017), GABA (Coda, Varis, Verni, Rizzello, & Katina, 2017)), and, foremost, bioactive peptides (Jakubczyk, Karaś, Złotek, Szymanowska, Baraniak, & Bochnak, 2019; Karkouch et al., 2017). The faba bean, due to its richness in health-promoting constituents, has high potential in the development of new nutraceuticals and biofunctional food ingredients.

Many recent studies have focused on the nutritional and bioactive properties of faba beans (Dugardin et al., 2020; Jakubczyk et al., 2019; Karkouch et al., 2017; León-Espinosa et al., 2016; Nosworthy et al., 2018). The aim of this review is, therefore, to summarize the latest key findings related to the nutritional qualities of faba beans, as well as the biofunctional and health-beneficial properties, with particular focus on those related to proteins (Figure 2-1).

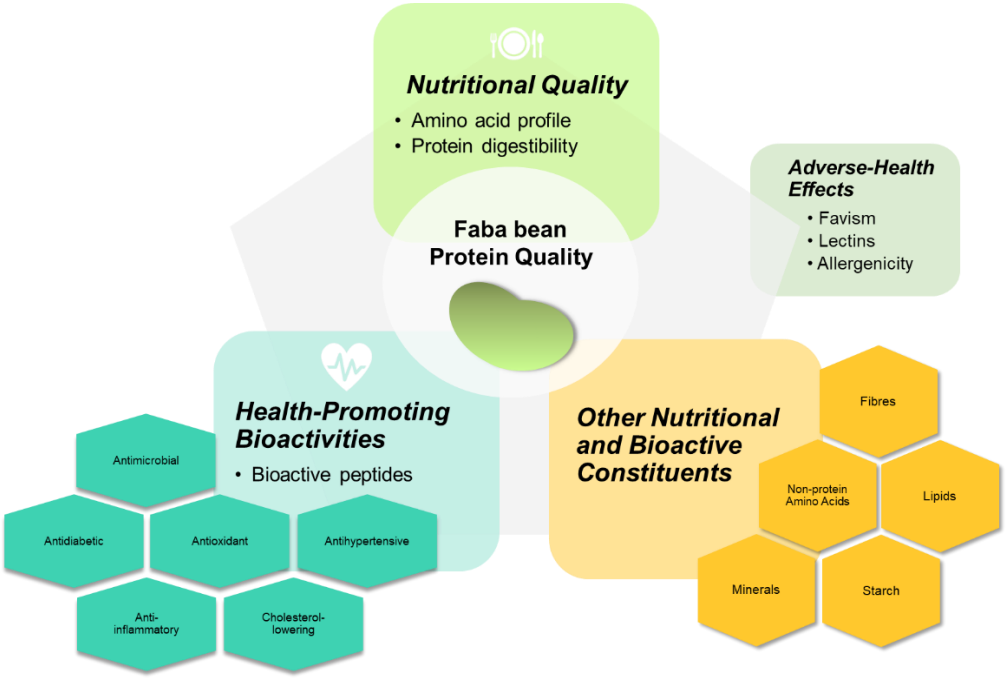

Figure 2-1. Faba bean protein quality is influenced by several factors, including nutritional qualities (amino acid profile and digestibility), health-promoting bioactivities, and other matrix constituents that can have beneficial (as well as adverse) nutritional and bioactive effects.

2.3 Faba Bean Proteins

The faba bean, as presented in Table 2-1, is a high protein pulse, with a higher protein content than peas and most pulses on the market, such as beans (22.17%), lentils (22.15%), and chickpeas

(19.53%) (Estefania et al., 2018), but lower than soy and lupin (40.0%) (Raikos et al., 2014). However, faba bean protein content is highly variable due to the substantial genetic diversity that exists among the species (Altaf Khan et al., 2015; Hood-Niefer et al., 2012; Ivarsson et al., 2018; Multari et al., 2016; Nosworthy et al., 2018). Indeed, several varieties have been developed via breeding over the years, depending on the consumer preferences in the region of origin, disease resistance, and the target market (Maalouf et al., 2019). Faba bean varieties are therefore highly heterogeneous in size (less than 0.3 g to more than 1 g per seed) (Purves et al., 2017), color, and shape (Maalouf et al., 2019; Wei, Wanasundara, & Shand, 2021), depending on the cultivating area and the environmental conditions during the cropping year (Barłóg, Grzebisz, & Łukowiak, 2019). Soil composition, atmospheric nitrogen fixation efficiency, and environmental stress, such as drought, are all factors that can affect faba bean protein content (Ntatsi et al., 2018; Senberga, Dubova, Alsina, & Strauta, 2017). Hood-Niefer et al. (2012) have demonstrated that the protein content of 11 faba bean varieties grown in three western Canadian regions during two different years (2006 and 2007) fluctuated from 27.5 to 32.4%.

Most faba bean proteins are globulin-types, which is common to most pulses (Boye et al., 2010). Plant proteins are classified into four main families, according to their solubilities in different solvents. Globulins are soluble in low-salt solutions, albumins in water, prolamins in 70% alcohol, and glutelins in alkaline solutions (Osborne, 1907). Globulin fractions account for 69.5 to 78.1% of total faba bean proteins, followed by glutelins (12.0 to 18.4%), prolamins (1.83 to 3.57%), and albumins (1.41 to 3.01%) (Alghamdi, 2009). The distributions of these proteins' fractions depend on the cultivars and the environmental conditions (Alghamdi, 2009).

Faba bean globulins are classified according to their sedimentation coefficients, into two major types, namely legumins (11S) and vicilins (7S) (Utsumi, 1992). Legumins are the most abundant globulins in faba beans, composing up to 55% of the seed proteins (Müntz, Horstmann, & Schlesier, 1999). They have hexameric structures, and each subunit is composed of acidic (α) and basic (β) peptide side chains that are retained together by a disulfide bond (Müntz et al., 1999). Vicilins have trimeric structures; each subunit is non-identical (Müntz et al., 1999). They are glycosylated and cysteine-free (Utsumi, 1992) and, therefore, are unable to form disulfide bonds. The legumins to vicilins ratio is an important factor in faba bean protein characterization, having an important impact on the functional properties of proteins (Martinez, Stone, Yovchev, Peter, Vandenberg, & Nickerson, 2016; Singhal, Stone, Vandenberg, Tyler, & Nickerson, 2016). The

legumin to vicilin ratio is affected by numerous factors, including cultivars, environmental conditions, and processing conditions. Warsame et al. Warsame, Michael, O'Sullivan, and Tosi (2020) found that the legumin to vicilin ratio ranged from 1 to 3 in 35 faba bean cultivars from across the world. Singhal, Stone, Vandenberg, Tyler, and Nickerson Singhal et al. (2016) reported that the legumin to vicilin ration ranged from 3.4 to 4.6 in the faba bean flour of seven Canadians cultivars cultivated in four different regions (Singhal et al., 2016), but the difference was not significant. That same ratio was reported to vary from 3.76 to 5.40 in faba bean protein concentrates obtained by the air classifications of five Canadian cultivars cultivated in two different regions (Martinez et al., 2016).

Faba bean legumins are more thermally stable than vicilins; their denaturing temperatures were reported to be 95.4 and 83.8 °C, respectively, for a protein concentration of 0.5 mg/mL (Kimura, Takako, Meili, Shiori, Maruyama, & Utsumi, 2008). Interestingly, denaturing temperatures of faba bean globulins were reported to be higher than pea and soy globulins (Kimura et al., 2008). Faba bean storage proteins have been studied recently with modern proteomic technics, and sixteen main storage proteins have been identified among six protein subunits of 97, 96, 64, 47, 38, and 32 kDa, with isoelectric points varying from 4.90 to 9.55 (Liu, Wu, Hou, Li, Sha, & Tian, 2017). Among them, globulins were the most abundant proteins in the 64, 42, 47, and 38 kDa subunits (Liu et al., 2017).

Faba bean proteins have several advantages regarding the techno–functional properties of food formulation. Such applications have been recently reviewed elsewhere (Rahate, Madhumita, & Prabhakar, 2021; Sharan et al., 2021).

2.3.1 Nutritional Quality

The nutritional qualities of proteins are determined by their amino acid compositions and their respective digestibility and bioavailability during the digestion process, fulfilling the dietary needs (Wang, Lin, Kan, Liu, Zeng, & Shen, 2012). Numerous methods have been used to quantify the nutritional qualities of faba beans. These methods are briefly described in the next section.

2.3.1.1 Assessment of Protein Nutritional Quality

Various metrics are used by regulatory agencies to rate protein sources and to regulate the protein quality claims of food products. Those metrics combine information relative to amino acid

composition and protein bioavailability to determine global protein quality. In Canada, the protein efficiency ratio (PER) is used, which is a measure of metabolizable protein efficacy (Canadian Food Inspection Agency, 2018). It is determined by a 28-day rat bioassay and consists of the ratio between weight gain and protein intake. The value is then normalized to a control to allow a study-to-study comparison (casein PER value is fixed to 2.5) (Health Canada, 1981). The Food and Drug Administration (FDA) uses the Protein Digestibility Corrected Amino Acid Score (PDCAAS), which is a ratio between the first essential limiting amino acid amount for a given food commodity and that same amino acid amount in a reference protein (amino acid score) (Marinangeli & House, 2017). The ratio is then corrected according to the total fecal protein digestibility. The maximum PDCAAS value is 1; values above are truncated (Marinangeli et al., 2017). The PDCAAS has been subsequently criticized because it does not consider the digestibility of each amino acid individually (FAO, 2013). Total fecal protein digestibility is also not entirely accurate since amino acids and short peptide absorption ends in the ileum, and unabsorbed proteins are further degraded in the large intestine by the microbiome (Schaafsma, 2012). Moreover, the truncated value does not provide enough information to evaluate the protein quality of a mixed diet (FAO, 2013) and to efficiently blend various protein sources in food applications to optimize amino acid composition (Marinangeli et al., 2017). The FAO has accordingly developed a new protein quality score based on these preoccupations, called the digestible indispensable amino acid score (DIAAS) (FAO, 2013). This indicator is not truncated and is calculated for each essential amino acid to consider their respective ileal digestibility compared to a reference protein. The suggested reference protein varies according to the age group studied (newborn, children, and adults) because the amino acid requirement is not the same during those different stages of life (FAO, 2013). The lowest DIAAS value among all essential amino acids of a specific food commodity corresponds to the global DIAAS value. DIASS values of 1 and above correspond to a high-quality protein and values between 0.75 and 0.99 correspond to a good-quality protein (FAO, 2013). The total fecal protein digestibility for PDCAAS and the ileal-amino acid digestibility for DIAAS are preferably assessed in humans or animal models (pigs and rats) (FAO, 2013). However, those assays are costly, invasive, ethically questionable, and time-consuming (Brodkorb et al., 2019). *In vitro* digestion assays are therefore widely used in the scientific literature to estimate protein digestibility. Although *in vitro* models are an estimation of the physiological complexity of *in vivo* digestion and not necessarily mimic the dynamic aspects of gastrointestinal digestion, strong correlations

between protein digestibility data obtained *in vivo* and *in vitro* have been observed, for purified protein of various origins (Sousa et al., 2023) and different complex food matrices (Bohn et al., 2018), which suggests the relevance of those digestion models. The above indicators have been used in the literature to quantify faba bean protein nutritional quality; results are discussed in the sections below.

2.3.1.2 Amino Acid Profile

The faba bean has an overall well-balanced amino acid profile that is similar to pea and soy (Table 2-2), containing a high amount of lysine, leucine, isoleucine, threonine, histidine, and aromatic amino acids (Małgorzata, Jerzy, & Ewa, 2018; Millar, Gallagher, Burke, McCarthy, & Barry-Ryan, 2019). However, sulfur-containing amino acids (methionine and cysteine) and tryptophan are present in lower amounts than soy (Małgorzata et al., 2018). This deficiency is common to most pulses (Boye et al., 2010) and is explained by the low content of those three amino acids in globulins (Carbonaro, Grant, & Cappelloni, 2005). Environmental conditions during cultivation have a significant impact on the faba bean amino acid profile (Barłóg et al., 2019). Interestingly, the faba bean amino acid profile is complementary to cereals that are deficient in lysine but contain high levels of methionine and cysteine (Mattila et al., 2018). It can be advantageous to blend faba beans with cereals in food product formulations to optimize the amino acid composition. There is growing interest for such applications in the scientific literature, e.g., Laleg et al. (2018) fortified wheat pasta with 35% of faba bean flour to enhance the essential amino acid content. This way, lysine, threonine, and branched aliphatic amino acid (leucine, isoleucine, and valine) content increased by 97, 23, and 10%, respectively. Furthermore, the protein efficiency ratio (PER) increased two-fold compared to wheat pasta (Laleg et al., 2018), which suggests that this combination is beneficial to increase protein quality. Coda et al. (2017) incorporated 30% of faba bean flour in wheat bread, which significantly increased the chemical score of lysine, threonine, and methionine compared to the control (wheat bread). Thus, these applications have excellent potential in the development of new food products that fulfill the nutritional needs of consumers and in improving product tastes and protein quality.

Table 2-2. Amino acid profiles of whole faba bean seeds compared to pea and soy.

	Faba Bean		Pea	Soy	Amino Acid Scoring Pattern [1]
	cv. Bobas (High tannin)	cv. Kasztelan (Low tannin)	cv. Solara	ND	
Histidine *	2.41	2.29	2.52	2.91	2
Isoleucine *	3.94	3.91	3.33	4.6	3.2
Leucine *	7.47	7.01	6.58	7.76	6.6
Lysine *	7.08	6.71	6.84	7.08	5.7
Methionine *	0.87	1.06	1.03	1.29	2.7 [2]
Cysteine	1.33	0.85	1.55	1.19	
Phenylalanine *	4.19	4.12	4.19	5.87	5.2 [3]
Tyrosine	2.78	2.59	3.16	3.65	
Threonine *	3.40	3.40	3.59	3.69	3.1
Tryptophan *	0.87	0.85	0.94	1.38	0.85
Valine *	4.31	4.12	3.89	4.64	4.3
Arginine	9.46	9.04	6.84	8.86	.
Alanine	4.15	4.03	4.27	4.39	.
Aspartic acid	10.74	10.4	10.68	11.98	.
Glutamic acid	16.51	16.26	16.92	17.88	.
Glycine	4.73	4.25	4.32	4.20	.
Proline	3.94	3.86	3.76	4.92	.
Serine	4.69	4.76	4.79	4.77	.
References	(Małgorzata et al., 2018)	(Małgorzata et al., 2018)	(Leterme, Monmart, & Baudart, 1990)	(Małgorzata et al., 2018)	(FAO, 2013)

*Essential amino acids. [1]Amino acid scoring pattern for children 6 months to 3 years, recommended for regulatory purposes by the FAO (2013). [2]Sulfur-containing amino acids (methionine and cysteine). [3]Aromatic amino acids (phenylalanine and tyrosine).

2.3.1.3 Protein Digestibility

Protein digestibility is a central element in protein quality assessment, by stating to what extent proteins are available for absorption during the digestion process. Plant-based protein digestibility depends on many factors, such as anti-nutritional content, cell wall integrity, particle size, protein structures, and protein interactions with the food matrix (Kashyap et al., 2019). Those elements are intrinsic to the specific food commodities but are also affected by food processing (Nosworthy et al., 2018). The effects of food processing and anti-nutritional content on protein digestibility and quality are discussed in the following sections.

Additionally, physiological factors, such as a consumer's age, also have crucial impacts on protein digestibility. In that respect, faba bean protein digestibility was recently evaluated in the context of infant nutrition (Roux, Chacon, Dupont, Jeantet, Deglaire, & Nau, 2020). The newborn digestive system is immature and the digestive fluid composition, enzyme activities, and pH in the digestive compartments are different than in adults (Hamosh, 1996). This might have a substantial impact on protein digestibility. Gilani and Sepehr (2003) have demonstrated that autoclaved-faba bean seed digestibility diminishes from 82 to 77% when assessed in young rats (5 weeks) compared to older rats (20 months), suggesting a significant impact of the age. Roux et al. (2020)

have replaced milk proteins by 50% plant-based proteins, such as pea, potato, rice, and faba bean in infant formulas. An *in vitro* digestion system that mimics the newborn physiology was used; the degree of hydrolysis and the amino acid bioaccessibility were assessed as indicators of protein digestibility. Amino acid bioaccessibility was defined as the percentage of free amino acids in the digestate compared to the total amino acids in the infant formula. Interestingly, the degree of hydrolysis and amino acid bioaccessibility of the infant formula enriched with either faba bean or pea proteins was not significantly different from the milk control. However, rice and potato protein enrichments significantly decreased protein digestibility, suggesting that pea and faba beans were good alternatives for such a formulation (Roux et al., 2020).

2.3.1.4 Effect of Processing on Protein Digestibility and Quality

Various processing strategies have been employed in the literature to increase faba bean protein digestibility and quality through biochemical and thermal processes.

2.3.1.4.1 Thermal Treatments

Reported thermal treatments in the literature included domestic processes, such as boiling, baking, and roasting, as well as industrial processes, such as extrusion cooking. Nosworthy et al. (2018) evaluated faba bean protein digestibility in rats and in vitro following extrusion, boiling, and oven-baking. Total fecal digestibility in rats ranged from 87.60 to 88.63% while in vitro total digestibility ranged from 76.79 to 82.22%, depending on the process used (Nosworthy et al., 2018). Faba bean showed the highest protein digestibility compared to various bean types, in which protein digestibility ranged from 57.58 to 87.41%. The authors also used total fecal protein digestibility to calculate the DIAAS values instead of the ileal amino acid digestibility. Most amino acids had a DIAAS value above 0.75, which corresponds to a good quality protein source (FAO, 2013), except for the sulfur-containing amino acids (0.54, 0.59, and 0.61 for extruded, boiled, and oven-baked faba bean flour, respectively) and tryptophan (0.70) following boiling. Aromatic amino acids (phenylalanine and tyrosine) and histidine had a DIAAS value above 1.00 for each process studied. They also reported the PDCAAS (58, 54, and 66%) and the PER (0.45, 0.85, and 0.66) for extruded, boiled, and baked faba beans, respectively. Thermal treatments can be beneficial for protein digestibility through their detrimental effects on heat-labile anti-nutrients, such as digestive protease inhibitors (trypsin and chymotrypsin inhibitors) (Shi et al., 2017).

Moreover, heat treatments can modify the native structures of proteins, affecting their digestibility. Carbonaro et al. (2005) demonstrated that intestinal digestibility in rats of purified faba bean globulins was significantly lower (78.79%) for thermally treated globulins (20 min at 120 °C) compared to the native globulins (95.08%) as a result of protein secondary structure changes during heat treatment (Estefania et al., 2018). Indeed, it was shown in various pulses that a high β-sheet content correlated to a decrease in protein digestibility due to the hydrophobic nature (Carbonaro, Maselli, & Nucara, 2012). β-sheet are rich in hydrophobic amino acids, such as tyrosine, phenylalanine, tryptophan, threonine, valine and isoleucine (Carbonaro, Maselli, & Nucara, 2012). The structural change depends on the temperature and the moisture used during thermal treatment. At 60% moisture and a temperature of 65 °C (annealing), faba bean protein digestibility increased from 76.23 to 82.43%, but decreased to 73.26% at 120 °C and 30% heat moisture (Estefania et al., 2018). Moreover, the α-helix and β-sheet ratios increased following annealing and decreased after heat moisture treatment.

2.3.1.4.2 Biochemical Processes

Faba bean protein digestibility could also be improved using biochemical processes, such as germination, fermentation, and enzymatic treatment. Those processes involved the use of various endogenous, microbial, or commercial proteases to partially degrade dietary proteins and facilitate their absorption during the digestion process (Coda et al., 2017; Rizzello, Verni, Koivula, et al., 2017). For instance, an increase in free amino acids up to 10-fold was observed during a solid phase fermentation of faba bean seeds with *Rhizopus oligosporus* used for faba bean tempeh preparation (Katarzyna Polanowska, Grygier, Kuligowski, Rudzińska, & Nowak, 2020).

Besides increasing protein content in bread by mixing 30% of faba bean to wheat flour, the addition of fermented faba bean flour significantly increased protein digestibility in bread (74%) compared to raw flour (64%) (Coda et al., 2017). Similar results were obtained in gluten-free corn-based fermented bread enriched with 50% of faba bean flour, where the protein digestibility increased from 53.9 to 72.3% (Sozer, Melama, Silbir, Rizzello, Flander, & Poutanen, 2019). For wheat pasta enriched with fermented faba bean flour, the *in vitro* protein digestibility increased from 49.2 to 54.3% and from 73.8 to 76.4% with a faba bean flour addition of 10 and 30%, respectively (Rizzello, Verni, Koivula, et al., 2017).

Berrazaga et al. (2018) prepared a hybrid-yogurt by mixing faba bean protein (47%) and milk protein (53%) that could compensate for faba bean essential amino acid deficiency while diminishing animal protein content. The protein gel was prepared by chemical acidification or fermentation and fed to rats to assess the incidence of the selected process on protein digestibility. Fermentation could increase total protein digestibility (+7%), growth rate (+35%), and PER ratio (1.6 times) compared to chemical acidification. Contrarily, the potential use of fermented chickpea and faba bean flour as food ingredients had no significant enhancement in protein digestibility during an *in vitro* digestion process (Chandra-Hioe, Wong, & Arcot, 2016). Those results suggest that fermentation conditions and microorganisms used are important factors that may influence fermentation efficiency to maximize protein quality.

Germination was also used in an attempt to improve faba bean protein quality. Setia et al. (2019) determined, *in vitro*, the PDCAAS of faba bean seeds that were raw (56.2%), soaked (52.9%), and germinated for 72 h (56.5%). Germination had no significant impact on protein quality. However, the first limiting amino acid was tryptophan for the raw and soaked seeds and surprisingly threonine for the germinated seeds. During germination, endogenous enzymes are activated to support plant growth, and storage proteins are used as energy supply and amino acid stock for enzyme synthesis, which may explain the amino acid profile variation (Setia et al., 2019).

2.3.1.5 Effects of Anti-Nutritional Content on Protein Digestibility and Quality

The presence of anti-nutrients, such as tannins, phytic acid, and trypsin inhibitors, have demonstrated a negative impact on faba bean protein digestibility.

2.3.1.5.1 Tannins

Tannins were shown to bind proteins and form insoluble complexes, reducing faba bean protein digestibility and bioavailability (Çalışkantürk Karataş et al., 2017; Ortiz, Centeno, & Treviño, 1993; Zduńczyk, Juśkiewicz, Wróblewska, & Flis, 2003). Tannin extracts from faba bean seed coats were shown to have higher precipitation potential against faba bean 11S globulins, followed by 7S globulins and 2S albumins and tannin-mediated precipitation occurring over a wide range of pH values (3 to 8) (Kosińska, Karamać, Penkacik, Urbalewicz, & Amarowicz, 2011). The faba bean mainly contains condensed tannins (proanthocyanidins), which are flavan-3-ol polymers. Their amounts can vary from 1.9 mg/g (Çalışkantürk Karataş et al., 2017) to 2586 mg/100 g

catechin equivalents (Weihua, Miao, Jing, Chuanxiu, & Yuwei, 2015). These important variations can be explained by the varietal differences, environmental conditions (Oomah et al., 2011), and quantification methods. Hydrolysable tannins (sugar esterified with a phenolic acid) are also present in faba beans, but at negligible amounts (Kosińska et al., 2011) compared to condensed tannins (58 mg/100 g of tannic acid equivalent) (Weihua et al., 2015). The most efficient way to decrease tannin content in faba bean is through the physical separation of the hull and the cotyledon. Based on reported studies, dehulling removes most (between 59.2 and 92.3%) of the phenolic compounds, including tannins (Alonso, Aguirre, & Marzo, 2000; Yu-Wei & Wei-Hua, 2013). Other processing, such as soaking, germination, and pressure-cooking, also proved to be effective household strategies used to reduce the levels of polyphenols and tannins in pulse-based foods, thereby enhancing the bioavailability of pulse proteins (Abdel-Aal et al., 2018; Erba et al., 2019; Lafarga, Villaró, Bobo, Simó, & Aguiló-Aguayo, 2019; Wei et al., 2021).

To eliminate nutritional quality issues related to tannins without using food processing strategies, low tannin faba bean varieties have been developed through breeding. This genetic trait is coded by two recessive genes (*zt-1* and *zt-2*) (Hou et al., 2018). These varieties can be easily distinguished from the wild type varieties by their smaller and lighter seeds and their white flowers. Condensed tannin content was shown to be 0.03 g/kg compared to 7.12 g/kg for a high tannin variety (Zduńczyk et al., 2003). The feeding of low tannin faba beans in rats significantly increases the protein efficiency ratio compared to high tannins (2.33 vs. 2.08), which confirms the nutritional improvement of low tannin varieties (Zduńczyk et al., 2003).

Although tannins have detrimental effects on protein digestibility, numerous health benefits are related to them. Faba bean tannins, obtained by acetone extraction and gel filtration chromatography, have demonstrated higher antioxidant activities (free radical scavenging and iron-reducing capacity) than low molecular weight phenolic compounds (Amarowicz et al., 2017). Faba bean tannin extracts, when fed to rats, showed a significant increase in serum HDL cholesterol and a slight decrease in total and LDL cholesterol, suggesting an improvement of the lipid profile (Zdunczyk, Frejnagel, Wróblewska, Juskiewicz, Oszmiański, & Estrella, 2002). Hence, tannins remain interesting healthy compounds even though they are classified as anti-nutrients. The beneficial health-related properties of various faba bean polyphenols were also reviewed in the literature (Turco, Ferretti, & Bacchetti, 2016).

2.3.1.5.2 Phytic Acid

Phytic acid (inositol hexaphosphate) is the main phosphorous storage found in faba bean cotyledons (Yu-Wei et al., 2013) and has a detrimental impact on protein digestibility by forming complexes with proteins and binding minerals, such as calcium, which are essential for digestive protease activity (Konietzny & Greiner, 2003). Phytic acid levels in faba beans vary among genotypes and environmental conditions (Oomah et al., 2011), and were reported to be higher than peas (Millar et al., 2019), lentils, and chickpeas, but similar to beans and soy (Shi et al., 2018). The effects of soaking and thermal treatments on phytic acid levels in faba beans varied among studies, from negligible changes to significant reductions due to differences in soaking and thermal conditions used (duration, pH, and pre-treatment) (Luo, Xie, Jin, Zhang, Wang, & He, 2013; Shi et al., 2018; Yu-Wei et al., 2013). In contrast, phytase hydrolysis reduced the phytic acid level in faba beans by 89% under optimized conditions (Rosa-Sibakov, Re, Karsma, Laitila, & Nordlund, 2018) and was efficient at increasing protein solubility and free amino acid levels during an *in vitro* digestion process, principally during the gastric phase (Rosa-Sibakov et al., 2018). Furthermore, germination and fermentation both had important reductions on phytic acid levels in faba beans and an increase in calcium availability through either endogenous or exogenous phytase actions, which can be beneficial to maximize digestive protease activities (Luo et al., 2013; Vidal-Valverde, Frias, Sotomayor, Diaz-Pollan, Fernandez, & Urbano, 1998).

2.3.1.5.3 Trypsin Inhibitor

Trypsin inhibitors decrease protein digestibility by inhibiting digestive protease activity in the gastrointestinal tract. Trypsin and chymotrypsin inhibitor activities were reported to be lower in faba beans than soy, beans, and chickpeas, and cooking was shown to reduce their activity levels below the limit of quantification (Shi et al., 2017). Extrusion-cooking was also shown to reduce trypsin inhibitor activity by 50% (Hejdysz, Kaczmarek, & Rutkowski, 2016). Trypsin inhibitors have been purified and characterized in faba beans. For instance, *Vicia faba* cv. *Giza 843* trypsin inhibitor (VFTI-G1) is a polypeptide of 15 kDa that has an inhibitory effect against both trypsin and chymotrypsin to a lower extent (Evandro Fei, Abdallah Abd Elazeem, Jack Ho, Clara Shui Fern, Saeed Saad, & Tzi Bun, 2011). Those inhibitory activities were reduced at temperatures above 60 °C (Evandro Fei et al., 2011). Nonetheless, faba bean trypsin inhibitors possess properties that make them appealing from a therapeutic point of view. VFTI-G1 (Bowman–Birk-type)

revealed anticancer properties, emphasized through an anti-proliferative effect demonstrated on a hepatocellular carcinoma cell line (HepG2) (Evandro Fei et al., 2011). Another trypsin and chymotrypsin inhibitor of 7.5 kDa (Bowman–Birk-type) from faba was shown to have fungicide properties (Ye, Ng, & Rao, 2001).

2.3.2 Faba Bean Health-Promoting Bioactive Properties

Bioactive peptides are short peptides of 2 to 20 amino acids that are either naturally present in the food matrix or released through protein hydrolysis (Sánchez & Vázquez, 2017). Those amino acid sequences have no specific activities while embedded in the initial protein structure but become highly active after protein hydrolysis (Sánchez et al., 2017). A broad range of bioactivities are attributed to peptides, such as antioxidant, antidiabetic, cholesterol-lowering, anti-inflammatory, anticancer, antihypertensive, opioid, and antimicrobial, among others (León-Espinosa et al., 2016; Sánchez et al., 2017; Zambrowicz et al., 2015). The relationship between chemical structure and bioactivity is still under investigation and varies according to the specific bioactivity (Karami & Akbari-Adergani, 2019; Manzanares, Gandía, Garrigues, & Marcos, 2019). However, the peptide length, charge, amino acid composition, and particular order, as well as the hydrophobic amino acid ratio content, are all factors of significant influence for many bioactivities (Karkouch et al., 2017; Lopez-Barrios, Gutierrez-Uribe, & Serna-Saldivar, 2014).

Bioactive peptides have been identified and isolated from a wide range of food commodities, including legumes that are considered significant sources (Lopez-Barrios et al., 2014). Some recent studies suggest that faba bean proteins have great bioactive potential (Table 2-3). Moreover, León-Espinosa et al. (2016) conducted a bioinformatics analysis using the BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008) to investigate potential bioactivities in main faba bean storage proteins. The algorithm used computes known bioactive fragment frequencies in a given protein sequence. This analysis revealed a high occurrence of potential antihypertensive, antioxidant, and other various biological activities. Legumin A was shown to have the highest bioactive fragment frequency, followed by convicilin and vicilin (León-Espinosa et al., 2016). However, the truly released fragments will highly depend on the enzymes used to hydrolyze the proteins and the hydrolysis efficiency, which need to be investigated through further analyses and experimental work.

Table 2-3. Bioactive peptides isolated from faba bean protein hydrolysates.

	Amino Acids Sequence	Bioactive Properties	Starting Material	Protein Hydrolysis Method	Protein Precursor	Hydrophobic Residue (%)	References
1	GGQHQQEEESEEQK	Antioxidant (DPPH assay) Antibiofilm (biofilm inhibition of *Pseudomonas aeruginosa PA14*)			Legumin	0	
2	GPLVHPQSQSQSN	Antioxidant (DPPH assay) Antityrosinase (tyrosinase inhibition assay)			Legumin	15	
3	LSPGDVLVIPAGYPVAIK	Antioxidant (DPPH, FRAP, and ferrous ion-chelating assays) Antibiofilm (biofilm inhibition of *Pseudomonas aeruginosa PA14*)	Faba bean protein isolate	Trypsin hydrolysis (18 h 37 °C)	Vicilin	56	(Karkouch et al., 2017)
4	VESEAGLTETWNPNHPELR	Antioxidant (DPPH assay), Antityrosinase (tyrosinase inhibition assay) Antibiofilm (biofilm inhibition of *Pseudomonas aeruginosa PA14*)			Legumin	26	
5	EEYDEEKEQGEEEIR	Antioxidant (DPPH assay) Antibiofilm (biofilm inhibition of *Pseudomonas aeruginosa PA14*)			Vicilin	13	
6	ELAFPGSAQEVDTLLENQK	Fungicide	Lentil, pea and faba bean flours mixed (1:1:1)	Veron® PS (6 h 30 °C)	Vicilin	36	(Rizzello, Verni, Bordignon, Gramaglia, & Gobbetti, 2017)
7	LSPGDVLVIPAGYPVAIK	Fungicide			Vicilin	50	
8	SAQ	Promoting lactic acid bacteria growth	Faba bean protein isolate	Alcalase (1 h 37 °C)	ND	ND	(P. Xiao, Liu, Rizwan ur, Kang, & Wang, 2015)
9	Peptide enriched fraction DALEPDNRIESEGGLIETWNPNNRQ FEEPQQSEQGEGR GSRQEEDEDEDE WMNYNDQIPVINNQLDQMPR RGEDEDDKEKRHSQKGES RLNIGSSSSDIYNPQAGR	Antioxidant (ABTS assay) Antihypertensive (ACE inhibition) Anti-inflammatory (LOX inhibition) Reduction of lipids absorption (pancreatic lipase inhibition)	Fermented faba bean flour	*In vitro* simulated gastrointestinal digestion	Legumin	ND	(Jakubczyk et al., 2019)
10	HLPSYSPSPQ	Promote muscle protein synthesis (increased phosphorylation S6 in skeletal muscle cells)	Faba bean protein powder	Food grade endopeptidase	ND	ND	(Corrochano et al., 2021)
11	TIKIPAGT	Anti-inflammatory (Reduced TNF-α in macrophages)					

2.3.2.1 2.2.1. Faba Bean Gastrointestinal Hydrolysates with Potential Beneficial Health-Related Bioactivities

Some studies (Ashraf et al., 2020; Corrochano et al., 2021; Dugardin et al., 2020; Felix, Cermeño, & FitzGerald, 2019; Jakubczyk et al., 2019; Karkouch et al., 2017; León-Espinosa et al., 2016;

Parya Samaei et al., 2020) have demonstrated that faba bean proteins hydrolyzed with gastrointestinal proteases have the potential to promote various beneficial health-related bioactivities that go beyond their nutritional properties. For now, most evidence concerning these health benefits are based on *in vitro* results. In this regard, Felix et al. (2019) studied the antioxidant, antihypertensive, and antidiabetic effects of a sunflower oil-based emulsion stabilized with a faba bean protein concentrate after an *in vitro* simulated gastrointestinal digestion with pepsin and Corolase PP® (bacterial endopeptidase mix). The free radical scavenging properties of the emulsion were enhanced following digestion when assessed with the ferric reducing antioxidant power (FRAP) assay, possibly due to the release of small peptides. Meanwhile, the oxygen radical absorbance capacity (ORAC) value decreased after digestion, showing that the free radical scavenging mechanisms of those peptides were better highlighted with the FRAP assay. The antihypertensive and antidiabetic properties of the emulsion also increased following digestion (Felix et al., 2019). Those properties were assessed through the angiotensin-converting enzyme (ACE) and dipeptidyl peptidase 4 (DPP-IV) inhibition assays. Following digestion, the enzyme inhibition activities of the emulsion increased from 23 to 60% for ACE and from 3 to 11% for DPP-IV (Felix et al., 2019). Those results suggest that highly bioactive peptides are potentially released from faba bean proteins during gastrointestinal digestion. However, further investigation is needed since those peptides were neither purified nor specifically characterized.

León-Espinosa et al. (2016) also observed antioxidant properties (2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) scavenging activities) of a faba bean protein isolate digested with either trypsin, chymotrypsin, or pancreatin. It was demonstrated that faba bean protein hydrolysis with trypsin was more efficient at liberating antioxidant peptides than chymotrypsin and pancreatin, which could be explained by their respective cutting sites that generated different peptides. Indeed, Parya Samaei et al. (2020) demonstrated that only 0.2% of the peptides identified were homologous when a faba bean protein digestate was prepared with either pepsin, trypsin, or alcalase. Moreover, when the enzymes were used in combination, only 26% of the peptides identified where homologous in the pepsin–trypsin and trypsin–pepsin digestates, which suggests the importance of the sequential enzyme order of addition. In that case, the usage of alcalase alone, and a combination of pepsin and trypsin, were the most efficient enzymes to maximize antioxidant activities. Moreover, numerous peptides with

sequences or fragments homologous to antioxidant peptides already identified were found in those faba bean digestates.

It was also demonstrated that faba bean protein trypsin digestate had a protective effect against colon cancer in mice by reducing the number of preneoplastic lesions induced by either a high-fat diet or azoxymethane injections (León-Espinosa et al., 2016). The lowest hydrolysate dose tested (10 mg/kg) was shown to have the highest effect. The same hydrolysate also caused a cholesterol-lowering effect, which was highlighted by the improvement of the lipidic profile. Dietary peptides can lower blood cholesterol by either decreasing exogenous cholesterol absorption in the intestine or in diminishing endogenous cholesterol biosynthesis. Ashraf et al. (2020) demonstrated that faba bean peptides (<3 kDa) obtained from the hydrolysis of a thermally treated protein isolate with pepsin and trypsin decreased cholesterol solubility into micelles and inhibited the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an important enzyme of the mevalonate pathway responsible for cholesterol synthesis. Its inhibition promotes the use of blood LDL cholesterol instead of de novo cholesterol synthesis (Ashraf et al., 2020). Those action mechanisms could explain the cholesterol-lowering effects of faba bean peptides observed *in vivo* by León-Espinosa et al. (2016).

Since fermentation is an effective process to enhance the bioactive properties of a food product, by releasing bioactive peptides through various proteolytic activities (Raveschot et al., 2018), Jakubczyk et al. (2019) recently studied the potential of fermented faba bean flour by *Lactobacillus plantarum* to produce bioactive peptides against metabolic syndrome during an *in vitro* digestion process that mimics human digestion. The digestion of the fermented flour was pursued by adding α-amylase, pepsin, and pancreatin subsequently. A peptide-rich fraction (peptide 9), as reported in Table 2-3, with antiradical activity (EC_{50} = 0.02 mg/mL) and inhibition potential against ACE (IC_{50} = 0.05 mg/mL), lipoxygenase (LOX) (IC_{50} = 0.10 mg/mL), and pancreatic lipase (IC_{50} = 0.46 mg/mL), was obtained. It was reported that inhibition of pancreatic lipase diminishes lipid absorption in the intestine and helps to restore calorie intake balance in patients with metabolic syndrome (Jawed et al., 2019). LOX is also known as an enzyme involved in the inflammatory response and chronic inflammation associated with various metabolic dysfunctions, including obesity and type II diabetes (Neels, 2013). Nevertheless, the identified peptides were not tested individually, and their specific bioactivities remain to be confirmed.

In their study, Karkouch et al. (2017) isolated five peptides (peptides 1 to 5 in Table 2-3) with either antioxidant, antityrosinase, or antibiofilm properties from faba bean proteins digested overnight with trypsin. One of these peptides was also isolated by Rizzello, Verni, Bordignon, et al. (2017) for its fungicide properties (peptide 3), revealing that bioactive peptides could have multifunctional properties. Among the peptides isolated, peptide 4 has the highest DPPH free radical scavenging activity with an effective concentration (EC_{50}) of 0.25 ± 0.02 mM. However, only peptide 3 has demonstrated a reducing capacity of Fe^{3+} to Fe^{2+} (EC_{50} = 0.31 ± 0.03 mM) and an iron-chelating activity (EC_{50} = 2.40 ± 0.31 mM). Interestingly, this peptide, with the highest hydrophobic amino acid residue ratio (56%), contributed to those properties (Karkouch et al., 2017). Chelation of transition metal ions contributes to peptide antioxidant properties because they act as catalysts in some free radical formation reactions (Santos, Alvarenga Brizola, & Granato, 2017; Wang, Hu, Nie, Yu, & Xie, 2016). Tyrosinase is an enzyme involved in melanin biosynthesis and tyrosinase activity abnormalities are associated with skin pigmentation diseases and even skin cancer (Karkouch et al., 2017). Peptide 4 has the highest tyrosinase inhibition capacity, with an IC_{50} of 0.14 ± 0.01 mM.

Other promising bioactivities were recently identified by Dugardin et al. (2020). It was shown that faba bean protein gastrointestinal hydrolysate could play a role in regulation of food intake in modulating the secretion of incretins, such as glucagon-like peptide-1 (GLP-I) and cholecystokinin (CCK) in a dose-dependent manner in a murine intestinal cell model. Nonetheless, the effect was inferior when compared to pea, potato, oat, and wheat protein hydrolysate. Interactions among faba bean protein with opioid receptors were also demonstrated, but the binding capacity decreased significantly after gastrointestinal digestion. Moreover, it was not investigated whether the hydrolysate had the capacity to cross the intestinal barrier to induce this effect *in vivo*. Interactions with opioid receptors in the portal vein could induce satiety and play a role in food intake regulation. Anti-inflammatory activity was also investigated, but no effect was observed. The faba bean hydrolysate was shown to have greater *in vitro* antihypertensive, antidiabetic, and antioxidant activity, when compared to oat, wheat, potato, and pea gastrointestinal hydrolysate (Dugardin et al., 2020).

Cal et al. (2020) have demonstrated that faba bean protein hydrolysate can play a beneficial role in skeletal muscle health in promoting skeletal protein synthesis and in preventing muscle loss caused by chronic inflammation. These activities could be beneficial to fight against sarcopenia

during aging, among others. Hydrolysate of various plant-based proteins (chickpeas, soy, Asian rice, and spirulina) were prepared with a food grade endopeptidase. Interestingly, the faba bean hydrolysate was the only one with a protein synthesis promoting activity (Cal et al., 2020). The activity of this novel functional ingredient was validated *in vitro* with cell models and *in vivo* in a pre-clinical study. The peptide profile of this complex functional food ingredient was screened for anti-inflammatory and protein synthesis promoting activity using a predictive machine learning approach (Corrochano et al., 2021). Two peptides were discovered, HLPSYSPSPQ and TIKIPAGT with protein synthesis promoting and anti-inflammatory activity, respectively. Moreover, both peptides were resistant to gastrointestinal digestion, when administered in the complex ingredient forms. They crossed the intestinal barrier and were resistant to human sera peptidases (Corrochano et al., 2021), which demonstrates excellent *in vivo* bioactive potential.

Thus, the faba bean protein could be considered a promising source of bioactive peptides. However, studies remain limited, and many more bioactive peptides need to be elucidated. Moreover, peptides released in a context that mimics human digestion have been scarcely investigated to date. Further studies are therefore required to evaluate the health benefits of introducing faba bean protein in the diet. Studies using more physiologically relevant assays, such as cellular or animal models, are required to confirm the results obtained with biochemical tests. Moreover, bioavailability, uptake, stability, and resistance to brush border and serum peptidases are factors that need to be addressed to determine if those peptides can reach their targeted sites of action in a sufficient concentration to induce respective beneficial health-related effects. Peptide absorbability depends on many factors, including length, charge, and hydrophobicity, as well as the food matrix composition (Sun, Acquah, Aluko, & Udenigwe, 2020). Fibre-rich matrices were shown to enhance bioactive peptide absorption and protect peptides against chemical degradation, whereas lipid-rich matrices substantially decreased peptide absorption (Sun et al., 2020). According to these facts, the faba bean, with its very low lipid content and high fibres levels, could be an optimal matrix to facilitate dietary peptides bioavailability and absorption. Further investigations are needed to validate this hypothesis.

2.3.2.2 Anti-Microbial Bioactivities of Faba Bean Enzymatic Hydrolysates

Faba bean protein hydrolysates have been produced in non-physiological conditions to create new biofunctional food ingredients. Rizzello, Verni, Bordignon, et al. (2017) have isolated two faba

bean peptides with fungicide properties from a commercial pulse flour mix (pea, lentil, and faba bean) hydrolysate obtained by a protease mix commonly used in the baking industry. Those two peptides (peptides 6 and 7) as reported in Table 2-3 had a high hydrophobic residue ratio (36 and 50%) in common. The mixed flour hydrolysate was added to the baking products and increased shelf life without impairing the organoleptic properties. This is a promising finding considering that one of the main problems encountered with bioactive peptide enrichment in food products is the development of an undesirable bitter taste (Iwaniak, Hrynkiewicz, Bucholska, Minkiewicz, & Darewicz, 2019). Karkouch et al. (2017) also identified four peptides in a faba bean trypsin hydrolysate with antimicrobial activities. These peptides had the ability to block biofilm formation by *Pseudomonas aeruginosa*; for peptides 3 and 5 (Table 2-3), the effect was dose dependent. Hydrophobic and basic residues seem to be important for this activity. Indeed, hydrophobic and basic amino acids can disrupt cell–cell interactions and, therefore, inhibit biofilm formation (Karkouch et al., 2017). Thus, faba bean protein hydrolysate could potentially be used as a biofunctional food ingredient acting as a natural preservative agent (Rizzello, Verni, Bordignon, et al., 2017). Similarly, a lactic acid bacteria (LAB) growth-promoting peptide was also obtained from faba bean proteins digested with alcalase (Xiao et al., 2015). This short peptide of only three amino acids (peptide 8), as reported in Table 2-3, could increase viable LAB count by one-log compared to the control, and act as a food ingredient to maintain the probiotic counts in various products (Xiao et al., 2015).

2.4 Other Nutritional and Bioactive Constituents of Faba Beans

2.4.1 Starch

Carbohydrates, similar to other plants belonging to the *Leguminosae* family, are the major constituents of faba beans, where the starch component counts for approximately 40% of the whole seed (Abdel-Aal et al., 2018). Li et al. (2019) reported that starch is conceptually characterized as rapidly digestible (if digested in less than 20 min), slow digestible (between 20 to 120 min), or resistant to digestion (more than 120 min). In the case of the raw faba bean, rapidly digestible starch was shown to account for 15.3%, slowly digestible for 34.5%, and resistant starch for 46.7%. Both slowly digestible and resistant starch help maintain the satiety feeling longer and contribute to a low glycemic index by flattening the blood glucose peak following food intake, thereby having a preventive effect against type II diabetes (Ramdath, Renwick, & Duncan, 2016). In addition,

resistant starch has a prebiotic effect; it is undigested and unabsorbed in the small intestine but fermented in the large intestine by the microflora into short-chain fatty acids, inducing health benefits in the large intestine, such as reducing inflammation and preventing colon cancer (DeMartino & Cockburn, 2020). Compared to cereals, the starch digestion rate in raw pulses is lower due to many factors, including starch granule morphology (large smooth rounded to oval shape granules (Mendes, Costa, Vicente, Oliveira, & Mafra, 2019)), crystallinity polymorph (C-type), and high amylose content, which reduces access to digestive enzymes (Dong & Vasanthan, 2020; Martens, Gerrits, Bruininx, & Schols, 2018).

There are about 20%–30% amylose (AM) and 70%–80% amylopectin (AP) in normal starch granules. Amylose is a linear glucose polymer (α-1,4 linkages), whereas amylopectin is a branched glucose polymer (α-1,4 and α-1,6 linkages). These two polymers can be combined into five different starch structural levels, including whole granule architecture (1–100 nm), growth rings (120–400 nm), blocklets (20–500 nm), amorphous and crystalline lamellae (9 nm), and AP and AM chains (0.1–1.0 nm) (Pérez & Bertoft, 2010). Physicochemical and structural properties of starches determine their applications in food and non-food industries. Amylose forms very compact structures, thus, it is hardly digestible (Bertoft, 2017). Differences in the levels of amylose in faba beans, varying from 18.6 to 44.4% of total starch, could be explained mainly by the analysis method used (enzymatic or potentiometric approach) (Dong et al., 2020; Mendes et al., 2019; Setia et al., 2019). Their levels were similar to other legumes (lentils and peas) but higher than corn and tapioca (Mendes et al., 2019).

Furthermore, the faba bean starch digestion rate is affected by cooking, reaching 88.1% digestibility within 20 min (Mendes et al., 2019), similar to other legumes and cereals (Dong et al., 2020). Enriching durum wheat pasta with 35% faba beans not only enhanced its protein and essential amino acid content and strengthened its protein network, but also resulted in a low glycemic and insulin index in healthy volunteer consumers (Greffeuille et al., 2015). Similarly, Tazrart, Lamacchia, Zaidi, and Haros (2016) demonstrated that the enrichment of fresh wheat pasta with faba bean flour decreased the pasta glycemic index in a dose-dependent manner. The glycemic index dropped from a value of 95.9 for the control to 91.9, 83.4, and 71.3 for pasta enriched with 10, 30, and 50% of faba bean flour, respectively. Moreover, the more faba bean flour added, the more resistant starch increased in the pasta, from 1.44 g/100 g to 1.86, 2.25, and 2.47 g/100 g, respectively. On the other hand, processing conditions for pasta preparation have a

substantial impact on pasta structure and, thus, digestibility (Greffeuille et al., 2015). During cooking, starch undergoes gelatinization, which irreversibly disrupts the starch granule structure (Wang & Copeland, 2013).

To increase native starch digestibility, various physical, chemical enzymatic, and biotechnological methods are applied. These techniques have been found to change the surface properties, polarity, and linearity of the molecular chains, the degree of substitution, the polymeric, granular, and crystalline structures, amylose to amylopectin ratio, solubility, viscosity, pasting, gelatinization, swelling, water absorption, and emulsifying properties of starch (Nawaz, Waheed, Nawaz, & Shahwar, 2020). Using chemical modification through cross-linking reactions (Dong et al., 2020) showed that phosphorylation of gelatinized faba bean starch decreased the digestion rate slightly, but further optimization of the phosphorylation reaction is required to optimize the effects (Dong et al., 2020). Another factor that can influence starch digestibility is the presence of α-amylase inhibitors. However, this activity was reported to be very low in faba beans (18.9 U/g in raw seeds compared to 248 U/g in raw kidney beans), highly heat-sensitive (Alonso et al., 2000), or completely absent (Yu-Wei et al., 2013).

2.4.2 Fibres

The faba bean is a very important source of both soluble and insoluble dietary fibres (non-starch polysaccharides), as shown in Table 2-1. Faba bean insoluble dietary fibres are mainly composed of hemicellulose (8.92 g/100 g), cellulose (8.33 g/100 g), and lignin (2.00 g/100 g) (Vidal-Valverde et al., 1998). Dietary fibre consumption is associated with many health benefits, including improvement of cholesterol profiles and preventive effects against diabetes, obesity (increasing satiety and maintaining it over time), and colon cancer (Mudryj, Yu, & Aukema, 2014). The recommended daily fibre intake in Canada is 25 g for women and 38 g for men (Abdullah, Gyles, Marinangeli, Carlberg, & Jones, 2015), but the actual fibre consumption is below those targets (19.1 g for men and 15.6 g for women) (Bélanger, Poirier, Jbilou, & Scarborough, 2014). The use of faba bean flour to improve the nutritional and functional features in food-making would certainly increase a consumer's recommended daily intake.

One of the health-promoting properties of fibre is related to its ability to bind bile acids during digestion and to decrease circulating LDL cholesterol. Bile acids are formed from cholesterol transformation in the liver and are indispensable to lipid digestion (Jesch & Carr, 2017). After

digestion, they are usually reabsorbed in the ileum; however, their sequestration by fibre prevents their reabsorption and promote the use of more blood LDL cholesterol to produce new bile acids (Jesch et al., 2017). In this regard, Çalışkantürk Karataş et al. (2017) assessed whole faba bean and faba bean hull fraction capacities to bind bile acids during an *in vitro* digestion process. Their results indicated that faba bean whole flour and the hull fraction had a bile acid-binding capacity of 14.6% and 282.6% (normalized to cholestyramine capacity), respectively. Interestingly, the bile acid-binding effect was attributed to tannin content rather than total fibre content (Çalışkantürk Karataş et al., 2017).

Another health-promoting property of fibre involves its prebiotic effects. Fibre is resistant to gastrointestinal digestion but can be fermented into short-chain fatty acids in the large intestine by the microflora, which prevents the growth of undesirable bacteria and contributes toward preventing colon cancer (Çalışkantürk Karataş et al., 2017; Gullón, Gullón, Tavaria, Vasconcelos, & Gomes, 2015). Çalışkantürk Karataş et al. (2017) assessed the potential of faba bean gastrointestinal digestion residue to promote gut microbiota fermentation. It was shown that faba bean digest residue promotes the formation of short-chain fatty acids, mainly acetic acid (56.9 µmol/100 mg residue), butyric acid (36.1 µmol/100 mg residue), propionic acid (23.9 µmol/100 mg residue), and valeric acid (8.8 µmol/100 mg residue). A similar pattern was obtained by Gullón et al. (2015), with the most abundant short-chain fatty acid being acetic acid, followed by butyric acid and propionic acid after 48 h of fermentation. Moreover, the faba bean gastrointestinal digest residue was shown to promote the growth of various healthy intestinal bacteria genera, such as Bifidobacterium, Lactobacillus, Enterococcus, Bacteroides, and Prevotella (Gullón et al., 2015). This growth-promoting effect can be attributed to dietary fibre and resistant starch, but also to α-galactosides that have well-established prebiotic effects. The faba bean was shown to contain an important amount of raffinose (4.8 g/kg), stachyose (10.1 g/kg), and verbascose (22.8 g/kg) (Fan, Zang, & Xing, 2015), which contribute to the whole seed prebiotic effect.

2.4.3 Lipids

The faba bean has a very low lipid content (Table 2-1), similar to peas and other pulses, but considerably lower than soy. The fatty acid profiles are comparable for these legumes and are composed mainly of beneficial monounsaturated (oleic acid) and polyunsaturated (linoleic acid) fatty acids. These unsaturated fatty acids have demonstrated beneficial health-related effects, such

as lipid profile improvement (an increase of sera HDL cholesterol) that contributes to heart disease prevention (Lunn & Theobald, 2006). Nonetheless, the faba bean is a minimal lipid source, which can be advantageous in food applications. Indeed, pulse flours and pulse-derived food ingredients could develop bitter and beany off-flavours during storage (Yang, Piironen, & Lampi, 2017), which is mainly attributed to lipid degradation by endogenous lipases and lipoxygenases (Yang et al., 2017). Endogenous lipoxygenase activity, in addition to its low-fat content, was reported to be lower in faba beans than in peas and soy, which decreases the risks of these degradation reactions during storage (Zhong-qing et al., 2016).

2.4.4 Minerals

The faba bean, in addition to being an excellent source of protein and starch, contains valuable mineral micronutrients. More precisely, it is a rich source of potassium, iron, and zinc (Eugeniusz R. Grela, Samolińska, Kiczorowska, Klebaniuk, & Kiczorowski, 2017; Millar et al., 2019). The faba bean contains a very low sodium amount (Eugeniusz R. Grela, Samolińska, et al., 2017), which is a desirable trait considering that high sodium consumption is associated with heart-disease preponderance (Farquhar, Edwards, Jurkovitz, & Weintraub, 2015). The primary issue with plant-derived minerals involves their poor bioavailability during the digestion process (Hever, 2016) due to anti-nutrients, such as oxalic and phytic acid. Oxalic and phytic acid, with their multiple acidic functional groups, can bind minerals to form insoluble salts in the intestine (phytate and oxalate, respectively), which decrease the uptake of essential minerals (Toledo, Brigide, López-Nicolás, Frontela, Ros, & Canniatti-Brazaca, 2019). Total oxalate content in the faba bean was shown to be 241.50 mg/100 g, which is similar to peas (244.65 mg/100 g) and lower than soy (370.49 mg/100 g) (Shi et al., 2018). Oxalate content can be partially reduced through soaking and cooking (Gad, el-Zalaki, Mohamed, & Mohasseb, 1982; Shi et al., 2018). Moreover, biochemical processes are suitable for decreasing phytic acid content in faba beans and increasing mineral bioavailability. For instance, germination significantly increased iron, copper, and calcium bioavailability, maintained that of manganese, but decreased zinc accessibility (Xie, Jin, Wang, & He, 2014). It was also shown that faba bean flour hydrolysis with phytase increases iron absorption in rats (Luo & Xie, 2012). Among the other factors that influence plant-derived iron bioavailability is the binding to phytoferritin, an iron storage protein. Recent studies suggest that phytoferritin-bound iron is protected from anti-nutrient precipitation due to protein coating (Liao, Yun, & Zhao,

2014) and it is more easily absorbed in the intestine (Perfecto, Rodriguez-Ramiro, Rodriguez-Celma, Sharp, Balk, & Fairweather-Tait, 2018). However, to exert this beneficial effect, phytoferritins have to resist the gastric digestion phase (Perfecto et al., 2018). Phytoferritins are composed of two subunits (H-1 and H-2); the H-2 subunit was shown to be more stable and resistant to pepsin hydrolysis than H1. Interestingly, faba bean phytoferritins have a higher proportion of H-2 subunits (H-1:H-2 ratio of 1:6) compared to soy (1:2) and peas (1:1), which suggest that faba bean phytoferritins have the potential to resist gastric conditions (Shaojun, Senpei, Luyao, Xin, Peng, & Guanghua, 2012), and thereby increase iron bioavailability. Further research is needed to confirm this hypothesis.

2.4.5 Non-Protein Amino Acids

The faba bean also contains a significant amount of non-protein amino acids that have beneficial health-related effects, particularly L-3,4-dihydroxyphenylalanine, also called levodopa or L-DOPA. L-DOPA, which is a dopamine precursor. Synthetic versions of this compound are widely used to improve motor functions in patients with Parkinson (Waller & Sampson, 2018). The synthetic version of L-DOPA causes many side effects, which explain the growing interest in finding natural sources (Mehran & B, 2013). It was demonstrated that the plasma levels of L-DOPA increased after the consumption of 250 g of cooked faba beans by healthy volunteers and patients with Parkinson's (Rabey, Vered, Shabtai, Graff, & Korczyn, 1992). The motor functions of patients with Parkinson's improved up to 4 h after faba bean ingestion, an effect that was similar to a treatment composed of 125 mg of L-DOPA and 12.5 mg of carbidopa. These results suggest that a realistic serving of faba bean contains a sufficient amount of L-DOPA to induce a clinical effect (Rabey et al., 1992). L-DOPA is naturally formed from tyrosine in the faba bean and accumulates in various organs of the plant, including the leaves (Etemadi, Hashemi, Randhir, ZandVakili, & Ebadi, 2018), flowers (Topal & Bozoglu, 2016), and seeds, where it reaches its highest concentrations while the plant is still immature. Mature dried seeds still contain an important amount (Etemadi et al., 2018; Purves et al., 2017). Purves et al. (2017) quantified L-DOPA in 42 faba bean cultivars with high genetic diversity in terms of seed size, tannins, and vicine and convicine content, and the L-DOPA content ranged from 0.09 to 1.15 mg/g (Purves et al., 2017). Other studies reported values in the same order of magnitude (Cardador-Martínez et al., 2012; K. Polanowska, Łukasik, Kuligowski, & Nowak, 2019). In addition to the cultivar,

environmental stress and processing can interfere with the accumulation of this specific secondary metabolite (Etemadi et al., 2018). L-DOPA was shown to be either completely or partially destroyed by thermal processes, such as boiling (Cardador-Martínez et al., 2012; Etemadi et al., 2018) and roasting (Cardador-Martínez et al., 2012). On the contrary, Abdel-Sattar et al. (2021) revealed that sprouting increased L-DOPA content of the faba bean. In their study, a methanolic extract of the faba bean was shown to have anti-Parkinson's effects in a mice model; the effects drastically improved after germination, which coincided with an increase in flavonoids, phenolic acids, saponins, and aromatic amino acids (Abdel-Sattar et al., 2021).

The faba bean also contains γ-aminobutyric acid (GABA), which is an inhibitory neurotransmitter amino acid that has blood pressure-lowering effects (Ma, Li, Ji, Wang, & Pang, 2015). It is a secondary metabolite formed from glutamic acid by glutamate decarboxylase, which accumulates in faba bean seeds due to environmental stress (Yang, Hui, & Gu, 2016; Yang, Wang, Yin, & Gu, 2015; R. Yang, Yin, & Gu, 2015; R. Yang, Yin, Guo, Han, & Gu, 2014). Germination (Li, Bai, Jin, Wen, & Gu, 2010) and fermentation (Coda et al., 2015; Coda et al., 2017; Katarzyna Polanowska et al., 2020) are both very useful processes to increase GABA in the faba bean. For instance, Coda et al. (2017) fortified wheat bread with either 30% raw or 30% of fermented faba bean flour. The addition of faba bean flour to the dough caused an increase of GABA content compared to the wheat dough. Fermentation of faba bean flour further increased GABA content from 89 to 315 mg/kg of flour. Coda et al. (2017) reported that the GABA content in a 50 g serving of bread containing fermented faba bean flour is a sufficient dose to trigger beneficial health effects.

2.5 Faba Bean Adverse Health Effects

2.5.1 Favism

Consumption of faba beans, despite the positive repercussions surrounding nutrition and the environment, might pose some health hazards to certain groups of consumers due to the presence of certain components. Faba beans contain pyrimidine glycosides vicine and convicine, which are precursors of the aglycones divicine and isouramil. These are the main factors of favism, a genetic condition that may lead to severe hemolysis after faba bean ingestion (Luzzatto & Arese, 2018; Rizzello et al., 2016). Favism is a hemolytic anemia that can be developed among people with a deficiency in glucose-6-phosphate dehydrogenase (G6PD). This enzyme deficiency affects around

330 million people worldwide, mainly in Africa, South America, the Mediterranean region, and South-East Asia (Nkhoma, Poole, Vannappagari, Hall, & Beutler, 2009). It is a recessive X-linked trait; thus, predominantly affecting men (Hagag, Badraia, Elfarargy, Elmageed, & Abo-Ali, 2018). Without G6PD, red blood cells are unable to re-establish the oxidative imbalance caused by isouramil and divicine, which leads to oxidative damage and hemolysis (Luzzatto et al., 2018). Ivarsson et al. (2018) analyzed 16 faba bean varieties, including low and high tannin varieties, and the values ranged from 6.64 to 7.90 g/kg and from 2.48 to 4.41 g/kg for vicine and convicine, respectively (Ivarsson et al., 2018). Low-vicine and convicine varieties (*vc*-) have been developed, and genetic markers have recently been identified to facilitate the distinction between the two genotypes (Khazaei et al., 2017). The genetic improvements to reduce vicine and convicine in faba bean are reviewed elsewhere (Khazaei et al., 2019). In low vicine and convicine varieties, the concentrations were reported to vary from 0.13 to 0.73 and 0.009 to 0.037 mg/g, respectively, a substantial decrease compared to standard varieties (Purves et al., 2017). It was recently demonstrated that the consumption of 500 g of *vc*-faba bean seeds per 70 kg of body weight did not cause oxidative damage or hemolysis in G6PD deficient patients. Those results suggest that *vc*-faba bean varieties are potentially safe for patients who are lacking G6PD, but further studies with more patients are required (Gallo et al., 2018). Moreover, new analytical procedures have been developed to quantify vicine and convicine in food products to assure food safety (Getachew, Vandenberg, & Smits, 2018; Purves, Khazaei, & Vandenberg, 2018; Purves et al., 2017).

2.5.2 Presence of Lectins

Faba beans also contain lectins, which are low molecular weight proteins (~18 kDa) (El-Araby, El-Shatoury, Soliman, & Shaaban, 2020) that have characteristic binding capacities against other proteins and sugars that cause agglutination of blood cells. Lectins, at low doses, interfere with nutrient digestion and decrease nutrient absorption. At high doses, they can trigger toxic effects and even death (Alatorre-Cruz et al., 2018). Interestingly, the activity of the lectin phytohemagglutinin (PHA) is lower in the raw faba bean (5.52 HU/mg) than in soy (692.82 HU/mg), beans (88.32 HU/mg), and lentils (11.01 HU/mg), but similar to peas (5.66 HU/mg) and higher than chickpeas (2.74 HU/mg) (Shi et al., 2018). However, heating processes completely inhibit PHA activity, which makes faba bean consumption perfectly safe (Shi et al., 2018). Partially purified and purified faba bean lectins were also shown to have antibacterial and

antifungal activities (El-Araby et al., 2020). Interestingly, the antifungal activities against *Candida albicans* were higher than pea and lentil activities. These results suggest that faba bean lectins could be used as natural antifungals.

2.5.3 Allergenicity

Faba bean proteins, as part of the legume family, can potentially induce hypersensitivity reactions. Thus, soybeans and peanuts are listed as priority allergens in North America (Gendel, 2012) and lupin is part of the main allergens list in Europe (Gendel, 2012). Many allergens have been identified in other legumes, such as lentils, peas, and beans (Smits, Le, Welsing, Houben, Knulst, & Verhoeckx, 2018). However, very little data are relatively available on faba bean allergens. A recent report compared the prevalence of sensitization to various legumes in a random group of allergic patients (n = 106) (Smits et al., 2021), and faba bean sensitization prevalence was among the lowest (5.7%), with green lentils (5.7%), when compared to black lentils (6.6%), white beans (7.5%), chickpeas (8.5%), soy (8.5%), blue lupine (8.5%), green peas (9.4%), white lupine (13.2%), and peanuts (14.2%).

A few reports published in the literature displayed clinical cases of allergic reactions to faba beans (Damiani et al., 2011; Mur Gimeno, Feo Brito, Martín Iglesias, Lombardero Vega, & Bautista Martínez, 2007; Rodríguez-Mazariego, Fuentes Aparicio, Bartolomé Zavala, Acevedo Matos, & Zapatero Remón, 2016). For instance, a woman in Italy experienced anaphylaxis shock after eating bread containing faba bean flour (Mur Gimeno et al., 2007). A 5-year-old boy experienced similar symptoms after eating a snack containing faba bean as well as other legumes and nuts (Rodríguez-Mazariego et al., 2016). An allergic reaction was also reported following faba bean handling by a farmer in Italy (Damiani et al., 2011). Allergenic specific responses to faba bean proteins were confirmed by a skin prick test (SPT) and specific IgE-binding protein identification in the three cases, suggesting that faba beans may contain numerous allergenic proteins and peptides.

Introducing a new dietary protein may induce allergenic reactions due to de novo sensitization or cross-reactivity with other allergens sharing structural similarities (Remington et al., 2018). Faba bean protein cross-reactivity with other legumes and vegetables (fenugreek, red kidney bean, red gram, green gram, chickpea, and black gram) have been reported (Kumar et al., 2014). As the integration of new protein sources in the diet can provoke adverse health effects, it is essential to

assess the allergenic potentials of novel protein sources before they are used in product formulations, to ensure public health safety (Remington et al., 2018). Faba bean allergen risks deserve further assessments and characterizations, as with all new sources of proteins.

2.6 Conclusion

The faba bean has excellent nutritional and environmental advantages, its high protein content, combined with its agronomic features, make it an 'up-and-coming' product that could be used to meet the shifting trends toward healthy eating and environmental consciousness. The faba bean has a balanced amino acid profile, which is complementary to cereal products, and its digestibility can be substantially increased through adequate processing strategies. Protein quality scores reported for faba beans remain lower than animal-based proteins, such as milk, eggs, and meat, which all have DIAAS value above 1.0 (Phillips, 2017). Nevertheless, with a diversified nutritious diet, amino acid requirements can be easily fulfilled from various plant sources (Mariotti & Gardner, 2019). Moreover, the classical definition of protein quality has been criticized because it does not take into account the whole food matrix composition that is known to have a significant influence on chronic disease preponderance and global health, which are major public health concerns (Katz, Doughty, Geagan, Jenkins, & Gardner, 2019). Katz et al. (2019) recommended the introduction of health and environmental (based on life-cycle assessment) dimensions to the standard PDCAAS, with either a ratings system or adjustment factors to rank protein sources, on a basis that considers up-to-date scientific knowledge and health and environmental concerns. A reform of the protein quality definition would benefit plant-based protein sources and, more specifically, pulses, by acknowledging their environmental and health advantages. The faba bean can contribute to a healthy diet with its high content of dietary fibre, resistant starch, and minerals, among others. The faba bean is also a source of bioactive peptides and components that may procure noteworthy health benefits. However, further research is needed to better understand the health benefits and risks (particularly the allergenicity) associated with faba bean consumption to help increase and solidify its place in the growing and challenging global plant-based market.

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CONNECTING STATEMENT I

Chapter II presented an extensive literature overview of the current body of knowledge on faba bean nutritional and bioactive properties. As demonstrated throughout this review, there is a lack of data on both these aspects for faba beans, particularly for the new Canadian varieties. A comprehensive portrait of faba bean nutritional value in comparison to other available legumes is required to clearly seize the opportunities of faba beans as alternative ingredients in food formulations.

Chapter III presents a comprehensive investigation of the nutritional attributes of three new Canadian faba bean varieties (Fabelle, Malik and Snowbird) in comparison to two control legumes (pea and soy). The impact of a conventional food thermal processing (boiling) on the nutritional quality was also investigated. The different legumes were compared based on their proximate composition (protein, starch, amylose, lipid and ash content), protein electrophoretic profile, antinutritional factors content, amino acid profile, amino acid scores, *in vitro* protein digestibility and *in vitro* Protein Digestible Indispensable Amino Acid Score (IV-DIAAS).

The results from this research were presented at the 2nd NIZO Plant Protein Functionality Conference and published in the journal *Food Research International*.

- Martineau-Côté, D., Achouri, A., Wanasundara, J., Karboune, S., & L'Hocine, L. (2022) Nutritional quality of processed Canadian faba bean (Vicia faba L.) flour assessed with a harmonized static *in vitro* gastrointestinal digestion system adapted to mimic intestinal brush border digestion. 2nd NIZO Plant Protein Functionality Conference, On-line, October 11-13, 2022.

- Martineau-Côté, D., Achouri, A., Pitre, M., Wanasundara, J., Karboune, S., & L'Hocine, L. (2023). Investigation of the Nutritional Quality of Raw and Processed Canadian Faba Bean (Vicia faba L.) Flours in Comparison to Pea And Soy Using a Human *In vitro* Gastrointestinal Digestion Model. *Food Research International*, 113264. doi:https://doi.org/10.1016/j.foodres.2023.113264

CHAPTER III. INVESTIGATION OF THE NUTRITIONAL QUALITY OF RAW AND PROCESSED CANADIAN FABA BEAN (*VICIA FABA* L.) FLOURS IN COMPARISON TO PEA AND SOY USING A HUMAN *IN VITRO* GASTROINTESTINAL DIGESTION MODEL

3.1 Abstract

Faba bean is an ancient legume that is regaining interest due to its environmental and nutritional benefits. Very little is known on the protein quality of the new faba bean varieties. In this study, the digestibility and the Digestible Indispensable Amino Acid Score (DIAAS) of the protein quality of three Canadian faba bean varieties (Fabelle, Malik and Snowbird) were compared to pea and soy using the harmonized *in vitro* digestion procedure developed by the International Network of Excellence on the Fate of Food in the Gastrointestinal Tract (INFOGEST). The impact of boiling on the nutritional quality of faba bean flours was also ascertained. Protein content in faba bean (28.7-32.5%) was lower than defatted soy (56.6%) but higher than pea (24.2%). Total phenolics and phytate content were higher (p <0.05) in faba bean (2.1-2.4 mg/g and 11.5-16.4 mg/g respectively) and soy (2.4 mg/g and 19.8 mg/g respectively) comparatively to pea (1.3 mg/g and 8.9 mg/g). Trypsin inhibitor activity was significantly higher (p <0.05) in soy (15.4 mg/g) comparatively to pea (0.7 mg/g) and faba bean (0.8-1.1 mg/g). The digestibility of free amino acids of raw faba bean flours ranged from 31-39% while the digestibility of total amino acids ranged from 38-39%. The *in vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS) of raw faba bean flours ranged from 13-16 (when calculated based on free amino acid digestibility) to 32-38 (when calculated based on total amino acid digestibility) and was in a similar range to pea (13-31) and soy (11-40). Boiling modified the protein electrophoretic profile and decreased trypsin inhibitor activity (30-86% reduction), while total phenolics and phytate content were unaffected. The IV-DIAAS significantly decreased in all boiled legumes, possibly due to an increased protein aggregation leading into a lower protein digestibility (18-32% reduction). After boiling, the nutritional quality of faba beans was significantly lower (p <0.05) than soy, but higher than pea. Our results demonstrate that faba bean has a comparable protein quality than other legumes and could be used in similar food applications.

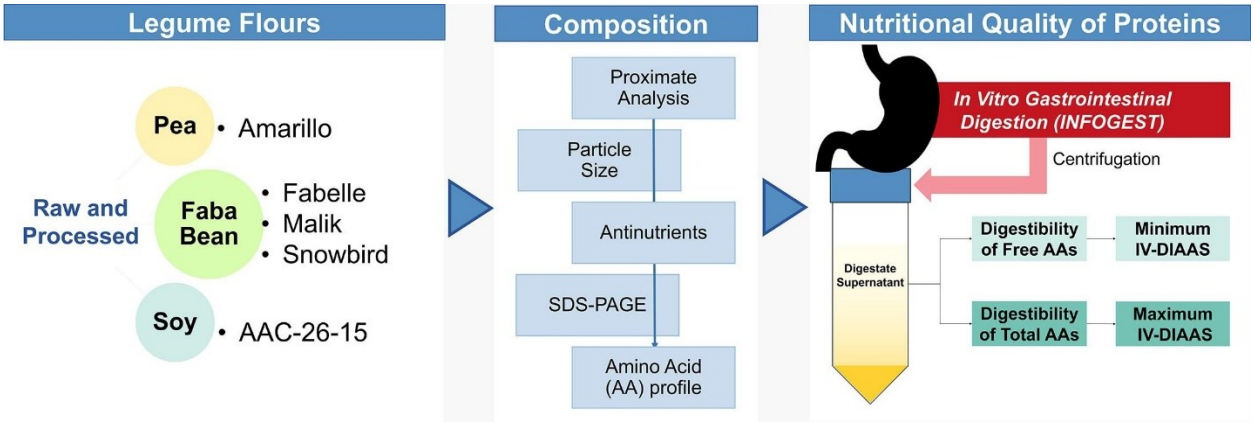

Figure 3-1. Graphical abstract

3.2 Introduction

In the context of climate change, population growth and diet-related diseases, the exploration of new sustainable high-quality protein sources with a minimal environmental footprint is required (Willett et al., 2019). In this context, pulse proteins are regarded as a promising alternative. The consumption of plant-derived protein sources, including pulses, is associated with a lower incidence of chronic illness such as cardiovascular disease and diabetes (Ferreira, Vasconcelos, Gil, & Pinto, 2021; Hafiz, Campbell, O'Mahoney, Holmes, Orfila, & Boesch, 2022; Willett et al., 2019) and is therefore highly recommended by the most recent Canadian food guide (Health Canada, 2019). However, plant-derived protein quality is usually lower than animal-based sources due to a lack of at least one essential amino acid (Gorissen et al., 2018), cell wall encapsulation of macronutrients (Rovalino-Córdova, Fogliano, & Capuano, 2019) and the presence of antinutritional factors such as phytate, enzyme inhibitors and tannins (Shi, Arntfield, & Nickerson, 2018; Shi, Mu, Arntfield, & Nickerson, 2017) that negatively affect protein digestibility. Nonetheless, protein digestibility of plant-based proteins can be improved with food processing strategies and amino acid requirements can be fulfilled by consuming a diversity of vegetal proteins throughout the day (Mariotti & Gardner, 2019).

Faba bean (*Vicia Faba* L.) is an ancient pulse that remains little known and underexploited in western countries (FAOSTAT, 2020). In Canada, faba bean production was first introduced in 1972 (Khazaei, Hawkins, & Vandenberg, 2021), but its production remained minor for decades (Statistics Canada, 2022). The faba bean annual production was 9,980 metric tons (on average) from 1991 to 2007, but it increased to reach 92,570 metric tons (on average) from 2014 to 2021 (Statistics Canada, 2022). This illustrates the recent and growing interest in the production of faba

bean due to its environmental, agronomic and nutritional benefits. Faba bean grows in various climatic conditions, has a higher protein content than pea, and is a rich source of fiber (Millar, Gallagher, Burke, McCarthy, & Barry-Ryan, 2019). It is also not part of the regulated allergens (Health Canada, 2018), gluten free (Sozer, Melama, Silbir, Rizzello, Flander, & Poutanen, 2019) and non-GMO (Calabrò et al., 2014). As most pulses, faba bean is limited in sulfur containing amino acids and tryptophan but is a rich source of lysine, leucine, isoleucine, threonine, histidine, and aromatic amino acids (Małgorzata, Jerzy, & Ewa, 2018; Millar et al., 2019). Canada produces a diversity of faba bean varieties differing in seed size, tannin, and vicine and convicine contents (Khazaei et al., 2021), that can be valuable sources of renewable and sustainable proteins for human consumption. Yet, the nutritional quality of faba bean protein remains largely unknown, particularly for these new Canadian varieties. Thus, a complete portrait of the nutritional value of faba bean is necessary to clearly seize the opportunities it has to offer in food applications.

This research aimed to assess the nutritional quality of three Canadian faba bean varieties (Fabelle, Malik and Snowbird) in comparison to the two most used legumes, pea and soy. The three faba bean cultivars included in the study had different quality traits: low-tannin (Snowbird); low vicine/convicine (Fabelle); and high tannin and high vicine/convicine (Malik), which are representative of the diversity among faba bean cultivars. Additionally, the impact of conventional food processing (boiling) on the nutritional quality of faba bean protein was also investigated. A harmonized *in vitro* gastrointestinal approach (Brodkorb et al., 2019) was adapted to evaluate amino acid digestibility and to compute the Digestible Indispensable Amino Acid Score (DIAAS), which is the recommended protein quality score by the Food and Agriculture Organization of the United Nations (FAO) since 2013 (FAO, 2013). This protein quality score is an improved version of the PDCAAS, which considers the ileal digestibility of each essential amino acid individually. The DIAAS is more representative of the real amino acid digestibility that occurs in the small intestine and there is a need to evaluate the nutritional value of faba bean considering this new approach. There is an increasing interest in using *in vitro* human digestion models for the assessment of the nutritional quality of food proteins (Nosworthy et al., 2023; Tavano, Neves, & da Silva Júnior, 2016), as *in vivo* studies are complex, time consuming, costly, and ethically questionable (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009; Mansilla et al., 2020). Very limited number of studies, however, have used these models to assess amino acid digestibility and DIAAS values (Ariëns et al., 2021; L'Hocine et al., 2023; Sousa et al., 2023). To the best of

our knowledge this is the first study reporting an *in vitro* DIAAS (IV-DIAAS) data for raw and processed faba bean, pea and soy flours.

3.3 Material and Methods

3.3.1 Material

Three dehulled faba bean cultivars (Fabelle Malik and Snowbird), one dehulled pea cultivar (Amarillo) and one dehulled soy cultivar (AAC-26-15) were used in this study. Faba bean cultivars Fabelle and Malik were provided by AGT Foods and Ingredients (Saskatoon, SK, CA), and Snowbird by W.A. Grain & Pulse Solutions (Innisfail, AB, CA). Certified yellow pea (CDC Amarillo) and soybean (Cdn #1, Variety AAC 26-25, Non-GMO & IP, Lot 261510504AT) were provided by Greenleaf Seeds (Tisdale, SK, CA) and Huron seeds (Clinton, ON, CA), respectively. Faba bean and pea samples were supplied as milled flours, and soybean as whole seeds.

Hydrochloric acid, sodium hydroxide, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), sodium bicarbonate, monosodium phosphate, disodium phosphate, phenol, tris, calcium chloride dihydrate, boric acid and sodium borate decahydrate were purchased from BioShop (Burlington, ON, Canada). Ethylenediaminetetraacetic acid (EDTA) was obtained from LECO (Saint Joseph, MI, USA). Ethanol, hexane, petroleum ether, dimethyl sulfoxide (DMSO), acetic acid, formic acid 88%, acetonitrile, methanol and norvaline were purchased from Fisher Scientific (Fair Lawn, NJ, USA). amino acid standard (5061-3330), borate buffer and ortho-phthalaldehyde (OPA) were purchased from Agilent Technologies (Mississauga, ON, Canada).

Trypsin from porcine pancreas (T0303), gallic acid, Folin-Ciocalteu's phenol reagent, Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), sodium azide, hydrogen peroxide solution (30%), hydrobromic acid 48%, tryptophan, glutamine, asparagine, cysteic acid and methionine sulfone were purchased from Sigma-Aldrich (St. Louis, MO, USA),

Pre-Cast Criterion Any KD gels, Laemmli buffer, Precision Plus Protein Dual Xtra (2–250 kDa molecular weight marker), β-mercaptoethanol, tris-glycine-SDS buffer and Coomassie blue G-250 were purchased from Bio-Rad (Hercules, CA, USA). The Pierce BCA assay kit was purchased from Thermo Fisher Scientific (San Jose, CA, USA). Total starch (K-TSTA), amylose and amylopectin (K-AMYL) and phytic acid (K-PHYT) assay kit were purchased from Megazyme (Bray, Wicklow, Ireland).

For the *in vitro* digestion procedure, α-amylase from porcine pancreas (A3176), pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545), porcine bile extract (B8631) and Bile Acid Assay Kit (MAK309) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade. Deionized water (Millipore) was used in all experiments.

3.3.2 Processing of Faba Bean, Pea and Soy Flours

Soy seeds were milled and defatted as described in Martineau-Côté, Achouri, Wanasundara, Karboune, and L'Hocine (2022). Briefly, the soy seeds were milled with a Brinkmann centrifugal grinding mill using a 0.2-mm rotary sieve with the addition of liquid nitrogen to prevent heating. The soy flour was then defatted through hexane extraction, according to the method of L'Hocine, Boye, and Arcand (2006) to obtain a fat content similar to the faba beans and peas. Faba bean, pea and soy flour were boiled following the procedure of Ma, Boye, Simpson, Prasher, Monpetit, and Malcolmson (2011). This process was selected, since it is a common and representative domestic processing for pulses. Briefly, the flour was hydrated in water (ratio 1:10) for 1 hour at room temperature under constant stirring and then boiled for 20 minutes. The cooked flour and cooking water were frozen at −40 °C, freeze-dried and milled once more to assure a particle size uniformity among the samples. All of the flour samples were stored at −20 °C in vacuum bags until needed.

3.3.3 Characterization of Faba Bean, Pea and Soy Flours

3.3.3.1 Proximate Analysis

Protein content was determined using the Dumas method (AOAC International, 1995) with a Vario MAX Cube (Elementar, Langenselbold, Germany) using a nitrogen to protein conversion factor of 6.25 and EDTA as standard. Fat content was measured with a Soxtec apparatus (Foss Tecator Soxtec System HT-6, 1043 extraction unit, Brampton, ON) according to the AACC method 30- 25.01 (Cereals & Grains Association, 1961b) through petroleum ether extraction. Moisture content was quantified following the AACC method 44-40.01 (Cereals & Grains Association, 1962) in drying 2 g of samples for 5 hours at 100 °C in a Fisher Isotemp Vacuum Oven (Fisher Scientific, Montreal, Qc, Canada), and ash content was determined using the AACC method 08-01-01 (Cereals & Grains Association, 1961a). Total starch was measured using the K-TSTA assay kit (Megazyme, Bray, Wicklow, Ireland) and amylose proportion in starch was

measured using the K-AMYL assay kit (Megazyme, Bray, Wicklow, Ireland), following the manufacturer protocol.

3.3.3.2 Electrophoretic Profile

Electrophoretic profile of raw and boiled legume flours were determined using SDS-PAGE in reducing and non-reducing conditions to assess the impact of boiling on the protein profiles. 10 mg of faba bean, pea and soy flours were suspended in 1 mL of Laemmli buffer (62.5 mmol/L Tris–HCl [pH 6.8], 25% glycerol, 2% SDS and 0.01% bromophenol blue, with or without the addition of 5% β-mercaptoethanol) and protein were extracted for 1 hour at room temperature under constant stirring. Soluble proteins were recovered by centrifugation (15 minutes at 16,000 x g). Supernatants were boiled for 5 minutes and cooled on ice. Ten micrograms of proteins were loaded per well into precast Bio-Rad Criterion AnykD gels (assuming that proteins are completely soluble in Laemmli buffer). A molecular weight marker of 2-250 kDa (Bio-Rad Precision Plus Protein Dual Xtra) was loaded concurrently. Gels were run at 100 V for 90 min in tris-glycine-SDS buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS) in a Criterion cell, stained with Coomassie blue G-250 for 1 hour and de-stained overnight in water. Image analysis was performed using a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA).

3.3.3.3 Particle Size Distribution

Particle size distribution was determined using a Mastersizer 2000 laser diffraction system (Malvern Instruments, Malvern, UK). Samples were hydrated in water (ratio 1:10) and added to the recirculating cell until a 10% obstruction value was obtained. The refractive index used was 1.52 and 1.33 for samples and dispersant (water), respectively.

3.3.4 Determination of Antinutritional Factors in Faba Bean, Pea and Soy Flours

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method following the procedure of Singleton, Orthofer, and Lamuela-Raventós (1999) as described in L'Hocine et al. (2023) with minor modification; total phenolics content (TPC) was expressed as mg of gallic acid equivalent per g of flour (on a dry basis) instead of ferulic acid equivalent.

Trypsin inhibitor activity (TIA) was determined using the method of Makkar, Siddhuraju, and Becker (2007) as described in L'Hocine et al. (2023) with minor modifications. 1 g of raw legume

flour was extracted in 50 mL of 0.01M NaOH for 3 hours at room temperature under constant stirring. A higher amount of flour (3.5 g) was used for the boiled samples due to a lower TIA activity in those samples. TIA was expressed as mg of pure trypsin inhibited per gram of sample on a dry basis.

Phytic acid content was determined following the method of McKie and McCleary (2016) using the K-PHYT assay kit (Bray, Wicklow, Ireland) as per the manufacturer protocol. Results were expressed as mg of phytic acid per gram of flour on a dry basis.

3.3.5 Evaluation of Amino Acid Digestibility of Faba bean, Pea and Soy Flours Using an *in vitro* Gastrointestinal Digestion Model

Amino acid digestibility of raw and boiled faba bean, pea and soy samples was determined using the static harmonized digestion procedure developed by the International Network of Excellence on the Fate of Food in the Gastrointestinal Tract (INFOGEST) (Brodkorb et al., 2019). Prior to digestion work, enzyme activity in amylase, pepsin and pancreatin was determined according to Brodkorb et al. (2019) and bile salts content in porcine bile extract was assessed using the Sigma-Aldrich bile assay kit (MAK309). Each batch of enzyme was tested to assure a constant enzyme-activity-to-meal ratios in the digestions.

For the *in vitro* gastrointestinal digestions, 0.4 g of flour was mixed with 0.6 g of water to reach the targeted consistency of tomato paste at the end of the oral phase (Brodkorb et al., 2019). For the oral digestion phase, hydrated flour was mixed in a ratio 1:1 with simulated salivary fluid (SSF) containing α-amylase from porcine pancreas (75 U/mL digestate) and digestion was conducted for 2 minutes at 37 °C under constant stirring. For the gastric digestion phase, the digestate was mixed in a ratio 1:1 with simulated gastric fluid (SGF) containing pepsin (2000 U/mL digestate). pH was adjusted to 3.0 by dropwise addition of 6 N HCl and digestion was continued for 2 hours at 37 °C under constant stirring. Gastric lipase was not added since lipids is a minor constituent in the sample studied (< 1%) comparatively to proteins and starch (Zhang, Noisa, & Yongsawatdigul, 2020). The digestates were diluted once more for the duodenal digestion phase in a ratio 1:1 with Simulated Intestinal Fluid (SIF) containing pancreatin from porcine mucosa (100 U trypsin activity/ mL digestate) and porcine bile extract (10 mM bile salts), pH was adjusted to 7.0 with 3 N NaOH and the digestate was incubated for 2 hours at 37 °C with constant stirring.

At the end of the digestion, samples were cooled on ice and protease inhibitor (5 mM AEBSF) was added to stop the digestion. The digestates were centrifuged (15,000 x g for 30 minutes at 4 °C) to recover the soluble fraction, which represents the bioavailable nutrients. 1 mL of the supernatant was kept as is and frozen at -80 °C for free amino acids quantification. The remaining supernatant volume was split in three equal fractions, frozen at -80 °C and freeze-dried for total amino acid determination (acidic hydrolysis, performic acid oxidation and alkaline hydrolysis). For free amino acid quantification in the legumes digestate supernatant, the later was filtered and injected as is.

3.3.6 Determination of Total Amino Acid Content in Legume Flours and *In vitro* Gastrointestinal Digestates

3.3.6.1 Acidic Hydrolysis for Total Amino Acid Quantification

The acidic hydrolysis was carried out as describe by Rutherfurd and Gilani (2009) to analyze 16 amino acids. Legume samples (~12 mg of protein) were mixed with 3 mL of 6 N HCl supplemented with 0.1% (w/v) phenol in glass test tubes. Tubes were flushed with nitrogen to prevent amino acid oxidation and incubated at 110 °C for 24 h. After the incubation period, the solvent was evaporated under a nitrogen flux. Amino Acids were quantified by reverse phase HPLC with on-line o-phthalaldehyde (OPA) derivatization following the method of Long (2015) as described in L'Hocine et al. (2023).

3.3.6.2 Performic Acid Oxidation for Methionine and Cysteine Quantification

For methionine and cysteine analysis, an oxidation step with performic acid was conducted before acidic hydrolysis to convert methionine to methionine sulfone and cysteine and cystine to cysteic acid, which are more stable derivatives. The method of Rutherfurd et al. (2009) was followed. Performic acid was freshly prepared in mixing ice cold 88% formic acid and 30% hydrogen peroxide in a ratio 9:1 for 30 minutes at room temperature. The mixture was cooled on ice for 30 minutes before usage. Legume samples (~12 mg of proteins) was mixed on ice with 3 mL of the performic acid solution and incubated for 16 hours at 4 °C. The next day, 450 µL of ice-cold 48% hydrobromic acid was added and the sample was further incubated on ice for 30 minutes. The solvent was evaporated under a nitrogen flux until dryness and the acidic hydrolysis was then performed as described in section 3.3.6.1.

Methionine sulfone and cysteic acid were quantified by reverse phase HPLC as described in L'Hocine et al. (2023) with modifications in the elution conditions, where the mobile phase B was adjusted to acetonitrile, methanol and water in a ratio of 20:60:20 (v:v:v). Calibration curves with methionine sulfone and cysteic acid were constructed and the elution times of each amino acid in analyzed samples were compared to those of the standard and the amount of each amino acid was calculated in mg/g based on the peak area.

3.3.6.3 Alkaline Hydrolysis for Tryptophan Analysis

An alkaline hydrolysis was performed following the method of Yust, Pedroche, Giron-Calle, Vioque, Millan, and Alaiz (2004) as described in L'Hocine et al. (2023) to determine tryptophan content. Tryptophan quantification was performed by reverse-phase HPLC using a Nova-Pack C18 column (Waters, Mississauga, ON). The mobile phase was composed of 25 mM sodium acetate buffer pH 6 supplemented with 0.02% sodium azide and acetonitrile in a ratio 9:1 (v: v). The elution was performed in isocratic mode. The absorbance was recorded at λ=280 nm. A calibration curve was constructed with tryptophan standards and tryptophan content was calculated based on peak area.

3.3.7 Calculation of the *In vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS) of Faba Bean, Pea and Soy Flours

IV-DIAAS values were calculated as described in L'Hocine et al. (2023). Free amino acid quantification in the supernatant was used to determine the minimal ileal digestibility (bioavailable free amino acids in the digestate) and the total amino acid quantification was used to obtain the maximal ileal digestibility. Those two values permit to obtain a plausible range of true amino acid digestibility since proteins are absorbed in the small intestine as free amino acids and as small peptides (Belović et al., 2011; Xiong, 2010). Minimal and maximal ileal digestibility of individual amino acid were therefore calculated as follows:

$$\text{In } vitro \text{ Ileal Amino Acid Digestibility } = \frac{\text{AA}_{\text{in the digestate (mg)}} - \text{AA}_{\text{in the blank (mg)}}}{\text{AA}_{\text{intake (mg)}}}$$

Where $\text{AA}_{\text{ in the digestate}}$ represents either free or total amino acids quantified in the digestate supernatant after the *in vitro* digestion. $\text{AA}_{\text{ blank}}$ refers to the digestive enzyme amino acid contribution, which was evaluated in performing blank digestions with water instead of flour. AA

$_{intake}$ is the amino acid content initially present in the legume flour. This calculus was repeated for each indispensable amino acid individually.

Minimal and maximal digestibility values were used to calculate the minimal and maximal IV-DIAAS, to obtain a range of plausible values. The IV- DIAAS was calculated with the following formula:

$$\text{IV-DIAAS (\%)} = \frac{\text{mg of indispensable digestible AA in 1 g of test protein}}{\text{mg of same AA in 1 g of reference protein}} \times 100$$

The IV-DIAAS was determined for three age groups (infant, children and older children, adolescent and adult) with their respective amino acid scoring patterns (FAO, 2013).

3.3.8 Statistical Analysis

Each analysis was performed in triplicate and results were expressed as mean ± standard deviation (SD). Data were analyzed through analysis of variance (ANOVA) (p <0.05) and the Turkey's honest significant difference (HSD) post-hoc test (p <0.05) using the XLSTAT software (Addinsoft, NY, USA) add-on to Microsoft Excel (Redmond, WA, USA) to determine significant differences.

3.4 Results and Discussion

3.4.1 Characterization of Faba Bean, Pea and Soy Flours

3.4.1.1 Proximate Composition

Proximate compositions of raw and boiled faba bean, pea and soy flour are reported in Table 3-1. Protein content varied significantly ($p < 0.05$) among the studied faba bean varieties and ranged from 28.7 to 32.5% for the raw flours, which is in accordance with previously reported values (27.5-32.4%) for faba bean (Hood-Niefer, Warkentin, Chibbar, Vandenberg, & Tyler, 2012). Snowbird variety had the highest protein content (32.5%), followed by Malik (29.9%) and Fabelle (28.7%). The three faba been varieties had significantly higher ($p < 0.05$) protein content than pea (24.2%) but lower than soy (56.6%).

Starch content was significantly higher in raw Fabelle flour (48.7%) comparatively to Malik (45.8%) and Snowbird (44.3%). The starch content in raw pea was similar to Fabelle and Malik ($p > 0.05$), but higher compared to Snowbird ($p < 0.001$). Amylose proportion was not significantly

different in faba bean and pea with contents varying from 22.1% to 23.2% ($p > 0.05$). Setia, Dai, Nickerson, Sopiwnyk, Malcolmson, and Ai (2019) have reported a starch content of 43.3% and 46.0% and an amylose proportion of 19.1% and 18.6% for faba bean (cv. Snowdrop) and pea (cv. Amarillo) respectively, which is in good accordance with our data. Gunasekera, Stoddard, and Marshall (1999) reported an amylose proportion varying from 17 to 29% in 72 faba bean samples. Fat content was comparable for raw faba bean, pea and defatted soy flours ranging between 0.8-1.2%). Ash content, however, was significantly higher for soy (6.2%), followed by faba bean (3.0-3.6%), then pea (2.6%).

Table 3-1. Proximate composition of raw and boiled faba bean, pea and soy flours.

Legume	Treatment	Protein (%)	Starch (%)	Fat (%)	Ash (%)	Moisture (%)	Amylose (%)[1]
		(Dry base)					
Faba bean							
Fabelle	Raw	28.7 (0.4)^{e}	48.7 (0.1)^{a}	1.03 (0.04)^{ac}	3.56 (0.03)^{d}	4.0 (0.6)^{b}	22.7 (0.5)^{cd}
	Boiled	27.4 (0.3)^{f}	47.1 (1.2)^{abc}	0.58 (0.03)^{de}	3.89 (0.03)^{c}	0.9 (0.6)^{de}	34.0 (1.5)^{a}
Malik	Raw	29.9 (0.4)^{d}	45.8 (0.5)^{bcd}	1.21 (0.16)^{a}	3.40 (0.07)^{ef}	4.7 (0.1)^{b}	22.1 (0.2)^{d}
	Boiled	29.5 (0.3)^{de}	45.2 (0.3)^{cd}	0.70 (0.01)^{ce}	3.40 (0.07)^{de}	2.6 (0.2)^{c}	29.7 (2.1)^{ab}
Snowbird	Raw	32.5 (0.6)^{c}	44.3 (1.2)^{d}	0.79 (0.17)^{bce}	3.02 (0.01)^{fg}	4.8 (0.3)^{b}	22.4 (1.5)^{d}
	Boiled	32.2 (0.2)^{c}	44.9 (1.8)^{cd}	0.45 (0.02)^{e}	3.34 (0.05)^{de}	2.1 (0.5)^{cd}	27.2 (1.7)^{bc}
Pea							
Amarillo	Raw	24.2 (0.2)^{g}	47.8 (0.5)^{ab}	1.03 (0.32)^{ac}	2.55 (0.07)^{h}	7.4 (0.1)^{a}	23.2 (1.7)^{cd}
	Boiled	24.0 (0.2)^{g}	49.0 (0.2)^{a}	0.88 (0.03)^{acd}	2.78 (0.09)^{gh}	1.7 (0.5)^{ce}	31.0 (2.6)^{ab}
Soy							
AAC-26-15	Raw	56.6 (0.3)^{a}	0.4 (0.1)^{e}	1.12 (0.08)^{ab}	6.16 (0.05)^{b}	5.0 (0.4)^{b}	.
	Boiled	55.1 (0.6)^{b}	0.3 (0.1)^{e}	0.47 (0.03)^{e}	6.65 (0.27)^{a}	0.8 (0.4)^{e}	.
***p*-value**	Legume	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**0.006**
	Treatment	**<0.001**	0.788	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	L×T[2]	**0.017**	0.072	**0.042**	0.285	**<0.001**	0.160

Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differs (p <0.05) as analyzed by two-way ANOVA and the Tukey's test; [1]The amylose content is expressed as a percentage of total starch; [2]L×T refers to Legume × Treatment interaction effect.

Boiling had generally no or little impact on protein and starch content, but significantly decreased ($p < 0.05$) the fat content, which could be explained by the heat indued formation of starch-lipid and/or protein-lipid complexes that are less easily extractable in petroleum ether (Ellefson, 2017). The ash content slightly increased after boiling. As expected, the starch amylose proportion increased in boiled faba bean and pea flours. This effect was also observed in maize, potatoes (Miyoshi, 2002), green bananas (Cordeiro, Veloso, Santos, Bonomo, Caliari, & Fontan, 2018) and tapioca (Chaiwanichsiri, 2016) after a wet heat treatment and was attributed to the degradation of

outer amylopectin chains. The degraded linear amylopectin chain could form a complex with amylose and cause an increase in apparent amylose content (Chaiwanichsiri, 2016; Cordeiro et al., 2018). However, the amylose increase could also be due to starch structural changes causing a method artefact.

3.4.1.2 Electrophoretic Profile

The protein electrophoretic profiles of raw and boiled faba bean, pea and soy flours were analysed by reducing and non-reducing SDS-PAGE (Figure 3-2). The electrophoretic profiles of the three faba bean varieties were similar under reducing conditions, showing protein bands ranging from ~12 kDa to 150 kDa. The four more intense groups of bands of ~75, ~50, ~37 and ~18-25 kDa are likely to correspond to the globulins, convicilin, vicilin, α-legumin and β-legumin, respectively (Ashraf et al., 2020; Singhal, Stone, Vandenberg, Tyler, & Nickerson, 2016; Vogelsang-O'Dwyer et al., 2020; Warsame, O'Sullivan, & Tosi, 2018). Globulins are the major storage protein fraction in faba bean, which accounts for up to ~80% of the seed proteins (Alghamdi, 2009). Under non-reducing conditions, three main clear band groups were present (~60-75, ~50 and ~45 kDa) that possibly corresponds to convicilin, vicilin and legumin respectively (Vogelsang-O'Dwyer et al., 2020). Legumins are composed of a basic (β) and acidic (α) polypeptide side chains that are linked together by a disulfide bond mostly observed under non-reducing conditions (Müntz, Horstmann, & Schlesier, 1999).

Boiling treatment had no noticeable effect on the electrophoretic pattern under reducing conditions. However, under non-reducing conditions a molecular weight shift of major protein bands was observed. The bands of ~12, ~50 and ~75 kDa disappeared and larger protein band above 250 kDa appeared at the top of the gel. Similar effect was observable for pea and soy flours. These results suggest the occurrence of protein aggregation through formation of new disulfide bonds during the thermal processed leading to higher molecular weight protein bands. For soy flour, defatting had no major impact on the electrophoretic pattern except for loss of intensity of low molecular weight bands (~2-5 kDa).

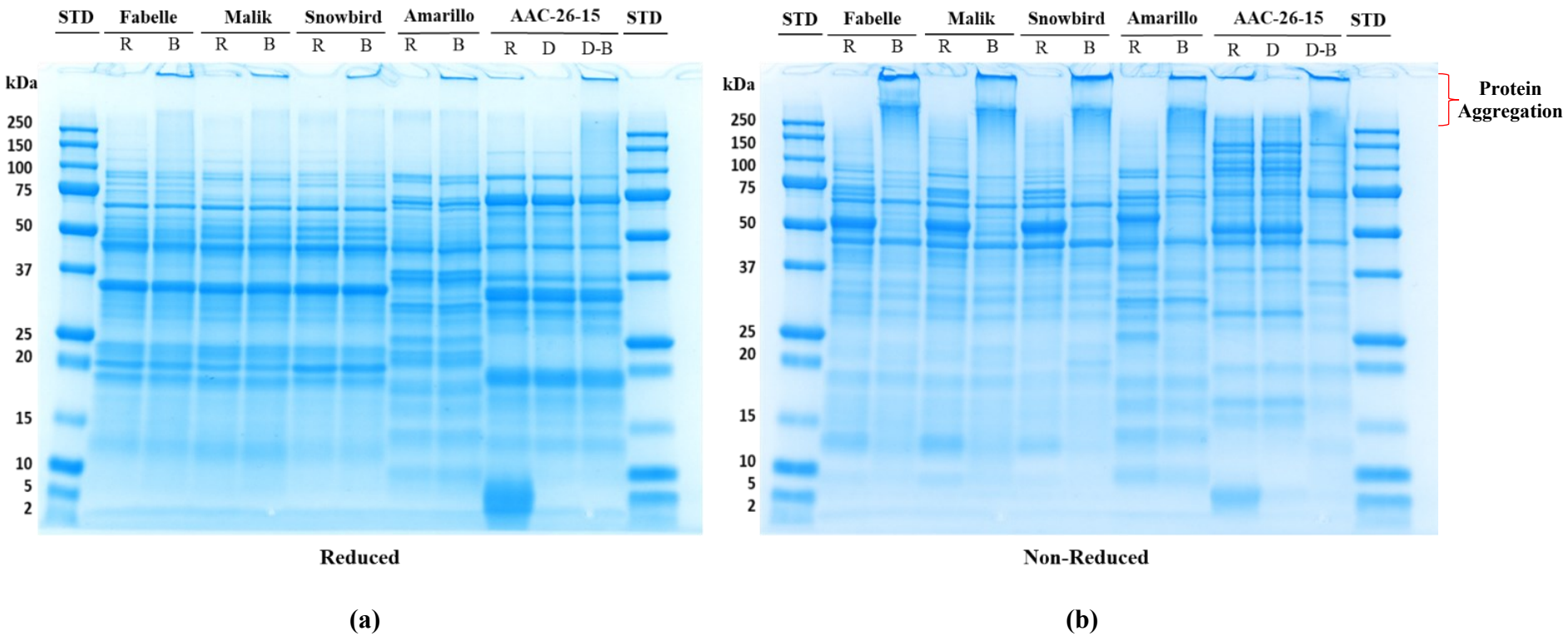

Figure 3-2. Electrophoretic profiles (SDS-PAGE) of faba bean, pea and soy flours; (a) Reducing conditions; (b) Non-reducing conditions; R, B and D refers to raw, boiled and defatted flours respectively.

3.4.1.3 Particle Size Distribution

Flour particle size has a significant impact on protein digestibility, since a smaller particle size increase the surface area between digestive enzymes and proteins (Paz-Yépez, Peinado, Heredia, & Andrés, 2019). In this study, each flour samples were milled using a 0.2 mm sieve to uniformize particle size after processing and to limit the effect of this parameter on protein digestibility. Volume percentiles (10th, 50th and 90th) of the particle size distribution are shown in Table 3-2. Raw legume flours had overall similar particle size distributions, with 10% of particles having a diameter ranging from 6 to 8 µm, 50% from 27 to 34 µm and 90% from 120 to 130 µm. Boiling caused a slight shift of the particle size distribution resulting in an increased of the 50th percentile (32-45 µm) and a decreased of the 90th percentile (89-119 µm). The observed particle size shift could be explained by protein aggregation, as shown previously in the electrophoretic profile. Nonetheless, samples particle size remained in a comparable order of magnitude (<0.2 mm).

Table 3-2. Volume percentiles of particle size distribution (µm) of raw and boiled faba bean, pea and soy flours.

Legume	Treatment	d (0.1)	d (0.5)	d (0.9)
Faba bean				
Fabelle	Raw	7.6 (0.7)^{bcd}	26.5 (1.9)^{f}	120 (14)^{ab}
	Boiled	7.1 (0.3)^{cd}	38.3 (1.5)^{abc}	95 (5)^{bc}
Malik	Raw	7.7 (0.4)^{bcd}	29.4 (1.4)^{def}	131 (14)^{a}
	Boiled	8.4 (0.5)^{ab}	41.0 (2.8)^{ab}	119 (9)^{ab}
Snowbird	Raw	8.3 (0.3)^{ab}	30.4 (0.4)^{def}	133 (10)^{a}
	Boiled	9.2 (0.4)^{a}	44.9 (2.5)^{a}	110 (12)^{abc}
Pea				
Amarillo	Raw	7.1 (0.2)^{d}	28.8 (0.8)^{ef}	130 (1)^{a}
	Boiled	6.8 (0.2)^{de}	32.3 (1.1)^{cdef}	89 (7)^{c}
Soy				
AAC-26-15	Raw	5.9 (0.2)^{e}	33.8 (5.8)^{cde}	132 (8)^{a}
	Boiled	8.2 (0.3)^{abc}	36.1 (0.8)^{bcd}	93 (1)^{bc}
***p*-value**	Legume	**<0.001**	**<0.001**	**0.013**
	Treatment	**<0.001**	**<0.001**	**<0.001**
	L x T[1]	**<0.001**	**<0.001**	0.089

Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differ (p <0.05) as analyzed by two-way ANOVA and the Tukey's test; [1]L×T refers to Legume × Treatment interaction effect.

3.4.2 Antinutritional Factors in Faba Bean, Pea and Soy Flours

Relevant compounds that are known to affect protein digestibility, namely, phytic acid, total phenolics and trypsin inhibitor were quantified in legume flours to subsequently evaluate their impact on observed protein digestibility (Table 3-3).

Table 3-3. Antinutritional factor content in raw and boiled faba bean, pea and soy flours.

Legume	Treatment	Total Phenolics (mg GA_{eq}[1]/g)	Trypsin Inhibitor Activity (mg/g)[2]	Phytic Acid (mg/g)
Faba bean				
Fabelle	Raw	2.38 (0.13)^{b}	1.08 (0.15)^{bc}	16.3 (1.1)^{b}
	Boiled	2.48 (0.04)^{b}	0.26 (0.01)^{c}	17.3 (0.02)^{b}
Malik	Raw	2.05 (0.06)^{c}	1.07 (0.14)^{bc}	11.5 (0.4)^{cd}
	Boiled	2.01 (0.06)^{c}	0.25 (0.06)^{c}	12.2 (0.3)^{c}
Snowbird	Raw	2.44 (0.08)^{b}	0.84 (0.03)^{bc}	12.2 (0.5)^{c}
	Boiled	2.45 (0.04)^{b}	0.28 (0.04)^{c}	11.4 (0.5)^{cd}
Pea				
Amarillo	Raw	1.31 (0.12)^{d}	0.71 (0.05)^{bc}	8.9 (0.2)^{e}
	Boiled	1.42 (0.03)^{d}	0.49 (0.07)^{c}	9.8 (0.5)^{de}
Soy				
AAC-26-15	Raw	2.39 (0.10)^{b}	15.4 (1.56)^{a}	19.8 (1.2)^{a}
	Boiled	2.72 (0.04)^{a}	2.15 (0.15)^{b}	19.7 (0.3)^{a}
***p*-value**	Legume	**<0.001**	**<0.001**	**<0.001**
	Treatment	**0.001**	**<0.001**	0.138
	L×T[3]	**0.006**	**<0.001**	0.062

Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differ (p <0.05) as analyzed by two-way ANOVA and the Tukey's test; [1]GA_{eq} refers to Gallic acid equivalent; [2] Trypsin inhibitor activity is expressed as mg of pure trypsin inhibited per gram of sample; [3] L × T refers to Legume × Treatment interaction effect.

3.4.2.1 Total Phenolics

Phenolic compounds, although being desirable for their health promoting properties (Matsumura, Kitabatake, Kayano, & Ito, 2023), can be classified as an antinutritional factor since they can form complexes with proteins and consequently reduce their solubility and the accessibility of certain amino acid residues for digestion (Cirkovic Velickovic & Stanic-Vucinic, 2018; Czubinski & Dwiecki, 2017). As shown in Table 3-3, total phenolic content was higher in raw faba bean flours (2.05-2.44 mg GAE/g) and soy (2.39 mg GAE/g) comparatively to pea (1.31 mg GAE/g) (p <0.05). Among the faba bean cultivars, the Malik variety had the lowest total phenolic content (2.05 mg GAE/g) (p <0.05). Interestingly, total phenolic content was not lower in the Snowbird variety compared to the two other faba bean varieties even though it is a low tannin cultivar. The

fact that the seeds have been dehulled can explain the obtained results since tannins are mainly located in the hull (Çalışkantürk Karataş, Günay, & Sayar, 2017). Total phenolic content was in the same range then previously reported values for dehulled faba bean [0.72 mg GAE/g (Alonso, Aguirre, & Marzo, 2000), 2.71 mg GAE/g (Boudjou, Oomah, Zaidi, & Hosseinian, 2013) and 4.00 mg GAE/g (Boukhanouf, Louaileche, & Perrin, 2016)]. Boiling had no significant impact ($p > 0.05$) on total phenolics content (except for soy).

3.4.2.2 Trypsin Inhibitor

Trypsin inhibitor is a protein found in legumes and other plant protein sources that is known for decreasing trypsin and in some cases chymotrypsin activity in the intestinal tract (Evandro Fei and Elazeem, 2011; Liener et al., 1988) resulting in a decrease of protein digestibility. The Trypsin inhibitor activity was importantly higher in raw soy four (15.43 mg/g) ($p < 0.05$) comparatively to pea (0.71 mg/g) and faba bean (0.84-1.08 mg/g) flours. A similar pattern was observed by Shi et al. (2017); they measured a trypsin inhibitor activity of 45.89 TIU/mg in raw soy, 3.16 TIU/mg in pea and 5.96-6.10 TIU/mg in faba bean. It is well documented that soy is one of the legume with the highest trypsin inhibitor activity (Avilés-Gaxiola, Chuck-Hernández, & Serna Saldívar, 2018; Savage & Morrison, 2003). Soy contains two type of trypsin inhibitor, the Kunitz (~20 kDa) and the Bowman-Birk (~8 kDa), whereas most pulses contain only the Bowman-Birk type (Avilés-Gaxiola et al., 2018). The Kunitz is a strong inhibitor against trypsin and the Bowman-Birk type can decrease activity of both trypsin and chymotrypsin (Gillman, Kim, & Krishnan, 2015). Trypsin inhibitor activity is heat sensitive (Evandro Fei et al., 2011), and as expected, its activity decreased after boiling. However, the only significant reduction ($p < 0.05$) was observed for soy (-86%). The absence of a statistically significant effect in faba bean and pea can be explained by the lower initial value compared to soy.

3.4.2.3 Phytic Acid

Phytic acid is the main phosphorous storage form in legumes (Gupta, Gangoliya, & Singh, 2015). At physiological pH, the phosphate groups possess negative charges and can form complexes with proteins and reduce their bioavailability for digestion and absorption (Konietzny & Greiner, 2003). Phytic acid content was lower in raw faba bean (12.2-16.4 mg/g) comparatively to soy (19.8 mg/g), but higher compared to pea (8.9 mg/g) ($p < 0.05$). Similar values were previously reported for phytic acid content in faba bean (19.65 mg/g), pea (9.93 mg/g) and soy (22.91 mg/g) (Shi et al.,

2018). Among the faba bean varieties, Fabelle had a significant higher phytic acid content (16.35 mg/g) (p <0.05) compared to Malik and Snowbird. Boiling had no impact on phytic acid content. Soaking and cooking were reported to decrease slightly phytate content in faba bean (Luo, Xie, Jin, Zhang, Wang, & He, 2013; L. Shi et al., 2018; Vidal-Valverde, Frias, Sotomayor, Diaz-Pollan, Fernandez, & Urbano, 1998; Yu-Wei & Wei-Hua, 2013) due to leaching and endogenous phytase activation. In this study the soaking/cooking water was kept to prevent nutrient loss and important variation of the nutritional value, which can explain the non-reduction of phytic acid.

3.4.3 Amino Acid Content in Faba Bean, Pea and Soy Flours

The amino acid profiles of legume flours are presented in Table 3-4. The amino acid composition of the three faba bean varieties were very similar, but some minor differences were observed. Fabelle had a higher alanine and threonine contents compared to Snowbird (p <0.05) and Malik had a higher alanine and lysine content compared to Snowbird (p <0.05). The obtained amino acid profiles for faba bean are comparable to the ones reported by Małgorzata et al. (2018), Nosworthy et al. (2018) and Mattila et al. (2018).

In comparison to the two other legumes, faba bean had generally a similar profile to pea with slight differences in lysine, phenylalanine and threonine contents, which were significantly higher in pea (p <0.05). The three raw faba bean varieties had, however, higher leucine content than pea and higher arginine content than pea and soy (p <0.05). Soy had a significantly (p <0.05) higher content of leucine, methionine, phenylalanine, tyrosine, tryptophan, glutamic acid and serine compared to faba bean. Boiling caused overall a slight decreased in amino acid content, but the difference was not significant (p >0.05) for most legume flours.

Table 3-4. Amino acid composition (g/100 g of proteins) of raw and boiled faba bean, pea and soy flours.

	Essential Amino Acids Mean (Standard Deviation)									Non-Essential Amino Acids Mean (Standard Deviation)							
	HIS	**ILE**	**LEU**	**LYS**	**MET[1]**	**PHE**	**THR**	**TRP[2]**	**VAL**	**ALA**	**ASX[3]**	**ARG**	**CYS[1]**	**GLX[3]**	**GLY**	**TYR**	**SER**
Fabelle																	
Raw	3.22^{ab}	3.80^{bc}	7.12^{b}	7.95^{cde}	0.99^{bc}	4.24^{bcd}	3.58^{bc}	1.17^{bcd}	4.27^{ab}	4.06^{a}	10.99^{abc}	9.00^{a}	1.54 ^{b}	17.78 ^{b}	3.96^{ab}	3.26^{bc}	4.77^{bc}
	(0.08)	(0.24)	(0.19)	(0.03)	(0.11)	(0.08)	(0.12)	(0.01)	(0.29)	(0.13)	(0.51)	(0.35)	(0.11)	(0.71)	(0.13)	(0.14)	(0.17)
Boiled	3.07 ^{b}	3.80^{bc}	6.92^{bc}	8.15^{cde}	0.88^{bc}	4.17^{cd}	3.52^{bcd}	1.11^{e}	4.25^{ab}	3.94^{ab}	10.55^{cd}	8.66^{a}	1.72^{ab}	16.87^{bc}	3.92^{ab}	3.24^{bc}	4.60^{cd}
	(0.06)	(0.43)	(0.22)	(0.33)	(0.07)	(0.13)	(0.08)	(0.02)	(0.44)	(0.08)	(0.14)	(0.28)	(0.18)	(0.28)	(0.18)	(0.07)	(0.18)
Malik																	
Raw	3.17^{ab}	3.89^{abc}	6.95^{bc}	8.23^{bcd}	0.89^{bc}	4.21^{cd}	3.46^{cd}	1.14^{cde}	4.42^{a}	4.00^{a}	11.20^{abc}	8.53^{ab}	1.46^{bc}	17.20^{b}	3.86^{ab}	3.21^{bc}	4.59^{cd}
	(0.14)	(0.22)	(0.14)	(0.09)	(0.09)	(0.19)	(0.09)	(0.01)	(0.14)	(0.17)	(0.42)	(0.34)	(0.12)	(0.65)	(0.18)	(0.09)	(0.11)
Boiled	2.78^{cd}	3.14^{d}	6.16^{d}	7.59^{de}	0.80^{c}	3.68^{e}	3.10^{e}	1.13^{cde}	3.55^{c}	3.54^{c}	9.79^{d}	7.43^{c}	1.62^{ab}	15.20^{c}	3.42^{c}	2.84^{de}	4.25^{de}
	(0.13)	(0.07)	(0.20)	(0.20)	(0.05)	(0.11)	(0.09)	(0.01)	(0.10)	(0.12)	(0.29)	(0.26)	(0.16)	(0.52)	(0.10)	(0.10)	(0.14)
Snowbird																	
Raw	2.97^{bc}	3.65^{cd}	6.73^{bc}	7.53^{ef}	1.13^{ab}	3.86^{de}	3.24^{de}	1.16^{bcde}	4.13^{abc}	3.62^{bc}	10.33^{cd}	8.56^{ab}	1.13^{c}	16.59^{bc}	3.66^{bc}	2.98^{cde}	4.40^{cde}
	(0.11)	(0.06)	(0.20)	(0.12)	(0.10)	(0.14)	(0.14)	(0.01)	(0.04)	(0.15)	(0.49)	(0.37)	(0.10)	(0.80)	(0.15)	(0.14)	(0.24)
Boiled	2.53^{d}	3.36^{cd}	6.20^{d}	6.88^{f}	0.82^{c}	3.56^{e}	3.00^{e}	1.12^{de}	3.70^{bc}	3.38^{c}	9.54^{d}	7.85^{bc}	1.59^{ab}	15.35^{c}	3.41^{c}	2.78^{e}	4.11^{e}
	(0.02)	(0.24)	(0.05)	(0.07)	(0.10)	(0.02)	(0.02)	(0.02)	(0.28)	(0.05)	(0.05)	(0.20)	(0.07)	(0.07)	(0.07)	(0.02)	(0.09)
Amarillo																	
Raw	2.96^{bc}	3.56^{cd}	6.53^{cd}	8.88^{ab}	1.01^{bc}	4.44^{bc}	3.82^{ab}	1.21^{b}	4.10^{abc}	4.05^{a}	10.96^{bc}	7.20^{c}	1.56^{b}	16.78^{bc}	3.96^{ab}	3.10^{bcd}	4.41^{cde}
	(0.10)	(0.08)	(0.22)	(0.45)	(0.17)	(0.17)	(0.11)	(0.02)	(0.06)	(0.12)	(0.35)	(0.22)	(0.14)	(0.41)	(0.14)	(0.10)	(0.14)
Boiled	2.96^{bc}	3.88^{abc}	6.77^{bc}	9.21^{a}	0.89^{bc}	4.61^{b}	4.03^{a}	1.17^{bc}	4.70^{a}	4.29^{a}	11.42^{abc}	7.58^{c}	1.94^{a}	17.65^{b}	4.24^{a}	3.33^{b}	4.61^{cd}
	(0.11)	(0.11)	(0.15)	(0.24)	(0.02)	(0.12)	(0.20)	(0.02)	(0.34)	(0.21)	(0.70)	(0.30)	(0.11)	(0.98)	(0.20)	(0.19)	(0.26)
AAC-26-15																	
Raw	3.39^{a}	4.37^{ab}	7.74^{a}	8.49^{bc}	1.32^{a}	5.41^{a}	4.10^{a}	1.51^{a}	4.41^{a}	4.26^{a}	12.10^{a}	7.39^{c}	1.92^{a}	19.98^{a}	4.03^{ab}	3.84^{a}	5.28^{a}
	(0.07)	(0.05)	(0.16)	(0.27)	(0.07)	(0.17)	(0.07)	(0.01)	(0.05)	(0.05)	(0.19)	(0.11)	(0.12)	(0.28)	(0.04)	(0.09)	(0.08)
Boiled	3.22^{ab}	4.45^{a}	7.67^{a}	8.27^{bc}	1.31^{a}	5.34^{a}	3.94^{a}	1.56^{a}	4.50^{a}	4.15^{a}	12.01^{ab}	7.26^{c}	1.76^{ab}	19.98^{a}	4.02^{ab}	3.70^{a}	5.09^{ab}
	(0.09)	(0.14)	(0.17)	(0.10)	(0.06)	(0.14)	(0.13)	(0.03)	(0.15)	(0.11)	(0.34)	(0.22)	(0.00)	(0.56)	(0.13)	(0.06)	(0.13)
***p*-value**																	
Legume	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Treatment	**<0.001**	0.100	**<0.001**	**0.028**	**0.001**	**0.004**	**0.008**	**0.007**	0.152	**0.008**	**0.005**	**0.001**	**<0.001**	**0.006**	0.099	**0.023**	**0.018**
L×T[4]	**0.005**	**0.002**	**<0.001**	**0.002**	0.108	**0.003**	**0.005**	**0.001**	**<0.001**	**0.002**	**0.007**	**0.002**	**0.003**	**0.004**	**0.003**	**0.002**	0.081

Amino acids are abbreviated with 3-letter code. Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differ (p <0.05) as analyzed by two-way ANOVA and the Tukey's test; [1] Methionine and cysteine were quantified separately as methionine sulfone and cysteic acid respectively. [2] Tryptophan was analyzed separately through alkaline hydrolysis; [3] Glutamine and asparagine are converted to glutamic acid and aspartic acid during the acidic hydrolysis. The reported values for these amino acids are the sum of asparagine and aspartic acid (aspx) and glutamine and glutamic acid respectively (glx); [4] L × T refers to Legume × Treatment interaction effect.

The amino acid scores (AAS) of faba bean, pea and soy flours were calculated based on the scoring pattern of children of 6 months-3 years (FAO, 2013) to gain an insight on how well the amino acid composition of these legume flours meet the nutritional needs in essential amino acids (Figure 3-3). For faba bean flours, the AAS were above the requirement for all the essential amino acids, except methionine and cysteine (SAA) and valine in the case of boiled flours. The deficiency in sulfur-containing amino acid for faba bean is well-documented (Nosworthy et al., 2018; Vogelsang-O'Dwyer et al., 2020). Raw pea flours were slightly deficient in SAA and valine, but this deficiency was not apparent after boiling. As expected, the AAS for soy was above the requirement for all indispensable amino acid, as it is well established that soy protein has a well-balanced amino acid profile that is comparable to animal-based proteins (Hertzler, Lieblein-Boff, Weiler, & Allgeier, 2020; Hughes, Ryan, Mukherjea, & Schasteen, 2011). All three legumes were very rich in lysine, making their amino acid profile complementary to cereals (Han et al., 2019).

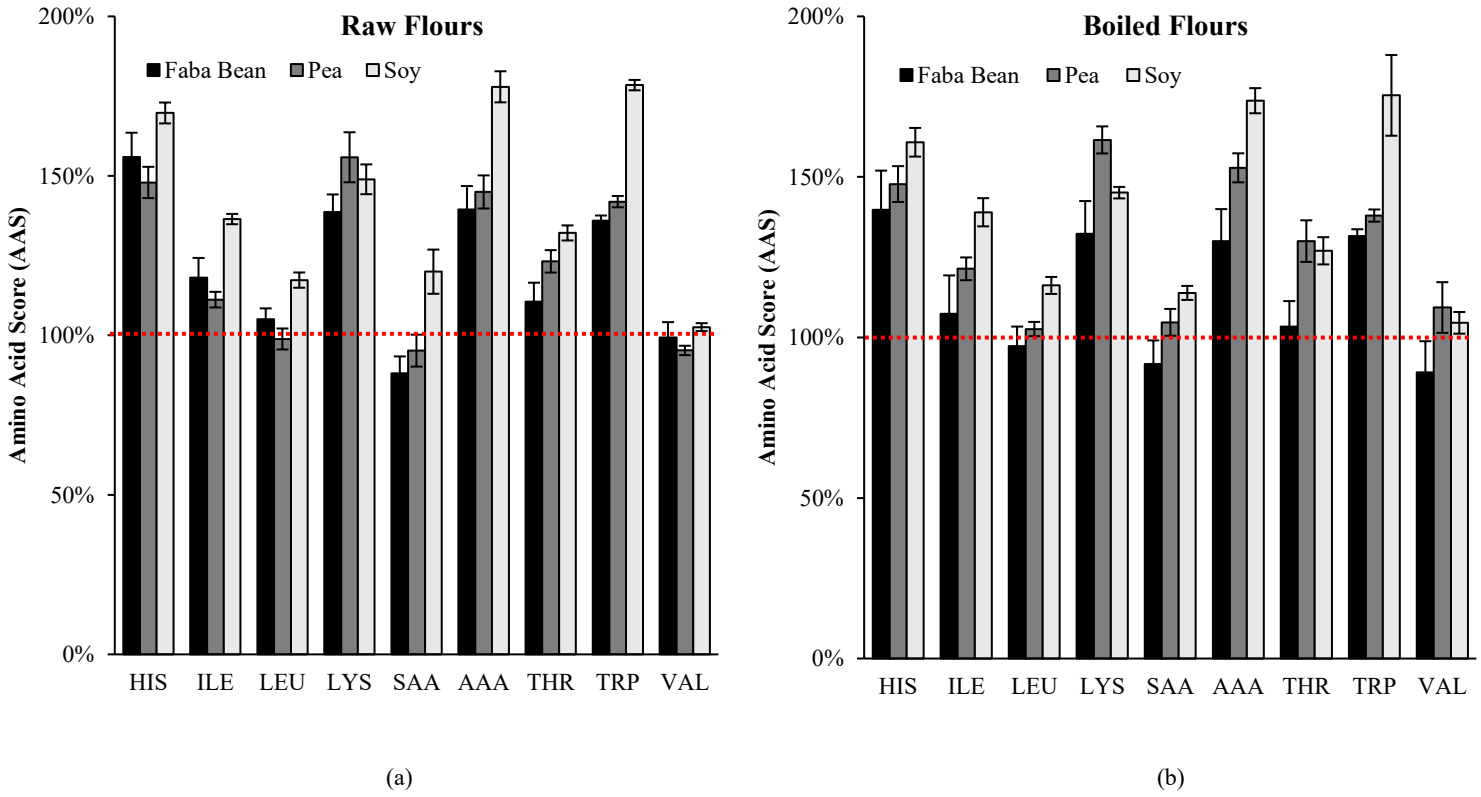

Figure 3-3. Amino acid score (AAS) of faba bean, pea and soy flours (Mean ± Standard deviation). Data for faba bean is the mean of the three varieties (Fabelle, Malik and Snowbird).The AAS were calculated using the the amino acid scoring pattern for children (6 months-3 years) (FAO, 2013); (a) raw flours; (b) boiled flours.

3.4.4 *In vitro* Amino Acid Digestibility of Faba Bean, Pea and Soy Flours

Protein digestibility of raw and boiled faba bean, pea and soy flours were determined after *in vitro* gastrointestinal digestion. Protein digestibility was evaluated through the sum of free amino acids digestibility and the sum of total amino acid digestibility (Figure 3-4). The quantification of free amino acids is an indicator of the minimum protein digestibility as a result of their direct availability for absorption, and the quantification of total amino acids is an indicator of the maximum protein digestibility since it includes all proteins present in the digestate in the form of free amino acids, small peptides and oligopeptides that will be absorbed in the small intestine. Quantification of free amino acid and total amino acid digestibility should therefore estimate a minimum and maximum range of protein digestibility values relative to in vivo data.

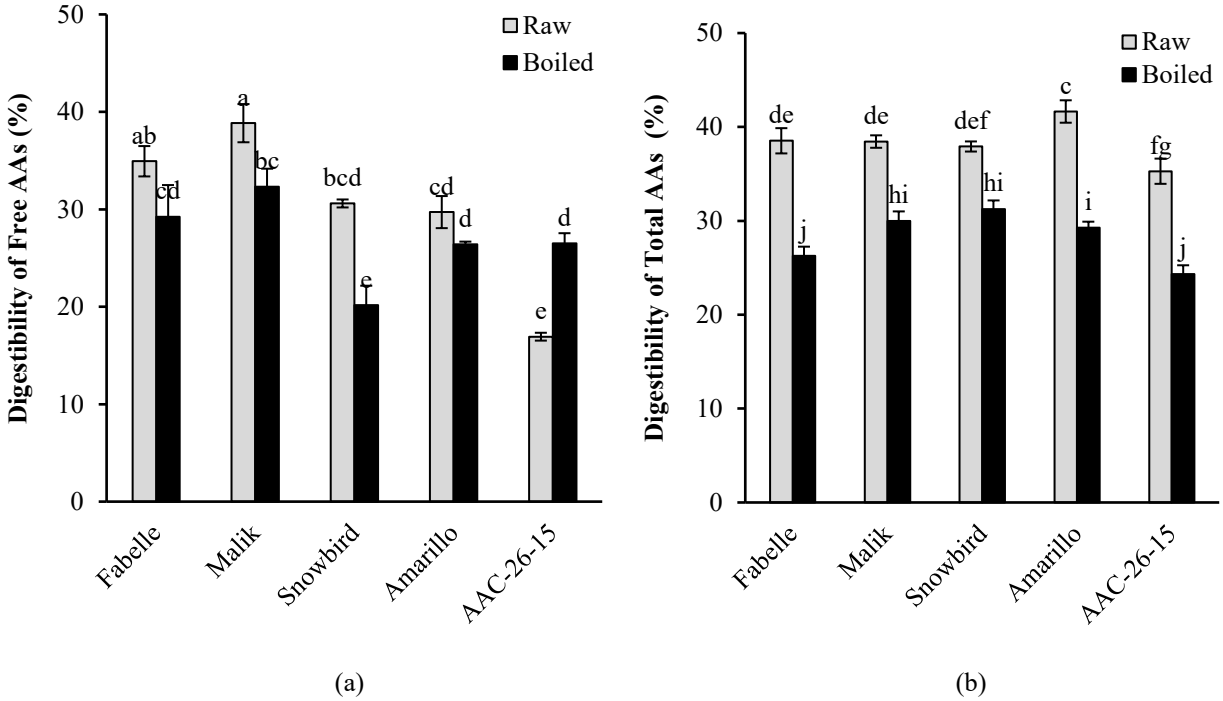

Figure 3-4. Protein Digestibility (Mean ± Standard deviation) of raw and boiled faba bean, pea and soy flour. Means without a common letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the Tukey's test; (a) free amino acid digestibility in the digestate supernatant; (b) total amino acid digestibility (including free amino acids, peptides and soluble proteins) in the digestate supernatant.

For raw flours, the sum of free amino acid digestibility was higher for the varieties Fabelle (35%), and Malik (39%) compared to pea (30%) and soy (17%) ($p < 0.05$). The sum of total amino acid digestibility was also higher in faba bean (37.9-38.5%) compared to soy (35%), but lower than pea

(41%). Interestingly, soy had the lowest calculated free (17%) and total (35%) amino acid digestibility, which could be due to its higher trypsin inhibitor activity compared to faba bean and pea (Table 3-3).

Boiling caused a significant decrease in free amino acids and total amino acids digestibility for the three faba bean varieties ($p < 0.05$). This could be due to heat induced protein unfolding and aggregation and interactions with the other constituents of the food matrix thereby limiting digestive enzyme accessibility. Reduction of faba bean protein digestibility (Carbonaro, Grant, & Cappelloni, 2005), as well as pulse protein solubility (Ma et al., 2011) after thermal processing were previously reported. Upon mild heating, proteins unfold in a reversible manner, resulting in a looser structure, which increases the contact area between dietary protein and digestive enzyme favoring protein digestibility (Joye, 2019). However, in harsher thermal conditions, proteins tend to unfold irreversibly exposing hydrophobic region which lead to the formation of new disulfide bound and protein aggregates (Visschers & de Jongh, 2005), as revealed by SDS-PAGE analysis under non-reducing conditions (Figure 3-2). Protein aggregates are large, compact, poorly soluble and therefore hardly digestible, since the accessibility to the hydrolysis sites of digestive enzyme is reduced. Boiling can also lead to the formation of poorly soluble protein-carbohydrates or protein-polyphenols complexes (Carbonaro, Grant, Cappelloni, & Pusztai, 2000), which could also be responsible for the observed decrease of protein digestibility at the boiling conditions used in this study. It is noteworthy, that in this study, the legumes were boiled as flours and not as whole seeds, which is likely to have an incidence on the matrix structure and thus protein digestibility. In whole seeds, protein and starch are separated as protein bodies and starch granules respectively (Xiong, Devkota, Zhang, Muir, & Dhital, 2022). Milling of the seeds disrupt this structure and increases interactions between protein and starch, which is likely to promote the formation of crosslinks upon heating. Besides, Ma et al. (2011) found that boiling lentil as flour led to the formation of an homogenous network of cross-linked protein and starch, while protein bodies and starch granules are still distinguishable when whole seeds were boiled (Aguilera, Esteban, Benítez, Mollá, & Martín-Cabrejas, 2009). This finding demonstrate the importance of process conditions in defining the impact on protein structure and nutritional properties.

Interestingly, the sum of free amino acid digestibility increased significantly after boiling ($p < 0.05$) for soy (from 17 to 27%) which could be due to the drastic reduction in trypsin inhibitor activity in boiled soy flour (as shown in Table 3-3). The very low content of starch in soy could

also explain the difference in the impact of heat treatment on the amount of free amino acid release during soy digestion for boiled soy flour versus the raw one compared to faba bean and pea. The sum of total amino acid digestibility of soy, however, followed the same trend as the faba bean and pea where boiled soy flour exhibited lower total amino acid digestibility

For the calculation of the IV-DIAAS, the digestibility of each amino acid was determined individually (Supplementary Table 3-1). For most amino acids, and as expected, free amino acids digestibility was generally lower than total amino acid digestibility. However, some inconsistencies were observed, which could be the result of some analytical bias. Indeed, unlike during quantification of free amino acids, the determination of total amino acids goes through acid hydrolysis of peptide bonds, which is well documented to affect differently amino acids. It is well known that all amino acid are not hydrolysed at the same rate in each protein sources and thus some are partially degraded, while others remained unhydrolyzed after the 24 hour hydrolysis period (Darragh, Garrick, Moughan, & Hendriks, 1996). For example, tyrosine digestibility was higher during free amino acid quantification comparatively to the total amino acid quantification. It has been reported that tyrosine can undergo halogenation during the acidic hydrolysis, which decreases its recovery, particularly in the case of plant matrices (Dahl-Lassen, van Hecke, Jørgensen, Bukh, Andersen, & Schjoerring, 2018).

3.4.5 IV- DIAAS of Faba Bean, Pea and Soy Flours

The IV-DIAAS was calculated for the three age groups as shown in Table 3-5. The *in vitro* Digestible Indispensable Amino Acid Ratio (IV-DIAAR) was calculated for each essential amino acid (Supplementary Table 3-2) and the DIAAS was calculated from the lowest ratio (Table 3-5), which correspond to the first limiting amino acid. Since the amino acid scoring pattern of children (6 months-3 years) is recommended by the FAO for regulatory purposes, the results for this age group are presented in Figure 3-5. In this work, we proposed to express the IV-DIAAS values within a minimum and maximum range values (as we calculated them using both free and total amino acids) since protein absorption is difficult to predict with *in vitro* procedures. Protein digestates are absorbed in the small intestine as free amino acids, di and tri-peptides and possibly as oligopeptides (Karaś, 2019). Therefore, quantifying both free and total amino acids could be a good way to represent the nutritional quality of protein by reflecting and taking into account important factors like extent of digestion, bioavailability and absorption of amino acids and bio

disponible peptides. Still, several factors, other than size, were shown to affect protein and peptide absorption. Primary and secondary structures, hydrophobicity, charge and food matrix composition have all an impact on protein absorption (Karaś, 2019). For raw faba bean flour, the minimum (based on free AAs content) and maximum (based on total AAs content) IV-DIAAS values ranged from 13-16 to 32-38, respectively. Among the three faba bean varieties, Malik had the highest minimum and maximum IV-DIAAS values (16 and 38), followed by Fabelle (15 and 35) and then Snowbird (13 and 32). The maximum IV-DIAAS of Malik and Fabelle was also significantly higher than that of raw pea flour (31) (p=0.0003 and p=0.041 respectively), but no significant differences were observed between pea and Snowbird (p >0.05). For raw soy flour, the minimum IV-DIAAS (11) was significantly lower than that of Malik (p <0.0001) and Fabelle (p <0.0001), while the soy maximum IV-DIAAS (40) was higher than pea (p <0.0001), and faba bean Fabelle (p =0.006) and Snowbird (p <0.0001) but not different than Malik (p =0.45). These results are in good agreement with previous studies that have demonstrated the higher protein quality of soy compared to pulses (Mathai, Liu, & Stein, 2017)

Table 3-5. *in vitro* Digestible Indispensable Amino acid Score (IV-DIAAS) of raw and boiled legume flours calculated based on free amino acid digestibility and total amino acid digestibility.

	Minimum IV-DIAAS (Free Amino Acid Digestibility)					
	Infant (0-6 months)		Child (6 months-3 years)		Older Child, Adolescent and Adult	
	DIAAS	Limiting AA	DIAAS	Limiting AA	DIAAS	Limiting AA
Fabelle						
Raw	12.1 (0.5)^{ab}	SAA	14.7 (0.6)^{ab}	SAA	17.9 (0.7)^{ab}	SAA
Boiled	9.1 (0.9)^{cde}	SAA	11.1 (1.1)^{cde}	SAA	13.1 (1.3)^{cde}	SAA
Malik						
Raw	13.1 (0.7)^{a}	SAA	16.0 (0.8)^{a}	SAA	18.8 (1.0)^{a}	SAA
Boiled	8.1 (0.3)^{de}	SAA	10.0 (0.4)^{de}	SAA	11.7 (0.5)^{de}	SAA
Snowbird						
Raw	10.6 (0.3)^{bc}	SAA	12.9 (0.4)^{bcd}	SAA	15.2 (0.4)^{bcd}	SAA
Boiled	7.1 (1.5)^{e}	SAA	8.7 (1.9)^{e}	SAA	10.2 (2.2)^{e}	SAA
Amarillo						
Raw	9.4 (0.3)^{cd}	THR	13.3 (0.4)^{abc}	THR	16.6 (0.4)^{abc}	THR
Boiled	2.8 (1.1)^{f}	THR	4.0 (1.6)^{f}	THR	5.0 (2.0)^{f}	THR
AAC-26-15						
Raw	7.6 (0.9)^{de}	THR	10.8 (1.2)^{cde}	THR	13.4 (1.5)^{cd}	THR
Boiled	7.3 (0.8)^{de}	THR	10.4 (1.2)^{cde}	THR	12.9 (1.5)^{cde}	THR
***p*-value**						
Legume	**<0.001**	.	**<0.001**	.	**<0.001**	.
Treatment	**<0.001**	.	**<0.001**	.	**<0.001**	.
L×T	**<0.001**	.	**<0.001**	.	**<0.001**	.
	Maximum IV-DIAAS (Total Amino Acid Digestibility)					
	Infant (0-6 months)		Child (6 months-3 years)		Older Child, Adolescent and Adult	
	DIAAS	Limiting AA	DIAAS	Limiting AA	DIAAS	Limiting AA
Fabelle						
Raw	28.8 (1.8)^{ab}	SAA	35.2 (2.1)^{bc}	SAA	41.3 (2.5)^{abc}	SAA
Boiled	7.0 (0.1)^{e}	TRP	13.9 (0.3)^{e}	TRP	17.9 (0.4)^{e}	TRP
Malik						
Raw	30.8 (1.0)^{a}	SAA	37.7 (1.2)^{ab}	SAA	44.2 (1.5)^{a}	SAA
Boiled	6.3 (0.4)^{e}	TRP	12.6 (0.8)^{e}	TRP	16.2 (1.1)^{e}	TRP
Snowbird						
Raw	26.1 (0.4)^{b}	SAA	32.0 (0.5)^{cd}	SAA	37.5 (0.6)^{c}	SAA
Boiled	5.8 (0.4)^{e}	TRP	11.6 (0.9)^{e}	TRP	15.0 (1.1)^{e}	TRP
Amarillo						
Raw	15.6 (1.0)^{d}	TRP	31.1 (1.9)^{d}	TRP	39.6 (1.8)^{bc}	TRP
Boiled	2.8 (0.4)^{f}	TRP	5.6 (0.8)^{f}	TRP	7.3 (1.0)^{f}	TRP
AAC-26-15						
Raw	28.6 (1.8)^{ab}	LEU	40.2 (0.4)^{a}	VAL	43.4 (0.5)^{ab}	VAL
Boiled	19.4 (0.7)^{c}	LEU	28.2 (1.1)^{d}	LEU	30.5 (1.1)^{d}	LEU
***p*-value**						
Legume	**<0.001**	.	**<0.001**	.	**<0.001**	.
Treatment	**<0.001**	.	**<0.001**	.	**<0.001**	.
L×T	**<0.001**	.	**<0.001**	.	**<0.001**	.

Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differ (p <0.05) as analyzed by two-way ANOVA and the Tukey's test. Minimal IV-DIAAS values were calculated based on free amino acid digestibility in the digestate supernatant and maximal IV-DIAAS value were calculated based on total amino acid digestibility in the digestate supernatant. The amino acid scoring patterns (mg/g of proteins) for each age group were as follows (FAO, 2013):
Infant: His-21, Ile-55, Leu-96, Lys-69, SAA-33, AAA-94, Thr-44, Trp-17 and Val-55
Child: His-20, Ile-32, Leu-66, Lys-57, SAA-27, AAA-52, Thr-31, Trp-8.5 and Val-43
Older child, adolescent and adult: His-16, Ile-30, Leu-61, Lys-48, SAA-23, AAA-41, Thr-25, Trp-6.6 and Val-40

Boiling caused a significant decrease ($p < 0.05$) of the IV-DIAAS for all three faba bean varieties, pea and soy, which is in concordance with the decrease in protein digestibility. The extent in the decrease in boiled soy IV-DIAAS values was less pronounced compared to pea and faba bean varieties, which could possibly relate to the difference in proximate composition between these legumes, as well as to differences in protein primary and secondary structures, hydrophobicity, charge that all have an impact on protein and amino acid digestibility (Karaś, 2019). Based on the maximum IV-DIAAS values, boiling had also a major impact on the identity of the limiting AAs, in raw faba bean they were determined as the sulfur containing amino acids (SAA), but in boiled faba bean, it was the tryptophan, which is known to be unstable during processing (Bellmaine, Schnellbaecher, & Zimmer, 2020). Tryptophan was also found to be the first limiting AA for both raw and boiled pea, while leucine was the first limiting AA for boiled soy (Supplementary Table 3-2). The same limiting amino acids were reported by Nosworthy et al. (2018) for faba bean in a rat bioassay, demonstrating the potential of *in vitro* approach to accurately evaluate the nutritional quality of proteins for comparison purposes.

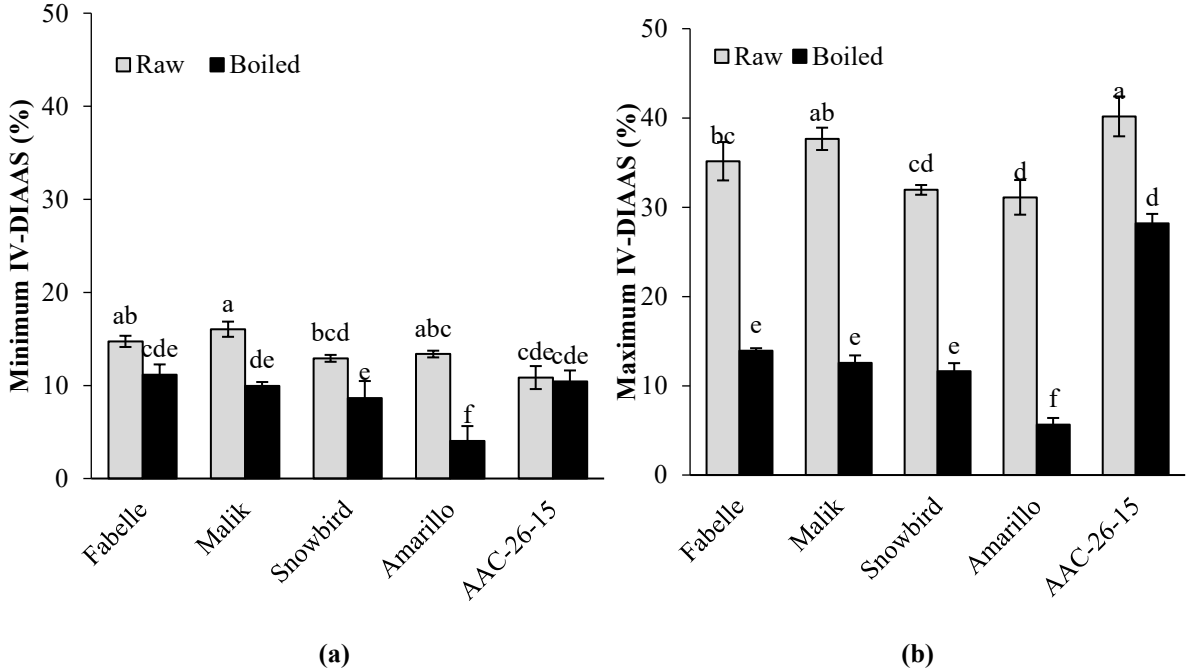

Figure 3-5. *In vitro* Digestible Indispensable Amino acid Score (IV-DIAAS) of raw and boiled legume flours calculated using the child scoring pattern (6 months-3years). Values are means ± standard deviation of three replicates. Means without a common letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the Tukey's test; (a) Minimal IV-DIAAS values were calculated based on free amino acid digestibility; (b) Maximal IV-DIAAS values were calculated based on total amino acid digestibility.

If compared to *in vivo* data, the IV-DIAAS for faba bean, pea and soy obtained in this study remains importantly lower, although following a similar trend. *In vivo* DIAAS values of 51 have been obtained for pea emulsions (Reynaud et al., 2021), 53-57 for whole boiled faba bean, 57 for whole cooked peas (Han, Moughan, Li, & Pang, 2020) and 89 for soy flour (Mathai et al., 2017). A possible explanation for the lower *in vitro* value is the absence of a jejunal-ileal digestion phase in the *in vitro* digestion approach (Picariello, Ferranti, & Addeo, 2016). Indeed, the intestinal digestion phase is performed using pancreatic proteases and does not contain brush border peptidases that are found in the jejunum and ileum. Over 20 different brush border peptidases are found in the human intestine brush border (Hooton, Lentle, Monro, Wickham, & Simpson, 2015; Picariello et al., 2016). Their activity increases longitudinally in the small intestine to reach a maximum at the ileum. The absence of a digestion phase mimicking brush border digestion is likely to underestimate protein digestibility (Brodkorb et al., 2019) and thus protein quality scores. The addition of this last digestion step seems of foremost importance for the accurate determination of protein quality scores *in vitro* and will need to be considered in future work.

3.5 Conclusions

In this study, *In vitro* Digestible Indispensable Amino Acid Scores (IV-DIAAS) were determined using the harmonized INFOGEST *in vitro* human digestion model, thereby enabling the assessment of the protein nutritional quality of three Canadian faba bean varieties in comparison to pea and soy, two of the most widely used plant proteins, considering the most recent recommendations of the FAO for the nutritional quality assessment of food protein sources. This study showed that faba bean Malik, a new developed variety, has a higher protein nutritional quality than Snowbird variety which is currently by far the most produced faba bean variety in Canada (Khazaei et al., 2021), thereby opening new opportunity for the production of faba bean with improved nutritional profile. Moreover, the ileal digestibility of raw faba bean was also superior to that of pea and soy. Boiling, however, at the studied conditions, negatively impacted the digestibility and the IV-DIAAS scores of all studied legumes, but to a higher extent those of faba bean and pea. This demonstrates the importance of studying the impact of food processing on protein nutritional quality to reflect the protein ingredient real capacity to fulfill nutritional requirements when used in formulation of processed products. In future work, other strategies should be considered to process faba bean flours in order to improve its nutritional value (Das et

al., 2022). Bio-processing and emerging food processing techniques, for instance, have the potential to improve protein digestibility while decreasing antinutritional factors, such as vicine and convicine (Rizzello et al., 2016) and fermentable oligo and di- monosaccharides, and polyols (Nyyssölä et al., 2021) that can be responsible for undesirable health effects. This could contribute to the development of value-added faba bean-based food ingredients. The protocol used in this study could be used to comparatively evaluate how conventional and novel food processing strategies can modulate the protein quality of faba beans, as well as others legumes.

In concluding, this study demonstrates the potential of faba bean to be used as a sustainable alternative source of good quality plant protein comparable to other most used sources pea and soy, and could be used in similar food applications and in complementarity to cereals to meet human nutritional requirements.

3.6 References

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3.7 Supplementary Materials

Supplementary Table 3-1. Amino acid digestibility of raw and boiled legume flours.

	Free Amino Acid Digestibility Mean (Standard Deviation)																
	Essential Amino Acids									Non-Essential Amino Acids							
	HIS	**ILE**	**LEU**	**LYS**	**MET**	**PHE**	**THR**	**TRP**	**VAL**	**ALA**	**ASX**	**ARG**	**CYS**	**GLX**	**GLY**	**TYR**	**SER**
Fabelle																	
Raw	34^{a}	30^{a}	63^{ab}	69^{ab}	34^{cde}	79^{a}	16^{a}	46^{ab}	23^{ab}	16^{b}	11^{bc}	74^{ab}	4^{c}	12^{b}	7^{bcde}	88^{b}	6^{de}
	(1.4)	(0.3)	(2.9)	(3.9)	(1.3)	(2.6)	(0.3)	(0.2)	(1.1)	(2.2)	(0.6)	(3.4)	(0.3)	(1.5)	(0.1)	(6.8)	(0.7)
Boiled	21^{bc}	19^{cd}	55^{bc}	54^{cd}	30^{defg}	63^{bc}	12^{cd}	33^{cd}	15^{def}	16^{b}	9^{cd}	68^{abc}	2^{c}	12^{b}	7^{de}	66^{de}	7^{de}
	(3.0)	(1.2)	(6.6)	(7.1)	(3.9)	(2.7)	(0.04)	(3.4)	(2.9)	(5.2)	(0.7)	(9.9)	(0.2)	(2.1)	(1.3)	(0.6)	(0.7)
Malik																	
Raw	38^{a}	33^{a}	68^{a}	72^{a}	42^{ab}	83^{a}	17^{a}	49^{a}	27^{a}	23^{a}	14^{a}	79^{a}	4^{c}	16^{a}	7^{bcd}	101^{a}	3^{f}
	(1.0)	(2.9)	(2.5)	(3.3)	(2.4)	(4.8)	(0.0)	(4.8)	(1.8)	(2.6)	(1.0)	(2.4)	(0.3)	(1.3)	(0.3)	(9.5)	(0.8)
Boiled	24^{b}	31^{a}	58^{bc}	60^{abc}	28^{efg}	67^{b}	13^{cd}	39^{bc}	18^{cde}	17^{ab}	11^{b}	74^{ab}	3^{c}	12^{b}	7^{cde}	81^{bc}	11^{b}
	(0.5)	(0.8)	(0.8)	(9.0)	(1.0)	(1.3)	(0.9)	(0.4)	(1.2)	(2.9)	(0.3)	(9.5)	(0.6)	(0.3)	(1.8)	(1.8)	(0.1)
Snowbird																	
Raw	18^{cde}	18^{cd}	52^{c}	56^{cd}	26^{fg}	67^{b}	15^{ab}	34^{cd}	21^{bc}	13^{b}	9^{de}	69^{abc}	5^{c}	13^{b}	10^{abc}	88^{b}	8^{cd}
	(0.7)	(1.6)	(1.4)	(2.4)	(0.8)	(1.2)	(1.0)	(2.3)	(0.3)	(0.5)	(0.4)	(0.5)	(0.6)	(0.4)	(0.4)	(1.3)	(0.7)
Boiled	15^{ef}	16^{d}	38^{d}	29^{e}	24^{g}	48^{d}	14^{bc}	25^{e}	12^{f}	14^{b}	6^{f}	48^{d}	3^{c}	7^{c}	4^{e}	44^{f}	10^{bc}
	(0.9)	(1.0)	(6.1)	(5.6)	(5.8)	(6.4)	(0.1)	(4.0)	(0.7)	(0.3)	(0.0)	(4.6)	(0.2)	(1.4)	(0.6)	(2.8)	(1.1)
Amarillo																	
Raw	17^{de}	23^{b}	55^{bc}	57^{bcd}	36^{bcd}	69^{b}	11^{d}	34^{cd}	16^{de}	12^{b}	11^{b}	64^{bc}	13^{b}	10^{bc}	12^{a}	73^{cd}	14^{a}
	(1.6)	(0.8)	(1.9)	(3.2)	(1.7)	(1.2)	(0.3)	(1.1)	(1.4)	(2.0)	(0.8)	(2.2)	(0.8)	(1.4)	(1.7)	(4.8)	(1.8)
Boiled	21^{bcd}	22^{b}	52^{c}	46^{d}	40^{abc}	65^{b}	3^{g}	43^{ab}	15^{ef}	13^{b}	9^{de}	57^{cd}	4^{c}	11^{b}	10^{ab}	58^{e}	12^{ab}
	(0.4)	(0.2)	(0.6)	(0.4)	(0.4)	(1.6)	(1.2)	(3.3)	(0.7)	(0.8)	(0.6)	(0.5)	(0.5)	(0.4)	(0.3)	(0.9)	(0.5)
AAC-26-15																	
Raw	13^{f}	17^{d}	30^{d}	27^{e}	33^{cdef}	34^{e}	8^{e}	30^{de}	18^{cde}	16^{b}	6^{f}	32^{e}	4^{c}	7^{c}	6^{de}	40^{f}	5^{ef}
	(0.8)	(0.8)	(0.7)	(0.4)	(0.6)	(0.7)	(0.9)	(1.5)	(0.9)	(0.7)	(0.3)	(0.4)	(2.0)	(0.3)	(0.3)	(2.8)	(0.3)
Boiled	22^{bc}	22^{bc}	50^{c}	47^{cd}	46^{a}	55^{cd}	6^{f}	35^{cd}	19^{bcd}	16^{b}	7^{e}	60^{bcd}	30^{a}	12^{b}	8^{bcd}	58^{e}	7^{de}
	(0.3)	(1.1)	(2.3)	(1.5)	(2.0)	(1.4)	(0.2)	(1.7)	(1.5)	(1.4)	(0.1)	(2.6)	(7.6)	(0.3)	(0.7)	(3.0)	(0.2)
***p*-value**																	
Legume	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Treatment	**<0.001**	**<0.001**	**0.036**	**<0.001**	0.352	**<0.001**	**<0.001**	**0.004**	**<0.001**	0.327	**<0.001**	0.201	**0.023**	0.060	**0.002**	**<0.001**	**<0.001**
L×T	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.054**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Supplementary Table 3-1. (continued…)

	Total Amino Acid Digestibility Mean (Standard Deviation)																
	Essential Amino Acids									Non-Essential Amino Acids							
	HIS	**ILE**	**LEU**	**LYS**	**MET**	**PHE**	**THR**	**TRP**	**VAL**	**ALA**	**ASX**	**ARG**	**CYS**	**GLX**	**GLY**	**TYR**	**SER**
Fabelle																	
Raw	53^{a}	51^{a}	47^{a}	40^{a}	26^{de}	51^{a}	45^{a}	59^{ab}	46^{ab}	35^{b}	30^{b}	35^{a}	45^{b}	31^{bc}	38^{b}	45^{b}	31^{ab}
	(0.6)	(0.1)	(0.5)	(1.6)	(0.4)	(0.7)	(0.7)	(2.3)	(0.1)	(2.2)	(2.5)	(2.0)	(3.5)	(1.7)	(2.4)	(2.0)	(2.4)
Boiled	43^{c}	32^{e}	30^{c}	22^{de}	28^{d}	42^{cd}	29^{ef}	11^{e}	28^{f}	28^{cde}	21^{e}	25^{c}	25^{c}	24^{d}	20^{e}	34^{cd}	21^{d}
	(1.8)	(0.7)	(1.2)	(1.4)	(0.6)	(0.6)	(1.1)	(0.2)	(0.1)	(1.7)	(0.8)	(1.8)	(0.4)	(1.3)	(0.6)	(3.1)	(1.1)
Malik																	
Raw	55^{a}	50^{a}	48^{a}	37^{a}	23^{e}	49^{ab}	46^{a}	52^{b}	45^{ab}	35^{b}	29^{bc}	36^{a}	51^{ab}	31^{bc}	39^{b}	53^{a}	31^{ab}
	(0.7)	(0.2)	(0.3)	(1.5)	(1.7)	(1.3)	(0.4)	(3.9)	(2.4)	(1.2)	(1.5)	(0.7)	(3.1)	(1.5)	(3.4)	(0.4)	(1.0)
Boiled	48^{b}	39^{bc}	35^{b}	25^{d}	27^{d}	44^{cd}	33^{de}	10^{e}	33^{d}	29^{cde}	24^{de}	31^{b}	28^{c}	28^{bcd}	22^{de}	40^{bc}	25^{cd}
	(0.6)	(1.3)	(1.1)	(1.5)	(1.2)	(1.9)	(2.3)	(0.6)	(0.1)	(1.1)	(0.8)	(1.3)	(2.2)	(1.7)	(2.5)	(1.5)	(2.1)
Snowbird																	
Raw	52^{a}	54^{a}	48^{a}	36^{ab}	22^{e}	48^{ab}	45^{ab}	62^{a}	47^{a}	33^{bcd}	28^{bc}	34^{ab}	53^{a}	31^{bc}	37^{b}	46^{b}	30^{ab}
	(2.1)	(0.4)	(0.2)	(0.2)	(1.7)	(0.3)	(0.1)	(4.6)	(0.8)	(2.5)	(0.3)	(1.9)	(1.2)	(0.4)	(0.7)	(5.0)	(0.5)
Boiled	55^{a}	37^{cd}	38^{b}	30^{c}	25^{de}	42^{d}	36^{cd}	9^{e}	33^{de}	35^{b}	26^{cd}	34^{ab}	23^{c}	26^{cd}	2^{e}	36^{c}	29^{ab}
	(1.2)	(2.4)	(3.0)	(2.0)	(1.1)	(1.4)	(3.1)	(0.7)	(3.0)	(0.7)	(1.2)	(1.5)	(0.5)	(5.8)	(5.5)	(1.3)	(0.3)
Amarillo																	
Raw	43^{c}	43^{b}	46^{a}	39^{a}	33^{c}	50^{a}	39^{bc}	22^{d}	41^{bc}	42^{a}	46^{a}	36^{a}	48^{ab}	41^{a}	5^{a}	41^{bc}	32^{a}
	(1.3)	(2.4)	(1.2)	(1.0)	(0.7)	(1.2)	(1.8)	(1.4)	(1.8)	(1.7)	(1.6)	(1.1)	(1.8)	(1.1)	(2.7)	(2.5)	(2.0)
Boiled	34^{d}	34^{de}	35^{b}	33^{bc}	38^{b}	46^{bc}	27^{f}	4^{e}	25^{f}	28^{de}	21^{e}	30^{b}	31^{c}	25^{cd}	29^{cd}	45^{b}	27^{bc}
	(0.2)	(0.5)	(1.0)	(1.4)	(1.2)	(1.1)	(1.9)	(0.5)	(1.2)	(1.2)	(0.4)	(0.2)	(2.4)	(0.7)	(1.4)	(2.1)	(0.7)
AAC-26-15																	
Raw	45^{bc}	40^{bc}	36^{b}	36^{ab}	44^{a}	38^{e}	33^{de}	38^{c}	40^{c}	33^{bc}	31^{b}	34^{ab}	47^{b}	33^{b}	36^{bc}	39^{bc}	31^{ab}
	(1.9)	(3.0)	(2.2)	(2.4)	(0.7)	(1.6)	(1.3)	(2.9)	(2.0)	(1.0)	(1.2)	(1.6)	(1.7)	(1.1)	(1.7)	(0.9)	(1.8)
Boiled	31^{d}	25^{f}	24^{d}	20^{e}	33^{c}	29^{f}	25^{f}	35^{c}	29^{ef}	24^{e}	22^{e}	23^{c}	46^{b}	23^{d}	16^{e}	29^{d}	21^{d}
	(1.0)	(1.2)	(0.9)	(0.5)	(1.8)	(1.0)	(3.0)	(3.4)	(0.5)	(3.5)	(0.2)	(2.0)	(2.5)	(1.2)	(1.3)	(2.8)	(1.3)
***p*-value**																	
Legume	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**
Treatment	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.062	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
L×T	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	**0.008**	**0.013**	**<0.001**	**0.004**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	0.389	**<0.001**	**<0.001**

Values are means (standard deviation) of three replicates. Means in a column without a common superscript letter differ (p <0.05) as analyzed by three-way ANOVA and the Tukey's test. Minimum digestibility was assessed through quantification of free amino acid in the digestate supernatant whereas maximum digestibility was assessed through total amino acid content in the digestate supernatant. Total amino acids include free amino acids, peptides and soluble proteins.

Supplementary Table 3-2. Minimum and Maximum *in vitro* Digestible Indispensable Amino acid Ratio (IV-DIAAR) of raw and boiled legume flours.

	Minimum IV- DIAAR Infant (0-6 months)									Maximum IV- DIAAR Infant (0-6 months)								
	HIS	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**	**HIS**	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**
Fabelle																		
Raw	0.52	0.21	0.46	0.80	**0.12**	0.66	0.13	0.31	0.17	0.81	0.35	0.35	0.46	**0.29**	0.39	0.37	0.41	0.36
	(0.021)	(0.002)	(0.021)	(0.045)	(0.005)	(0.034)	(0.003)	(0.001)	(0.009)	(0.009)	(0.000)	(0.004)	(0.019)	(0.018)	(0.010)	(0.006)	(0.016)	(0.001)
Boiled	0.31	0.13	0.40	0.64	**0.09**	0.50	0.10	0.22	0.11	0.62	0.22	0.22	0.25	0.20	0.31	0.23	**0.07**	0.21
	(0.043)	(0.008)	(0.047)	(0.084)	(0.009)	(0.010)	(0.000)	(0.022)	(0.022)	(0.027)	(0.005)	(0.009)	(0.016)	(0.003)	(0.011)	(0.009)	(0.001)	(0.001)
Malik																		
Raw	0.57	0.23	0.49	0.86	**0.13**	0.72	0.13	0.33	0.21	0.83	0.35	0.35	0.44	**0.31**	0.40	0.36	0.35	0.36
	(0.015)	(0.021)	(0.018)	(0.039)	(0.007)	(0.048)	(0.000)	(0.032)	(0.014)	(0.010)	(0.001)	(0.002)	(0.018)	(0.010)	(0.006)	(0.003)	(0.026)	(0.019)
Boiled	0.32	0.18	0.37	0.66	**0.08**	0.50	0.09	0.26	0.12	0.63	0.22	0.22	0.28	0.20	0.31	0.23	**0.06**	0.21
	(0.006)	(0.005)	(0.005)	(0.099)	(0.003)	(0.003)	(0.006)	(0.003)	(0.008)	(0.007)	(0.007)	(0.007)	(0.016)	(0.012)	(0.009)	(0.016)	(0.004)	(0.001)
Snowbird																		
Raw	0.25	0.12	0.36	0.61	**0.11**	0.55	0.11	0.23	0.16	0.73	0.36	0.35	0.39	**0.26**	0.34	0.33	0.42	0.35
	(0.010)	(0.010)	(0.010)	(0.026)	(0.003)	(0.009)	(0.008)	(0.016)	(0.003)	(0.030)	(0.003)	(0.001)	(0.003)	(0.004)	(0.017)	(0.001)	(0.032)	(0.006)
Boiled	0.18	0.10	0.25	0.29	**0.07**	0.31	0.09	0.17	0.08	0.66	0.22	0.25	0.30	0.17	0.32	0.24	**0.06**	0.22
	(0.011)	(0.006)	(0.039)	(0.056)	(0.015)	(0.033)	(0.001)	(0.026)	(0.005)	(0.014)	(0.015)	(0.020)	(0.020)	(0.004)	(0.020)	(0.021)	(0.004)	(0.020)
Amarillo																		
Raw	0.24	0.15	0.37	0.73	0.17	0.57	**0.09**	0.24	0.12	0.60	0.28	0.31	0.50	0.33	0.37	0.34	**0.16**	0.31
	(0.022)	(0.005)	(0.013)	(0.041)	(0.009)	(0.020)	(0.003)	(0.008)	(0.010)	(0.019)	(0.016)	(0.008)	(0.013)	(0.006)	(0.014)	(0.015)	(0.010)	(0.013)
Boiled	0.29	0.16	0.37	0.61	0.13	0.52	**0.03**	0.30	0.12	0.47	0.24	0.25	0.43	0.27	0.38	0.25	**0.03**	0.21
	(0.006)	(0.001)	(0.004)	(0.005)	(0.003)	(0.006)	(0.011)	(0.023)	(0.006)	(0.002)	(0.004)	(0.007)	(0.018)	(0.017)	(0.011)	(0.017)	(0.004)	(0.010)
AAC-26-15																		
Raw	0.20	0.13	0.24	0.33	0.16	0.36	**0.08**	0.27	0.14	0.72	0.31	**0.29**	0.44	0.45	0.38	0.31	0.34	0.32
	(0.014)	(0.006)	(0.006)	(0.005)	(0.014)	(0.014)	(0.009)	(0.013)	(0.007)	(0.031)	(0.024)	(0.018)	(0.029)	(0.013)	(0.013)	(0.012)	(0.026)	(0.016)
Boiled	0.19	0.13	0.24	0.32	0.15	0.35	**0.07**	0.27	0.14	0.48	0.20	**0.19**	0.24	0.38	0.28	0.22	0.32	0.23
	(0.013)	(0.006)	(0.006)	(0.004)	(0.013)	(0.014)	(0.008)	(0.013)	(0.007)	(0.015)	(0.010)	(0.007)	(0.006)	(0.018)	(0.014)	(0.027)	(0.031)	(0.004)

Supplementary Table 3-2. (Continued…)

	Minimum IV- DIAAR Child (6 months-3 years)									Maximum IV- DIAAR Infant (6 months-3 years)								
	HIS	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**	**HIS**	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**
Fabelle																		
Raw	0.55	0.36	0.67	0.97	**0.15**	1.19	0.19	0.63	0.22	0.85	0.61	0.51	0.56	**0.35**	0.70	0.52	0.81	0.45
	(0.022)	(0.004)	(0.031)	(0.054)	(0.006)	(0.062)	(0.004)	(0.003)	(0.011)	(0.009)	(0.001)	(0.005)	(0.023)	(0.021)	(0.018)	(0.008)	(0.032)	(0.001)
Boiled	0.32	0.22	0.58	0.78	**0.11**	0.91	0.14	0.43	0.15	0.66	0.38	0.31	0.31	0.25	0.55	0.33	**0.14**	0.27
	(0.046)	(0.014)	(0.069)	(0.102)	(0.011)	(0.018)	(0.000)	(0.045)	(0.028)	(0.028)	(0.008)	(0.013)	(0.020)	(0.004)	(0.019)	(0.012)	(0.003)	(0.001)
Malik																		
Raw	0.60	0.40	0.72	1.04	**0.16**	1.30	0.19	0.65	0.27	0.87	0.61	0.51	0.53	**0.38**	0.72	0.51	0.70	0.46
	(0.016)	(0.035)	(0.026)	(0.047)	(0.008)	(0.086)	(0.000)	(0.064)	(0.019)	(0.011)	(0.002)	(0.003)	(0.022)	(0.012)	(0.011)	(0.005)	(0.052)	(0.025)
Boiled	0.34	0.31	0.54	0.79	**0.10**	0.91	0.13	0.52	0.15	0.66	0.38	0.32	0.34	0.25	0.56	0.33	**0.13**	0.27
	(0.007)	(0.008)	(0.008)	(0.120)	(0.004)	(0.005)	(0.009)	(0.005)	(0.010)	(0.008)	(0.013)	(0.011)	(0.019)	(0.015)	(0.016)	(0.023)	(0.008)	(0.001)
Snowbird																		
Raw	0.27	0.21	0.53	0.74	**0.13**	1.00	0.16	0.46	0.20	0.77	0.63	0.50	0.47	**0.32**	0.62	0.47	0.84	0.45
	(0.011)	(0.018)	(0.015)	(0.032)	(0.004)	(0.016)	(0.011)	(0.032)	(0.003)	(0.031)	(0.005)	(0.002)	(0.003)	(0.005)	(0.030)	(0.001)	(0.063)	(0.008)
Boiled	0.18	0.16	0.36	0.35	**0.09**	0.56	0.13	0.33	0.10	0.69	0.38	0.36	0.36	0.21	0.57	0.35	**0.12**	0.28
	(0.012)	(0.010)	(0.057)	(0.067)	(0.019)	(0.059)	(0.001)	(0.052)	(0.006)	(0.015)	(0.025)	(0.029)	(0.024)	(0.005)	(0.037)	(0.030)	(0.009)	(0.026)
Amarillo																		
Raw	0.26	0.25	0.54	0.89	0.21	1.02	**0.13**	0.48	0.15	0.63	0.48	0.45	0.61	0.40	0.67	0.49	**0.31**	0.39
	(0.023)	(0.009)	(0.019)	(0.050)	(0.011)	(0.037)	(0.004)	(0.016)	(0.013)	(0.020)	(0.027)	(0.011)	(0.016)	(0.008)	(0.025)	(0.022)	(0.019)	(0.017)
Boiled	0.30	0.27	0.53	0.73	0.16	0.95	**0.04**	0.59	0.16	0.50	0.41	0.36	0.52	0.33	0.69	0.35	**0.06**	0.27
	(0.006)	(0.002)	(0.006)	(0.006)	(0.003)	(0.011)	(0.016)	(0.045)	(0.008)	(0.003)	(0.006)	(0.010)	(0.022)	(0.021)	(0.020)	(0.024)	(0.008)	(0.013)
AAC-26-15																		
Raw	0.21	0.23	0.35	0.39	0.19	0.65	**0.11**	0.53	0.18	0.76	0.54	0.42	0.54	0.55	0.68	0.44	0.68	**0.40**
	(0.014)	(0.011)	(0.008)	(0.006)	(0.017)	(0.025)	(0.012)	(0.026)	(0.009)	(0.032)	(0.041)	(0.026)	(0.035)	(0.015)	(0.024)	(0.017)	(0.052)	(0.021)
Boiled	0.20	0.23	0.35	0.38	0.18	0.64	**0.10**	0.55	0.18	0.50	0.35	**0.28**	0.29	0.46	0.50	0.31	0.64	0.30
	(0.013)	(0.011)	(0.008)	(0.005)	(0.016)	(0.024)	(0.012)	(0.027)	(0.009)	(0.015)	(0.017)	(0.011)	(0.007)	(0.022)	(0.026)	(0.038)	(0.062)	(0.005)

Supplementary Table 3-2. (Continued…)

	Minimum IV- DIAAR Older Child, Adolescent and Adult									Maximum IV- DIAAR Older Child, Adolescent and Adult								
	HIS	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**	**HIS**	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**
Fabelle																		
Raw	0.68	0.38	0.73	1.15	**0.17**	1.51	0.23	0.81	0.24	1.07	0.65	0.55	0.66	**0.41**	0.89	0.65	1.05	0.49
	(0.028)	(0.004)	(0.033)	(0.065)	(0.007)	(0.079)	(0.005)	(0.004)	(0.012)	(0.012)	(0.001)	(0.006)	(0.027)	(0.025)	(0.023)	(0.010)	(0.041)	(0.001)
Boiled	0.41	0.23	0.63	0.92	**0.13**	1.16	0.17	0.56	0.16	0.82	0.40	0.34	0.37	0.29	0.70	0.41	**0.18**	0.29
	(0.057)	(0.015)	(0.074)	(0.121)	(0.013)	(0.023)	(0.000)	(0.057)	(0.030)	(0.035)	(0.009)	(0.014)	(0.024)	(0.005)	(0.025)	(0.015)	(0.004)	(0.001)
Malik																		
Raw	0.74	0.43	0.78	1.23	**0.19**	1.65	0.23	0.84	0.29	1.09	0.65	0.55	0.63	**0.44**	0.91	0.64	0.90	0.50
	(0.020)	(0.038)	(0.028)	(0.056)	(0.010)	(0.109)	(0.000)	(0.082)	(0.020)	(0.013)	(0.002)	(0.004)	(0.026)	(0.015)	(0.014)	(0.006)	(0.067)	(0.027)
Boiled	0.42	0.33	0.59	0.94	**0.12**	1.16	0.16	0.67	0.16	0.83	0.41	0.35	0.40	0.29	0.70	0.40	**0.16**	0.29
	(0.008)	(0.008)	(0.008)	(0.142)	(0.005)	(0.007)	(0.011)	(0.006)	(0.010)	(0.010)	(0.014)	(0.012)	(0.023)	(0.017)	(0.021)	(0.028)	(0.011)	(0.001)
Snowbird																		
Raw	0.33	0.22	0.57	0.88	**0.15**	1.27	0.20	0.59	0.22	0.96	0.67	0.54	0.56	**0.37**	0.78	0.58	1.09	0.48
	(0.013)	(0.019)	(0.016)	(0.038)	(0.004)	(0.021)	(0.013)	(0.041)	(0.004)	(0.039)	(0.005)	(0.002)	(0.004)	(0.006)	(0.038)	(0.001)	(0.081)	(0.008)
Boiled	0.23	0.17	0.39	0.42	**0.10**	0.72	0.16	0.43	0.11	0.87	0.41	0.39	0.43	0.25	0.73	0.43	**0.15**	0.30
	(0.015)	(0.011)	(0.062)	(0.080)	(0.022)	(0.075)	(0.001)	(0.067)	(0.007)	(0.019)	(0.027)	(0.031)	(0.029)	(0.006)	(0.047)	(0.037)	(0.011)	(0.028)
Amarillo																		
Raw	0.32	0.27	0.58	1.05	0.25	1.30	**0.17**	0.61	0.17	0.79	0.51	0.49	0.73	0.47	0.85	0.60	**0.40**	0.42
	(0.029)	(0.010)	(0.021)	(0.059)	(0.013)	(0.046)	(0.004)	(0.021)	(0.014)	(0.024)	(0.028)	(0.012)	(0.019)	(0.009)	(0.032)	(0.027)	(0.025)	(0.018)
Boiled	0.38	0.29	0.58	0.87	0.18	1.20	**0.05**	0.76	0.17	0.62	0.44	0.39	0.62	0.39	0.88	0.44	**0.07**	0.29
	(0.008)	(0.002)	(0.007)	(0.007)	(0.004)	(0.014)	(0.020)	(0.058)	(0.008)	(0.003)	(0.006)	(0.011)	(0.026)	(0.025)	(0.025)	(0.030)	(0.010)	(0.014)
AAC-26-15																		
Raw	0.26	0.24	0.38	0.47	0.22	0.83	**0.13**	0.69	0.19	0.95	0.58	0.45	0.64	0.64	0.87	0.55	0.87	**0.44**
	(0.018)	(0.011)	(0.009)	(0.007)	(0.020)	(0.032)	(0.015)	(0.034)	(0.010)	(0.040)	(0.044)	(0.028)	(0.042)	(0.018)	(0.030)	(0.022)	(0.067)	(0.022)
Boiled	0.25	0.24	0.38	0.46	0.21	0.81	**0.13**	0.71	0.20	0.63	0.37	**0.30**	0.34	0.54	0.64	0.39	0.82	0.32
	(0.017)	(0.012)	(0.009)	(0.006)	(0.019)	(0.031)	(0.015)	(0.035)	(0.010)	(0.019)	(0.018)	(0.011)	(0.008)	(0.025)	(0.033)	(0.047)	(0.079)	(0.006)

Values are means (standard deviation) of three experiments. Minimal IV-DIAAR values were calculated based on free amino acid digestibility and maximal IV-DIAAR value were calculated based on total amino acid digestibility. The lowest IV-DIAAR value in a row, corresponding to the first limiting amino acid, is printed in bold-faced type. The amino acid scoring patterns (mg/g protein) for each age group were as follows (FAO, 2013):

Infant: His-21, Ile-55, Leu-96, Lys-69, SAA-33, AAA-94, Thr-44, Trp-17 and Val-55

Child: His-20, Ile-32, Leu-66, Lys-57, SAA-27, AAA-52, Thr-31, Trp-8.5 and Val-43

Older child, adolescent and adult: His-16, Ile-30, Leu-61, Lys-48, SAA-23, AAA-41, Thr-25, Trp-6.6 and Val-40

[1]SAA=Sulfur amino acid (methionine and cysteine); [2]AAA=Aromatic amino acid (tyrosine and phenylalanine).

CONNECTING STATEMENT II

Chapter III presented a comprehensive study of the nutritional quality of three Canadian faba bean varieties (Fabelle, Malik and Snowbird) in comparison to pea and soy. From this study, it was demonstrated that the IV-DIAAS calculated from *in vitro* digestibility were underestimated compared to publish *in vivo* data. The lack of a jejunal-ileal digestion phase in the *in vitro* digestion model might contribute to underestimating protein digestibility.

In Chapter IV, the digestion procedure was further improved to include an additional digestion phase to mimic brush border digestion. This digestion phase was performed with a commercially available porcine aminopeptidase as a replacement of porcine brush border membrane extract, which lack standardization and accessibility. The *in vitro* digestibility and IV-DIAAS of raw and boiled faba bean, pea and soy flours as well as two well-characterized dairy proteins (whey protein isolate and casein) were re-assessed by the modified digestion procedure. The obtained *in vitro* protein quality data were compared to related available *in vivo* data to judge for the physiological relevance of the improved model.

The results from this research were presented at the 2nd NIZO Plant Protein Functionality Conference published in the journal *Food Research International*.

- Martineau-Côté, D., Achouri, A., Wanasundara, J., Karboune, S., & L'Hocine, L. (2022) Nutritional quality of processed Canadian faba bean (Vicia faba L.) flour assessed with a harmonized static *in vitro* gastrointestinal digestion system adapted to mimic intestinal brush border digestion. 2nd NIZO Plant Protein Functionality Conference, On-line, October 11-13, 2022.

- Martineau-Côté, D., Achouri, A., Pitre, M., Karboune, S., & L'Hocine, L. (2024). Improved in vitro gastrointestinal digestion protocol mimicking brush border digestion for the determination of the Digestible Indispensable Amino Acid Score (DIAAS) of different food matrices. *Food Research International*, 178, 113932. https://doi.org/https://doi.org/10.1016/j.foodres.2024.113932.

CHAPTER IV. IMPROVED *IN VITRO* GASTROINTESTINAL DIGESTION PROTOCOL MIMICKING BRUSH BORDER DIGESTION FOR THE DETERMINATION OF THE DIGESTIBLE INDISPENSABLE AMINO ACID SCORE (DIAAS) OF DIFFERENT FOOD MATRICES

4.1 Abstract

The Digestible Indispensable Amino Acid Score (DIAAS) is the new gold standard method for the assessment of protein nutritional quality. The DIAAS is evaluated with *in vivo* models, that are complex, constraining and costly. There is still no established method to assess it in vitro. In this study, we proposed to add a jejunal-ileal digestion phase to the standardized *in vitro* gastrointestinal digestion protocol developed by the International Network of Excellence on the Fate of Food in the Gastrointestinal Tract (INFOGEST protocol) to mimic brush border digestion and to enable DIAAS assessment *in vitro* in a more physiologically relevant manner. This jejunal-ileal digestion phase was performed with a porcine intestinal aminopeptidase as an alternative to brush border membrane extract, which is more difficult to obtain in a standardized way. This modified INFOGEST protocol was applied to various food matrices (faba bean, pea and soy flours, whey protein isolate and caseins) and the results were compared to published *in vivo* data to assess the model's physiological relevance. The addition of the jejunal-ileal digestion phase led to a significant ($p < 0.05$) increase of 31 and 29% in free and total amino acid digestibility, respectively, and of 83% on average for the *in vitro* DIAAS for all food matrices. Although the *in vitro* DIAAS remained underestimated compared to the *in vivo* ones, a strong correlation between them was observed ($r = 0.879$, $p=0.009$), stating the relevance of this last digestion phase. This improved digestion protocol is proposed as a suitable alternative to evaluate the DIAAS *in vitro* when *in vivo* assays are not applicable.

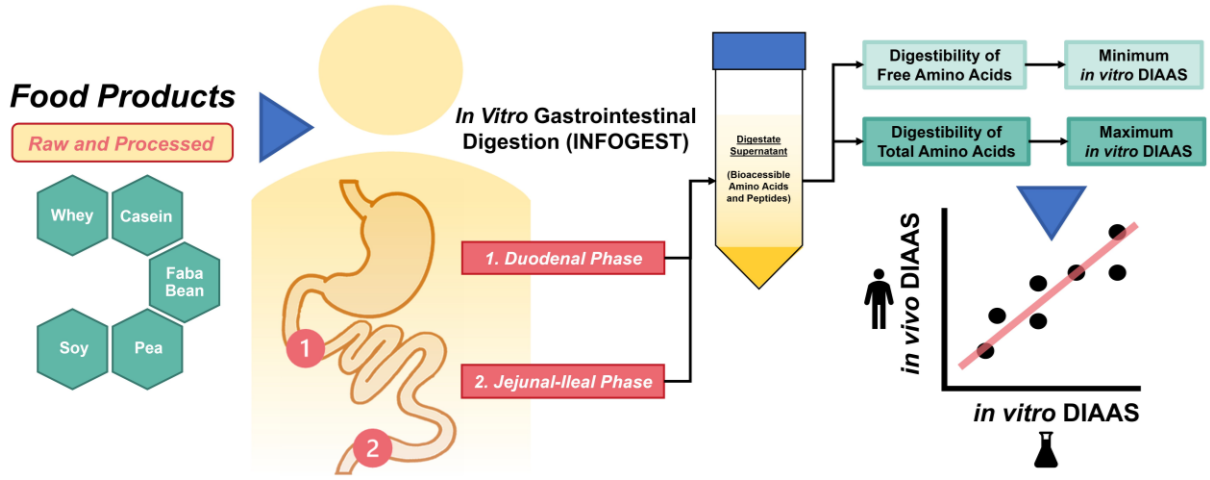

Figure 4-1. Graphical abstract

4.2 Introduction

Evaluation of protein nutritional quality is of foremost importance to evaluate the potential of a protein source to fulfill dietary needs in essential amino acids. Nutritional quality of protein sources is influenced by two main parameters, namely the amino acid composition and their respective digestibility. Indeed, the amino acid profile must match the nutritional requirement pattern, and proteins have to be bioavailable for their absorption during the digestion process. Nutritional scores, such as the Protein Digestibility Corrected Amino Acid Score (PDCAAS) and the Digestible Indispensable Amino Acid Score (DIAAS) take into account both parameters and are used as protein quality indicators to regulate protein claims on food products (FAO, 2013).

The DIAAS is the new gold standard method for the evaluation of protein quality that was proposed by the FAO in 2013 (FAO, 2013), as a replacement of the PDCAAS (FAO/WHO, 1991). The most significant advantage of the DIAAS compared to PDCAAS is the consideration of true ileal digestibility of each essential amino acid individually instead of total fecal protein digestibility (FAO, 2013). Amino acids are thus considered as individual nutrients, which is more representative of amino acid bioavailability during gastrointestinal digestion. Upon recommendation of the FAO working group, new protein scoring reference patterns were also introduced and separated in three age groups, infants (birth to 6 months), children (6 months to 3 years) and older children, adolescent and adults, to better meet amino acid requirement for each age group.

The DIAAS has to be determined *in vivo*, where the pig model is preconized since it is more representative of the human digestive system (FAO, 2013). Experiments using this animal model are costly, laborious, not widely accessible, and ethically questionable, particularly at a time where the use of animals in science need to be replaced and reduced as much as possible. As many novel protein sources are entering the market at a fast-growing rate (Ismail et al., 2020), new methodologies are required to evaluate protein quality scores. A promising alternative is the use of *in vitro* gastrointestinal digestion procedures.

The INFOGEST standardized static *in vitro* gastrointestinal digestion protocol (Brodkorb et al., 2019) mimics human digestion (Minekus et al., 2014) and is proposed as a robust *in vitro* alternative to human and animal models. This digestion procedure provides results that are physiologically relevant and reproducible from a study to another (Egger et al., 2019). The INFOGEST protocol comprises three digestion phases (oral, gastric and intestinal) and uses a

constant ratio of food and digestive fluid. The conditions used during each digestion phase (pH, duration, digestive fluid composition and enzymatic activities) are a scientific consensus based on up-to-date physiological data. Interest in the use of the INFOGEST protocol to evaluate the digestibility of amino acids in order to evaluate the DIAAS *in vitro* (IV-DIAAS) has increased recently (Ariëns et al., 2021; Fu et al., 2023; Komatsu et al., 2023; L'Hocine et al., 2023; Sousa et al., 2023). This protocol lacks, however, a standardized jejunal-ileal digestion phase, which represents an important limitation for the determination of true ileal amino acid digestibility (Picariello et al., 2016). The intestinal digestion phase is performed using pancreatic proteases and does not contain brush border peptidases that are found in the jejunum and ileum. More than 20 different brush border peptidases are found in the human intestine brush border (Hooton et al., 2015; Picariello et al., 2016). Their activity increases longitudinally in the small intestine to reach a maximum at the ileum (Picariello et al., 2016). Brush border peptidases are mostly aminopeptidases, but also include carboxypeptidases, dipeptidases and endopeptidases (Hooton et al., 2015). These peptidases further hydrolyze the oligopeptides generated in the stomach and duodenum into free amino acids and small absorbable peptides. Without this last digestion phase, protein digestion remains incomplete, and therefore protein nutritional quality is underestimated (Minekus et al., 2014). In a previous research, we have observed that in the absence of a jejunal-ileal phase, the standardized INFOGEST protocol, although pertinent, underestimated digestibility of amino acids and the IV-DIAAS of various legume flours (faba bean, pea and soy) compared to available *in vivo* data (Martineau-Côté et al., 2023).

Several authors have used a complex enzymatic mix extracted from porcine intestinal brush border membrane (BBM) to simulate jejunal-ileal digestion for various applications (Asledottir et al., 2019; Claude et al., 2019; Di Stasio et al., 2017; Hausch et al., 2002; Mamone, Picariello, et al., 2019; Mamone, Sciammaro, et al., 2019; Shan et al., 2002). BBM, however, is not commercially available and its composition is subjected to variability from an animal to another and from the enzyme extraction method used. Another proposed alternative to BBM as a source of various peptidases is the rat intestinal acetone powder (Garcia-Campayo et al., 2018). This product is commercially available; however, the rat digestive system is considered a less suitable model to the human's one as further distant compared to pigs (Deglaire & Moughan, 2012), inferring that the brush border enzymes from pig would be more representative of the human ones. Indeed, an alignment of the aminopeptidases sequences from human, rat and pigs by UniProt ID, shows a

slightly higher homology between pig and human (79.4%) compared to rat and human (78.01%), while the homology between pig and rat is the lowest (76.77 %) (analysis not shown). Consequently, and given that enzymes from pigs are prioritized throughout the INFOGEST protocol, we evaluated, in the present study, the use of a commercial intestinal porcine aminopeptidase as an alternative to BBM to simulate brush border digestion, with the aim to achieve better standardization, repeatability and accessibility. Aminopeptidases are the main hydrolases found in the jejunum and ileum and are active on a broad range of substrates (Picariello et al., 2016; Turner, 2013; Woodley, 2009). It is therefore hypothesized that the addition of the aminopeptidase will lead to a significant improvement of protein digestibility and consequently an improvement of the IV-DIAAS.

The impact of the addition of the aminopeptidase-based jejunal-ileal digestion phase on the IV-DIAAS of various food matrices, namely raw and thermally treated faba bean, soy and pea flours and milk protein products (whey protein isolate and micellar caseins) were evaluated. The IV-DIAAS obtained with and without this last digestion phase were compared to published *in vivo* data to evaluate the physiological relevance of the proposed model. To the best of our knowledge, this is the first study that included a jejunal-ileal digestion phase to evaluate the DIAAS *in vitro*.

4.3 Materials and Methods

4.3.1 Materials

Three dehulled faba bean cultivars (Fabelle Malik and Snowbird), one dehulled pea cultivar (Amarillo) and one dehulled soy cultivar (AAC-26-15) were used in this study. Faba bean cultivars Fabelle and Malik were provided by AGT Foods and Ingredients (Saskatoon, SK, CA), and Snowbird by W.A. Grain & Pulse Solutions (Innisfail, AB, CA). Certified yellow pea (CDC Amarillo) and soybean (Cdn #1, Variety AAC 26-25, Non-GMO & IP, Lot 261510504AT) were provided by Greenleaf Seeds (Tisdale, SK, CA) and Huron seeds (Clinton, ON, CA), respectively. Faba bean, pea and defatted soybean flours were prepared and thermally treated (boiled) as previously described in Martineau-Côté et al. (2022). The flours were dispersed in water (ratio 1:10) for 1 hour at room temperature under constant stirring and then boiled for 20 minutes. The boiled flours and cooking water were frozen at −40 °C, freeze-dried and milled to assure a particle size uniformity among the samples. All of the flour samples were stored at −20 °C in vacuum bags until needed. Whey protein isolate (WPI) and micellar caseins were purchased from Canadian

Protein (Windsor, Ontario, Canada) and used as is. The protein content was 86.5% and 79.4% for WPI and caseins, respectively. The complete proximate composition of the legume flours can be found in Martineau-Côté et al. (2023).

Hydrochloric acid, sodium hydroxide, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), L-leucine, monosodium phosphate, disodium phosphate, sodium dodecyl sulfate (SDS), were purchased from BioShop (Burlington, ON, Canada). Trinitrobenzenesulfonic acid (TNBS) 5% (w/v) in methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Alpha-amylase from porcine pancreas (A3176), pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545), porcine bile extract (B8631), bile acid assay kit (MAK309) and L-leucine-p-nitroanilide were purchased from Sigma-Aldrich (St. Louis, MO). Native porcine peptidase (Nate-0548, ≥ 500 U of leucyl aminopeptidase activity.g^{-1}) was purchased from Creative Enzyme (Shirley, NY, USA). All chemicals and reagents used were of analytical grade. Deionized water was used in all experiments.

4.3.2 Evaluation of Amino Acid and Protein Digestibility of Food Matrices Using an *in vitro* Gastrointestinal Digestion Model

4.3.2.1 In vitro Gastrointestinal Digestion Protocol

Amino acid and protein digestibility of food matrices (raw and boiled legume flours, WPI and caseins) were determined using the static harmonized gastrointestinal digestion procedure developed by the INFOGEST international network (Brodkorb et al., 2019). The legume flours (0.4 g, corresponding to 90-220 mg of proteins) were dispersed in water (0.6 g) prior to the oral phase to reach the targeted consistency of tomato paste at the end of the oral phase (Brodkorb et al., 2019). The milk protein products (WPI and caseins) were dispersed in water to reach a concentration of 60 mg.mL^{-1} of proteins (Santos-Hernández et al., 2020). The dispersed protein products and the flours (1 g) were then mixed in a ratio 1:1 with simulated salivary fluid (SSF) and the oral, gastric and duodenal digestion phases were conducted as previously described (Martineau-Côté et al., 2023). For comparison, all samples were digested without and with the addition of the jejunal-ileal digestion phase.

4.3.2.2 Addition of a last digestion phase to mimic brush border digestion (jejunal-ileal digestion phase)

To simulate digestion that occurs at the brush border in the jejunum and ileum, a last digestion phase was added to the INFOGEST protocol. At the end of the duodenal digestion phase, the pH was adjusted to 7.2 and the aminopeptidase (13 mU leucine-aminopeptidase activity/mL digestate) was added. The digestate was incubated for another 4 hours at 37°C under constant stirring. The digestion conditions for the jejunal-ileal phase (pH, digestion time and enzyme activity) were selected based on previous studies conducted with porcine BBM (Di Stasio et al., 2017; Hausch et al., 2002; Mamone, Picariello, et al., 2019; Mamone, Sciammaro, et al., 2019; Shan et al., 2002). Leucine aminopeptidase activity in porcine intestinal peptidase was evaluated following the procedure of Hausch et al. (2002) and one unit was defined as the consumption of 1 µmol of leucine-p-nitroanilide per minute at 30°C and pH 8.0. Each batch of enzyme was tested to assure a constant enzyme-activity-to-meal ratios in the digestions.

At the end of all digestions (with and without the jejunal-ileal digestion phase), samples were cooled on ice and 5 mM AEBSF (protease inhibitor) was added to stop the digestion. The digestates were centrifuged (15 000 x g for 30 minutes at 4°C) to recover the soluble fraction, representing the bioaccessible phase. 1 mL of the supernatant was kept as is and frozen at -80°C for the assessment of free amino acids and degree of hydrolysis. The remaining supernatant volume was split in three equal fractions, frozen at -80°C and freeze-dried for total amino acid determination (acidic hydrolysis, performic acid oxidation and alkaline hydrolysis). For free amino acid quantification the supernatant was filtered and injected as is.

4.3.2.3 Preparation of the Digestate Samples for Amino Acid Analysis

At the end of all digestions (with and without the jejunal-ileal digestion phase), samples were rapidly cooled on ice to reduce enzyme activity and 5 mM AEBSF (protease inhibitor) was added to inhibit trypsin and chymotrypsin activity. The digestates were centrifuged (15,000 x g for 30 min at 4 °C) to recover the soluble fraction, representing the bioavailable phase. One mL of the supernatant was kept as is and immediately frozen at -80 °C for the assessment of free amino acids and degree of hydrolysis. Upon thawing in ice bath, the sample was used right away to limit any further protein hydrolysis of the sample by the other proteases from pancreatin and the aminopeptidase. For free amino acid analysis, the sample was filtered and injected as is. For the

total amino acid determination, the remaining digestate supernatant volume was split in three equal fractions, frozen at -80 °C and freeze-dried for acidic hydrolysis, performic acid oxidation and alkaline hydrolysis.

4.3.3 Determination of Amino Acid Content in Food Matrices and *in vitro* Gastrointestinal Digestates

Amino acids were quantified in food matrices and in the digestate supernatant to evaluate amino acid digestibility. An acidic hydrolysis was performed to quantify 14 amino acids. Sulfur-containing amino acids and tryptophan were analyzed separately through performic acid oxidation and alkaline hydrolysis, respectively. All amino acids were quantified as previously described (Martineau-Côté et al., 2023).

4.3.4 Evaluation of the Degree of Protein Hydrolysis of Food Matrices

The evaluation of the degree of protein hydrolysis was carried out following the method of Adler-Nissen (1979) as described in L'Hocine et al. (2023) to evaluate to what extend the various food matrices were hydrolyzed during the *in vitro* digestion procedure. Digestate supernatants were appropriately diluted in SDS 1% (m/v) and L-leucine standards (32-262 $mg.L^{-1}$) were prepared in the same solvent. Ten µL of standard and diluted samples were added to a 96 well microplate in triplicate and 80 µL of 0.2125 M phosphate buffer pH 8.2 was added to each well. Eighty µL of 0.1% (m/v) TNBS reagent (diluted in water) was added to each well and the plate was incubated at 50 °C for 1 h. After the incubation period, 160 µL of 0.1 N HCl was added to stop the reaction and the absorbance was recorded at λ=340 nm. Free amino groups in the samples were expressed as mg of leucine equivalent using the L-leucine standard curve. Those values were used to calculate the degree of hydrolysis (DH), which was defined as the proportion of peptide bonds cleaved during the *in vitro* digestion procedure, using the following formula:

$$DH\ (\%) = \frac{(AN_2 - AN_1)}{N_p}$$

Where AN_2, AN_1 and N_p are the primary amino group content expressed as mg of leucine equivalent normalized to the protein content, in the digestate (after enzyme contribution subtraction), in the undigested samples (no enzyme) and in the totally hydrolysed sample, respectively. Enzyme contribution was evaluated by performing digestions with water instead of

samples. Totally hydrolysed samples were prepared in acidic conditions (6 N HCl) and incubated at 110 °C for 24 h.

4.3.5 Calculation of the *in vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS) of Food Matrices

Amino acid digestibility and IV-DIAAS was calculated as previously described (L'Hocine et al., 2023). Briefly, free amino acid quantification in the digestate supernatant was used to determine the minimal digestibility and minimal IV-DIAAS, whereas the total amino acid quantification was used to obtain the maximal digestibility and the maximal IV-DIAAS. Total amino acid quantification included free amino acid, oligopeptides and soluble proteins.

Minimal and maximal jejunal-ileal digestibility of individual amino acids were therefore calculated as follows:

$$\text{In } vitro \text{ Ileal Amino Acid Digestibility } = \frac{\text{AA}_{\text{in the digestate (mg)}} - \text{AA}_{\text{in the blank (mg)}}}{\text{AA}_{\text{intake (mg)}}}$$

Where $AA_{\text{in the digestate}}$ represents either free or total amino acid quantified in the digestate supernatant after the *in vitro* digestion. $AA_{\text{in the blank}}$ refers to the digestive enzyme amino acid contribution, which was evaluated in performing blank digestions with water instead of protein meal. AA_{intake} is the amino acid content initially present in the protein meal . This calculus was repeated for each indispensable amino acid individually.

Minimal and maximal digestibility values were used to calculate the minimal and maximal IV-DIAAS, to obtain a range of plausible values. The IV- DIAAS was calculated with the following formula:

$$\text{IV-DIAAS (\%)} = \frac{\text{mg of indispensable digestible AA in 1 g of test protein}}{\text{mg of same AA in 1 g of reference protein}} \times 100$$

The IV-DIAAS was calculated using the protein scoring pattern for children (6 months to 3 years), which is the protein scoring pattern recommended by the FAO for reglementary purposes for all foods and population group, except for infant formula (FAO, 2013).

4.3.6 Statistical Analysis

Each analysis was performed in triplicate and results were expressed as mean ± standard deviation (SD). Data were analysed through analysis of variance (ANOVA) ($p < 0.05$) using the XLSTAT

software (Addinsoft, NY) and the Turkey's honest significant difference (HSD) post-hoc test ($p < 0.05$) add-on to Microsoft Excel (Redmond, WA) to determine significant differences. Pearson correlation coefficients were calculated to investigate the relationship between the obtained IV-DIAAS and *in vivo* DIAAS collected from the literature.

4.4 Results and Discussion

4.4.1 Impact of the Jejunal-Ileal Digestion Phase on Protein Digestibility of the Different Food Matrices

Protein digestibility was evaluated for all selected food matrices at the end of the duodenal and the jejunal-ileal digestion phases, through the assessment of the degree of hydrolysis, free amino acid and total amino acid digestibility (Figure 4-2). A porcine aminopeptidase was selected to perform the jejunal-ileal digestion phase, since it is the most abundant enzymatic activity in the human small intestine (Langguth et al., 1997) and in BBM (Mamone & Picariello, 2023). The degree of hydrolysis quantifies the hydrolysis of the peptide bond, but it does not give an indication on the resulting protein hydrolysis products (free amino acids, small peptides are oligopeptides). Since digested proteins are absorbed in the small intestine as free amino acids, small peptides and oligopeptides (Belović et al., 2011; Xiong, 2010), quantification of free amino acid digestibility is an indicator of minimal protein digestibility and quantification of total amino acid digestibility is an indicator of maximal protein digestibility.

The results, as shown in Figure 4-2, indicated that the addition of a digestion phase mimicking brush border digestion resulted in a significant increase ($p < 0.05$) of protein digestibility for all studied food matrices. Indeed, the digestibility of total amino acid increased significantly ($p < 0.05$) in all food matrices by 29% on average, and that of free amino acid by 31% on average, except for boiled soy, for which free amino acid digestibility was unchanged. The free amino acid digestibility of soy was among the lowest of the investigated food product, which may be explained by its higher protein content and differences in the food matrix composition. Moreover, previous research has showed that the amino acid digestibility of the different legume flour was decreased after boiling, as a result of protein aggregation (Martineau-Côté et al., 2023). Thereby, possibly modifying the digestibility pattern and the amount and profile of free amino acids released. The digestibility of the two dairy protein products were the highest at both digestion endpoint, which is consistent with *in vivo* data (Mathai et al., 2017). Interestingly, despite the increasing trend of

the degree of hydrolysis at the end of the ileal phase, the observed increment was not found significant for all the food matrices studied, which can be explained by the low precision of the method (Rutherfurd, 2019).

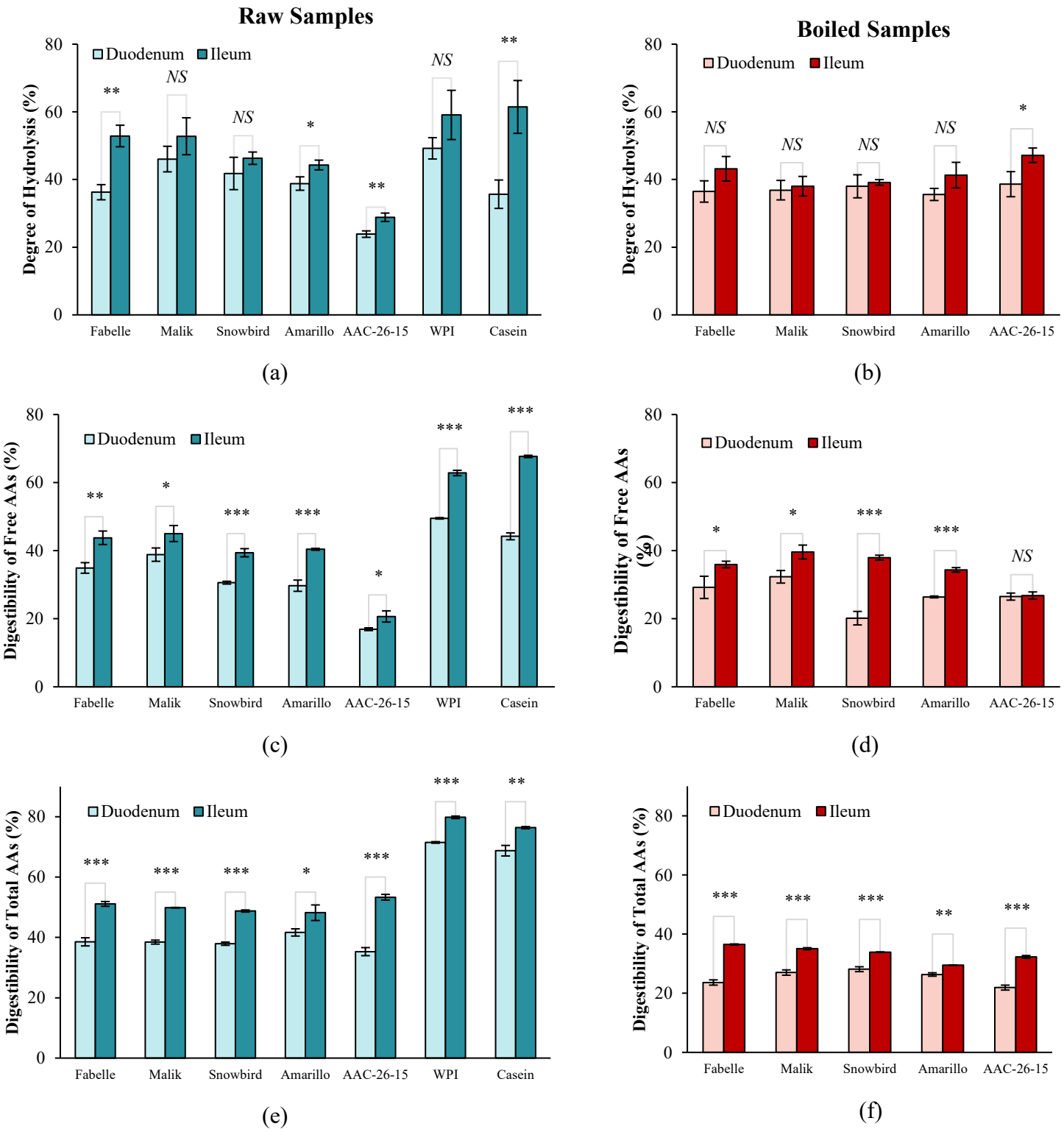

Figure 4-2. *In vitro* Protein digestibility (mean ± standard deviation) of faba bean flours (Fabelle, Malik and Snowbird), pea flour (Amarillo), soy flour (AAC-26-15), whey protein isolate (WPI) and caseins at different digestion endpoints. Protein digestibility at the end of the duodenal and jejunal-ileal digestion phase was compared by one way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant); (a) Degree of hydrolysis of raw samples; (b) Degree of hydrolysis of boiled samples; (c) Digestibility of free amino acid of raw samples, (d) Digestibility of free amino acid of boiled samples; (e) Digestibility of total amino acid of raw samples; (f) Digestibility of total amino acid of boiled samples.

The degree of hydrolysis at the end of the jejunal-ileal digestion phase was 59% and 61% for WPI and caseins, respectively. These data were lower than those reported in a study conducted with BBM, where the degree of hydrolysis of WPI and sodium caseinate was 71% and 77%, respectively (Picariello et al., 2015). This difference in the degree of hydrolysis values could be due to the presence of multiple hydrolyzing enzymes in the BBM, versus the aminopeptidase in the present study. Differences in the digestion experimental conditions could also explain this discrepancy, as the digestion protocol conditions used by Picariello et al. (2015) did not correspond exactly to those of the INFOGEST protocol

4.4.2 Impact of the Jejunal-Ileal Digestion Phase on the IV-DIAAS of Food Matrices

The IV-DIAAS of food matrices was determined at the end of the duodenal digestion phase (INFOGEST protocol without modification) and at the end of the jejunal-ileal digestion phase (modified protocol) to evaluate the impact of the addition of this last digestion phase on the IV-DIAAS (Table 4-1 and Figure 4-3). The IV-DIAAS was calculated using the protein reference scoring pattern for child (6 months to 3 years), which is the recommended protein of reference for regulatory purposes (FAO, 2013). Since there is no standardized protocol to mimic amino acid absorption *in vitro,* a minimum IV-DIAAS was calculated based on free amino acid digestibility and a maximum IV-DIAAS was calculated based on total amino acid digestibility to obtain a plausible range of IV-DIAAS. The results indicated that the addition of the jejunal-ileal digestion phase significantly increased ($p < 0.05$) the minimum IV-DIAAS in all the food matrices studied. Similarly, the maximum IV-DIAAS also increased significantly ($p < 0.05$), with the exception of raw pea and soy flours. On average, both the minimum IV-DIAAS and maximum IV-DIAAS increased substantially by 85% and 81% respectively.

Table 4-1. Minimum and Maximum *in vitro* Digestible Indispensable Amino Acid Ratio (IV-DIAAR) of food products determined at different digestion endpoints.

	Minimum IV-DIAAR Mean (Standard Deviation)								
	HIS	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**
Fabelle									
Raw									
Duodenum	0.55 (0.02)	0.36 (0.00)	0.67 (0.03)	0.97 (0.05)	**0.15 (0.01)**	1.19 (0.06)	0.19 (0.00)	0.63 (0.00)	0.22 (0.01)
Ileum	0.55 (0.05)	0.65 (0.04)	0.80 (0.04)	0.96 (0.06)	**0.27 (0.01)**	1.19 (0.08)	0.32 (0.00)	0.91 (0.11)	0.49 (0.01)
Boiled									
Duodenum	0.32 (0.05)	0.22 (0.01)	0.58 (0.07)	0.78 (0.10)	**0.11 (0.01)**	0.91 (0.02)	0.14 (0.00)	0.43 (0.04)	0.15 (0.03)
Ileum	0.52 (0.01)	0.26 (0.01)	0.59 (0.01)	1.20 (0.01)	**0.16 (0.00)**	0.76 (0.12)	0.21 (0.01)	0.80 (0.01)	0.21 (0.02)
Malik									
Raw									
Duodenum	0.60 (0.02)	0.40 (0.04)	0.72 (0.03)	1.04 (0.05)	**0.16 (0.01)**	1.30 (0.09)	0.19 (0.00)	0.65 (0.06)	0.27 (0.02)
Ileum	0.58 (0.03)	0.68 (0.04)	0.76 (0.02)	0.98 (0.06)	**0.26 (0.02)**	1.12 (0.03)	0.35 (0.03)	0.79 (0.06)	0.55 (0.03)
Boiled									
Duodenum	0.34 (0.01)	0.31 (0.01)	0.54 (0.01)	0.79 (0.12)	**0.10 (0.00)**	0.91 (0.01)	0.13 (0.01)	0.52 (0.01)	0.15 (0.01)
Ileum	0.53 (0.08)	0.47 (0.18)	0.59 (0.02)	1.06 (0.06)	**0.19 (0.01)**	0.84 (0.10)	0.22 (0.00)	0.67 (0.03)	0.21 (0.01)
Snowbird									
Raw									
Duodenum	0.27 (0.01)	0.21 (0.02)	0.53 (0.01)	0.74 (0.03)	**0.13 (0.00)**	1.00 (0.02)	0.16 (0.01)	0.46 (0.03)	0.20 (0.00)
Ileum	0.43 (0.01)	0.44 (0.02)	0.66 (0.02)	0.83 (0.02)	**0.22 (0.01)**	1.04 (0.05)	0.28 (0.01)	0.59 (0.06)	0.41 (0.01)
Boiled									
Duodenum	0.18 (0.01)	0.16 (0.01)	0.36 (0.06)	0.35 (0.07)	**0.09 (0.02)**	0.56 (0.06)	0.13 (0.00)	0.33 (0.05)	0.10 (0.01)
Ileum	0.46 (0.01)	0.55 (0.01)	0.51 (0.01)	0.83 (0.02)	**0.13 (0.00)**	0.94 (0.04)	0.18 (0.00)	0.53 (0.01)	0.34 (0.01)
Amarillo									
Raw									
Duodenum	0.26 (0.02)	0.25 (0.01)	0.54 (0.02)	0.89 (0.05)	0.21 (0.01)	1.02 (0.04)	**0.13 (0.00)**	0.48 (0.02)	0.15 (0.01)
Ileum	0.35 (0.02)	0.57 (0.03)	0.65 (0.00)	1.13 (0.00)	**0.32 (0.01)**	1.06 (0.02)	0.34 (0.02)	0.46 (0.03)	0.48 (0.02)
Boiled									
Duodenum	0.30 (0.01)	0.27 (0.00)	0.53 (0.01)	0.73 (0.01)	0.16 (0.00)	0.95 (0.01)	**0.04 (0.02)**	0.59 (0.05)	0.16 (0.01)
Ileum	0.47 (0.02)	0.48 (0.04)	0.60 (0.03)	0.96 (0.01)	0.19 (0.01)	1.06 (0.02)	**0.15 (0.02)**	0.55 (0.03)	0.36 (0.02)
AAC-26-15									
Raw									
Duodenum	0.21 (0.01)	0.23 (0.01)	0.35 (0.01)	0.39 (0.01)	0.19 (0.02)	0.65 (0.03)	**0.11 (0.01)**	0.53 (0.03)	0.18 (0.01)
Ileum	0.30 (0.04)	0.32 (0.03)	0.40 (0.02)	0.42 (0.05)	0.23 (0.12)	0.60 (0.01)	**0.19 (0.02)**	0.26 (0.05)	0.40 (0.02)
Boiled									
Duodenum	0.20 (0.01)	0.23 (0.01)	0.35 (0.01)	0.38 (0.01)	0.18 (0.02)	0.64 (0.02)	**0.10 (0.01)**	0.55 (0.03)	0.18 (0.01)
Ileum	0.40 (0.02)	0.39 (0.02)	0.61 (0.02)	0.71 (0.06)	0.28 (0.00)	0.95 (0.06)	**0.15 (0.01)**	0.67 (0.09)	0.26 (0.02)
WPI[3]									
Duodenum	**0.13 (0.02)**	0.67 (0.01)	1.24 (0.01)	1.60 (0.00)	0.99 (0.03)	0.70 (0.01)	0.97 (0.02)	1.29 (0.00)	0.55 (0.01)
Ileum	**0.23 (0.02)**	0.78 (0.01)	1.29 (0.01)	1.68 (0.01)	1.26 (0.01)	0.72 (0.02)	1.00 (0.01)	1.59 (0.07)	0.63 (0.01)
Casein									
Duodenum	0.60 (0.01)	0.40 (0.02)	0.89 (0.01)	0.99 (0.05)	0.65 (0.01)	1.15 (0.05)	**0.37 (0.01)**	0.81 (0.06)	0.42 (0.02)
Ileum	0.72 (0.02)	**0.54 (0.01)**	1.09 (0.02)	1.34 (0.03)	0.85 (0.01)	1.39 (0.03)	0.76 (0.01)	1.40 (0.06)	0.84 (0.01)

Table 4-1. (continued…)

	Maximum IV-DIAAR Mean (Standard Deviation)								
	HIS	**ILE**	**LEU**	**LYS**	**SAA[1]**	**AAA[2]**	**THR**	**TRP**	**VAL**
Fabelle									
Raw									
Duodenum	0.85 (0.01)	0.61 (0.00)	0.51 (0.01)	0.56 (0.02)	**0.35 (0.02)**	0.70 (0.02)	0.52 (0.01)	0.81 (0.03)	0.45 (0.00)
Ileum	0.85 (0.01)	0.73 (0.01)	0.59 (0.00)	0.66 (0.02)	**0.49 (0.00)**	0.73 (0.00)	0.59 (0.00)	0.83 (0.01)	0.53 (0.01)
Boiled									
Duodenum	0.66 (0.03)	0.38 (0.01)	0.31 (0.01)	0.31 (0.02)	0.25 (0.00)	0.55 (0.02)	0.33 (0.01)	**0.14 (0.00)**	0.27 (0.00)
Ileum	0.73 (0.00)	0.57 (0.00)	0.50 (0.00)	0.48 (0.01)	0.36 (0.00)	0.63 (0.00)	0.46 (0.01)	**0.33 (0.01)**	0.43 (0.00)
Malik									
Raw									
Duodenum	0.87 (0.01)	0.61 (0.00)	0.51 (0.00)	0.53 (0.02)	**0.38 (0.01)**	0.72 (0.01)	0.51 (0.00)	0.70 (0.05)	0.46 (0.02)
Ileum	0.87 (0.00)	0.73 (0.00)	0.59 (0.00)	0.63 (0.00)	**0.48 (0.00)**	0.71 (0.00)	0.57 (0.00)	0.76 (0.03)	0.52 (0.01)
Boiled									
Duodenum	0.66 (0.01)	0.38 (0.01)	0.32 (0.01)	0.34 (0.02)	0.25 (0.01)	0.56 (0.02)	0.33 (0.02)	**0.13 (0.01)**	0.27 (0.00)
Ileum	0.59 (0.01)	0.48 (0.01)	0.41 (0.01)	0.44 (0.01)	0.34 (0.01)	0.52 (0.01)	0.40 (0.00)	**0.31 (0.02)**	0.38 (0.01)
Snowbird									
Raw									
Duodenum	0.77 (0.03)	0.63 (0.00)	0.50 (0.00)	0.47 (0.00)	**0.32 (0.01)**	0.62 (0.03)	0.47 (0.00)	0.84 (0.06)	0.45 (0.01)
Ileum	0.82 (0.00)	0.70 (0.01)	0.55 (0.00)	0.57 (0.02)	**0.43 (0.00)**	0.65 (0.01)	0.55 (0.01)	0.73 (0.00)	0.49 (0.01)
Boiled									
Duodenum	0.69 (0.02)	0.38 (0.02)	0.36 (0.03)	0.36 (0.02)	0.21 (0.01)	0.57 (0.04)	0.35 (0.03)	**0.12 (0.01)**	0.28 (0.03)
Ileum	0.55 (0.00)	0.49 (0.00)	0.41 (0.01)	0.40 (0.00)	0.33 (0.00)	0.49 (0.00)	0.38 (0.01)	**0.29 (0.04)**	0.37 (0.00)
Amarillo									
Raw									
Duodenum	0.63 (0.02)	0.48 (0.03)	0.45 (0.01)	0.61 (0.02)	0.40 (0.01)	0.67 (0.03)	0.49 (0.02)	**0.31 (0.02)**	0.39 (0.02)
Ileum	1.06 (0.01)	0.71 (0.04)	0.50 (0.03)	0.82 (0.10)	0.54 (0.01)	0.85 (0.03)	0.55 (0.04)	**0.33 (0.01)**	0.58 (0.05)
Boiled									
Duodenum	0.50 (0.00)	0.41 (0.01)	0.36 (0.01)	0.52 (0.02)	0.33 (0.02)	0.69 (0.02)	0.35 (0.02)	**0.06 (0.01)**	0.27 (0.01)
Ileum	0.48 (0.01)	0.43 (0.01)	0.42 (0.01)	0.43 (0.03)	0.35 (0.01)	0.52 (0.01)	0.39 (0.00)	**0.23 (0.01)**	0.33 (0.01)
AAC-26-15									
Raw									
Duodenum	0.76 (0.03)	0.54 (0.04)	0.42 (0.03)	0.54 (0.04)	0.55 (0.02)	0.68 (0.02)	0.44 (0.02)	0.68 (0.05)	**0.40 (0.02**)
Ileum	1.03 (0.01)	0.59 (0.00)	**0.43 (0.00)**	0.49 (0.02)	0.64 (0.01)	0.57 (0.02)	0.77 (0.01)	1.04 (0.02)	0.53 (0.01)
Boiled									
Duodenum	0.50 (0.02)	0.35 (0.02)	**0.28 (0.01)**	0.29 (0.01)	0.46 (0.02)	0.50 (0.03)	0.31 (0.04)	0.64 (0.06)	0.30 (0.01)
Ileum	0.60 (0.01)	0.46 (0.00)	0.43 (0.01)	0.48 (0.01)	0.80 (0.01)	0.61 (0.01)	0.45 (0.00)	0.81 (0.02)	**0.36 (0.00)**
WPI[3]									
Duodenum	**0.36 (0.01)**	1.01 (0.02)	0.83 (0.01)	1.71 (0.01)	1.42 (0.02)	0.77 (0.02)	1.33 (0.00)	1.64 (0.03)	0.61 (0.01)
Ileum	**0.59 (0.00)**	1.11 (0.01)	1.03 (0.01)	1.73 (0.00)	1.62 (0.01)	0.72 (0.01)	1.40 (0.01)	2.27 (0.05)	0.72 (0.01)
Casein									
Duodenum	**0.57 (0.02)**	0.67 (0.02)	0.88 (0.03)	0.77 (0.07)	0.91 (0.02)	1.32 (0.01)	0.82 (0.02)	1.29 (0.02)	0.63 (0.02)
Ileum	0.90 (0.02)	0.85 (0.00)	0.99 (0.01)	1.07 (0.00)	1.15 (0.01)	1.43 (0.01)	1.01 (0.02)	1.73 (0.02)	**0.80 (0.00)**

Minimal IV-DIAAR values were calculated based on free amino acid digestibility and maximal IV-DIAAR values were calculated based on total amino acid digestibility. The lowest IV-DIAAR value in a row, corresponding to the first limiting amino acid, is printed in bold-faced type. The amino acid scoring pattern (mg/g protein) was as follows (FAO, 2013):

Child: His-20, Ile-32, Leu-66, Lys-57, SAA-27, AAA-52, Thr-31, Trp-8.5 and Val-43

[1]SAA=Sulfur amino acid (methionine and cysteine); [2]AAA=Aromatic amino acid (tyrosine and phenylalanine); [3]Whey Protein Isolate.

The first limiting amino acids obtained for the different food matrices are in good agreement with the literature (Table 4-1). In the case of maximum IV-DIAAS, the first limiting amino acid was sulfur-containing amino acids for faba bean raw samples and tryptophan for faba bean boiled samples. Both tryptophan and sulfur-containing amino acids have been reported for faba bean as first limiting amino acids (Herreman, Nommensen, Pennings, & Laus, 2020; Nosworthy et al., 2018; Setia, Dai, Nickerson, Sopiwnyk, Malcolmson, & Ai, 2019). For pea, the first limiting amino acid was tryptophan for both raw and boiled samples, which again is in good agreement with previous studies that reported either tryptophan or sulfur-containing amino acids as first limiting amino acid for pea (Herreman et al., 2020; Nosworthy et al., 2017; Setia et al., 2019). For WPI, the first limiting amino acid was histidine, as widely reported in the literature (Herreman et al., 2020; Mathai, Liu, & Stein, 2017). Noteworthy, the first limiting amino acid for faba bean, pea and WPI remained the same with or without the addition of the jejunal-ileal digestion phase.

For caseins, however, the first limiting amino acid was histidine at the end of the duodenal digestion phase and valine at the end of the jejunal-ileal digestion phase. Caseins are known as complete proteins and are not supposed to be lacking in any amino acid (Herreman et al., 2020). With the addition of the jejunal-ileal digestion phase, the DIAAR of caseins have increased for all amino acids (28% increase on average) and were close to 1.0 or above, which suggest that the addition of this digestion phase leads to more physiologically relevant results.

In the case of soy, the first limiting amino acid observed was either valine or leucine, which differs from what is usually reported being either methionine or cysteine. However, as reported by Herreman et al. (2020), the scores of sulfur-containing amino acids, valine and leucine in soy products are not far from each other with average DIAAS of 91 ± 11.5, 95 ± 7.3 and 102 ± 6.1 respectively, which is consistent with our results. Soy proteins are reported as a nearly complete protein, with a DIAAS close to 100. As presented in Table 4-1, the IV-DIAAS for soy remain low for several amino acids, even with the addition of the jejunal-ileal digestion phase. A possible explanation is the ratio of soy flour and enzyme that have been used in this study. Indeed, according to the INFOGEST protocol, it is recommended to use a constant meal to digestive fluid ratio, regardless of the food substrate composition. Since defatted soy flour has significantly higher protein content (56.6%) compared to faba bean flours (28.7-32.5%) and pea flour (24.2%), the protein digestibility may have been reduced for soy flour, by the lower ratio enzyme/protein during the *in vitro* digestion. Indeed, a recent report (Rieder et al., 2021) showed that increasing the

proportion of caseins in a casein suspension, while keeping the amount of digestive enzyme constant lead to an important reduction of protein digestibility after the duodenal phase using the INFOGEST protocol. This shows the importance of a better standardization and adaptation of the INFOGEST protocol to the protein content of the food matrix.

Data for the calculation of minimum IV-DIAAS, showed that the first limiting amino acids were methionine and cysteine for faba bean flour both at the end of the duodenal and jejunal-ileal digestion phases, which is consistent with the literature (Herreman et al., 2020). For WPI, the first limiting amino acid was histidine at the end of both digestion phases, which again is in good agreement with published *in vivo* data (Herreman et al., 2020; Mathai et al., 2017). In the case of pea, the first limiting amino acid was threonine at the end of the duodenal digestion phase for both raw and boiled samples, and sulfur-containing amino acids at the end of the jejunal-ileal digestion phase for raw sample only. The first limiting amino acid reported for pea flour are usually tryptophan or sulfur-containing amino acids (Herreman et al., 2020; Nosworthy et al., 2017; Setia et al., 2019), demonstrating that the addition of the jejunal-ileal digestion phase lead to a more physiologically relevant result since the same first limiting amino acid was identified as *in vivo.* The first limiting amino acid for soy was threonine at the end of both the duodenal and the jejunal-ileal digestion phase. For caseins, it was threonine at the end of the duodenal phase and isoleucine at the end of the jejunal-ileal digestion phase. The IV-DIAAS for soy and caseins were underestimated compared to *in vivo* data. For the boiled samples, the obtained first limiting amino acids for the minimum IV-DIAAS are generally less representative of *in vivo* data compared to those obtained with the maximum IV-DIAAS. The minimum IV-DIAAS is herein considered as an underestimation of the real DIAAS since only free amino acids are quantified. Globally, the first limiting amino acid as determined by the maximum IV-DIAAS is in better agreement with that obtained with *in vivo* data.

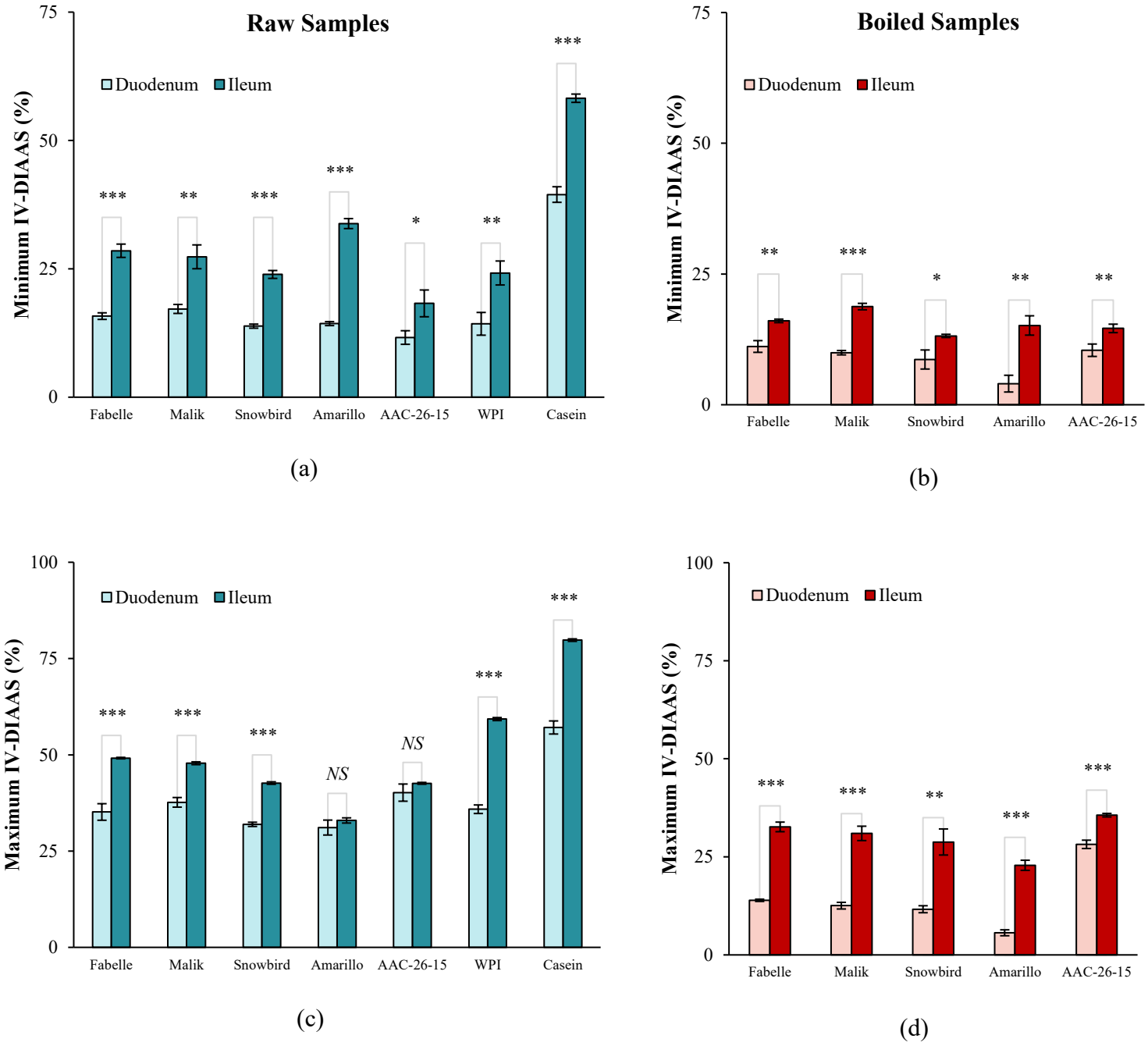

Figure 4-3. Minimum and maximum IV-DIAAS (mean ± standard deviation) of faba bean flours (Fabelle, Malik and Snowbird), pea flour (Amarillo), soy flour (AAC-26-15), whey protein isolate (WPI) and caseins at different digestion endpoints. The protein reference pattern of children (6 months to 3 years) was used for IV-DIAAS calculation. Minimum IV-DIAAS was calculated based on free amino acid digestibility and maximum IV-DIAAS was calculated based on total amino acid digestibility. IV-DIAAS at the end of the duodenal and jejunal-ileal digestion phase was compared by one way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant); (a) Minimum IV-DIAAS of raw samples; (b) Minimum IV-DIAAS of boiled samples; (c) Maximum IV-DIAAS of raw samples; (d) Maximum IV-DIAAS of boiled samples.

4.4.3 Comparison between the IV-DIAAS and *in vivo* DIAAS of the Different Food Matrices

The obtained IV-DIAAS at the end of the duodenal (standardized INFOGEST protocol) and at the end of the jejunal-ileal (modified INFOGEST protocol) were compared to *in vivo* DIAAS collected from the literature to evaluate the physiological relevance of the modified digestion protocol. Only *in vivo* DIAAS obtained in pig or human models were retained (Table 4-2). The IV-DIAAS for raw soybean were excluded from the comparison since no *in vivo* data were available.

Table 4-2. *in vivo* DIAAS of the different food matrices collected from the literature[1]

	DIAAS (6 months-3 years)				
	Mean	**SD[2]**	**Min[3]**	**Max[4]**	**n[5]**
Raw Faba Bean	58	.	56	59	2
Boiled Faba Bean	53	.	.	.	1
Raw Pea	65	6	54	77	13
Boiled Pea	57	.	.	.	1
Soybean Meal[6]	85	13	59	99	13
Whey Protein Isolate and Concentrate	96	9	88	107	4
Casein	126	17	113	145	3

[1] Data retrieved from Berrocoso et al. (2015); Cervantes-Pahm and Stein (2008); Centraal Veevoeder Bureau (CVB) (2016); Gottlob et al. (2006); Grosjean et al. (2000); Kong et al. (2014); Lee et al. (2020); Liu et al. (2016); Liu et al. (2014); National Research Council (2012); Sauvant et al. (2004); Son et al. (2019) (as cited in Herreman et al. (2020)), Guillin et al. (2022); Han et al. (2020); Mathai et al. (2017). [2]The DIAAS are based on the amino acid scoting pattern of children of 6 months to 3 years (FAO, 2013)
[3]SD: standard deviation; [4]Min: minimal value; [5]Max: maximal value, [6]n; number of data; [7]defatted and heat treated soybean.

As presented in Figure 4-4, the IV-DIAAS obtained at the end of the jejunal-ileal digestion phase were closer to the *in vivo* data for all food matrices in comparison to the IV-DIAAS obtained at the end of the duodenal digestion phase. Moreover, the maximum IV-DIAAS were in better agreement with the *in vivo* values, although they remained underestimated. Several factors could explain the underestimation of the IV-DIAAS. For instance, the evaluation of amino acid digestibility requires the subtraction of the digestive enzymes contribution to the amino acid pool, which is done in performing a blank digestion. However, in presence of low or no substrate, the digestive enzymes are more prone to autolysis (Atallah et al., 2020), which can result in an overestimation of their amino acid contribution. It is also possible that the digestion by the aminopeptidase is not as extensive as the multiple enzymatic activity of the BBM in *vivo*. Finally, it is important to note that the static *in vitro* digestion procedure remains a simplification of the

complex dynamic *in vivo* digestion which can explain such disparities between *in vivo* and *in vitro* data.

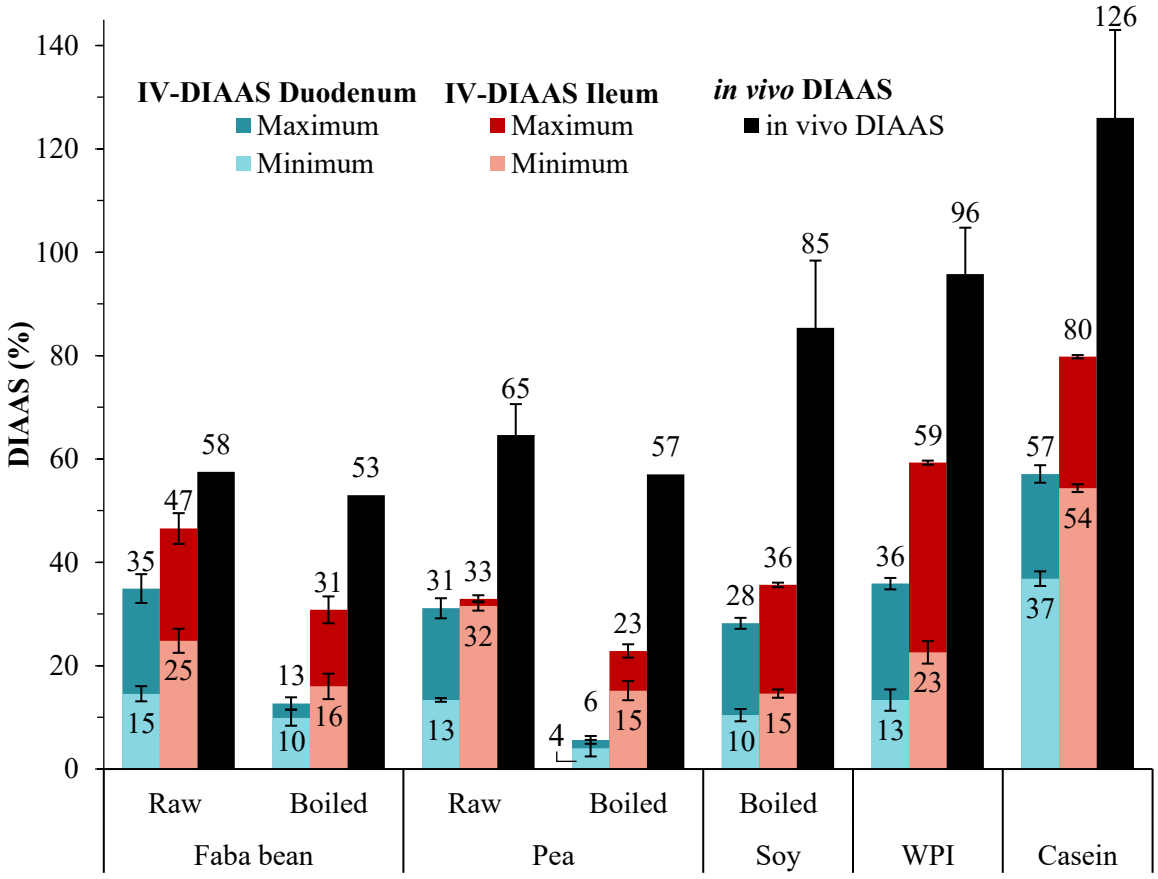

Figure 4-4. Comparison between IV-DIAAS determined at the end of the duodenal digestion phase (standardized INFOGEST Protocol), jejunal-ileal digestion phase (modified INFOGEST protocol) and *in vivo* DIAAS data collected from the literature. The minimum IV-DIAAS was calculated based only on the free amino acid digestibility and the maximum IV-DIAAS was calculated based on total amino acid digestibility (including free amino acid, oligopeptides and soluble proteins). The scoring pattern of children (6 months to 3 years) was used for the calculation of the IV-DIAAS.

Although the IV-DIAAS was an underestimation of the *in vivo* DIAAS, strong correlations between the *in vitro* and *in vivo* data were observed (Figure 4-5). Statistically significant correlations were determined between *in vivo* DIAAS and the minimum IV-DIAAS ($r = 0.818$) and the maximum IV-DIAAS ($r = 0.819$) at the end of the duodenal digestion phase. However, stronger correlations were found between *in vivo* DIAAS and the maximum IV-DIAAS at the end of the jejunal-ileal digestion phase, as shown by the Pearson coefficient closer to 1.0 ($r = 0.879$) and the lower *p*-value ($p = 0.009$). This indicates that among the investigated data, the maximum IV-DIAAS determined with the jejunal-ileal digestion phase is more physiologically relevant to *in*

vivo conditions. This can be attributed to the fact that the minimum IV-DIAAS is an underestimation of the *in vivo* DIAAS, as it was calculated solely based on free amino acid digestibility, leading to a less representative identification of the first limiting amino acid, as discussed in section 4.4.2. One limitation of this result is that the correlation evaluation was based on published *in vivo* DIAAS, and not on *in vivo* data obtained on the same food matrices and analysis protocols. As shown in Table 4-2, there are wide variations in the *in vivo* DIAAS from one study to another for the same food product, possibly as a result of cultivar differences, processing conditions and methodology. These variations are particularly noticeable for raw pea and soy meal, where the *in vivo* DIAAS ranged from 54 to 77 and 59 to 99 respectively. For this reason, future research would need to consider measurement of DIAAS *in vivo* and *in vitro* on same samples and analytical methodologies to generate more robust correlation data. Noteworthy, the amino acid digestibility and DIAAS of dairy proteins (WPI and caseins) were higher compared to legume flours, as found by *in vivo* studies (Mathai et al., 2017), showing the pertinence of the modified *in vitro* digestion model for comparison purposes of protein quality.

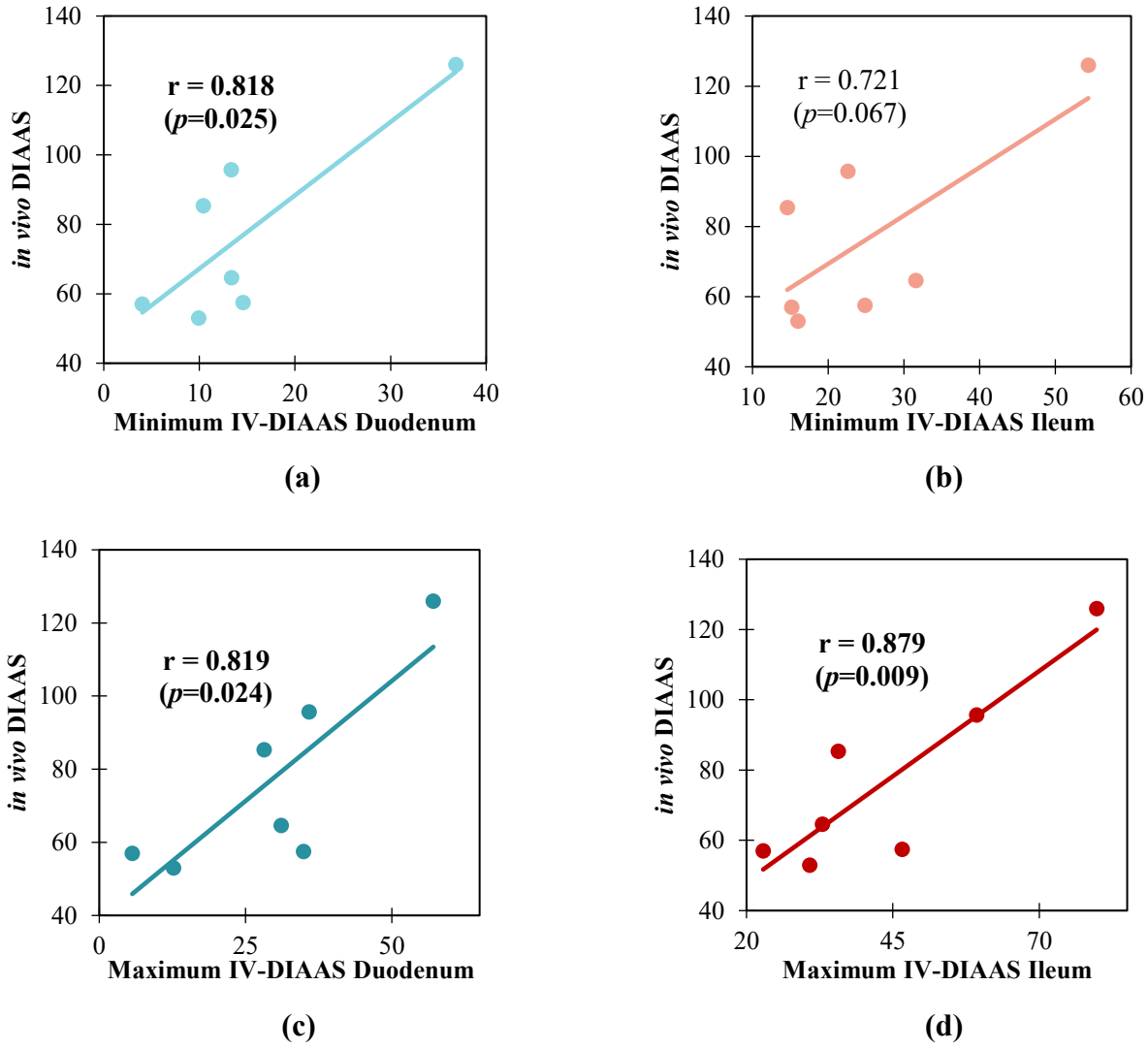

Figure 4-5. Correlations between the obtained *in vitro* DIAAS and *in vivo* DIAAS collected from the literature at different digestion endpoints. All DIAAS were calculated using the protein scoring pattern for children (6 months to 3 years). The *in vivo* DIAAS were determined either in pig or human. ; (a) Minimum IV-DIAAS at the end of the duodenal digestion phase; (b) Minimum IV-DIAAS at the end of the jejunal-ileal digestion phase; (c) Maximum IV-DIAAS at the end of the duodenal digestion phase; (d) IV-DIAAS at the end of the jejunal-ileal digestion phase.

4.5 Conclusions

In this work, we have proposed the addition of a jejunal-ileal digestion phase to the standardized INFOGEST protocol using a commercially available porcine aminopeptidase to assess amino acid digestibility and determine *in vitro* DIAAS of various raw and processed (boiled) food ingredients, including faba bean, pea, soy, caseins and whey protein isolate. The proposed modified protocol, is a simplified, reproducible and easily standardizable alternative to the protocol using porcine brush border membrane extract, which is not commercially available and which requires time

consuming extraction, purification and characterisation steps. Our results showed that the addition of the aminopeptidase-based jejunal-ileal phase resulted in over 30% increase of free and total amino acid digestibility and up to 83% increase in IV-DIAAS for all studied food matrices, leading to *in vitro* data that strongly correlated to *in vivo* data. This study asserts the relevance and importance of the jejunal-ileal phase in the development of a robust and relevant *in vitro* gastro-intestinal digestion model. It further demonstrates that the use of commercial peptidases could be a suitable alternative to brush border membrane extract to mimic protein digestion that occurs in the jejunum and ileum. This protocol could be further improved in the future by including other enzymes of the jejunum and ileum that may promote protein digestion, such as glycosidases and lipases. Glycosidases are important enzymes of the brush border membrane (Mamone & Picariello, 2023) and their presence may further improved protein digestion, as starch and protein interactions can reduce availability of digestive enzyme to their hydrolysis site, leading to a reduced protein digestibility. Moreover, a consensus on the digestion conditions (pH, duration, enzyme to substrate ratio) still need to be established and their physiological relevance ascertained. Nonetheless, given the complexity of jejunal and ileal digestion (Mackie et al., 2020; Verhoeckx et al., 2019), the herein selected conditions reproducibly resulted in more relevant IV-DIAAS that correlated well with available published *in vivo* data. In concluding, the proposed improved *in vitro* INFOGEST digestion protocol could be a valuable *in vitro* tool that enable a primary evaluation of the digestibility and quality of novel protein sources and processed food products, prior to *in vivo* study.

4.6 References

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CONNECTING STATEMENT III

Chapter III and IV gave a comprehensive overview of faba beans protein nutritional quality in comparison to pea and soy. However, dietary proteins can procure health benefits that go beyond fulfilling nutritional needs. These health benefits can be attributed to the release of bioactive peptides after gastrointestinal digestion. Limited data on faba bean protein bioactive properties following gastrointestinal digestion are available.

In this vein, Chapter V presents a comprehensive investigation of the bioactive properties of the three new Canadian faba bean varieties (Fabelle, Malik and Snowbird) in comparison to two control legumes (pea and soy) to complement the protein nutritional quality assessment. The improved *in vitro* digestion procedure adapted in Chapter IV was applied to mimic more closely physiological conditions of protein digestion. Only processed legume flours (i.e. boiled) were compared, since they are more representative on how these legumes are typically consumed. After digestion, the supernatants of the legume flour digestates were filtered on a 3 kDa membranes to recover bioacessible peptides. The 3 kDa permeates of gastrointestinal digestates were screened for antioxidant, antihypertensive and antidiabetic activities using a combination of *in silico*, *in vitro* and cellular models. Most potent faba bean bioactive peptides were further purified and identified.

The results from this research were presented at the 2021 International AOCS Annual Meeting & Expo and at the 2023 CCARM International Trainee Symposium in Agri-Food, Nutrition and Health. This research was also published in the *International Journal of Molecular Sciences*.

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ingredient with antioxidant and antihypertensive properties [Oral Presentation]. 2023 CCARM International Trainee Symposium in Agri-Food, Nutrition and Health.

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CHAPTER V. HEALTH BENEFICIAL BIOACTIVITIES OF FABA BEAN FLOURS GASTROINTESTINAL (*IN VITRO*) DIGESTATE IN COMPARISON TO SOYBEAN AND PEA

5.1 Abstract

Faba beans are a promising emerging plant-based protein source to be used as a quality alternative to peas and soy. In this study, the potential health beneficial activities of three Canadian faba bean varieties (Fabelle, Malik and Snowbird) were investigated after *in vitro* gastrointestinal digestion and compared to two commonly used legumes (peas and soy). The results revealed that the faba beans had a higher antioxidant activity than peas when assessed with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays, except for the Fabelle variety. In the oxygen radical absorbance capacity (ORAC) and the iron chelating assays, the faba beans had a lower antioxidant activity than soy. Interestingly, Fabelle and Snowbird showed a higher antioxidant effect than the peas and soy at the cellular level. The antihypertensive properties of Fabelle and Malik varieties were significantly higher than peas but lower than soy. The *in vitro* antidiabetic activity was higher for soy, but no differences were found at the cellular level. The faba bean peptides were further fractionated and sequenced by mass spectrometry. Eleven peptides with *in silico* predicted bioactivities were successfully identified in the faba bean digestate and support validating the health-promoting properties of peptides. The results demonstrate the bioactive potential of faba beans as a health-promoting food ingredient against non-communicable diseases.

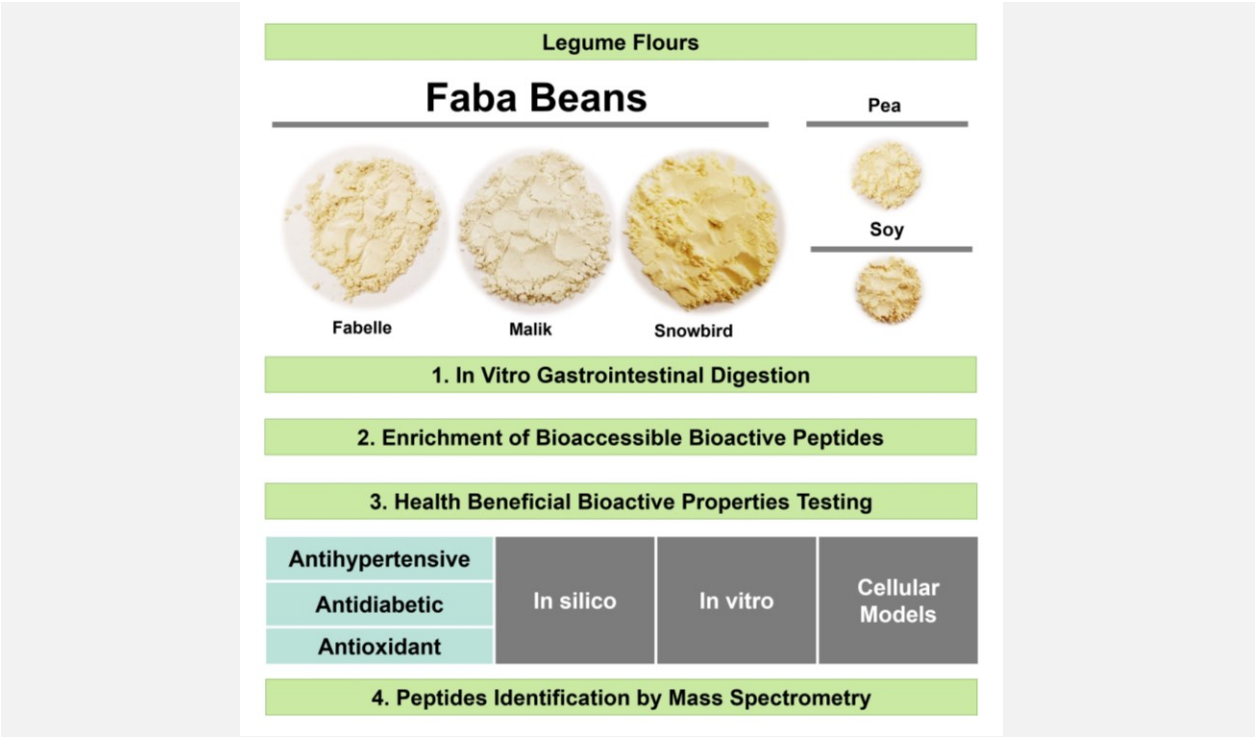


Figure 5-1. Graphical abstract

5.2 Introduction

The demand for plant-based protein sources keeps increasing to respond to environmental, ethical, health, economic and food security challenges. In these circumstances, faba beans have retained attention, due to various desirable environmental, agronomic and nutritional characteristics (Multari, Stewart, & Russell, 2015). Indeed, faba bean production has largely increased in the last few years, from 4.5 million tons in 2014 to 5.7 million tons in 2020 (FAOSTAT, 2020). Faba beans have a high protein content (~30%) (Raikos, Neacsu, Russell, & Duthie, 2014) and are a rich source of fiber, resistant starch, minerals and vitamins (Millar, Gallagher, Burke, McCarthy, & Barry-Ryan, 2019). Besides, novel cultivars with a low anti-nutrient content (vicine/convicine and tannins) are available (Hou et al., 2018; Khazaei et al., 2019), which further improves its nutritional value and application potential.

Faba beans are also a rich source of bioactive compounds, namely dietary fibers (Çalışkantürk Karataş, Günay, & Sayar, 2017; Gullón, Gullón, Tavaria, Vasconcelos, & Gomes, 2015), L-DOPA (Abdel-Sattar et al., 2021; Rabey, Vered, Shabtai, Graff, & Korczyn, 1992) and polyphenols (Boudjou, Oomah, Zaidi, & Hosseinian, 2013; Johnson, Skylas, Mani, Xiang, Walsh, & Naiker, 2021), that have been associated with health benefits. The faba bean proteins could also play an important role for their health-promoting activities, by releasing small bioactive peptides after gastrointestinal digestion. The food-derived bioactive peptides possess various bio-functional capacities and can intervene in various physiological processes (Belović, Mastilović, Torbica, Tomić, Stanić, & Džinić, 2011). Their bioactive properties are beneficial in fighting against non-communicable diseases, including type II diabetes, hypertension, obesity and cancer, among others (León-Espinosa et al., 2016; Sánchez & Vázquez, 2017; Zambrowicz et al., 2015). The faba bean protein hydrolysates have antioxidant (Ashraf et al., 2020; Felix, Cermeño, & FitzGerald, 2019; Jakubczyk, Karaś, Złotek, Szymanowska, Baraniak, & Bochnak, 2019; Karkouch et al., 2017; León-Espinosa et al., 2016), antidiabetic (Felix et al., 2019), antihypertensive (Felix et al., 2019), cholesterol-lowering (Ashraf et al., 2020; León-Espinosa et al., 2016), anticancer (León-Espinosa et al., 2016), anti-inflammatory (Jakubczyk et al., 2019) and food intake regulation properties (Dugardin et al., 2020) identified to date. However, the faba beans' bioactive peptides that are released in the physiological context of gastrointestinal conditions have been scarcely investigated to date.

In this regard, the purpose of this study was to assess the health-promoting bioactive properties of three new Canadian faba bean varieties (Fabelle, Malik and Snowbird) after *in vitro* gastrointestinal digestion in comparison to two commonly used legumes, namely, peas and soy. The three faba bean cultivars included in the study had different quality traits: low-tannin (Snowbird); low vicine/convicine (Fabelle); and high tannin and high vicine/convicine (Malik). The processed flours were digested *in vitro* using a standardized gastrointestinal INFOGEST digestion protocol, to which an ileal digestion phase was added to mimic brush border digestion. The digestates were filtered on a 3 kDa molecular weight cut-off membrane to recover peptides and small bioactives. The collected filtrates were assessed for antioxidant, antidiabetic and antihypertensive activities with a combination of *in vitro* and cell-based assays. The bioactive properties were selected based on the results of an *in silico* analysis. The faba bean peptides with the highest activity were further fractionated and identified by mass spectrometry.

5.3 Materials and Methods

5.3.1 Materials

Three dehulled faba bean cultivars (Fabelle, Malik and Snowbird), one dehulled pea cultivar (Amarillo) and one dehulled soy cultivar (AAC-26–15) were used in this study. The faba bean and pea samples were supplied as milled flours, and soybean as whole seeds. The faba bean cultivars, Fabelle and Malik, were provided by AGT Foods and Ingredients (Saskatoon, SK, Canada), and Snowbird by W.A. Grain & Pulse Solutions (Innisfail, AB, Canada). The certified yellow pea (CDC Amarillo) and soybean (Cdn #1, Variety AAC 26-25, Non-GMO and IP, Lot 261510504AT) were provided by Greenleaf Seeds (Tisdale, SK, Canada) and Huron seeds (Clinton, ON, Canada), respectively.

The hexane, sulfuric acid, dimethyl sulfoxide (DMSO) and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The ethanol was purchased from Commercial Alcohols (Brampton, ON, Canada).

The hydrochloric acid, sodium hydroxide, Glucose, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), Phosphate-buffered saline (PBS), sodium bicarbonate, monosodium phosphate, sodium chloride and Tris-HCl were purchased from BioShop (Burlington, ON, Canada).

The 2,2′Azobis (2-methylpropionamidine) dihydrochloride (AAPH), Trolox, fluorescein, 2′-7′-Dichlorofluorescein diacetate (DCFH-DA), Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC), Dipeptidyl Peptidase IV (CD26) from Porcine Kidney, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, ferrous chloride, ferrozine, borax, angiotensin-converting enzyme (ACE) from rabbit lung (A6778), N-Hippuryl—His—Leu hydrate (HHL) substrate (H1635), trifluoroacetic acid (TFA), Gallic Acid, Folin–Ciocalteu and phenol were purchased from Sigma-Aldrich (St. Louis, MO, USA)

For the *in vitro* digestion procedure, α-amylase from porcine pancreas (A3176), pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545), trypsin from porcine pancreas (T0303), α-chymotrypsin from bovine pancreas (C7762), porcine bile extract (B8631) and the Bile Acid Assay Kit (MAK309) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The native porcine peptidase (Nate-0548) was purchased from Creative Enzyme (Shirley, NY, USA).

For the cell culture, Dulbecco's Modification of Eagle's Medium (DMEM) containing 4.5 g/L glucose, with phenol-red and without sodium pyruvate, 200 mM L-Glutamine, Dulbecco's Phosphate-Buffered-Saline (D-PBS) without Ca^{2+} and Mg^{2+}, Nonessential Amino Acid Solution 100×, heat-inactivated fetal bovine serum (FBS), 5000 IU penicillin and 5000 µg/ml streptomycin solution, Trypsin solution (0.05%) containing 0.53 mM EDTA in HBSS and Trypan blue (0.4%) were purchased from Wisent Bioproducts (Saint-Jean-Baptiste, QC). Hank's Balanced Salt Solution (HBSS) was purchased from Gibco (Thermo Fisher Scientific, San Jose, CA, USA). The Caco-2 cells (ATCC® HTB-37™, passage 18) were purchased from ATCC (Manassas, VA, USA). The Pierce BCA Assay kit was purchased from Thermo Fisher Scientific (San Jose, CA, USA) and the Cell Titer-Glo 2.0 kit was purchased from Promega (Fitchburg, WI, USA).

All of the chemicals and reagents used were of analytical grade and deionized water (Millipore) was used in all of the experiments.

5.3.2 Sample Preparation

The soy seeds were milled with a Brinkmann centrifugal grinding mill using a 0.2-mm rotary sieve with the addition of liquid nitrogen to prevent heating. The soy flour was then de-oiled through hexane extraction, according to the method of L'Hocine, Boye, and Arcand (2006) to obtain a fat

content similar to the faba beans and peas. The faba bean, pea and soy flours were cooked (thermally treated through boiling), which is a representative domestic processing for legume preparation, following the procedure of Ma, Boye, Simpson, Prasher, Monpetit, and Malcolmson (2011). The flour was hydrated in water (ratio 1:10) for 1 hour at room temperature under constant stirring and then boiled for 20 minutes. The cooked flour and cooking water were frozen at −40 °C, freeze-dried and milled once more to assure a particle size uniformity among the samples. All of the flour samples were stored at −20 °C in vacuum bags until needed.

5.3.3 Adaptation of the *In vitro* Gastrointestinal Digestion Procedure for Cell Culture

The gastrointestinal digestion was simulated using the INFOGEST harmonized static *in vitro* digestion procedure (Brodkorb et al., 2019) with some modifications. Indeed, Brodkorb et al. (2019) suggested investigating whether the bile salts and protease inhibitor concentration interfere with the cell model, since those two elements can compromise cell viability. The preliminary studies were conducted to investigate the impact of those elements on the specific cell line used in this work, the Caco-2 cells. Three parameters were considered: the enzyme used during the intestinal digestion phase (pancreatin or a mix of trypsin (100 U/mL digest); α-chymotrypsin (25 U/mL digest) and α-amylase (200 U/mL digest), with or without the addition of a jejunal-ileal digestion phase), the bile salts' concentration (1 or 10 mM) and the protease inhibitor (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride or AEBSF) concentration (0.25, 1 or 5 mM). The digestions were performed with one faba bean variety (Fabelle) in triplicate and one parameter was varied at the time.

5.3.4 *in vitro* Gastrointestinal Digestion of Legume Flours

Once the adjusted digestion conditions for the cell culture were obtained, the *in vitro* gastrointestinal digestate of the different legume flours was prepared accordingly. Prior to digestion work, the enzyme activity of amylase, pepsin, pancreatin, trypsin and chymotrypsin were determined, according to Brodkorb et al. (2019) procedures and the bile salts content in the porcine bile extract was assessed, using the Sigma-Aldrich Bile Assay Kit (MAK309). The leucine aminopeptidase activity in the porcine intestinal peptidase was evaluated, following the procedure of Hausch, Shan, Santiago, Gray, and Khosla (2002) and one unit was defined as the consumption of 1 μmol of leucine-*p*-nitroanilide per minute at 30 °C and pH 8.0.

Briefly, 1.2 g of flour was mixed with 1.8 g of water to reach the targeted consistency of tomato paste at the end of the oral phase. For the oral digestion phase, the hydrated flour was mixed in a ratio 1:1 with simulated salivary fluid (SSF) containing α-amylase from the porcine pancreas (75 U/mL digestate) and digestion was conducted for 2 minutes at 37 °C under constant stirring. For the gastric digestion phase, the digestate was mixed in a ratio 1:1 with simulated gastric fluid (SGF), containing pepsin (2000 U/mL digestate). The pH was adjusted to 3.0 by the dropwise addition of 6 N HCl, and the digestion was continued for 2 hours at 37 °C under constant stirring. The gastric lipase was not added since lipid is a minor constituent in the sample studied (<1%) compared to the proteins and starch. The digestates were diluted once more for the duodenal digestion phase, in a ratio 1:1 with the Simulated Intestinal Fluid (SIF) containing pancreatin from the porcine mucosa (100 U trypsin activity/mL digestate) and the porcine bile extract (10 mM bile salts), the pH was adjusted to 7.0 with 3 N NaOH and the digestate was incubated for 2 hours at 37 °C with constant stirring. To simulate the digestion that occurs at the brush border in the jejunum and ileum, the pH was adjusted to 7.2 and a Native Porcine Peptidase (13 mU leucine-aminopeptidase activity/mL digestate) was added. The digestate was incubated for another 4 hours at 37 °C under constant stirring.

At the end of the digestion, the samples were cooled on ice and the protease inhibitor (1 mM AEBSF) was added to stop the digestion. The digestates were centrifuged at 15,000× *g* for 30 minutes at 4 °C and the supernatants were filtered using an Amicon ultrafiltration system equipped with a 3 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane (END Millipore, Billerica, MA) to recover the small molecular weight peptides. Peptides stability during the ultrafiltration step was verified in quantifying the free amino groups using the 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) method of Adler-Nissen (1979), with modifications by Mason (2019).

The permeates' osmolality was measured and the permeates were diluted in water to reach a physiological osmolality of 285–300 mOsm/kg, using a Micro-Osmometer (Model 3320; Advanced Instruments INC., Norwood, Massachusetts). Their pH was also adjusted to a physiological value of 7.3. The diluted 3 kDa permeates were analyzed for their protein content with the Pierce Bicinchoninic Acid (BCA) protein assay kit, using bovine serum albumin as standard and frozen at −80 °C until further use. A portion of the frozen permeates was freeze-dried and stored at −20 °C until further characterization.

5.3.5 *In Silico* Prediction of Bioactive Fragments Released during Gastrointestinal Digestion of Legume Proteins

The potential bioactive fragments released during the gastrointestinal digestion of the major faba bean, pea and soy storage proteins were investigated using an *in silico* analysis. The accession numbers of the protein used for the analysis are displayed in Table 5-1. The globulins' sequences were hydrolyzed *in silico* with pepsin (pH > 2) (EC 3.4.23.1), trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) simultaneously to simulate gastrointestinal digestion with the Bioactive Peptide Database of University of Warmia and Mazury (BIOPEP-UWM) enzyme tools (Minkiewicz, Iwaniak, & Darewicz, 2019). The frequency of the peptide fragments' release with the selected enzyme activity values (A_E) for each of the selected protein and bioactivities were automatically calculated by the BIOPEP-UWM algorithm (Minkiewicz et al., 2019) and retrieved on 14 July 2022.

Table 5-1. UniProtKB Protein accession numbers of faba bean, pea and soy main storage proteins used for the *in silico* analysis.

Faba Bean (*Vicia faba*)			
Proteins		Gene	Accession Number
Legumin (11S)	Legumin B	LEB4	P05190
		LEB2	P16078
		LEB6	P16079
		LEB7	P16080
	Legumin A	A1	Q03971
		A2	Q99304
Vicilin (7S)	Vicilin	.	P08438
	Convicilin	.	B0BCL8
		.	B0BCL7
Pea (*Pisum sativum*)			
Proteins		Gene	Accession Number
Legumin (11S)	Legumin B	LEGJ	P05692
		LEGK	P05693
		LEGB	P14594
	Legumin A	A	P02857
		A2	P15838
Vicilin (7S)	Vicilin	.	P13918
	Convicilin	CVA	P13915
		CVB	P13919
Soy (*Glycine max*)			
Proteins		Gene	Accession Number
Glycinin (11S)	Glycinin-G1	GY1	P04776
	Glycinin-G2	GY2	P04405
	Glycinin-G3	GY3	P11828
	Glycinin-G4	GY4	P02858
	Glycinin-G5	GY5	P04347
β-Conglycinin (7S)	β-Conglycinin-α'	CG-1	P11827
	β-Conglycinin-α	CG-3	P0DO16
	β-Conglycinin β	CG-4	P25974

5.3.6 Characterization of the 3 kDa Permeate of Legume Digestates

5.3.6.1 Molecular Weight Distribution of Peptides by Size Exclusion HPLC

The molecular weight distribution profile of the peptides present in the 3 kDa permeate of legume digestate was determined by size exclusion chromatography, following the procedure of Achouri, Boye, Belanger, Chiron, Yaylayan, and Yeboah (2010) with modifications by Mason, L'Hocine, Achouri, Pitre, and Karboune (2020). An Agilent-1200 Series HPLC system (Agilent Technologies Canada, Inc., Mississauga, ON, USA), equipped with a Enrich SEC-70 column (10 × 300 mm) (Bio-Rad Laboratories, Mississauga, ON, USA), was used. The samples from the 3 kDa permeate of legume digestate (2.5 µL) were loaded on the column and eluted with 10-mM phosphate buffered saline, with 154-mM NaCl (pH 7.4) at a flow rate of 0.5 mL/min. The absorbance was monitored at λ = 220 nm. The standards, including aprotinin (6.511 kDa), vitamin B12 (1.355 kDa), Gly-Gly-Gly (tripeptide, 189 Da) and carnosine (dipeptide, 226 Da), were used to evaluate the samples' molecular weight distribution.

5.3.6.2 Composition Analysis of the 3 kDa Permeate of Legume Digestates

The protein content in the 3 kDa permeate of legume digestate was determined according to the Dumas method (AOAC International, 1995) with a Vario MAX Cube (Elementar, Langenselbold, Germany) using a nitrogen to protein conversion factor of 6.25 and EDTA as standard.

The total phenolic content was determined by the Folin–Ciocalteu method following the procedure of Singleton, Orthofer, and Lamuela-Raventós (1999) with modifications. A total of 50 mg of freeze-dried permeate was extracted in 1 mL of 70% ethanol containing 1% (v/v) 12 N HCl, in the dark, at room temperature and under constant stirring for 2 hours. The samples were then centrifuged (16,000× *g* for 15 minutes) and filtrated on 0.45 µm polyethersulfone (PES) filter (Canadian Life Science, Peterborough, ON, USA). The gallic acid standard (50–500 mg/L) was prepared in the same solvent. A total of 200 µL of the samples and standard were mixed with 1.5 mL of Folin–Ciocalteu reagent (diluted 1:10 in water) and incubated in the dark for 5 minutes. Then, 1.5 mL of 7.5% (m/v) sodium bicarbonate solution was added, and the samples were incubated for 75 minutes in the dark at room temperature. The samples were centrifuged (6000× *g* for 15 minutes) and the absorbance was read at λ = 750 nm. The total phenolics content (TPC) was expressed as mg of gallic acid equivalent per g of 3 kDa permeate of legume digestates (on a dry basis).

The total carbohydrate content was determined, following the method of DuBois, Gilles, Hamilton, Rebers, and Smith (1956). The freeze-dried permeates were solubilized in water (10 mg/mL). A total of 2 mL of each sample and standard (glucose 0-50 mg/L) were added to a glass test tube in duplicate. A total of 50 µL of phenol 80% (m/m) was added to each sample and standard, followed by 5 mL of sulfuric acid 95.5%. The samples were incubated at room temperature for 10 minutes and then incubated in a water bath at 25 °C for 10 minutes. The samples were cooled to room temperature and the absorbance was recorded at λ = 490 nm. The results were expressed as g of glucose equivalent per 100 g of freeze-dried 3 kDa permeates.

5.3.7 *In vitro* Antioxidant and Chelating Activities of the 3 kDa Permeate of Legume Digestates

5.3.7.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

The DPPH assay was performed following the method of Orona-Tamayo, Valverde, Nieto-Rendón, and Paredes-López (2015), as described in Mason et al. (2020) with slight modifications. A total of 100 µL of diluted 3 kDa permeate of the legume digestate (100–6000 µg peptides/mL) was mixed with 100 µL of 0.2 mM DPPH (prepared in ethanol 100% and stored at −20 °C) in a clear 96-well clear microplate and incubated for 30 minutes at 37 °C in the dark. The absorbance was read at λ = 517 nm with an Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT, USA). The free-radical scavenging capacity was calculated as follows, after background subtraction:

$$\text{DPPH radical scavenging capacity (\%)} = 1 - \frac{A_{sample}}{A_{control}}$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of DPPH in absence of antioxidants. The EC_{50} value was reported, which was defined as the required peptide concentration to scavenge 50% of the DPPH free radicals. The EC_{50} was calculated using a non-linear regression with a four-parameter logistic (4PL) curve of the DPPH free-radical scavenging capacity plotted against its respective peptide concentration.

5.3.7.2 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Scavenging Assay

The ABTS assay was performed following the method of Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999), as described in Mason et al. (2020). The day before the assay, a

solution containing 7 mM ABTS and 2.45 mM of potassium persulfate was prepared in water and incubated overnight at room temperature in the dark to generate the free radicals. The next day, the solution was diluted in ethanol 100% to reach an absorbance value of 0.70 ± 0.02 at λ = 734 nm. A total of 50 µL of the 3 kDa permeate of legume digestates (10–400 µg peptides/mL) was mixed with 180 µL of the ABTS solution in a 96 well clear flat-bottom plate, and incubated for 6 minutes at room temperature in the dark. The absorbance was read at λ = 734 nm with an Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT, USA) microplate reader. The free-radical scavenging capacity was calculated as follows, after background subtraction:

$$ABTS\ radical\ scavenging\ capacity\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of ABTS in the absence of antioxidant. The EC_{50} value was reported, which was defined as the required peptide concentration to scavenge 50% of the ABTS free radicals. The EC_{50} was calculated using a non-linear regression with a 4PL curve of the ABTS free-radical scavenging capacity, plotted against its respective peptide concentration.

5.3.7.3 Oxygen Radical Absorption Capacity (ORAC) Assay

The ORAC assay was performed following the method of Tomer, McLeman, Ohmine, Scherer, Murray, and O'Neill (2007), as described in Mason et al. (2020). All of the solutions were prepared in a 75 mM phosphate buffer pH 7.4. A total of 25 µL of appropriately diluted 3 kDa permeate of legume digestates and Trolox standard (6.25–50 µM) were loaded in a 96-well black plate with a clear bottom. The outermost wells of the 96-well plates were not used to prevent the plate-edge effects. A total of 150 µL of 96 nM fluorescein solution was added to each well and the plate was incubated for 30 minutes at 37 °C. After the incubation period, 25 µL of 79.65 mM 2,'2-azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical initiator, was added to each well using an automatic injector and the fluorescence was recorded every minute for 90 minutes ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 520 nm), using a Synergy HTX microplate reader (Bio-Tek, Winooski, VT, USA). The area under the curve (AUC) was calculated for the samples, standards and blanks with the Gen5 Data Analysis Software (BioTek Instruments, Inc., Winooski, VT, USA), using the following regression equation:

$$AUC = 1 + \frac{RFU_1}{RFU_0} + \frac{RFU_2}{RFU_0} + \frac{RFU_3}{RFU_0} + \cdots \frac{RFU_{90}}{RFU_0}$$

where RFU_0 is the initial fluorescence and RFU_x is the relative fluorescence at each time points. Then, the net AUC was calculated as follows:

$$Net\ AUC = AUC_{standards/samples} - AUC_{blank}$$

The net AUC value of the Trolox standards was used to build a standard curve. The antioxidant capacity of the samples was expressed as µmol of Trolox equivalent per mg of proteins.

5.3.7.4 Iron Chelating Activity

The iron-chelating activity assay was performed following the procedure of Orona-Tamayo et al. (2015), as described in Mason et al. (2020). A total of 50 µL of diluted 3 kDa permeate (25–1000 µg peptides/mL) of the legume digestates was mixed with 25 µL of 0.25 mM ferrous chloride and 25 uL of 0.625 mM ferrozine in a clear 96-well plate. The plate was incubated for 10 minutes in the dark at room temperature and the absorbance was recorded at λ = 562 nm. The percentage of iron chelating was calculated as follows:

$$Iron\ Chelating\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the iron-ferrozine complex in the absence of chelating peptides. The EC_{50} value was reported, which is defined as the required peptide concentration to chelate 50% of the ferrous ions. The EC_{50} was calculated using a non-linear regression, with a 4PL curve of the iron chelating plotted against its respective peptide concentration.

5.3.8 *In vitro* Antihypertensive Activity (Angiotensin-Converting Enzyme Inhibition Activity)

The antihypertensive activity was evaluated using the Angiotensin-Converting Enzyme (ACE) inhibition assay of Barbana and Boye (2011), as described in Mason et al. (2020) with slight modifications. All of the solutions were prepared in a 1 mM borate buffer pH 8.3 containing 0.3 M NaCl. A total of 10 µL of appropriately diluted 3 kDa permeate of legume digestates (100–6000 µg peptides/mL) was mixed with 10 µL of ACE (8 mU; ACE from rabbit lung, A6778; Sigma-Aldrich, St. Louis, MO, USA) in a test tube and incubated for 10 minutes at 37 °C. Fifty µL of 1 mM Hippury-L-histidyl-L-Leucine (HHL) was added and the incubation was continued for 30 minutes. At the end of the incubation period, 85 µL of HCL 1N par was added to stop the reaction.

The reaction mixture was analyzed by reverse-phase HPLC, using a 4.60 × 250 mm Aqua C18 column (5-µm pore size 125 Å; Phenomenex, Torrance, CA, USA). The samples were eluted with 50% (v/v) methanol in water containing 0.1% TFA at a flow rate of 0.5 mL/min for 15 min. The absorbance was monitored at λ = 228 nm. The peak area of HHL was recorded and the *ACE inhibition* was calculated, as follows:

$$ACE\ Inhibition\ (\%) = \left(1 - \frac{(PA_a - PA_b)}{(PA_a - PA_c)}\right) \times 100$$

where PA_a is the peak area of the control (HHL alone, corresponding to 100% inhibition); PA_b is the peak area of the sample (HHL, ACE and inhibitory peptides) and PA_c is the peak area of the reaction blank (HHL and ACE, corresponding to 0% inhibition). The IC_{50} value was reported, which is defined as the required peptide concentration to inhibit 50% of ACE activity. The IC_{50} was calculated using a non-linear regression with a 4PL curve of the ACE inhibition (%) plotted against its respective peptide concentration.

5.3.9 *In vitro* Antidiabetic Activity (Dipeptidyl Peptidase-IV Inhibition Assay)

The *in vitro* Dipeptidyl Peptidase-IV (DPP-IV) inhibition assay was performed following the procedure of Caron, Domenger, Dhulster, Ravallec, and Cudennec (2017), with slight modifications. Then, 50 µL of diluted 3 kDa permeate of legume digestate (100–6000 µg peptides/mL) was mixed with 25 µL of Dipeptidyl Peptidase IV (CD26) from Porcine Kidney (0.018 U/mL) and 50 µL of 0.1 M Tris-HCl buffer pH 8.0 in a 96-well black plate with a clear bottom and incubated at 37 °C for 5 minutes. Then, 50 µL of Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) 1 mM was added and the fluorescence was recorded at 37 °C after 30 minutes (λ_{ex} = 350 λ_{em} = 450), using a Synergy HTX plate reader (Bio-Tek, Winooski, VT, USA). The peptides, enzymes and substrates were all diluted in a 0.1 M Tris-HCl buffer at pH 8.0. The *DPP-IV inhibition* was calculated as follows, after background subtraction:

$$DPP\text{-}IV\ Inhibition\ (\%) = \left(1 - \frac{RFU_{sample}}{RFU_{control}}\right) \times 100$$

where RFU_{sample} is the fluorescence of the samples and $RFU_{control}$ is the fluorescence of the control (DPP-IV with a buffer instead of inhibitory peptides). The IC_{50} value was reported, which is defined as the required peptide concentration to inhibit 50% of the DPP-IV activity. The IC_{50} was

calculated using non-linear regression with a 4PL curve of the DPP-IV inhibition (%) against its respective peptide concentration.

5.3.10 Cell Culture

The Caco-2 cells were cultivated in growth medium, which was composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, 1% non-essential amino acids and 2 mM L-glutamine and incubated at 37 °C in an atmosphere containing 5% CO_2. The cells were sub-cultivated once a week at 80–90% confluence using a trypsin–EDTA solution and the culture medium was changed every 2–3 days. The cells were subcultured three times prior to bioactivity assessment to enable cell phenotype stabilization (Hubatsch et al., 2007). The cells between passage 22 and 32 were used in this study. In every assay, the outermost wells of the 96-well plates were not used to prevent plate-edge effects.

5.3.11 Cell Viability

The cell viability following incubation with a different concentration of 3 kDa permeate of each legume digestate (3000, 3500 and 4000 µg peptide/mL) was verified using the Cell Titer-Glo 2.0 kit (Promega, Fitchburg, Wisconsin), which is a luminescent based method that quantify ATP as an indicator of cell viability. The cells were seeded in growth medium in a 96-well black plate with a clear bottom at a density of 6.5×10^4 cells/well for 48 hours to reach confluence. On the day of the assay, the medium was discarded, the cells were washed with 100 µL of Dulbecco's Phosphate-Buffered-Saline (D-PBS) and then incubated with 100 µL of 3 kDa permeate of the legume digestates diluted in DMEM for 1 hour at 37 °C. The DMEM was used as a negative control and the H_2O_2 (20%) as a positive control. After the incubation period, the plate was equilibrated at room temperature for 30 minutes and 100 µL of Cell Titer-Glo® 2.0 Reagent (equilibrated previously at room temperature) was added per well. The plate was shaken for 2 minutes to provoke cell lysis, then incubated 10 minutes at 22 °C and the luminescence (L) was recorded using a Synergy HTX plate reader (Bio-Tek, Winooski, VT, USA). The cell viability was expressed as follows, after background subtraction:

$$Cell\ viability\ (\%) = \left(\frac{L_{sample}}{L_{control}}\right) \times 100$$

where L_{sample} is the luminescence of the sample and $L_{control}$ is the luminescence of the cells incubated in DMEM (corresponding to 100% viability).

5.3.12 Cellular Antioxidant Assay (CAA) in an Intestinal Cell Model

The cellular antioxidant assay was performed following the methods of Wan, Liu, Yu, Sun, and Li (2015) and Kellett, Greenspan, and Pegg (2018), with modifications. Briefly, the Caco-2 cells were cultivated in growth medium and seeded in a 96-well black plate with a clear bottom at a density of 6.5×10^4 cells/well for 48 hours to reach confluence. On the day of the assay, the medium was discarded, and the cells were washed with 100 µL D-PBS. Then, the cells were incubated for 1 hour at 37 °C with different concentrations of 3 kDa permeate of legume digestate (3000, 3500 and 4000 µg peptides/mL) diluted in DMEM containing 25 µM (final concentration) of Dichloro-dihydro-fluorescein diacetate (DCFH-DA), which is used as a marker of intracellular oxidation. Once DCFH-DA enters the cells, it is diacetylated by intracellular esterase into 2′,7′-dichlorodihydrofluorescein (DCFH) that is more easily oxidizable. The 3 kDa permeate of blank digestions (digestion solutions and enzyme without legume flour) was used as a control, since the digestion medium was shown to interfere in the assay. The DMEM was used as the blank. The DCFH-DA (25 µM final concentration) was also added to the control and blank wells. After the incubation period, the samples were discarded, and 100 µL of a 600 µM AAPH solution, a peroxyl radical initiator, prepared in Hank's Balanced Salt Solution (HBSS) was added to the sample and control wells. The HBSS without AAPH was added to the blank wells. Once the DCFH was oxidized into dichlorofluorescein (DCF) by the free radicals, the probe became fluorescent. The fluorescence (λ_{ex} = 485 nm, λ_{em} = 520 nm) was reordered every minute for 1 hour at 37 °C, using a Synergy HTX plate reader (Bio-Tek, Winooski, VT). The antioxidant capacity was expressed as the Cellular Antioxidant Activity (*CAA*) unit, which was calculated as follows:

$$CAA\ (\%) = \left(1 - \frac{\int SA}{\int CA}\right) \times 100$$

where *SA* is the sample curve of relative fluorescence over time and *CA* is the control curve of relative fluorescence over time. The area under the curve of the samples and controls were calculated by the Gen5 software (Bio-Tek, Winooski, VT, USA) after the blank and initial fluorescence reading subtraction.

5.3.13 Dipeptidyl Peptidase-IV (DPP-IV) Inhibition in an Intestinal Cell Model

A cellular model was also used to verify the inhibitory effect of the faba bean-, pea- and soy-derived peptides against DPP-IV in more physiologically relevant conditions. The experiment was carried out as described by Lammi et al. (2016), with modifications. The Caco-2 cells were plated in a growth medium 48 h before the assay at a density of $5 \cdot 10^4$ cells/well in a 96-well black plate with a clear bottom. On the day of the assay, the medium was discarded, and cells were washed with 100 µL D-PBS. The cells were then incubated with different concentrations of 3 kDa permeate of legume digestate (3000, 3500 and 4000 µg/mL) diluted in DMEM, for 2 hours at 37 °C. The DMEM was used as the control. After incubation, the medium was discarded, the cells were washed with D-PBS and 100 µL of 50 µM Gly-Pro-AMC prepared in D-PBS was added per well. The PBS without the Gly-Pro-AMC was used for the background subtraction. The plate was incubated for 10 minutes at 37 °C and the fluorescence ($\lambda_{excitation}$ = 350 nm, $\lambda_{emission}$ = 450 nm) was recorded, using a Synergy HTX plate reader (Bio-Tek, Winooski, VT). The DPP-IV inhibition was expressed as follows, after background subtraction:

$$DPP\text{-}IV\ Inhibition\ (\%) = \left(1 - \frac{RFU_{sample}}{RFU_{control}}\right) \times 100$$

where RFU_{sample} is the fluorescence of the samples and $RFU_{control}$ is the fluorescence of the control.

5.3.14 Faba Bean Peptides Fractionation and Sequencing by Mass Spectrometry

Based on the results of the *in vitro* and cell culture bioactivity assays, the 3 kDa permeates of the Fabelle digestates were further fractionated by size exclusion chromatography, as described in Section 5.3.6.1. The obtained fractions were freeze-dried and tested again for their antioxidant and antihypertensive activity. The peptides present in each fraction were identified by tandem mass spectrometry. To do so, the freeze-dried samples were solubilized in 5% (v/v) acetonitrile and 0.2% (v/v) formic acid and loaded onto a C18 precolumn (0.3 mm × 5 mm) followed by separation on a reversed-phase column (150 µm × 150 mm) with a linear gradient from 10% to 30% (v/v) acetonitrile and 0.2% (v/v) formic acid at a 600-nL/min flow rate for 56 min, using an Ultimate 3000 HPLC system (Eksigent, Dublin, CA, USA) connected to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Each of the full MS spectrum that was acquired at a resolution of 70,000 was followed by 12 tandem-MS (MS/MS) spectra on the

most abundant multiple-charged precursor ions. The tandem-MS experiments were performed using collision-induced dissociation at a relative collision energy of 27%.

All of the MS/MS data were analyzed using PEAKS Studio (Bioinformatics Solutions, Waterloo, ON, Canada; version 10.6). The PEAKS Studio search settings were: *Vicia faba* database (UniProt/SwissProt); nonspecific digestion enzyme; fragment ion mass tolerance of 0.0100 Da; parent ion tolerance of 10.0 ppm; carbamidomethylation of cysteine as a fixed post-translational modification and acetylation, oxidation, deamidation and phosphorylation as variable post-translational modifications

Scaffold (version 5.0.1, Proteome Software Inc., Portland, OR, USA) was used to validate the MS/MS-based peptide and protein identifications. The peptide identifications were accepted if they could be established at a greater than 95.0% probability by the Peptide Prophet algorithm (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) with Scaffold delta-mass correction. The protein identifications were accepted if they could be established at a greater than 99.0% probability and contained at least one identified peptide. The protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003).

The faba bean peptides identified by mass spectrometry were searched in the BIOPEP-UWM database to screen for potential bioactive properties.

5.3.15 Statistical Analysis

Each analysis was performed in triplicate and the results were expressed as mean ± standard deviation (SD). The data were analyzed through analysis of variance (ANOVA) ($p < 0.05$) followed by the Tukey's honest significant difference (HSD) post-hoc test ($p < 0.05$), using the XLSTAT software (Addinsoft, NY, USA) add-on to Microsoft Excel (Redmond, WA, USA) to determine the significant differences. The Pearson correlation coefficients were calculated to investigate the relationship between free radical scavenging capacities determined by different assays and the relationship between the antioxidant activities in the 3 kDa permeates of legume digestate and the total phenolic content (TPC).

5.4 Results and Discussion

5.4.1 Optimization of the *In vitro* Digestion Model for Subsequent Cell Culture Experiments

The impact of different *in vitro* digestion conditions on cell viability after incubation with the faba bean 3 kDa permeates was investigated, with the aim of determining the digestion conditions that enable acceptable cell viability, while maintaining as much as possible the standardized digestion conditions of the INFOGEST protocol. Three main digestion factors were considered: (1) the bile salts concentrations; (2) the intestinal enzymes used (pancreatin or trypsin, chymotrypsin and α-amylase, with or without the addition of a purified aminopeptidase to mimic brush border digestion) and (3) the protease inhibitor concentration. A two-way ANOVA table (Table 5-2) was generated to explore the impact of the three factors of interest, the peptide concentrations and their interaction on cell viability at the end of the incubation period.

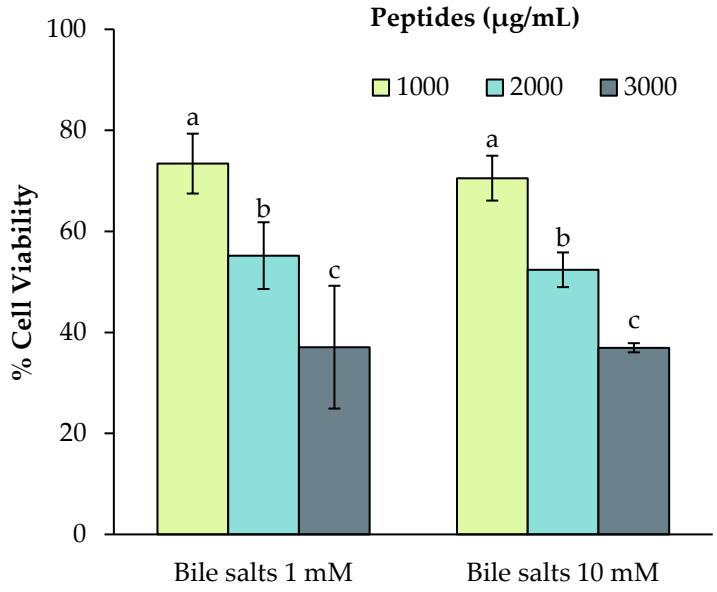
Table 5-2. Statistical significance of the studied digestion conditions and faba bean peptide concentration on cell viability as assessed by two-way ANOVA.

Factor	*p*-value [1]
Bile salt	
Bile salt concentration	0.285
Peptide concentration	**<0.0001**
Bile salt concentration × Peptide concentration	0.774
Intestinal enzyme	
Intestinal enzyme used	**<0.0001**
Peptide concentration	**<0.0001**
Intestinal enzyme used × Peptide concentration	**<0.0001**
Protease inhibitor	
Protease inhibitor concentration	**<0.0001**
Peptide concentration	**<0.0001**
Protease inhibitor concentration × Peptide concentration	**0.004**

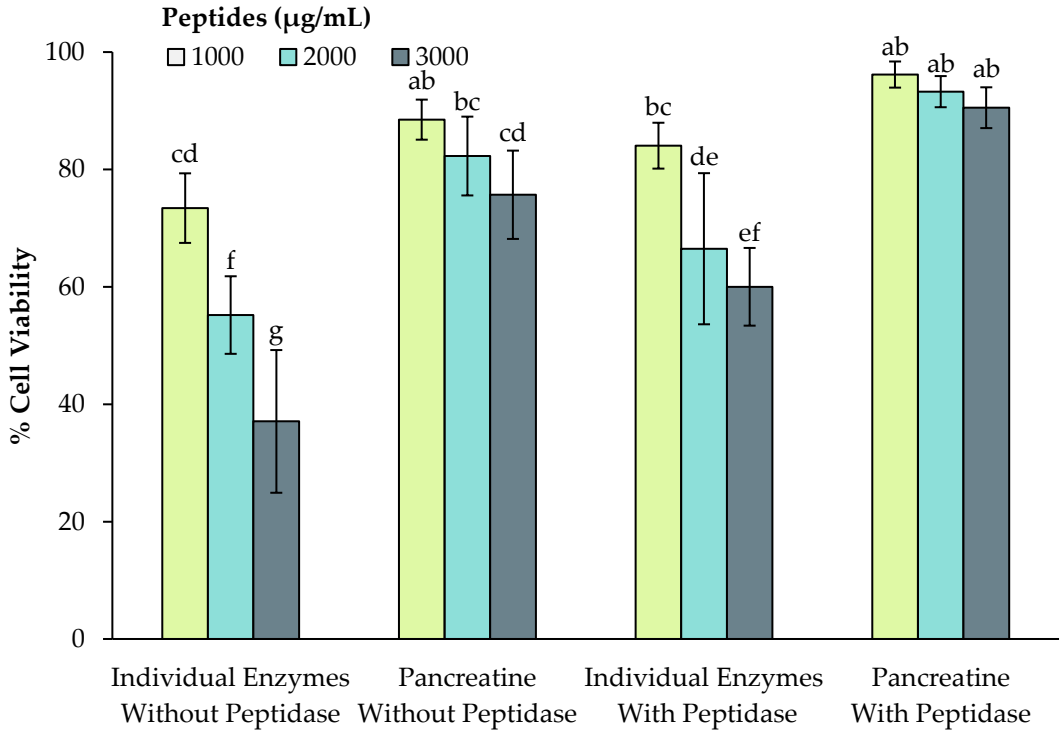
[1] Values significant at the 5% level are printed in bold-faced type.

The results revealed that the bile salt concentration had no significant impact on the cell viability in the range of the peptide concentrations tested (Figure 5-2). Moreover, the use of pancreatin extract instead of individual pancreatic enzymes (namely α-amylase, trypsin and chymotrypsin) improved significantly the cell viability ($p < 0.05$) (Figure 5-2). This result may be explained by the low purity of the porcine commercial pancreatic α-amylase used. Moreover, the pancreatic extract incorporates a broader range of pancreatic proteases (elastase, carboxypeptidase A and B), which is more physiologically relevant. The addition of the porcine intestinal peptidase to mimic brush border digestion improved the cell viability, to a value above 90% for the three peptide

concentrations tested. This effect could be explained by the increase in protein digestibility, leading to a better peptide and amino acid availability for cell growth and viability.



(**a**)



(**b**)

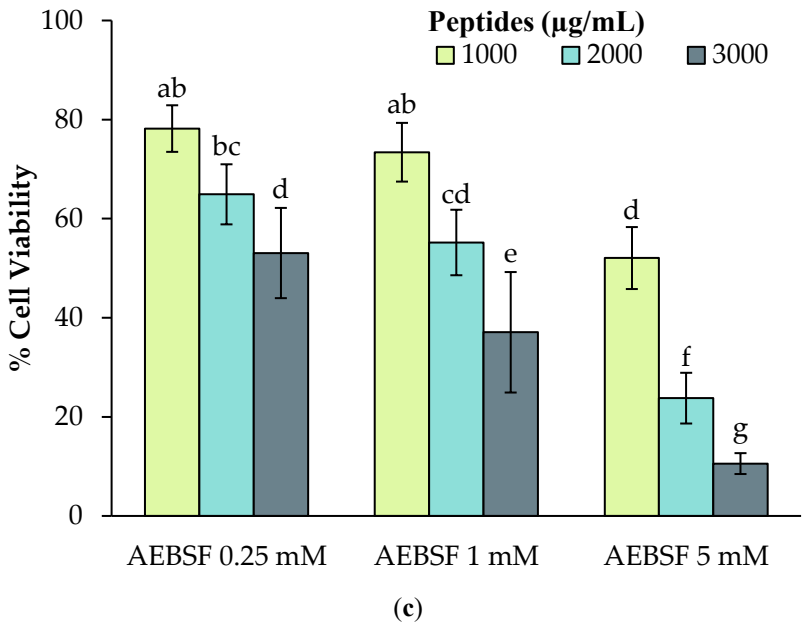


Figure 5-2. The impact of various concentrations (1000, 2000 and 3000 µg peptides/mL) of 3 kDa permeate of faba bean digestate (variety Fabelle) obtained in different digestion conditions on Caco-2 cell viability. The digestions were performed with different (**a**) bile salts concentrations; (**b**) different intestinal enzyme combinations; (**c**) different protease inhibitor (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)) concentrations. Data are expressed as mean ± standard deviation of three experiments and means without a common letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the Tukey's test.

The concentration of the protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), used to stop the digestion also had a significant effect on cell viability (Figure 5-2). AEBSF reacts with the hydroxyl group of the serine side chain residue, forming a sulfonyl cross-link (Narayanan & Jones, 2015), thereby irreversibly inactivating the serine proteases (Narayanan et al., 2015). Three doses of AEBSF were tested in the digestate: 0.25 mM (the recommended maximal dose for cell experiments (Akter et al., 2021)); 1 mM (the recommended dose in the first version of the INFOGEST protocol (Minekus et al., 2014)) and 5 mM (the recommended dose in the INFOGEST 2.0 protocol (Brodkorb et al., 2019)). The AEBSF dose was increased in the second version of the INFOGEST protocol version, to inactivate the residual protease activity that was found in the digestate samples (Egger et al., 2016). The use of the 0.25 mM and 1 mM AEBSF dose had a similar impact on the cell viability (except for the 3000 ug/mL peptide dose). However, the 5 mM AEBSF dose had a significant ($p < 0.05$) detrimental effect on the cell viability and is therefore not suitable for cell experiments. Since the residual protease activity was found in the digestate when the 1 mM AEBSF dose was used, we investigated whether the ultrafiltration step could remove these residual activities. Indeed, with 1 mM finale

concentration of AEBSF in the digestate, 3.96 mU of residual leucine-aminopeptidase activity per mL was measured in the digestate supernatant, and no remaining activity was measured in the 3 kDa permeate. Moreover, the peptide stability in the permeate was verified during the ultrafiltration step by quantifying the free amino groups using the 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) method. No significant differences in the free amino group content were observed (p = 0.113). Therefore, the use of AEBSF at a final concentration of 1 mM was retained. Denaturing conditions were not considered to inhibit digestive enzyme activity in order to preserve the native structure of proteins after digestion.

In light of these results, the use of 10 mM bile salt, pancreatin with the addition of a purified amino peptidase to mimic brush border digestion and 1 mM AEBSF during *in vitro* digestion were retained as well-suited conditions to enable the cell model assays, while maintaining the physiological conditions of protein digestion. The cell viability was measured after incubation with the 3 kDa permeates of each legume digestate that were obtained at these optimal digestion conditions (Figure 5-3). No significant differences were found in cell viability for all of the legumes at the three different tested peptide concentrations.

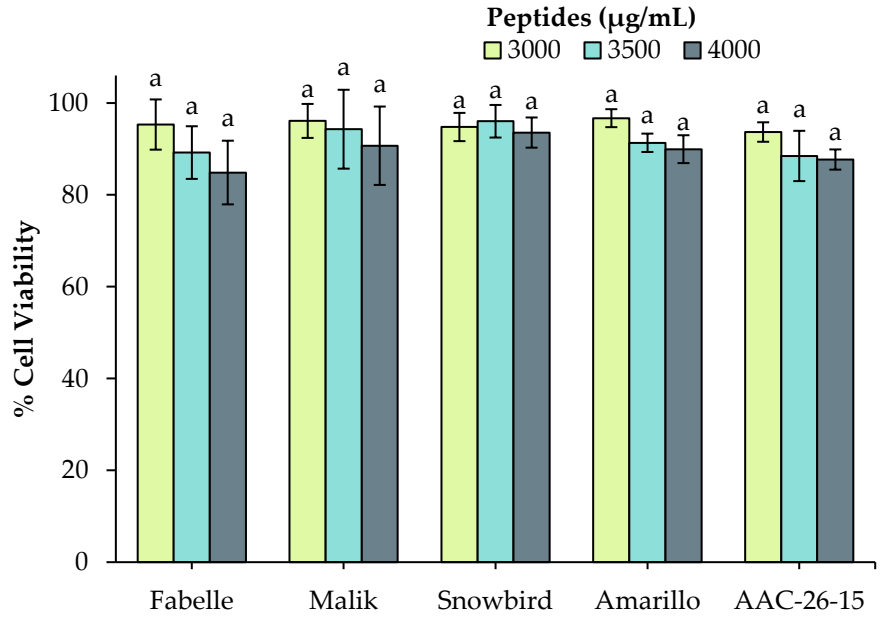
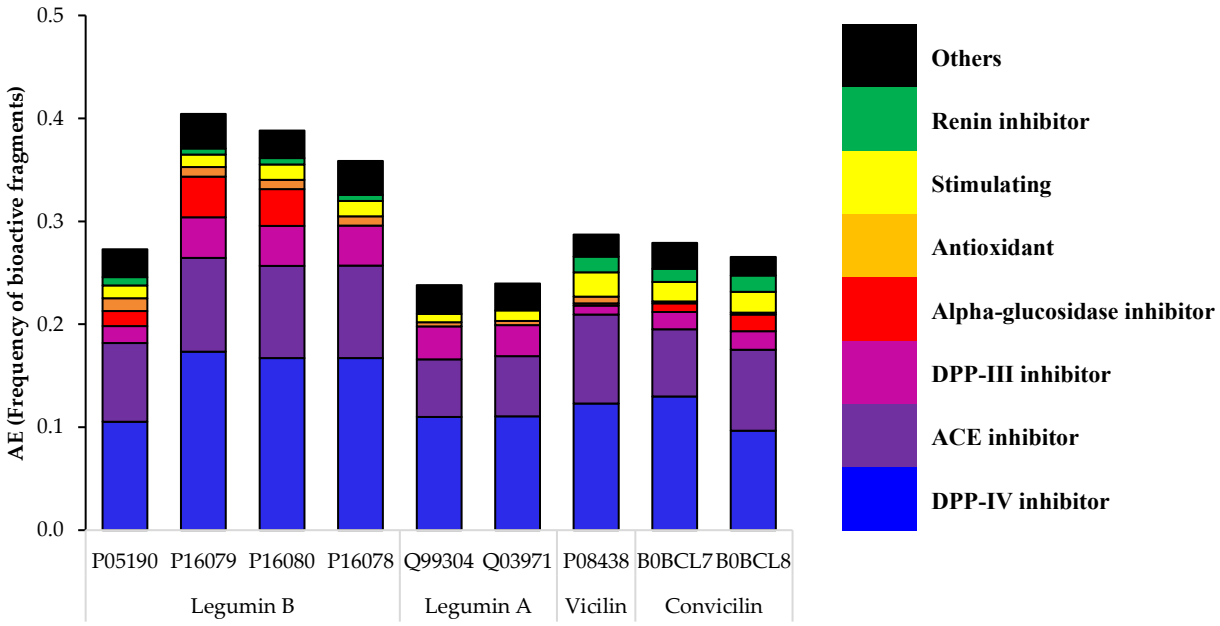


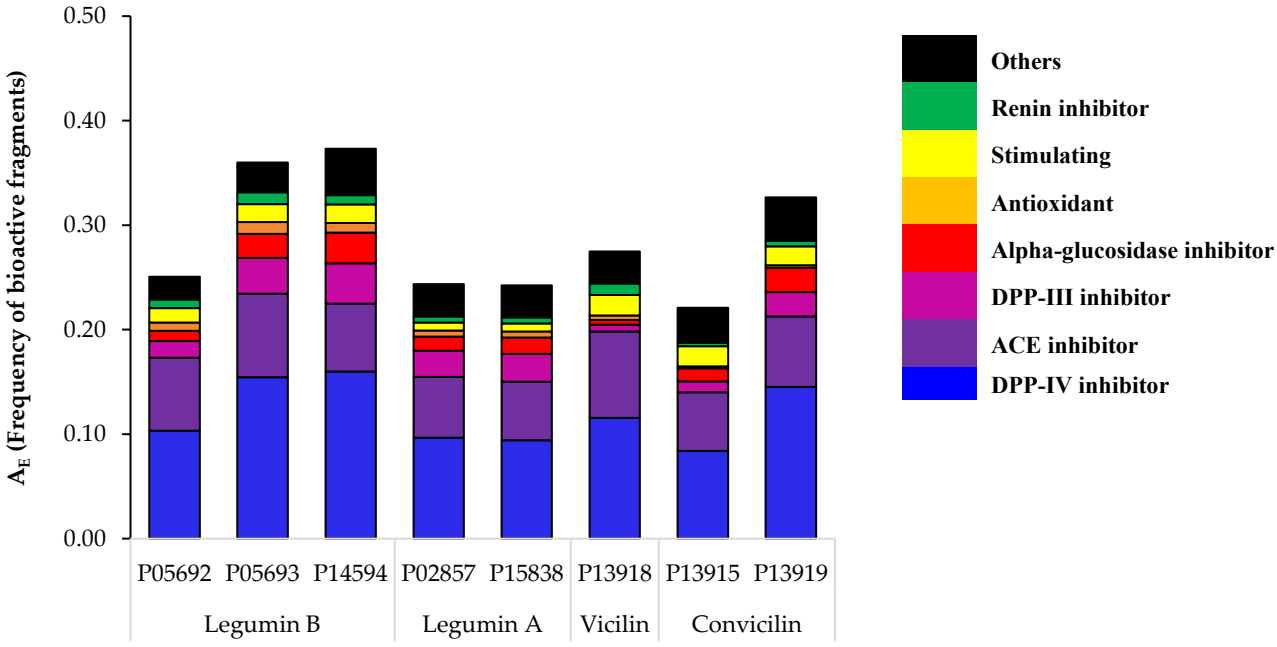
Figure 5-3. Impact of different concentrations (3000, 3500 and 4000 µg peptides/mL) of 3 kDa permeate of legume digestates obtained in the optimized digestion conditions (AEBSF 1 mM, bile salt 10 mM, pancreatin and peptidase) on cell viability. Data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the Tukey's test.

5.4.2 2.2. *In silico* Prediction of Bioactive Fragments Released during Gastrointestinal Digestion of Legume Proteins

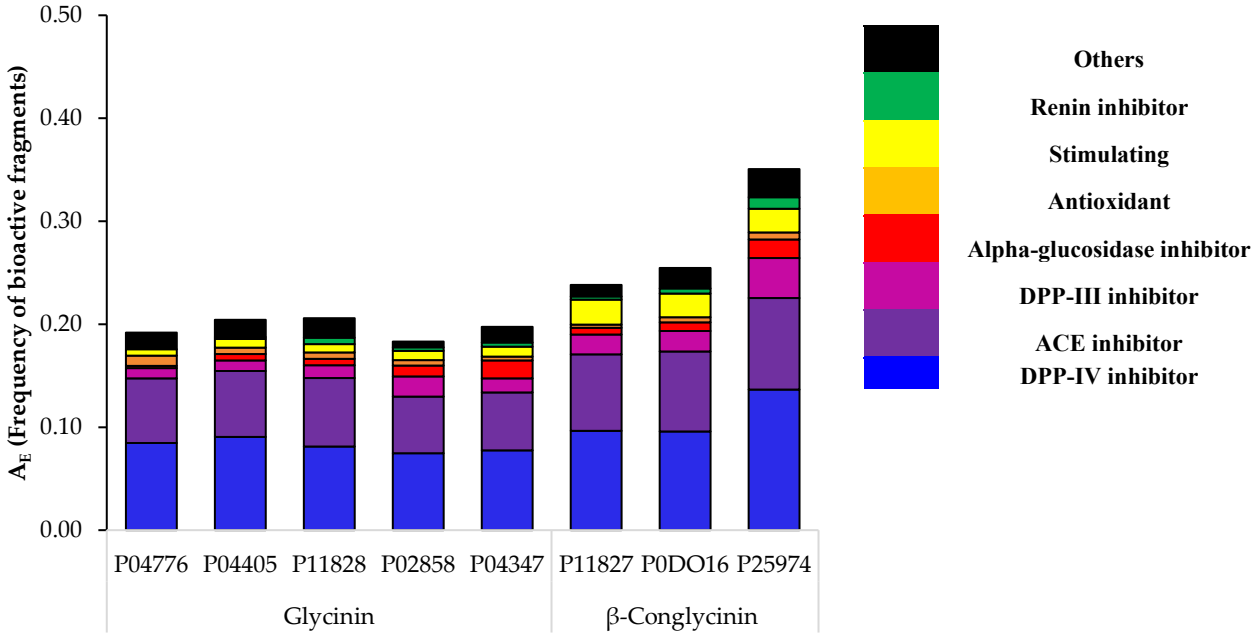
An *in silico* analysis was used as a first screening tool to investigate the potential bioactive fragments released during the gastrointestinal digestion of the faba bean, pea and soy main storage proteins. As shown in Figure 5-4, the bioactive fragments' frequency (A_E) pattern was quite similar for the three legumes, which can be explained by protein sequence similarities. For faba beans and peas, Legumin B had the highest frequency of bioactive fragments, and for soy it was β-Conglycinin. The Dipeptidyl Peptidase-IV (DPP-IV) and the Angiotensin-Converting Enzyme (ACE) inhibitor were the most frequent fragments released by the selected enzymes for the three legumes. The antioxidant fragments were also present, but in a smaller proportion. It is important to note that certain peptide bioactive properties were more studied in the literature than others (M. Dziuba & Dziuba, 2010), therefore more peptide sequences with those given activities have been ascertained. When the analysis was conducted, there were 1084 ACE inhibitor, 772 antioxidant and 432 DPP-IV inhibitory fragments out of 4485 peptides listed in the Bioactive Peptide Database of University of Warmia and Mazury (BIOPEP-UWM database). The fact that less DPP-IV inhibitory fragments are listed, while they are the most frequent fragments found in the three legumes' globulins, suggest that this is a promising bioactivity to study further. Interestingly, the frequency of the antioxidant fragments is similar to that of the more rarely studied bioactivities, such as stimulating (87), Dipeptidyl Peptidase-III (DPP-III) inhibitor (66), alpha glucosidase inhibitor (34) and renin inhibitor (41). Nonetheless, one needs to consider that the A_E parameter is an indication of the bioactive fragment frequency and not the bioactivity intensity. Experimental work is therefore needed to complement those results and target the most promising bioactivities. Besides, the *in silico* hydrolysis pattern is only based on the protein primary structure, and thus the bioactive fragments obtained experimentally are likely to differ to some extent.


AE (Frequency of bioactive fragments)
0.5
0.4
0.3
0.2
0.1
0.0
P05190 P16079 P16080 P16078 Q99304 Q03971 P08438 B0BCL7 B0BCL8
Legumin B
Legumin A
Vicilin
Convicilin
Others
Renin inhibitor
Stimulating
Antioxidant
Alpha-glucosidase inhibitor
DPP-III inhibitor
ACE inhibitor
DPP-IV inhibitor

(a)


A_E (Frequency of bioactive fragments)
0.50
0.40
0.30
0.20
0.10
0.00
P05692 P05693 P14594 P02857 P15838 P13918 P13915 P13919
Legumin B
Legumin A
Vicilin
Convicilin
Others
Renin inhibitor
Stimulating
Antioxidant
Alpha-glucosidase inhibitor
DPP-III inhibitor
ACE inhibitor
DPP-IV inhibitor

(b)



(**c**)

Figure 5-4. *In silico* prediction of bioactive fragments released during gastrointestinal digestion of (**a**) faba bean; (**b**) pea; (**c**) and soy main storage proteins.

Other interesting bioactivities were also present in the faba bean, pea and soy globulins, including alpha-glucosidase inhibitor, renin inhibitor and stimulating fragments. These fragments contribute to the overall antihypertensive and antidiabetic potential of the globulins. The renin is the first enzyme of the RAS system that catalyzes the conversion of angiotensinogen into angiotensin 1 (Peach, 1977), while the alpha-glucosidase is an enzyme of the intestinal brush border that is responsible for the digestion of the oligosaccharides into absorbable monosaccharides. The alpha-glucosidase inhibitors are used in diabetes treatment to delay carbohydrate intestinal digestion and reduce glucose uptake (Bischoff, 1995). The "stimulating" bioactivity is a very broad category that included any peptide that stimulates biological processes that do not yet have a category of their own. Among the bioactive fragments identified in this study, the fragments were found that stimulate glucose uptake and release of vasoactive substances.

The DPP-III inhibitory fragments were also found. DPP-III is a peptidase widely distributed in the human body, that is responsible for opioid peptides' (enkephalins) degradation (Kumar et al., 2016). The DPP-III inhibitors have therefore excellent therapeutic potential in pain management. DPP-III's overall physiological role is not yet fully understood, but its role in cancer, oxidative

stress defense (Kumar et al., 2016) and blood pressure regulation (Pang et al., 2016) have been listed.

In the light of these results, the antidiabetic, antihypertensive and antioxidant activities were selected to be further investigated, as these bioactivities are associated with the health benefits of foremost importance.

5.4.3 Characterization of the 3 kDa Permeate of Legume Digestates

In this study, the *in vitro* gastrointestinal digestates were filtered on a 3 kDa membrane to collect the small molecular weight peptides. It is well established that the peptide bioactivity potency is highly affected by the amino acid chain length (Acquah, Chan, Pan, Agyei, & Udenigwe, 2019), and that the peptides with the highest activity are usually composed of 2–20 amino acids (Sánchez et al., 2017). The efficiency of the ultrafiltration step to remove the remaining large soluble proteins was verified by size exclusion HPLC (Figure 5-5). The size exclusion HPLC analysis of the faba bean (Fabelle variety) digestate before and after the 3 kDa cut-off ultrafiltration demonstrates the removal of the high molecular weight proteins and polypeptides (>17 kDa). These could be the remaining digestive enzymes and the undigested faba bean proteins. Moreover, the antihypertensive activity of the faba bean digestate shows an important increase after ultrafiltration, as demonstrated by the significant decrease ($p < 0.05$) in the ACE inhibition IC_{50} (Figure 5-5). This indicates that the 3 kDa cut-off ultrafiltration is a valuable approach to recover most of the potent bioactive peptides.

The size exclusion HPLC profiles of the five legumes' 3 kDa permeates are shown in Figure 5-6. There were no noticeable differences among the faba beans, pea and soy profiles. All of the patterns were composed of three main peaks with retention times of ~22.5, 26.5 and 36.5 minutes. The first peak had a retention time overlapping the carnosine (~226 Da) and Gly-Gly-Gly (~189 Da). This peak is likely to correspond with the small peptides with molecular weights in the same range as these standards. The two other peaks had higher retention times than the dipeptide carnosine standard, therefore probably corresponding to the amino acid residues.

Since the peptides are not the only potential bioactive constituent of the legume digestates found in the targeted molecular weight range (<3 kDa), a composition analysis of the 3 kDa permeates was performed (Table 5-3). The peptides accounted for 34.4–38.4%, 32.9% and 54.5% of the 3 kDa permeate content for the faba bean, pea and soy, respectively, while the carbohydrates

accounted for 43.5–45.2, 46.7 and 16.6 g of the glucose equivalent/g, respectively. Similar concentrations of the total polyphenols, ranging from 4.43 mg to 4.97 mg of gallic acid equivalent/g were found in all of the three studied legumes permeate. The observed differences in the carbohydrate and protein contents among the faba bean, pea, and soy 3 kDa permeate is most probably related to the differences in the seeds' composition. The polyphenols and carbohydrates also reported bioactive properties (Al Shukor et al., 2013; H. Chen, Liu, Zhu, Xu, & Li, 2010; Endringer, Oliveira, & Braga, 2014; Martínez-Villaluenga & Frías, 2014; Singh, Singh, Kaur, & Singh, 2017; Y. Zhang, Pechan, & Chang, 2018), and could therefore also contribute to the exhibited 3 kDa permeates bioactivities.

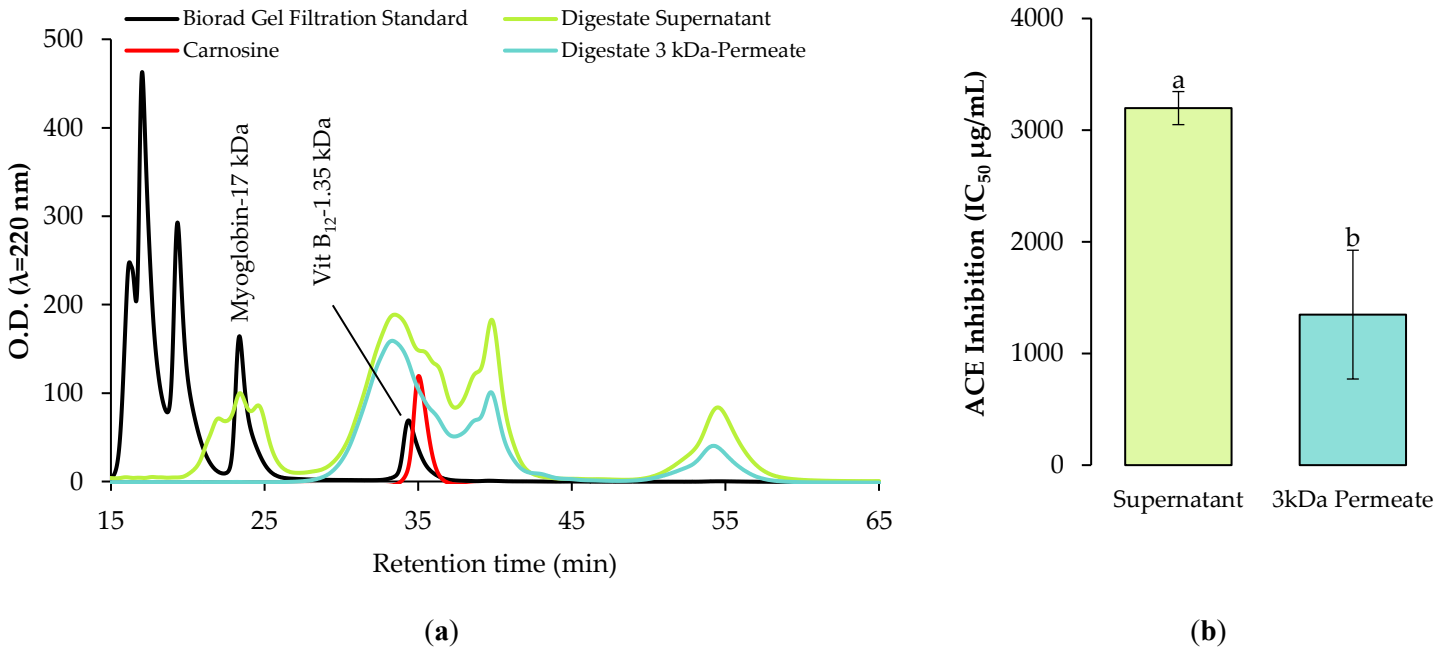


Figure 5-5. (**a**) Molecular weight distribution of peptides; and (**b**) exhibited antihypertensive activity of the faba bean digestate (variety Fabelle) before and after 3 kDa cut-off ultrafiltration. The ACE inhibition data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

Table 5-3. Composition analysis of the 3 kDa permeate of legume digestates.

	% Proteins	Total Polyphenol (mg. Gallic Acid Equivalent/g) (Dry Base)	Total Carbohydrates (g Glucose Equivalent/100 g)
Fabelle	34.37 ± 0.22 [b]	4.81 ± 0.19 [a]	43.5 ± 1.6 [b]
Malik	36.11 ± 0.47 [c]	4.97 ± 0.25 [a]	44.0 ± 2.0 [b]
Snowbird	38.42 ± 0.27 [d]	4.80 ±0.31 [a]	45.2 ± 0.5 [b]
Amarillo	32.87 ±0.17 [a]	4.43 ±0.17 [a]	46.7 ± 1.5 [b]
AAC-26-15	54.49 ± 0.54 [e]	4.81 ±0.51 [a]	16.6 ±0.8 [a]

Data are expressed as mean ± standard deviation and means in a column without a common letter differ (p <0.05) as analyzed by one-way ANOVA and the Tukey's test.

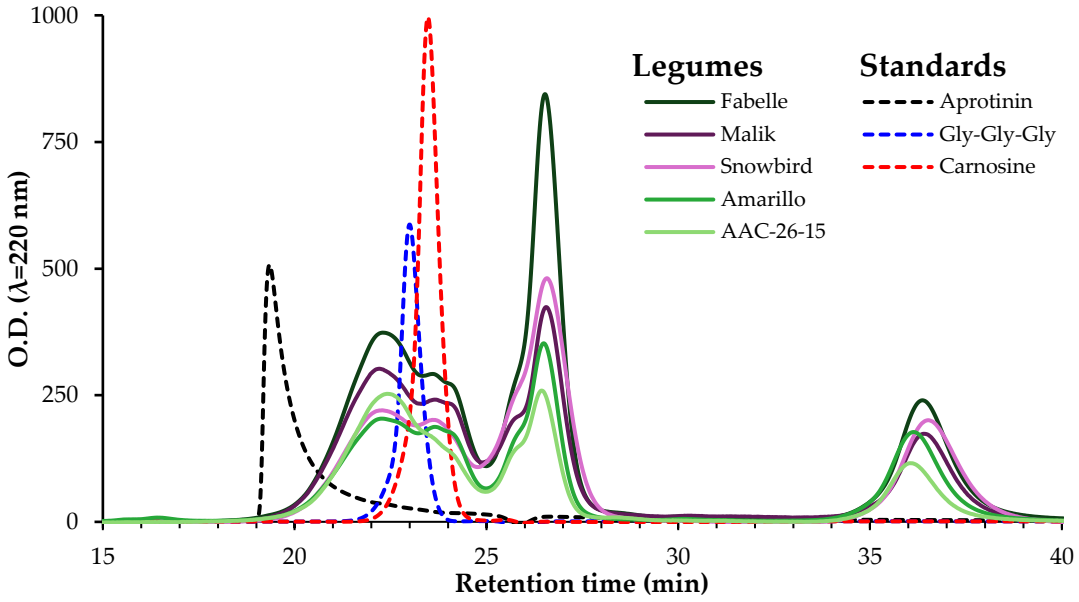


Figure 5-6. Molecular weight distribution of peptides in the 3 kDa permeate of legume digestates as assessed by size exclusion HPLC.

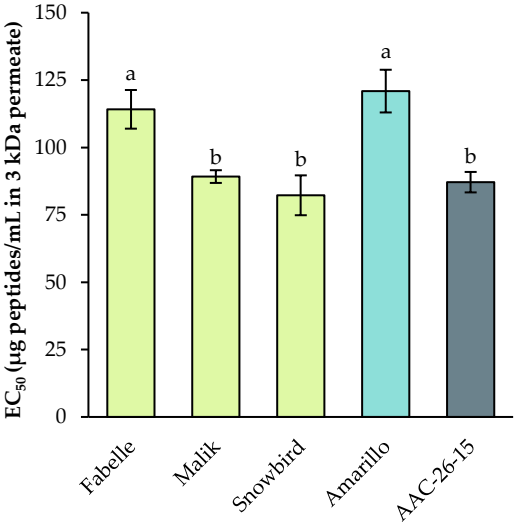
5.4.4 Bioactive Properties of the 3 kDa Permeate of Legume Digestates

5.4.4.1 Antioxidant and Chelating Activities

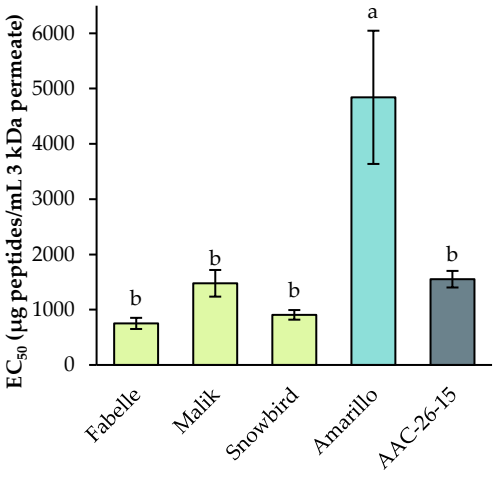
Various assays, based on different mechanisms of action, were used to assess the antioxidant activity of the 3 kDa permeate of the legume digestates (Kedare & Singh, 2011; Moniruzzaman, Khalil, Sulaiman, & Gan, 2012; Re et al., 1999; Watanabe et al., 2012). Indeed, the antioxidants can quench free radicals either through single electron transfer (SET), hydrogen atom transfer (HAT) or a combination of both (Liang & Kitts, 2014). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay are free-radical quenching assays that are mainly SET-based (Gulcin, 2020; Zhong & Shahidi, 2015). On the other hand, the oxygen radical absorbance capacity (ORAC) assay is HAT-based. The antioxidants can also prevent the free radicals' formation through the chelation of transition metal ions that act as

catalysts in free radicals' formation, such as the Fenton reaction (Santos, Alvarenga Brizola, & Granato, 2017; Wang, Hu, Nie, Yu, & Xie, 2016). The iron chelating assay measures this preventive capacity of antioxidants.

In the four antioxidant assays conducted *in vitro*, no significant differences were found among the faba beans varieties (Figure 5-7), except for the ABTS assay in which the Fabelle variety had a significantly lower activity ($p < 0.05$). Overall, the faba bean varieties had either a lower or similar antioxidant power compared to the soy, and a similar or higher antioxidant power than the pea. In the DPPH assay, the EC_{50} of the peas was significantly higher than the soy and faba beans ($p < 0.05$), suggesting a lower antioxidant activity. In the ABTS assay, the same tendency was observed, except for the Fabelle variety, which had a similar EC_{50} than the peas and a higher EC_{50} than the Malik, Snowbird and soy. In the ORAC assay, a different outcome was observed, where the antioxidant activity of the soy was higher compared to the faba beans and peas. Besides, there was a significant correlation between the DPPH and ABTS assay results ($r = 0.522$; $p = 0.046$), but not with the ORAC assay, which is expected due to its different mechanism of action. For the iron chelating assay, there were no significant differences between the EC_{50} of the faba beans and peas. However, the EC_{50} of the soy was significantly lower ($p < 0.05$), which suggest a higher chelating activity.



(**a**)



(**b**)

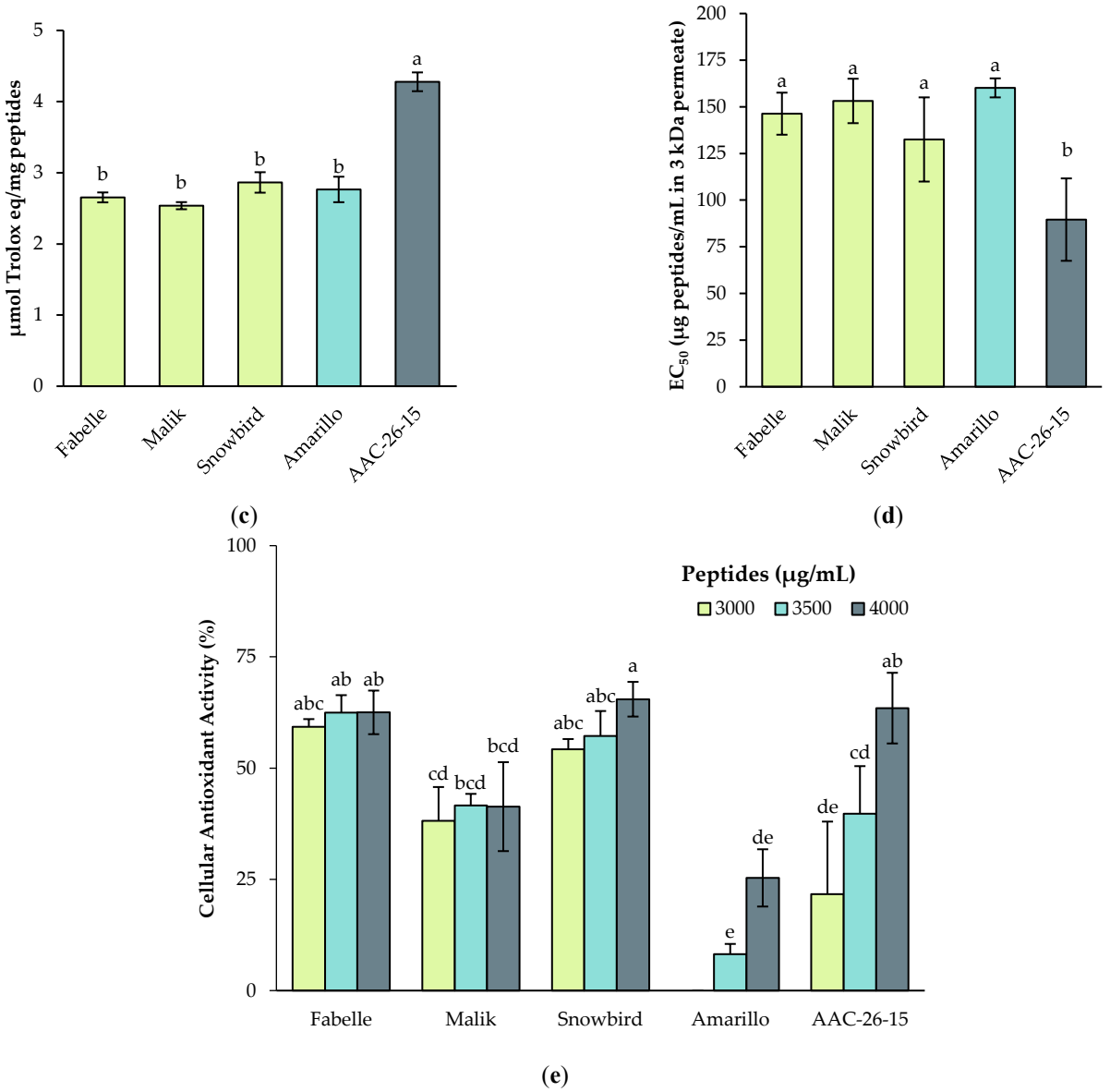


Figure 5-7. Antioxidant activity of the 3 kDa permeate of legume digestates as assessed by (**a**) the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay; (**b**) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay; (**c**) the oxygen radical absorbance capacity (ORAC); (**d**) the iron chelating assay; and (**e**) the cellular antioxidant assay (CAA). Data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

The ABTS EC_{50} value obtained for the studied faba bean varieties were lower than the reported values by Jakubczyk et al. (2019) for a 3 kDa permeate of an *in vitro* gastrointestinal digestate of fermented faba bean seeds. In their study, the EC_{50} value ranged from 990 to 3510 µg/mL, depending on the fermentation conditions used, whereas in this study the EC_{50} varied from 82 to 114 µg/mL. For the iron chelating activity, the EC_{50} obtained by Parya Samaei et al. (2020) for the faba bean protein hydrolyzed with Alcalase, pepsin and/or trypsin were lower (EC_{50} = 3.50–8.62

μg/mL) than the levels obtained in this study (EC_{50} = 132–153 μg/mL). The DPPH assay results were in the same order of magnitude as the results obtained by Karkouch et al. (2017) for the peptide fractions separated by strong cation-exchange chromatography obtained from a faba bean trypsin hydrolysate. The DPPH scavenging ranged from 40 to 85% when the peptide fractions were tested at 1000 μg/mL whereas, in this study, 50% DPPH scavenging was obtained at concentrations varying from 752 to 1477 μg/mL. Nonetheless, the disparities in the faba bean varieties, sample digestion conditions and antioxidant assay procedures make comparisons difficult between studies.

The antioxidant activity of the 3 kDa permeates was also assessed, using a cellular model to evaluate the antioxidant activity in a more physiologically relevant manner. The cellular antioxidant assay considers diverse mechanisms, including direct free-radical quenching and the stimulation of antioxidant enzyme actions, in experimental conditions that take into account cell uptake and metabolism (Kellett et al., 2018; Wolfe & Liu, 2007). The Caco-2 cell line was used, which is a well-established model of small intestine enterocytes. At the highest peptide concentration tested (4000 μg/mL), the peas had a lower antioxidant activity compared to the soy and faba beans ($p < 0.05$), as shown by a lower Cellular Antioxidant Activity (CAA) value. Among the faba bean varieties, Snowbird had a significant higher CAA value compared to Malik. For the faba bean varieties, the CAA value was similar for the three concentrations tested, which suggest that these concentrations were in the upper-plateau of the dose–response curve. However, for the soy and pea, the CAA values significantly decreased with concentration and reached no effect in the case of the peas at the lowest dose tested. At the lowest peptide concentration tested (3000 μg/mL), the CAA value was significantly higher for the Fabelle and Snowbird variety compared to the soy and pea. At the same concentration, the CAA value of the Malik variety was similar to the soy but higher than pea. Those results suggest that at lower doses, the antioxidant effect of the faba bean peptides is higher compared to the pea and soy peptides. This outcome is slightly different than the results observed with the *in vitro* assays, where the soy had overall better antioxidant activity compared to the faba beans. This effect could possibly be explained by a better bioavailability and/or a better resistance to cell metabolism of the faba bean peptides compared to the pea and soy peptides. It is also possible that the faba bean-derived peptides intervene in cell signaling antioxidant pathway, such as the activation of the nuclear factor erythroid 2–related factor 2 (Nrf2) (Ma, 2013). Further research will be needed to investigate this hypothesis. It is also

possible that the faba bean peptides had a better activity in this particular assay. The dissimilarities between *in vitro* antioxidant assays and CAA results have been observed by Wan et al. (2015) as well. They found that the *in vitro* ORAC and CAA results were not in good agreement for numerous purified phytochemicals. However, there was a strong correlation between CAA and *in vivo* ORAC results (ORAC values of rat plasma after the intake of antioxidants), which state that the CAA result was a better indicator of *in vivo* antioxidant effect.

To the best of our knowledge, this is the first time that the cellular antioxidant activity of the faba bean hydrolysate have been assessed. More generally, the data relative to the cellular antioxidant activity of a complex food hydrolysate are very limited. Chen et al. (2019) have measured the cellular antioxidant activity of the 10-kDa permeate of common bean milk and yogurt *in vitro* gastrointestinal digestates in Caco-2 cells and have obtained CAA values ranging from 25 to 35%, depending on the doses tested (0.1–0.5 mg/mL). Torres-Fuentes, Contreras, Recio, Alaiz, and Vioque (2015) have obtained CAA values ranging from 10 to 30% for chickpea proteins hydrolyzed with pepsin and pancreatin, depending on the dose tested (0.5–5 mg/mL). Zhang, Noisa, and Yongsawatdigul (2020) investigated the cellular antioxidant activity of the *in vitro* gastrointestinal digestate of tilapia protein and tilapia protein hydrolysate prepared with Alcalase in HepG2 cells. They obtained CAA values varying from 30 to 50%, depending on the doses tested (0.1–5.0 mg/mL) and the hydrolysate preparation conditions. The CAA values obtained in this study for the Fabelle and Snowbird faba bean varieties (~60%) are higher than the reported values for common bean milk/yogurt and tilapia proteins, while the CAA values for the Malik variety were in the same range (~40%). Nonetheless, the differences in the experimental conditions for the *in vitro* gastrointestinal digestion, as well as the cell culture conditions, make comparisons quite difficult from one study to another.

The antioxidant effects of the legume 3 kDa permeates are possibly caused by a synergistic effect of the peptides and other bioactive constituents. For instance, the polyphenols have well-established free radical scavenging and chelating activities (Chaieb, González, López-Mesas, Bouslama, & Valiente, 2011). However, our results did not show any significant correlations between the total polyphenol content (TPC)/g of the proteins in the 3 kDa permeate of the legume digestate and the DPPH and ABTS scavenging activity and the CAA values. Moreover, there was a significant inverse correlation between the TPC content/g of proteins and the iron chelating activity ($p < 0.0001$) and the ORAC score ($p = 0.00037$), meaning that the lowest TPC content/g

of the proteins in the 3 kDa permeates lead to the highest antioxidant activity. Since the TPC content was similar among the five studied legumes, it can be inferred that the bioactive peptides are possibly responsible in a larger proportion for the observed differences in the antioxidant effect among the samples.

5.4.4.2 Antihypertensive Activity

The antihypertensive activity of the legume 3 kDa permeates was evaluated by means of the Angiotensin-Converting Enzyme (ACE) inhibition assay (Figure 5-8). There were no significant differences for the ACE inhibition activity among the faba bean varieties. The soy had a significantly lower IC_{50} compared to the faba beans and peas, which means a higher antihypertensive activity. The Fabelle and Malik varieties had a significantly higher antihypertensive activity compared to the pea, but the Snowbird variety was similar to the pea. The obtained IC_{50} for the faba beans were in the same range as the values obtained by Jakubczyk et al. (2019) for a 3 kDa permeate of an *in vitro* gastrointestinal digestate of fermented faba bean seeds. In their study, the IC_{50} varied from 1010 to 2920 ug/mL, depending on the fermentation condition used, whereas in this study the values varied from 1348 to 1884 µg/mL. Dugardin et al. (2020) obtained a lower IC_{50} value for an *in vitro* gastrointestinal digestate of faba bean protein isolate (IC_{50} = 52 ug/mL), which can be explained by experimental conditions disparities, such as the lower ACE unit used in their study (0.05 U/mL) compared to ours (0.8 U/mL). Moreover, the faba bean protein isolate could possibly have a higher digestibility rate than the faba bean flour, leading to a higher content of the smaller and highly bioactive peptides.

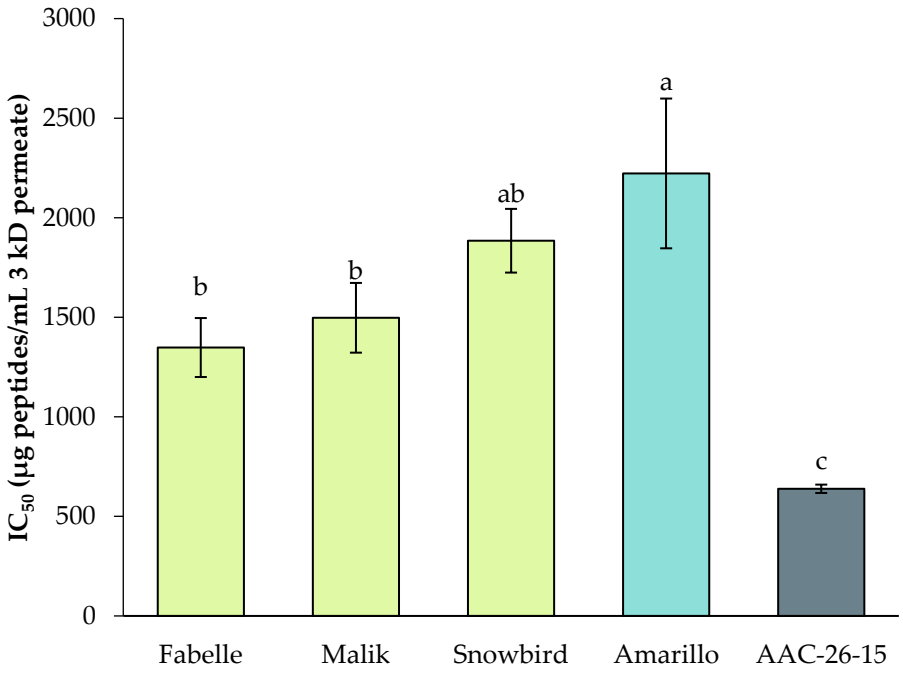


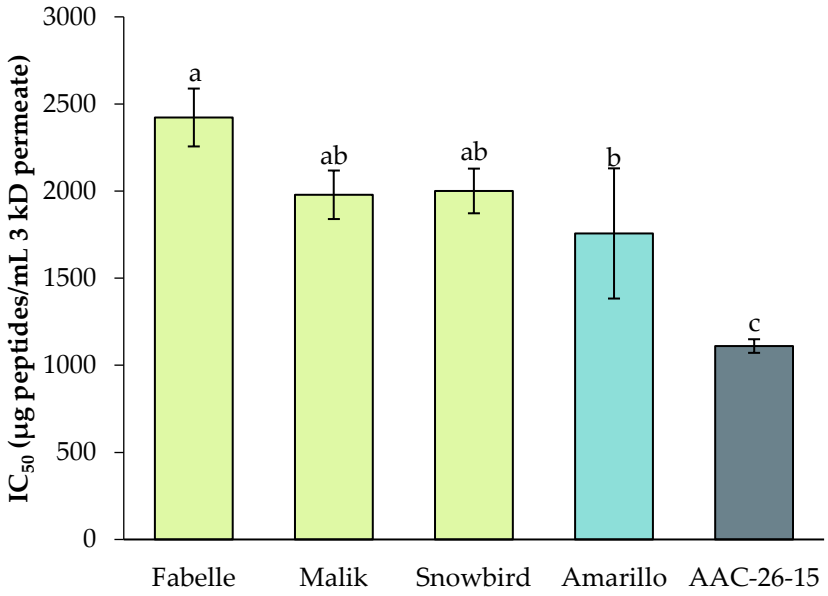
Figure 5-8. Antihypertensive activity (Angiotensin-Converting Enzyme inhibition) of the 3 kDa permeate of legume digestates. Data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

5.4.4.3 Antidiabetic Activity

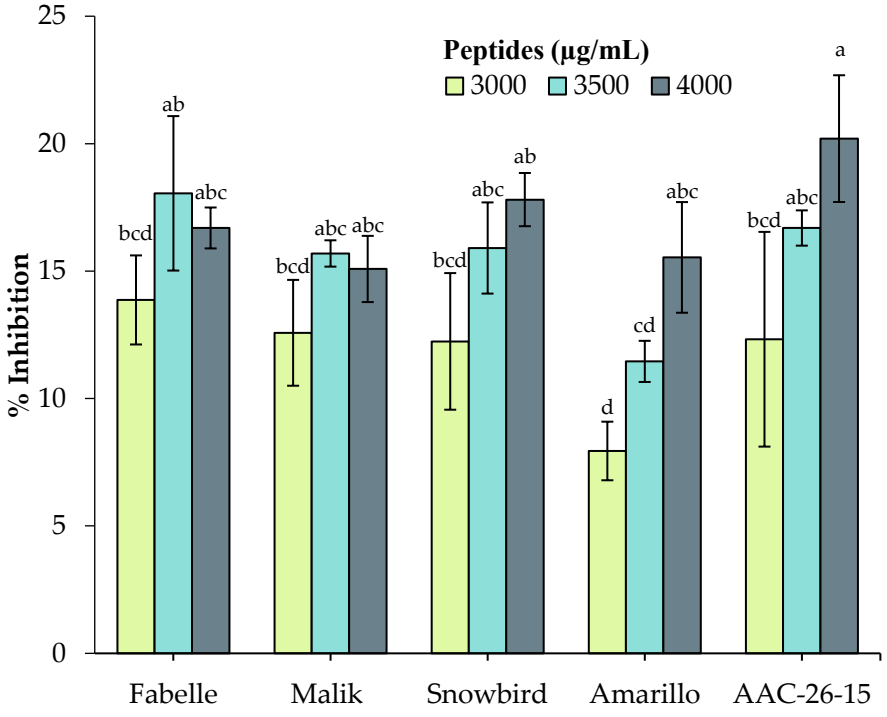
The antidiabetic activity of the legume 3 kDa permeates was assessed, using both *in vitro* and cellular Dipeptidyl Peptidase-IV (DPP-IV) inhibition assays. DPP-IV is a peptidase with multiple functions in the body and is found in the intestinal enterocytes' membrane, among others. The Caco-2 cells that express DPP-IV are therefore a relevant model to study the inhibitory potential of the dietary bioactive peptides. In addition, they have the advantage of mimicking the cell metabolism and proteolysis that occur at the brush border, with a cell viability and experimental conditions that are closer to the physiological context (Aiello, Li, Boschin, Bollati, Arnoldi, & Lammi, 2019; Caron et al., 2017; Lammi, Bollati, Ferruzza, Ranaldi, Sambuy, & Arnoldi, 2018; C. Zhang, Liu, Chen, & Luo, 2018).

In the *in vitro* assay, the IC_{50} of the soy was significantly lower compared to the faba beans and peas, which demonstrate a higher antidiabetic potency (Figure 5-9). This result followed the same trend as the ACE inhibition assay, in which the soy IC_{50} was the lowest compared to the faba beans and peas. There were no significant differences among the faba bean varieties. However, the Fabelle variety had a significantly higher IC_{50} compared to the peas and therefore a lower antidiabetic activity. Dugardin et al. (2020) obtained a lower IC_{50} value for an *in vitro*

gastrointestinal digestate of faba bean protein isolate (IC_{50} = 540 ug/mL) compared to this study, where the IC_{50} for the faba beans varied from 1979 to 2400 µg/mL. As above, this variation can be explained by the differences in the sample nature (faba bean flour versus protein isolate) and the digestion conditions. For instance, the gastrointestinal digestion procedure of Dugardin et al. (2020) included only a gastric and duodenal digestion phase, whereas ours included an additional jejunal–ileal digestion phase. The addition of this last digestion phase mimics more closely the physiological conditions of protein digestion, and is likely to have an impact on the peptide profile found in the digestate (Mamone, Picariello, Ramondo, Nicolai, & Ferranti, 2019).



(**a**)



(**b**)

Figure 5-9. Antidiabetic activity (Dipeptidyl-Peptidase-IV inhibition) of the 3 kDa permeate of legume digestates: (**a**) *in vitro* assay with a purified Dipeptidyl-Peptidase-IV from porcine kidney; (**b**) inhibition assay in a cellular model. Data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

In the cell model (Figure 5-9), there were no significant differences among the faba beans, peas and soy. However, as observed in the Cellular Antioxidant Assay, there was a dose–response effect observed for peas and soy; the DPP-IV inhibition effect was significantly lower at 3000 µg/mL compared to 4000 µg/mL, and there were no significant differences among the studied faba bean protein concentrations. Again, this effect may be explained by a better bioavailability and/or better resistance to the brush border peptidase degradation of the faba bean peptides compared to the pea and soy peptides. This hypothesis deserves further investigation.

Nonetheless, the percentage of DPP-IV activity inhibition in the cell model remains lower compared to the *in vitro* results. In the cell-based assay, the percentage of activity inhibition ranged from 15 to 20% at 4000 µg/mL, versus 68 to 90% in the *in vitro* assay at the same concentration. The same trend was observed by Caron et al. (2017), where the IC_{50} of a bovine hemoglobin gastrointestinal digestate was 10 times higher in the cell model (16.02 mg/mL) compared to the *in vitro* assay (1.62 mg/mL), meaning that the activity was ten times lower in the cell assay compared to the *in vitro* assay. Aiello et al. (2019) also found the same tendency for a spirulina protein hydrolysate. Lacroix and Li-Chan (2015) demonstrated that porcine DPP-IV was more easily inhibited by the peptides as compared to the human DPP-IV, which can explain this finding. This observation may also be explained by a further hydrolysis of the bioactive peptides once incubated with Caco-2 cells, which express numerous peptidases of the intestinal brush border. This finding reasserts the importance of investigating the bioactive properties with cell models and not exclusively with *in vitro* assays, to obtain a more realistic picture of the potential bioactivities *in vivo*.

5.4.5 Peptides Fractionation and Sequencing

Based on the results of the *in vitro* and cellular bioactivity assays, the Fabelle variety was selected to be investigated further, since it stood out for its antihypertensive and antioxidant activities. Moreover, this new variety contains a low amount of the anti-nutrients vicine, convicine and tannins, which represents an important advantage for food applications. The Fabelle 3 kDa permeate was fractionated by size exclusion HPLC and three peptide fractions were recovered (Figure 5-10). The three collected fractions were tested again for their antioxidant and antihypertensive activities. The results indicated that F1 had the highest antihypertensive potency, followed by F2 and F3 (Figure 5-11). F2 had the highest free radical scavenging activity through SET, as demonstrated by the results of the ABTS assay, while F2 and F3 were equal for their free

radical scavenging activity through HAT, as indicated by the results of the ORAC assay (Table 5-4). The F3 was the fraction with the highest iron chelating activity (Table 5-4).

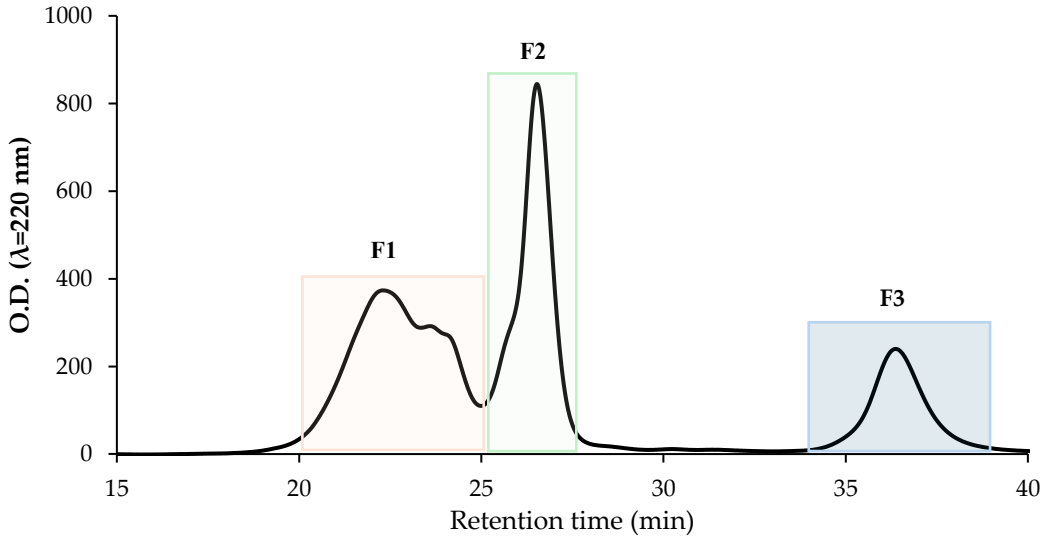


Figure 5-10. Peptide fractionation of the 3 kDa permeate of Fabelle *in vitro* gastrointestinal digestate by size exclusion HPLC. F1, fraction 1; F2, fraction 2; F3, fraction 3.

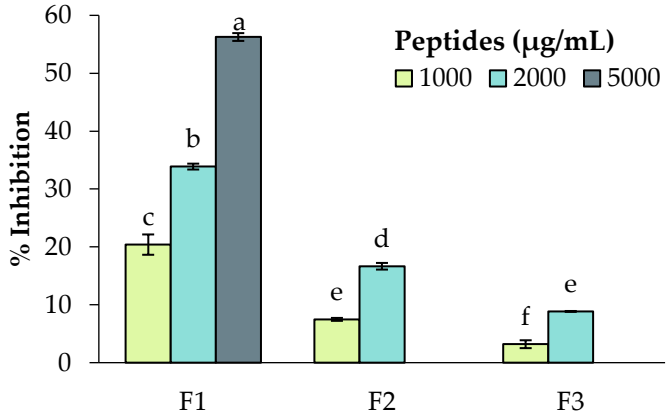


Figure 5-11. Angiotensin-Converting Enzyme inhibition of faba bean (variety Fabelle) peptide-enriched fractions (F1, F2 and F3). Data are expressed as mean ± standard deviation and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

Table 5-4. Antioxidant activity of faba bean (variety Fabelle) peptide-enriched fractions (F1, F2 and F3).

	ABTS [1] (% Scavenging)	Iron Chelating [2] (% Chelating)	ORAC (µmol Trolox eq/mg Peptides)
F1	41.5 ±10.4 [c]	67.6 ± 2.7 [b]	0.7 ± 0.5 [b]
F2	98.9 ±0.8 [a]	54.1 ±1.8 [c]	3.2 ± 0.6 [a]
F3	71.4 ± 15.3 [b]	88.5 ±1.9 [a]	3.1 ± 0.1 [a]

[1] Fractions were tested at 40 µg/mL; [2] Fractions were tested at 5 µg/mL; Data are expressed as mean ± standard deviation and means with different letter in a column are statistically different ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test.

Interestingly, the fractionation caused either a loss or an increase in the bioactive activities compared to the unfractionated 3 kDa permeates. In the case of the ACE inhibition, a 50% inhibition was obtained at 1348 μg/mL for the unfractionated Fabelle 3 kDa permeate, whereas the inhibition varied from 8 to 34% for the fractions at 2000 μg/mL. It is possible that the peptides present in the different fractions had a synergistic effect on ACE, which explains the activity loss at the fractions' level. It is also possible that the fractionation removed the other bioactive constituents that contributed to the overall effect against ACE activity. On the contrary, the ABTS scavenging, and iron chelating activities highly increased after fractionation. The same tendency was observed by Jakubczyk et al. (2019); in their study, the EC_{50} for the ABTS scavenging was 0.99 mg/mL before fractionation and decreased to 0.02–0.1 mg/mL after fractionation. The removal of the other constituent of the 3 kDa permeate with antagonist effect could explain this outcome. In the case of ORAC, F2 and F3 had a higher activity compared to the complete 3 kDa permeate, but F1 had a lower activity, suggesting that the peptides responsible for this antioxidant effect were mostly eluted in F2 and F3.

In order to obtain a better understanding of the observed differences in the bioactive properties of the three fractions, the peptide profile of each fraction was identified by LC-MS/MS and database searching (Table 5-5). Eleven unique peptides were identified, 11 in F1, 1 in F2 and none in F3. One peptide was present in both F1 and F2, as presented in Table 5-5. This is most probably due to some peak overlapping during the size exclusion chromatography, as previously observed by Torres-Fuentes et al. (2015). Most of the peptides were found in F1, which is in accordance with the determined protein content of each fraction. Approximately 70% of the initial proteins of the 3 kDa permeate were recovered in F1, 20% in F2 and 10% in F3. The peptides were composed of 9 to 11 amino acid residues, with molecular weights ranging from 888 Da to 1336 Da, which is in the typical range of highly active peptides (Sánchez et al., 2017). Seven identified peptides were from the globulin storage proteins (legumin, vicilin and convicilin). Those peptides are likely to contribute significantly to the faba bean protein bioactive properties, since the globulins account for up to 80% of the faba bean seed proteins (Alghamdi, 2009).

Table 5-5. LC-MS/MS identified faba bean peptides and their respective precursor proteins found in the peptide-enriched fractions of the 3 kDa permeate of Fabelle *in vitro* gastrointestinal digestate.

Peptide Sequence	Fraction	Observed Mass (Da)	Calculated Mass (Da)	ppm [1]	Precursor Protein	Protein Accession Number	Fragment Location
N[2]YDEGSEPR	F1	1066.421	1066.420	0.43	Convicilin	B0BCL8	29–37
PVNRPGEPQ	F1	992.507	992.504	2.60	Vicilin	I0B569	152–160
LDNIN[2]ALEPDH	F1	1250.578	1250.578	−0.19	Legumin B	P05190.1	35–45
TETWNPNHPE	F1	1223.522	1223.521	1.21	Legumin B	P05190.1	52–61
TETWNPNHPEL	F1	1336.606	1336.605	0.88	Legumin B	P05190.1	52–62
EEEDEDEPR	F1	1146.436	1146.431	3.64	Legumin	Q43673	327–335
KEEEDEDEPR	F1	1274.530	1274.526	3.06	Legumin	Q43673	326–335
VIPTEPPH	F1	888.470	888.471	−0.70	Tonoplast intrinsic protein 32	A0A024NRI7	155–162
VIPTEPPHA	F1	959.508	959.508	−0.13	Tonoplast intrinsic protein 32	A0A024NRI7	155–163
VVIPTEPPHA	F1	1058.577	1058.576	0.55	Tonoplast intrinsic protein 32	A0A024NRI7	154–163
VVIPTEPPH	F1 and F2	987.540	987.539	1.07	Tonoplast intrinsic protein 32	A0A024NRI7	154–162

Amino acids are abbreviated with 1 letter code; [1] Mass error was expressed in ppm and calculated as follow: $\frac{Oberserved\ mass - Calculated\ mass}{Obserevd\ mass} \times 10^6$; [2] Deamidation of asparagine residue

The relationship between the peptide chemical structure and bioactivity is not yet well understood (Karami & Akbari-Adergani, 2019; Manzanares, Gandía, Garrigues, & Marcos, 2019). However, the peptide length, charge, amino acid composition and the particular order and the presence of the hydrophobic residues are all of the factors influencing the bioactivity potency (Karkouch et al., 2017; Lopez-Barrios, Gutierrez-Uribe, & Serna-Saldivar, 2014). The potential bioactive properties of the LC-MS/MS identified peptides were screened *in silico*, using the BIOPEP-UWM database and the results are presented in Table 5-6. All of the identified peptides contained fragments with an inhibitory effect against ACE and DPP-IV, and four of them also had antioxidant fragments, which corroborated our experimental findings. The results (Table 5-6) revealed that the peptide PVNRPGEPQ has a very promising bioactive potential, especially for ACE and DPP-IV inhibition. Indeed, the fragments with these specific activities are present in high frequency and those fragments are highly active, as revealed by the high B parameter values. Noteworthy, this peptide was found in F1, which was the fraction with the highest measured ACE inhibitory activity. The peptides VIPTEPPH, VIPTEPPHA, VVIPTEPPH and VVIPTEPPHA contains as well a high frequency of ACE and DPP-IV inhibitor fragments, however, their predicted activity is lower. The peptides EEEDEDEPR and KEEEDEDEPR contain a low frequency of ACE inhibitor fragments, but have a high predicted activity.

The peptide TETWNPNHPEL has the highest antioxidant fragment frequency among the identified peptides. Indeed, this peptide was previously identified by Torres-Fuentes et al. (2015) in a pepsin-pancreatin chickpea protein hydrolysate for its antioxidant and chelating activity. The

finding of the same peptide in the present study suggests that the *Fabaceae* storage proteins are highly conserved. Although the F2 and F3 had the highest measured antioxidant activity overall, only one peptide was successfully identified and no *in silico* predicted antioxidant activity was associated with it. Still, this peptide contains a histidine, glutamic acid and threonine residue that are well known for their contribution to the iron-chelating activity (Xiong, 2010). Histidine is also implicated in free radical scavenging (Xiong, 2010). The peptides identified in F2 and F3 remain very limited. The faba bean proteome is incomplete which makes the peptide identification challenging. It is also possible that F2 and F3 contained very low-molecular weight peptides and the free amino acids that were not detected. Still, the chelating activity and ABTS scavenging activity of F1 was importantly higher than the complete 3 kDa permeate, which indicate an excellent antioxidant activity that can be attributed to the peptides.

Other bioactivities were also identified, namely the anti-amnestic, antithrombotic, regulating stomach mucosal membrane activity, DPP-III inhibitor, renin inhibitor, alpha-glucosidase inhibitor and stimulating activities. Noteworthy, most of these activities were identified as well in the *in silico* screening of the faba bean storage proteins. The alpha-glucosidase inhibition activity of the faba bean ethanol extract (Di Stefano, Tsopmo, Oliviero, Fogliano, & Udenigwe, 2019), and the germinated and fermented faba beans *in vitro* gastrointestinal digestates (Loizzo, Bonesi, Leporini, Falco, Sicari, & Tundis, 2021) were also reported by others, which is in agreement with our finding. The other identified activities, however, were not so far experimentally investigated in faba beans.

Table 5-6. *In silico* prediction of bioactive properties of the LC-MS/MS identified faba bean peptides using the BIOPEP-UWM database.

Peptide Sequence	Fraction	Potential Bioactivity [1]	Bioactive Fragments	A [2]	B [3] (μM^{-1})
NYDEGSEPR	F1	ACE inhibitor	PR, GS, EG, NY	0.44	0.03
		Stimulating[5]	SE	0.11	.
		DPP-IV inhibitor	EP, EG, NY, YD	0.44	.
		DPP-III inhibitor	PR	0.11	.
PVNRPGEPQ	F1	Anti-amnestic	PG	0.11	.
		ACE inhibitor	GEP, RP, GE, PG, PQ	0.56	0.36
		Antithrombotic	PG	0.11	.
		Regulating [4]	PG	0.11	.
		DPP-IV inhibitor	RP, EP, GE, NR, PG, PQ, PV, VN	0.89	4.96
		DPP-III inhibitor	GE	0.11	.
		Renin inhibitor	NR	0.11	.
LDNINALEPDH	F1	ACE inhibitor	ALEP	0.09	1.44×10^{-5}
		DPP-IV inhibitor	EP, AL, DN, IN, NA	0.45	1.03×10^{-4}
TETWNPNHPE	F1	ACE inhibitor	TE, HP	0.2	.
		Antioxidant	TW	0.1	.
		Alpha-glucosidase inhibitor	PE	0.1	3.99×10^{-6}
		DPP-IV inhibitor	HP, NP, WN, ET, NH, PN, TE, TW	0.8	3.35×10^{-3}
		DPP-III inhibitor	HP, PE	0.2	.
TETWNPNHPEL	F1	ACE inhibitor	TE, HP	0.18	.
		Antioxidant	EL, PEL, TW, TETWNPNHPEL	0.36	.
		Alpha-glucosidase inhibitor	PE	0.09	3.63×10^{-6}
		DPP-IV inhibitor	HP, NP, WN, ET, NH, PN, TE, TW	0.73	3.04×10^{-3}
		DPP-III inhibitor	HP,PE	0.18	.
EEEDEDEPR	F1	ACE inhibitor	PR	0.11	0.03
		Stimulating [5]	EEE, EE	0.33	.
		DPP-IV inhibitor	EP	0.11	.
		DPP-III inhibitor	PR	0.11	.
KEEEDEDEPR	F1	ACE inhibitor	PR, KE	0.2	0.02
		Stimulating [5]	EEE, EE	0.3	.
		DPP-IV inhibitor	EP, KE	0.2	.
		DPP-III inhibitor	PR	0.1	.
VIPTEPPH	F1	ACE inhibitor	IP, TE, PT, PP, PH	0.63	9.62×10^{-4}
		Alpha-glucosidase inhibitor	PP	0.13	6.93×10^{-6}
		DPP-IV inhibitor	PP, IP, EP, PH, PT, TE, VI	0.88	3.26×10^{-4}
VIPTEPPHA	F1	ACE inhibitor	IP, TE, PT, PP, PH	0.56	8.55×10^{-4}
		Antioxidant	PHA	0.11	.
		Alpha-glucosidase inhibitor	PP	0.11	6.16×10^{-6}
		DPP-IV inhibitor	PP, HA, IP, EP, PH, PT, TE, VI	0.89	2.90×10^{-4}
VVIPTEPPHA	F1	ACE inhibitor	IP, TE, PT, PP, PH	0.5	7.69×10^{-4}
		Antioxidant	PHA	0.1	.
		Alpha-glucosidase inhibitor	PP	0.1	5.55×10^{-6}
		DPP-IV inhibitor	PP, VV, HA, IP, EP, PH, PT, TE, VI	0.9	2.61×10^{-4}
VVIPTEPPH	F1 and F2	ACE inhibitor	IP, TE, PT, PP, PH	0.56	8.55×10^{-4}
		Alpha-glucosidase inhibitor	PP	0.11	6.16×10^{-6}
		DPP-IV inhibitor	PP, VV, IP, EP, PH, PT, TE, VI	0.89	2.90×10^{-4}

Amino acids are abbreviated with 1 letter code; [1] Potential bioactivities for each peptide were determined using the BIOPEP-UWM database (Minkiewicz et al., 2019); [2] The parameter A represents the occurrence frequency of a fragment with a given bioactivity: $A = \frac{a}{N}$, where a is the number of fragments with a given bioactivity and N is the number of amino acid residues in the peptide sequence (J. Dziuba, Iwaniak, & Minkiewicz, 2003); [3] The parameter B represents the potential biological activity of the peptide: $B = \frac{\sum_{i=1}^{k} \frac{a_i}{EC_{50}i}}{N}$, where a is the number of repetitions of a given fragment with a given activity, EC_{50} is its respective half maximum activity (μM) and k is the number of different fragments with a given bioactivity and N is the number of amino acid residues (J. Dziuba et al., 2003). The B parameter is only calculated if EC_{50} data are available. The higher the B value is, the higher the predicted bioactivity is. A and B were automatically computed by the BIOPEP algorithm; [4] Peptide regulating the stomach mucosal membrane activity; [5] Peptide stimulating vasoactive substance releas

5.5 Conclusions

In this study, the beneficial health potential of faba bean flour was investigated and compared to two commonly used legumes (soy and pea) through the screening of bioactive properties resulting from the physiological context of the *in vitro* gastrointestinal digestion. The *in vitro* assays revealed that the faba bean flour digestates had either a similar or better bioactive activity compared to the pea digestates, and a similar or lower bioactive activity compared to soy. Nonetheless, the faba bean varieties showed a higher antioxidant activity and antidiabetic activity in the cell-based assays, which suggest that the faba bean peptides may have a better bioavailability or a better activity *in vivo*. This hypothesis will require further confirmation. Fabelle, one of the three studied faba bean varieties, stood out for its higher antioxidant and antihypertensive activity. Eleven peptides with excellent *in silico* predicted activity were identified by mass spectrometry, which confirm that the peptide played an important role in the observed bioactive activities. As there is a growing interest for health-promoting functional food ingredients in the food industry (Zaky, Simal-Gandara, Eun, Shim, & Abd El-Aty, 2022), our results demonstrate that faba beans have an excellent bioactive potential that complements their nutritional interest quality and therefore present a high potential for use in the development of new functional and nutraceutical ingredients in food applications. Further investigation of these health-promoting bioactivities with *in vivo* models and in humans are required to confirm these findings.

5.6 References

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CONNECTING STATEMENT IV

Chapter V revealed that faba bean peptides derived from *in vitro* gastrointestinal digestion have a high potential as health-promoting agents, particularly in terms of antioxidant and antihypertensive activities. Although faba bean peptides generated after gastrointestinal have important health-promoting potential, it is still needed to identify which faba bean peptides are responsible for such bioactive effects and to deepen the understanding their modes of actions.

To that end, in Chapter VI, faba bean peptides derived from gastrointestinal digestion were chemically synthesised and tested individually for antioxidant and antihypertensive activity. Their mechanisms of actions were further studied with a combination of *in vitro*, cellular and computational studies.

The results from this research were presented at the 2023 BÉNÉFIQ congress and published in the *Journal of Agricultural and Food Chemistry:*

- Martineau-Côté, D. Achouri, A., Pitre, M., Karboune S. & L'Hocine, L. (2023, October 4-5). [Poster Presentation]. Faba bean (*Vicia faba* L.) flour as a health-promoting functional food ingredient with antioxidant and antihypertensive properties after in vitro gastrointestinal digestion. BÉNÉFIQ congress, Centre des congrès de Québec, Québec, Canada.
- Martineau-Côté, D., Achouri, A., Karboune, S., & L'Hocine, L. (2024). Antioxidant and Angiotensin-Converting Enzyme Inhibitory Activity of Faba Bean-Derived Peptides After In Vitro Gastrointestinal Digestion: Insight into Their Mechanism of Action. *J Agric Food Chem*. https://doi.org/10.1021/acs.jafc.4c00829.

CHAPTER VI. ANTIOXIDANT AND ANGIOTENSIN-CONVERTING ENZYME INHIBITORY ACTIVITY OF FABA BEAN-DERIVED PEPTIDES AFTER *IN VITRO* GASTROINTESTINAL DIGESTION : INSIGHT INTO THEIR MECHANISM OF ACTION

6.1 Abstract

Faba bean flour after *in vitro* gastrointestinal showed important antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities. In the present study, 11 faba bean-derived peptides were synthesized to confirm their bioactivities and provide a deeper understanding of their mechanisms of action. The results revealed that 7 peptides were potent antioxidants, namely NYDEGSEPR, TETWNPNHPEL, TETWNPNHPE, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH. Among them, TETWNPNHPEL had the highest activity in the ABTS (EC_{50} = 0.5 ± 0.2 mM) and DPPH (EC_{50}= 2.1 ± 0.1 mM) assays ($p < 0.05$), whereas TETWNPNHPE had the highest activity ($p < 0.05$) in the ORAC assay (2.84 ± 0.08 mM Trolox equivalent/mM). Synergistic and/or additive effects were found when selected peptides (TETWNPNHPEL, NYDEGSEPR and VVIPTEPPHA) were combined. Four peptides were potent ACE inhibitors, where VVIPTEPPH (IC_{50}= 43 ± 1 μM) and VVIPTEPPHA (IC_{50} = 50 ± 5 μM) had the highest activity ($p < 0.05$), followed by VIPTEPPH (IC_{50}= 90 ± 10 μM) and then VIPTEPPHA (IC_{50} = 123 ± 5 μM) ($p < 0.05$). These peptides were noncompetitive inhibitors, as supported by kinetic studies and a molecular docking investigation. This study demonstrated that peptides derived from faba beans have multifunctional bioactivities, making them a promising food functional and nutraceutical ingredient.

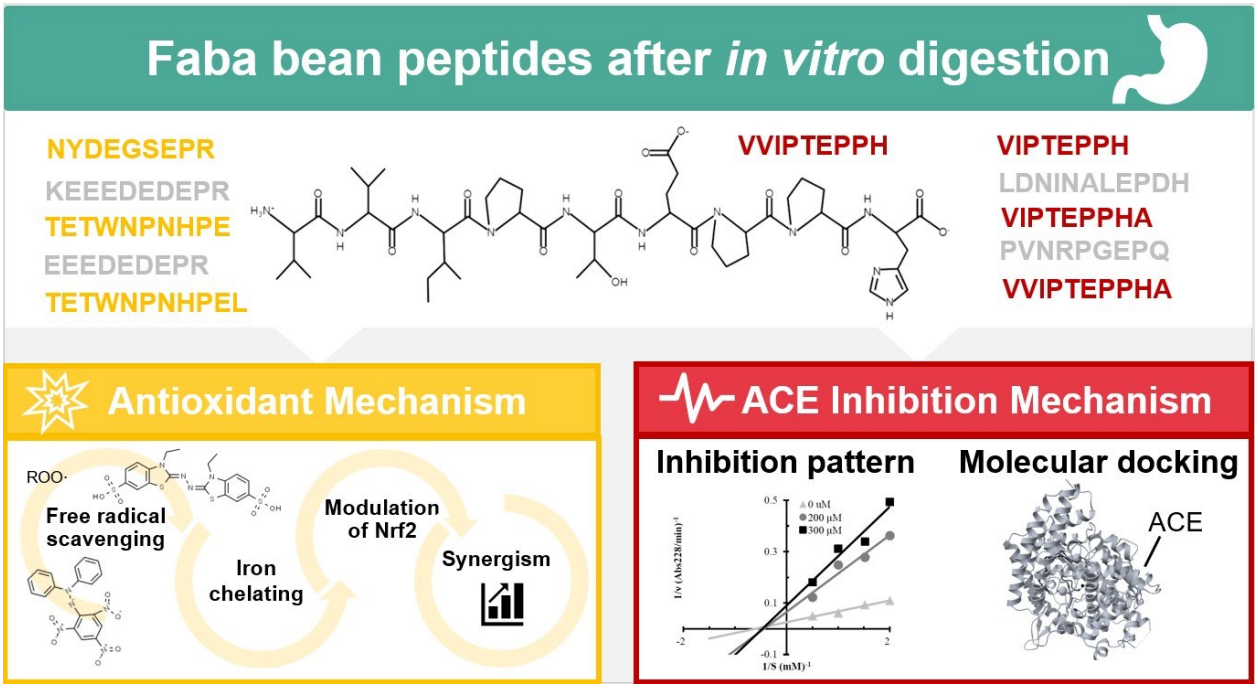


Figure 6-1. Graphical abstract.

6.2 Introduction

According to the World Health Organization (WHO), noncommunicable diseases are the world-leading cause of premature death, with cardiovascular diseases, being the leading cause of mortality, followed by cancer and respiratory disease (WHO, 2021). Hypertension is a well-recognized risk factor for cardiovascular diseases (Kokubo & Matsumoto, 2017) that can be managed through medication and a healthy lifestyle.

An important pharmacological target for the treatment of hypertension is the angiotensin-converting enzyme (ACE). ACE is a zinc dipeptidyl carboxypeptidase that plays a critical role in the regulation of blood pressure and cardiovascular function through the renin-angiotensin-aldosterone system (RAAS) (Riet et al., 2015) and the kallikrein-kinin system (KKS) (See et al., 2016). In the RAAS, ACE hydrolyzes angiotensin I to form the potent vasoconstrictor angiotensin II, while in the KKS, ACE converts bradykinin, a potent vasodilator to an inactive fragment (See et al., 2016). Numerous synthetic ACE inhibitors, such as captopril and linosipril, among others, have been used for decades to treat hypertension.

In addition to RAAS deregulation, increased oxidative stress has also been linked to the development of hypertension and noncommunicable diseases (Rodrigo et al., 2011). Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense system, leading to an imbalance of the redox cellular signaling pathways and thus molecular damages (Griendling et al., 2021). Oxidative stress can result in endothelial and renal damage, vascular dysfunction, and cardiovascular fibrosis, all of which are known to play a role in the development of hypertension (Griendling et al., 2021). The new generation of drugs with dual ACE inhibition and antioxidant effects are, therefore, regarded promising alternatives to treat high blood pressure and prevent cardiovascular diseases (Dandona et al., 2007; Ghatage et al., 2021).

Concomitant with medication, a healthy lifestyle can have a protective effect against hypertension (Lelong et al., 2019). In this vein, recent reports have suggested that pulse consumption is associated with a blood pressure-lowering effect (Ferreira et al., 2021; Guo et al., 2020; Jayalath et al., 2014). Several pulse components, such as phenolic compounds, γ-aminobutyric acid (GABA), and dietary fibers, are believed to contribute to this hypotensive effect (Acquah et al., 2021). The release of antioxidant and ACE inhibitor peptides after gastrointestinal digestion of pulse proteins also has the potential to contribute to this beneficial health effect.

Faba bean (*Vicia faba* L.) is an emerging high-quality and sustainable pulse protein source with promising health benefits (Martineau-Côté, Achouri, Karboune, et al., 2022). In a previous work, we demonstrated that faba bean flours after *in vitro* gastrointestinal digestion (Martineau-Côté et al., 2024) have a high antioxidant and ACE inhibitory effect, which could play a role in hypertension management (Martineau-Côté, Achouri, Wanasundara, et al., 2022). The faba bean peptides present in the *in vitro* gastrointestinal digestate complex mixture (Martineau-Côté et al., 2024) were enriched through a 3 kDa cutoff membrane ultrafiltration followed by preparative size exclusion chromatography and sequenced by mass spectrometry. The obtained peptide-enriched fractions maintained a high antioxidant and ACE inhibition effect, demonstrating that these health-beneficial bioactivities were related to peptides (Martineau-Côté, Achouri, Wanasundara, et al., 2022).

The objective of the present work was to further ascertain the antioxidant and ACE inhibitory activities of faba bean-derived peptides and to gain a new understanding of their mode of action. To this end, the highly active faba bean peptides (Martineau-Côté, Achouri, Wanasundara, et al., 2022) were chemically synthesized (Table 6-1) and tested individually for antioxidant and ACE inhibition activity. The mechanisms of action of antioxidant peptides were investigated using a combination of *in vitro* and cellular antioxidant assays. The mechanisms of action of ACE inhibitor peptides were investigated through enzyme kinetic studies to determine the inhibition pattern and molecular docking to assess and compare their potential binding mode to ACE. This is the first study reporting an in-depth investigation of the mechanisms of action of faba bean-derived bioactive peptides after gastrointestinal digestion with multifunctional and synergistic activities, using a combination of *in silico*, *in vitro,* and cellular models.

Table 6-1. List of synthesized peptides identified from faba bean flour *in vitro* gastrointestinal digestate (Martineau-Côté, Achouri, Wanasundara, et al., 2022).

Peptides	Parent Protein	Protein Accession Number	Fragment Location	% Hydrophobic Residue[1]
NYDEGSEPR	Convicilin	B0BCL8	29–37	11.11
PVNRPGEPQ	Vicilin	I0B569	152–160	44.44
LDNINALEPDH	Legumin B	P05190.1	35–45	45.45
TETWNPNHPEL			52–61	36.36
TETWNPNHPE			52–62	30.00
EEEDEDEPR	Legumin	Q43673	327–335	11.11
KEEEDEDEPR			326–335	10.00
VIPTEPPH	Tonoplast intrinsic protein 32	A0A024NRI7	155–162	62.50
VIPTEPPHA			155–163	66.67
VVIPTEPPHA			154–163	70.00
VVIPTEPPH			154–162	66.67

[1]Hydrophobic and uncharged residue are phenylalanine (F), isoleucine (I), leucine (L), methionine (M), valine (V), tryptophan (W), alanine (A), and proline (P).

6.3 Materials and Methods

6.3.1 Chemicals

The peptides derived from faba bean flour gastrointestinal digestate, NYDEGSEPR, PVNRPGEPQ, LDNINALEPDH, TETWNPNHPEL, TETWNPNHPE, EEEDEDEPR, KEEEDEDEPR, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH (Table 6-1) were synthesized by Biomatik (Kitchener, Ontario, Canada). Their purity (>98%) and quality were checked by reverse-phase HPLC (>98%) and mass spectrometry analysis.

Angiotensin-converting enzyme (ACE) from rabbit lung (A6778), N-hippuryl-His-Leu hydrate (HHL) (H1635) and captopril were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For cell culture, minimum essential medium (MEM), nonessential amino acid solution 100×, heat-inactivated fetal bovine serum (FBS), 5000 IU penicillin, and 5000 µg/mL streptomycin solution, were purchased from Wisent Bioproducts (Saint-Jean-Baptiste, QC, Canada). Sodium pyruvate (100 mM) was purchased from Cytiva (Uppsala, Sweden). Geneticin (50 mg/mL) was purchased from Gibco (Thermo Fisher Scientific, San Jose, CA, USA). Antioxidant response element (ARE) reporter-HepG2 cells and the One-Step™ Luciferase Assay System were purchased from BPS Bioscience (San Diego, CA, USA). All chemicals and reagents used were of analytical grade. Deionized water was used in all of the experiments.

6.3.2 Antioxidant Mechanism of Faba Bean Derived-Peptides

6.3.2.1 In vitro Antioxidant Assays

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Oxygen Radical Absorption Capacity (ORAC), and the iron chelating assays were performed following the methods of Orona-Tamayo, Valverde, Nieto-Rendón, and Paredes-López (2015), Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999), Tomer, McLeman, Ohmine, Scherer, Murray, and O'Neill (2007) and of Orona-Tamayo et al. (2015), respectively, as described in Martineau-Côté, Achouri, Wanasundara et al. (2022). For the DPPH, ABTS, and iron chelating assays, the peptides were first screened at a high dose (10 mM). Peptides with antioxidant activity were then tested at different concentrations (0.1-10 mM) to evaluate the dose-response effect. The results were expressed as the half-maximal effective concentration (EC_{50}), which was defined as the required peptide concentration leading to 50% scavenging or chelating activity. The EC_{50} values were calculated using a four parameter logistic curve regression, and Trolox was used as a positive control. For the ORAC assay, the results were expressed as μmol of Trolox equivalent per mM of peptides.

6.3.2.2 Investigation of Potential Additive, Synergistic and/or Antagonist Interactions of Faba Bean-Derived Antioxidant Peptides

Since the synthesized faba bean derived-peptides had individually lower antioxidant activities than the complete faba bean flour digestate with the DPPH and ABTS assays, we investigated whether some peptide combinations were additive or synergistic. To this end, the method of Chou and Talalay (1984) was used to determine the combination index (CI) and the dose reduction index (DRI) of selected peptides combinations. The peptides were tested individually and in combination using a constant ratio (i.e. the ratio of their EC_{50}) at various concentrations. The DPPH and ABTS assays were performed as described in Section 6.3.2.1.

Data analysis was performed using the CompuSyn software (Zhang et al., 2016) (ComboSyn Inc.) to calculate the CI and the DRI values at various levels of free radical scavenging activity (Fa). The CI values were used to determine the type of interaction between peptides, where $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergism, additivity, and antagonism, respectively. DRI represents the dose reduction fold that can be achieved for a given peptide when used in combination. DRI values above 1 indicate that dose reduction is favorable whereas a value below 1 indicates that dose reduction is unfavorable.

6.3.2.3 Modulation of the Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2/ARE) Cellular Pathway by Faba Bean-Derived Antioxidant Peptides

The most potent antioxidant peptides identified with *in vitro* antioxidant assays were tested at the cellular level using the Nrf2-ARE live cell assay as described by Vigliante, Mannino, and Maffei (2019) with minor modifications. The HepG2 cells transfected with a firefly luciferase gene under the control of ARE were routinely cultivated in growth medium, which was composed of MEM supplemented with 10% FBS, 1% nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin and streptomycin solution and 600 µg/mL Geneticin at 37 °C in an atmosphere containing 5% CO_2. Cells were subcultivated at 90% confluence using a split ratio of 1:5. Cells between passages 3 and 11 were used in the experiments.

The activation of the Nrf2-ARE pathway by faba bean derived-peptides was investigated both in basal conditions and in the presence of oxidative stress (H_2O_2 0.25 mM). To this end, 4×10^4 cells in 45 µL of growth medium without Geneticin were added to 96-well white microplates with clear bottoms. Five µL of faba bean derived-peptides were added in triplicate to reach a final concentration of 1, 0.5 or 0.05 mM, with or without 0.25 mM H_2O_2. Tert-butylhydroquinone (TBHQ) was used as a positive control, and assay medium with and without 0.25 mM H_2O_2 was used as negative controls. The plates were incubated for 18 h at 37 °C in an atmosphere containing 5% CO_2.

The next day, the activation of the Nrf2-ARE pathway was quantified using the One-Step™ Luciferase Assay System (BPS Bioscience, San Diego, CA, USA) as described by the manufacturer. Briefly, 100 µL of the luciferase assay working solution equilibrated at room temperature was added to each well. The plate was incubated for 15 min at room temperature with constant stirring. Luminescence was recorded with a Synergy HTX microplate reader (Bio-Tek, Winooski, VT, USA). ARE modulation was expressed as a fold increase compared to the negative control using the following formula after background subtraction:

$$ARE - Mediated\ Gene\ Expression\ (Fold\ increase\ \%) = \frac{L_{sample}}{L_{control}} \times 100 \quad \text{(eq 1)}$$

where L_{sample} is the relative luminescence reading of the cells treated with the faba bean-derived peptides and $L_{control}$ is the relative luminescence reading of untreated cells.

6.3.3 ACE Inhibition Mechanism of Faba Bean Derived-Peptides

6.3.3.1 In vitro ACE inhibitory activity

ACE inhibition activity was measured following the protocol of Barbana and Boye (2011) as described in Martineau-Côté, Achouri, Wanasundara, et al. (2022). ACE from rabbit was used for *in vitro* testing, since rabbit and human ACE are nearly homologous, and their active site are highly similar (Chamata et al., 2020).

6.3.3.2 Determination of ACE Inhibition Pattern

A kinetic study was performed following the procedure of Barbana et al. (2011) to determine the inhibition pattern of four faba bean peptides with ACE inhibition activity (VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH). The initial rate of reaction was measured with different HHL (0.5-2 mM) and peptides concentrations. Lineweaver-Burk double reciprocal plots were built to identify the inhibition pattern.

6.3.3.3 Elucidation of the Peptide Binding Mode by Molecular Docking

Molecular docking was used to identify the potential binding mode of faba bean derived-peptides (VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH) to ACE. The peptide structures were created using PEP-FOLD 3.5 (Lamiable et al., 2016). The crystal structure of the C-domain of somatic human ACE (PDB: 4APH, resolution: 1.99 Å) was retrieved from the RCSB protein databank (https://www.rcsb.org). The PDB file was edited to remove any molecules except the protein chain, the zinc ion, the two chlorine ions and angiotensin II. Angiotensin II was kept in the docking simulation, since the kinetic study revealed that the four faba bean peptides were noncompetitive inhibitors, meaning that they can bind ACE whether or not the substrate is binding the active site. Angiotensin II was used to simulate the enzyme substrate since no crystal structure of Angiotensin I with ACE is available.

The most probable binding sites between ACE and the four peptides were predicted using HPEPDOCK (Zhou et al., 2018), a global flexible peptide protein docking software. Global docking enables a blind docking simulation on the whole protein chain when the binding site is unknown. The most probable model for each peptide was selected based on the lowest HPEPDOCK docking score. The ACE and faba bean derived-peptide complexes were further analyzed with Ligplot+ (Laskowski

& Swindells, 2011) to identify molecular interactions. Molecular graphics were produced with UCSF ChimeraX (Pettersen et al., 2021).

The peptide protein docking procedure was validated with two controls, Angiotensin II and the bradykinin-potentiating peptide b (BPPb). Angiotensin II and BPPb, were extracted from their co-crystallized structure with ACE (PDB 4APH and 4APJ, respectively) and re-docked with ACE. The docked poses were compared to the crystal structure through root mean square deviation (RMSD) and comparison of the molecular interaction stabilizing the complexes.

6.3.4 Statistical Analysis

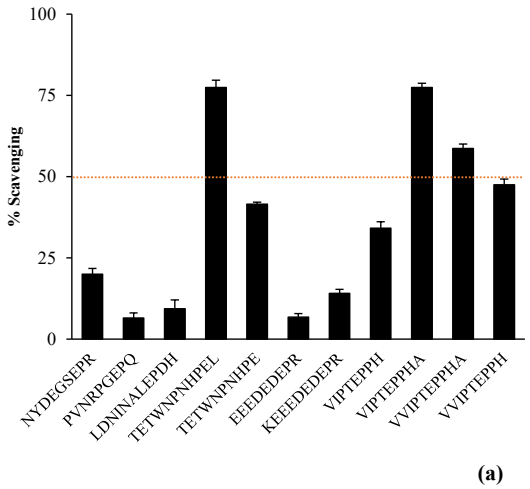
Each analysis was performed in triplicate and the results were expressed as the mean ± standard deviation (SD). The data were analyzed through analysis of variance (ANOVA) (p <0.05) followed by the Tukey's honest significant difference (HSD) posthoc test (p <0.05) or the Dunnett's posthoc test, using the XLSTAT software (Addinsoft, NY, USA) add-on to Microsoft Excel (Redmond, WA, USA) to determine significant differences.

6.4 Results and Discussion

6.4.1 Antioxidant Mechanism of Faba Bean Derived-Peptides

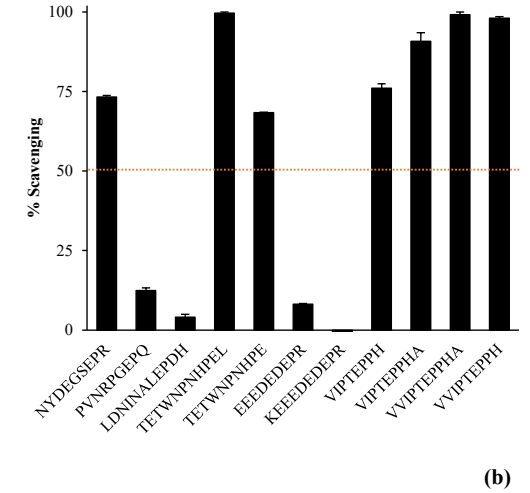
6.4.1.1 In vitro Antioxidant Activity of Faba Bean-Derived Peptides

The 11 faba bean derived-peptides (Table 6-1) were first tested for *in vitro* antioxidant activity (Figure 6-2). Among them, 7 peptides were potent free radical scavengers, when assessed with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and/or Oxygen Radical Absorption Capacity (ORAC) assay (Figure 6-2), namely TETWNPNHPEL, TETWNPNHPE, NYDEGSEPR, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH. These 7 peptides had in their primary sequence an amino acid recognized for free radical scavenging activity such as tryptophan (W), tyrosine (Y), and/or histidine (H) (Esfandi et al., 2019), which may partly explain these antioxidant properties.



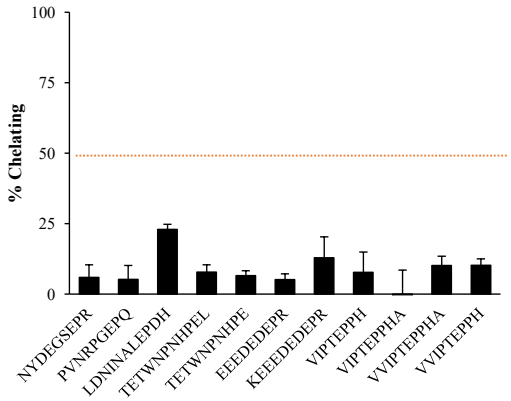
(a)

	DPPH (EC_{50})	
	mM	mg/mL
NYDEGSEPR	-	-
PVNRPGEPQ	-	-
LDNINALEPDH	-	-
TETWNPNHPEL	2.1 ± 0.1^{c}	2.8 ± 0.1^{c}
TETWNPNHPE	-	-
EEEDEDEPR	-	-
KEEEDEDEPR	-	-
VIPTEPPH	-	-
VIPTEPPHA	6.2 ± 0.3^{b}	5.9 ± 0.3^{b}
VVIPTEPPHA	8.1 ± 0.4^{a}	8.5 ± 0.4^{a}
VVIPTEPPH	-	-
Trolox	*0.02 ± 0.01*	*0.005 ± 0.003*



(b)

	ABTS (EC_{50})	
	mM	mg/mL
NYDEGSEPR	2.8 ± 0.5^{c}	3.0 ± 0.5^{bc}
PVNRPGEPQ	-	-
LDNINALEPDH	-	-
TETWNPNHPEL	0.5 ± 0.2^{d}	0.7 ± 0.2^{d}
TETWNPNHPE	4.2 ± 0.2^{b}	5.2 ± 0.2^{a}
EEEDEDEPR	-	-
KEEEDEDEPR	-	-
VIPTEPPH	5.7 ± 0.3^{a}	5.0 ± 0.2^{a}
VIPTEPPHA	3.7 ± 0.1^{b}	3.5 ± 0.3^{b}
VVIPTEPPHA	2.9 ± 0.1^{c}	3.1 ± 0.1^{bc}
VVIPTEPPH	2.8 ± 0.2^{c}	2.8 ± 0.2^{c}
Trolox	*0.064 ± 0.005*	*0.016 ± 0.001*



(c)

	ORAC	
	(µMol TE eq / mM)	(µMol TE eq / mg)
NYDEGSEPR	557 ± 5^{c}	523 ± 5^{c}
PVNRPGEPQ	6.0 ± 0.1^{d}	6.0 ± 0.1^{d}
LDNINALEPDH	57 ± 1^{d}	46 ± 1^{d}
TETWNPNHPEL	2482 ± 39^{b}	1856 ± 29^{b}
TETWNPNHPE	2838 ± 78^{a}	2318 ± 64^{a}
EEEDEDEPR	2.0 ± 0.1^{d}	1.5 ± 0.1^{d}
KEEEDEDEPR	3.0 ± 0.3^{d}	2.3 ± 0.2^{d}
VIPTEPPH	56 ± 2^{d}	63 ± 2^{d}
VIPTEPPHA	47 ± 3^{d}	49 ± 3^{d}
VVIPTEPPHA	49 ± 2^{d}	46 ± 2^{d}
VVIPTEPPH	59 ± 1^{d}	60 ± 1^{d}

(d)

Figure 6-2. Antioxidant activity (mean ± standard deviation) of faba bean derived-peptides was assayed with **(a)** the DPPH, **(b)** the ABTS, **(c)** the iron chelating and, **(d)** the ORAC assay. For the DPPH, the ABTS and the iron chelating assay, a first screening was performed at a high peptide concentration (10 mM) and the EC_{50} was determined for the peptides with an activity. For the ORAC assay, the data were expressed as µMol of Trolox equivalent . Means without a common letter differ ($p < 0.05$), as analyzed by one-way ANOVA and Tukey's test.

The peptides TETWNPNHPEL and TETWNPNHPE had a very high activity in the ORAC assay, which was 2.5 to 2.8 times higher than Trolox on a molar basis (Figure 6-2). The presence of tryptophan (W) and histidine (H) in these peptide sequences is likely to contribute to this free radical scavenging property. The activities of TETWNPNHPEL and TETWNPNHPE, when expressed in µMol of Trolox equivalent per mg of peptides (1.9 and 2.3 µMol Trolox eq/mg, respectively) were higher than the activity measured in the faba bean peptide-enriched fraction (0.7 µMol Trolox eq/mg) and slightly lower than the activity measured in the complete 3 kDa permeate of faba bean digestate (2.7 µMol Trolox eq/mg). This finding means that TETWNPNHPEL and TETWNPNHPE are very important contributors of this activity in the complete 3 kDa permeate of faba bean digestate. The peptide NYDEGSEPR also had a high activity in the ORAC assay, which was equivalent to half that of Trolox on a molar basis. The dipeptide NY, present at the N-terminal extremity of NYDEGSEPR, was shown to have a strong free radical scavenging activity in the ORAC assay (3246 µMol TE eq/mM) and in the ABTS assay (EC_{50}=8.3 µM) (Du et al., 2019). This peptide fragment is, therefore, undoubtedly an important contributor to the activity of NYDEGSEPR. The significantly higher activity of TETWNPNHPEL and TETWNPNHPE compared to NYDEGSEPR, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH (Figure 6-2) (p <0.05) could be explained by their respective amino acid composition, since tryptophan (W) was shown to have a higher free radical scavenging activity in the ORAC assay (2790 µMol TE eq/mM) compared to tyrosine (Y) (1020 µMol TE eq/mM) and histidine (78 µMol TE eq/mM) (Torkova et al., 2015).

None of the identified faba bean peptides were potent iron chelators (Figure 6-2). This finding was surprising since a high-chelating activity was measured in the 3 kDa permeate of faba bean flour digestate. The synthesized peptides were tested at a high concentration (10 mM), corresponding to ~9,000 to ~13,000 µg/mL. These concentrations are superior to the EC_{50} of the digestate permeate (146 µg/mL) (Martineau-Côté, Achouri, Wanasundara, et al., 2022), indicating that the iron chelating activity of the permeate might be explained by the contribution of smaller peptides that were not detected or other bioactive components , such as polyphenols.

Seven peptides showed potent antioxidant activity with the ABTS assay (NYDEGSEPR, TETWNPNHPEL, TETWNPNHPE, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH) and among these, three exhibited high activity with the DPPH assay (TETWNPNHPEL, VIPTEPPHA, and VVIPTEPPHA) (Figure 6-2). TETWNPNHPEL was the most potent antioxidant peptide in both assays. Interestingly, the EC_{50} (mg/mL) values of these 7 peptides were higher than those of the 3 kDa

permeate of faba bean flour digestate. The EC_{50} of the individual peptides ranged from 0.7 to 5.2 mg/mL and 2.8 to 8.5 mg/mL, compared to 0.1 and 0.8 mg/mL for the 3 kDa permeate of faba bean flour digestate, in the ABTS and DPPH assays, respectively. Therefore, the activities of the individual peptides were at least 4 times lower than the complete 3 kDa permeate of faba bean digestate. This finding means that the activity measured in the complete permeate digestate is likely the result of an additive or synergistic effect of a combination of these peptides. Moreover, the contribution of other bioactive constituents of the faba bean matrix in the permeate digestate, such as polyphenols and oligosaccharides, cannot be excluded. Indeed, the crude characterization of the faba bean digestate 3 kDa permeate was performed in a previous study (Martineau-Côté, Achouri, Wanasundara, et al., 2022), and in addition to peptides (34.4 g/100 g), it contained 4.8 mg/g of total polyphenols (expressed as gallic acid equivalent) and 43.5 g/100 g of total carbohydrates (expressed as glucose equivalent).

Since the activities of TETWNPNHPEL and TETWNPNHPE were 2.5 to 2.8 times higher than Trolox in the ORAC assay but 8 to 66 times lower than Trolox in the ABTS assay, it can be hypothesized that their mechanism of free radical scavenging is essentially based on hydrogen atom transfer (HAT) rather than single electron transfer (SET). Similar results were obtained for VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH, whose activities were 17 to 21 times lower than Trolox in the ORAC assay, compared to 44 to 89 times lower in the ABTS assay, favoring a HAT-based mechanism. Interestingly, the results showed that minor modifications of the amino acid sequence led to important variations in the antioxidant potency of faba bean derived-peptides in the ABTS and DPPH assays (Figure 6-2). For instance, the leucine residue at the C-terminal position of TETWNPNHPE**L** seems to be crucial in the antioxidant activity, since its removal resulted in a significant increase ($p < 0.05$) of the EC_{50} by a factor of 8.4 in the ABTS assay and a loss of activity in the DPPH assay. This finding is in good agreement with a previous report, where the fragments EL and PEL demonstrated strong free radical scavenging activity in the DPPH assay, when present at the C-terminal extremity of a casein derived-peptide (Suetsuna et al., 2000). Moreover, this leucine residue at the C-terminal extremity increases the percentage of hydrophobic residue (Table 6-1), which is known to favorably affect the antioxidant activity (Karkouch et al., 2017; Petsantad et al., 2020). Similarly, the alanine residue at the C-terminal extremity of VIPTEPPH**A** and VVIPTEPPH**A** was revealed to be essential in the DPPH assay, since its removal caused a decrease in activity in VIPTEPPH and VVIPTEPPH, respectively. This could be attributed to the fact that the fragment PHA has demonstrated strong antioxidant activity (Saito et al., 2003). Moreover, the additional valine residue at the N-terminal

extremity of **V**VIPTEPPHA and **V**VIPTEPPH caused a significant increase of the antioxidant potency in the ABTS assay compared to VIPTEPPHA and VIPTEPPH, respectively ($p < 0.05$). This is in good agreement with previous reports, indicating that the presence of hydrophobic amino acids at the N-terminal extremity of peptides such as valine, alanine, leucine and isoleucine is an important contributor to free radical scavenging properties (Abeynayake et al., 2022; Karkouch et al., 2017). Moreover, the additional valine at the N-terminal extremity of **V**VIPTEPPHA and **V**VIPTEPPH increased the % of hydrophobic residue in the peptide sequence compared to VIPTEPPHA and VIPTEPPH, respectively (Table 6-1), further supporting the importance of the hydrophobic residue in the antioxidant activity. In the same vein, it is noted that VIPTEPPH had the lowest free radical scavenging activity in the ABTS and DPPH assays, coinciding with the lowest percentage of hydrophobic residue in its sequence compared to VVIPTEPPHA, VVIPTEPPH, and VIPTEPPHA.

6.4.1.2 Investigation of Potential Additive, Synergistic and/or Antagonist Effect of Selected Faba Bean-Derived Antioxidant Peptides Combinations

Since the synthesized peptides had individually lower antioxidant activity in the ABTS and DPPH assays than the complete 3 kDa permeate of faba bean *in vitro* gastrointestinal digestate, we investigated whether some of these peptides were having additive or synergistic effects using the Chou et al. (1984) method. Several studies have reported a lower antioxidant activity of peptides enriched fractions and/or synthesized peptides compared to the complete protein hydrolyzate, suggesting synergistic interactions between the different peptides (Jahanbani et al., 2016; Jia et al., 2022; Vásquez-Villanueva et al., 2016). Nonetheless, very few studies have investigated the synergistic and antagonistic interactions of specific peptide combinations to gain a better insight into this phenomenon (Jia et al., 2022; Song et al., 2015). The mechanisms behind such interactions between peptides are widely unknown. Combinations of the most potent faba bean-derived antioxidant peptides with different amino acid chains were tested. The selected combinations for the ABTS assay were VVIPTEPPHA and TETWNPNHPEL, VVIPTEPPHA and NYDEGSEPR, TETWNPNHPEL and NYDEGSEPR, and finally VVIPTEPPHA, TETWNPNHPEL and NYDEGSEPR. For the DPPH, the combination of VIPTEPPHA and TETWNPNHPEL was tested (Figure 6-3).

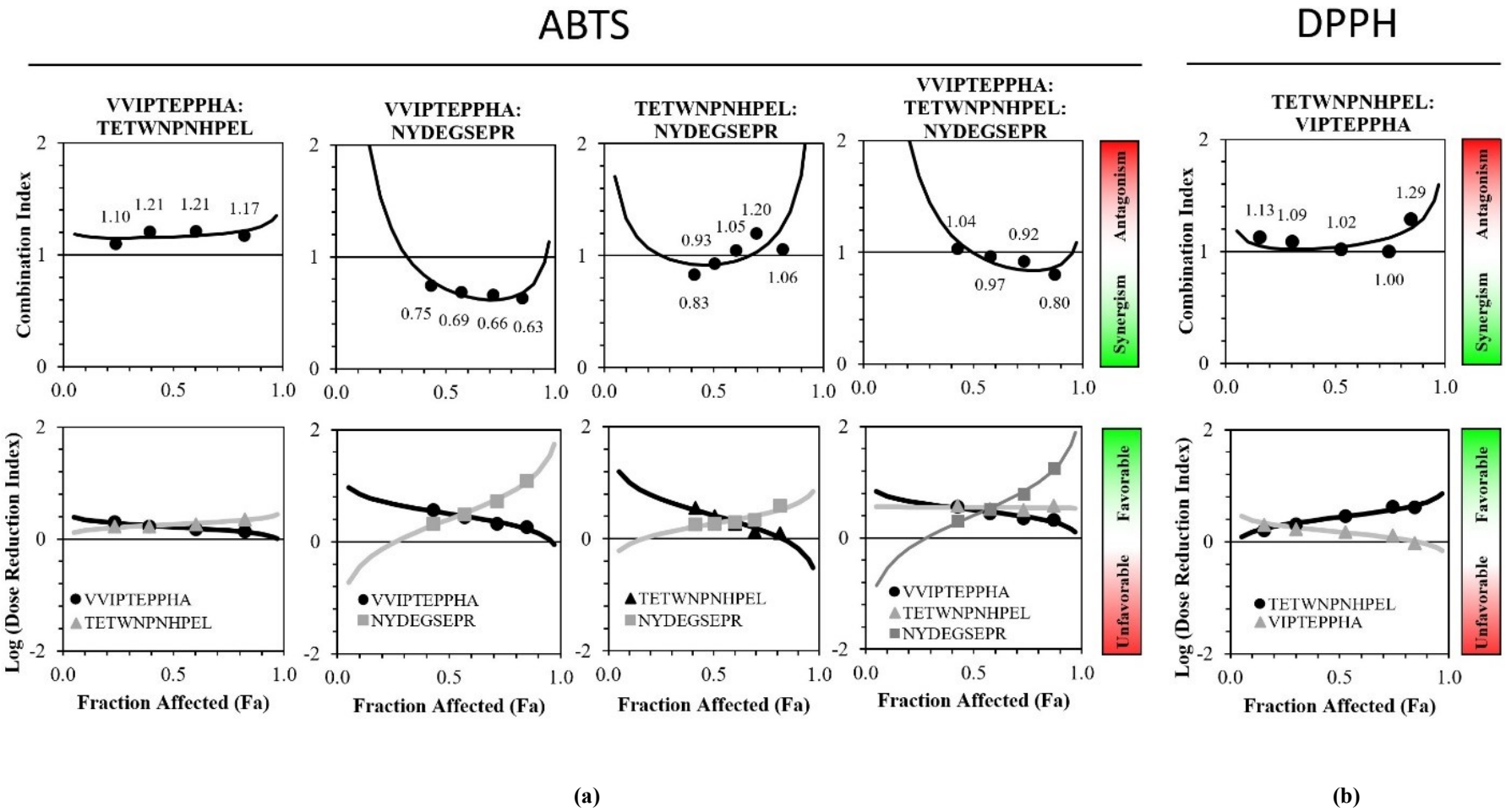


Figure 6-3. Combination index (CI) and dose reduction index (DRI) at different levels of free radical scavenging activity (F_a) of faba bean-derived peptide combinations; **(a)** ABTS assay; **(b)** DPPH assay; the CI and the DRI were calculated based on the method of Chou et al. (1984) (Chou et al., 1984) with the CompuSyn software (Zhang et al., 2016). The dots represent the experimental data, and the lines are the fitted data. Synergistic effects are defined as CI < 1, additive effects are CI = 1, and antagonistic effects are CI > 1

The combination index plot for each peptide combination was generated to identify synergistic, additive, and/or antagonist interactions (Figure 6-3) at different levels of free radical scavenging activity. A CI value < 1 indicates synergism, CI = 1 indicates additivity and CI > 1 indicate antagonism. The level of synergism and antagonism can also be evaluated based on the CI value, as a CI value < 0.1 shows very strong synergism, 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.7-0.85 moderate synergism, 0.85-0.90 slight synergism, 0.9-1.1 additive, 1.1-1.2 slight antagonism, 1.2-1.45 moderate antagonism, 1.45-3.3 antagonism, 3.3-10 strong antagonism and >10 very strong antagonism (Chou, 2006).

For the combination of VVIPTEPPHA and TETWNPNHPEL in the ABTS assay, the CI values of the four data points ranged from 1.10 to 1.21, showing an additive to a slight antagonist interaction. As these two peptides have a high proportion of hydrophobic residue (Table 6-1), it can be hypothesized that once combined, hydrophobic interactions are formed, decreasing the availability of tryptophan and histidine to scavenge the ABTS radical. On the contrary, the combination of VVIPTEPPHA and NYDEGSEPR was synergistic for the four data points. The peptide NYDEGSEPR is more hydrophilic than VVIPTEPPHA (Table 6-1), which may decrease peptide interactions. The combination of TETWNPNHPEL and NYDEGSEPR was additive for 3 data points, moderately synergistic for one point, and slightly antagonistic for one point, showing that the type of interaction is dependent on the level of free radical scavenging activity. Since the interaction was mainly additive, it means that the activities of the two peptides are independent, indicating that both reacted with the ABTS radical in a similar manner and that there are limited interactions between the two peptides. This indicates that both peptides react with the ABTS radical in a similar manner and that there are limited interactions between the two peptides. Contrarily to our results, Jia et al. (2022) found strong synergism between a tryptophan (VAGW) and a tyrosine (LLLYK)-containing peptide in the ABTS assay, meaning that the particular position of these reactive amino acids in the peptide sequence and the surrounding amino acids greatly impact the type of interactions between peptides. More generally, in their study (Jia et al., 2022), tryptophan-containing peptides (VAGW and APPAMW) displayed a synergistic interaction with a broad variety of antioxidant peptides. This was attributed to the specific location of the tryptophan residue (i.e., the C-terminal position).

When the three faba bean-derived peptides were combined, the interaction was additive, except for the last data point, where moderate synergism was observed. Since the combination of VVIPTEPPHA and TETWNPNHPEL was slightly antagonistic, the combination of VVIPTEPPHA and NYDEGSEPR

was synergistic, and the combination of TETWNPNHPEL and NYDEGSEPR was additive, it can be inferred that the combination of the three peptides canceled the synergistic and antagonistic interactions, leading to a global additive effect.

The dose reduction index (DRI) plots were also generated (Figure 6-3) to evaluate whether the peptide concentration could be reduced when it was in combination to reach the same level of effect. A DRI of less than one is considered unfavorable, meaning that the peptide dose needs to be increased when used in combination to reach the same level of activity. On the contrary, a DRI above 1 means that dose reduction is favorable and that the peptide concentration can be reduced when used in combination to reach the same level of activity. As log (DRI) is plotted in Figure 6-3, log (DRI) < 0 is unfavorable, and log (DRI) > 0 is favorable. For all peptide combinations tested, the DRI was favorable, as explained by the additive and synergistic effects. The slightly antagonistic interactions were not sufficient to make the DRI unfavorable. The peptide dose could be reduced by 1.25 up to 18-fold when used in combination to reach the same level of free radical scavenging activity. This finding confirms that the lower EC_{50} measured in the complete 3 kDa permeate of the faba bean flour digestate can be attributed to the additive and synergistic effects of the different peptides. Therefore, the seven potent antioxidant peptides identified in the ABTS assay can be considered important contributors to the overall effect of the antioxidant activity of faba bean flour after gastrointestinal digestion.

For the DPPH assay, the interaction of VIPTEPPHA and TETWNPNHPEL was mostly additive and become slightly antagonistic at low and high levels of free radical scavenging activity. It can be hypothesized that the high proportion of hydrophobic residues in these peptide sequences (Table 6-1) increases peptide interactions and decreases the availability of reactive residues, tryptophan and histidine to quench the DPPH radical, particularly at high peptide concentrations. Dose reduction was favorable, which again confirms that the lower EC_{50} measured in the complete 3 kDa permeate of faba bean flour digestate can be attributed to the additive effect of the different peptides.

6.4.1.3 Modulation of the Nuclear Factor Erythroid 2-Related Factor 2/Antioxidant Response Element (Nrf2/ARE) Cell-Signaling Pathway by Faba Bean-Derived Antioxidant Peptides

In addition to direct free radical scavenging, antioxidant peptides can have other modes of action, leading to a protective effect against oxidative stress. One of these process is the modulation of antioxidant cell-signaling pathways. We therefore investigated this potential mode of action of faba

bean-derived antioxidant peptides through the Nrf2-ARE live cell assay using a luciferase reporter-gene system. The nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that induces the expression of several genes that are part of the cell defense system against oxidative stress. When oxidative stress occurs, Nrf2 dissociates from the Kelch-like ECH-associated protein 1 (Keap1) and is translocated from the cytosol to the nucleus where it binds the antioxidant response element (ARE), an enhancer found in the promoter of several antioxidative enzyme genes, such as the superoxide dismutase (SOD1), the glutathione reductase (GR) and the thioredoxin 1 (Trx1), among others (Ma, 2013). Some food-derived antioxidant peptides were shown to activate this pathway by disrupting the interaction between Nrf2 and Keap1 (Mirdamadi et al., 2021; Tonolo et al., 2020) and thus causing the translocation of Nrf2 to the nucleus.

We investigated whether the most potent faba bean-derived antioxidant peptides could modulate the Nrf2-ARE signaling antioxidant pathway and thus complement their free radical scavenging properties. Modulation of this cellular antioxidant pathway was tested both in basal conditions and in the presence of oxidative stress (0.25 mM H_2O_2). As shown in Figure 6-4, none of the tested peptides caused a significant increase in ARE-mediated gene expression ($p > 0.05$). This result remains in good agreement with previous studies, where free radical scavenging properties and cell-signaling antioxidant properties were not necessarily correlated (Tonolo et al., 2020). It is also possible that these peptides failed to induce a cell-signaling effect because of their poor stability and *in vitro* bioavailability. The bioavailability of these peptides will have to be confirmed with subsequent investigations. From these data, we can conclude that the principal mode of action of faba bean-derived antioxidant peptides is through free radical scavenging and not the modulation of the Nrf2 cell signaling pathway or metal ion chelation. Although a dual mechanism of HAT and SET was found for free radical scavenging, the results indicated that the HAT mechanism was favored compared to that of SET. The antioxidant activity of faba bean peptides will need to be tested with *in vivo* assays to confirm the present findings.

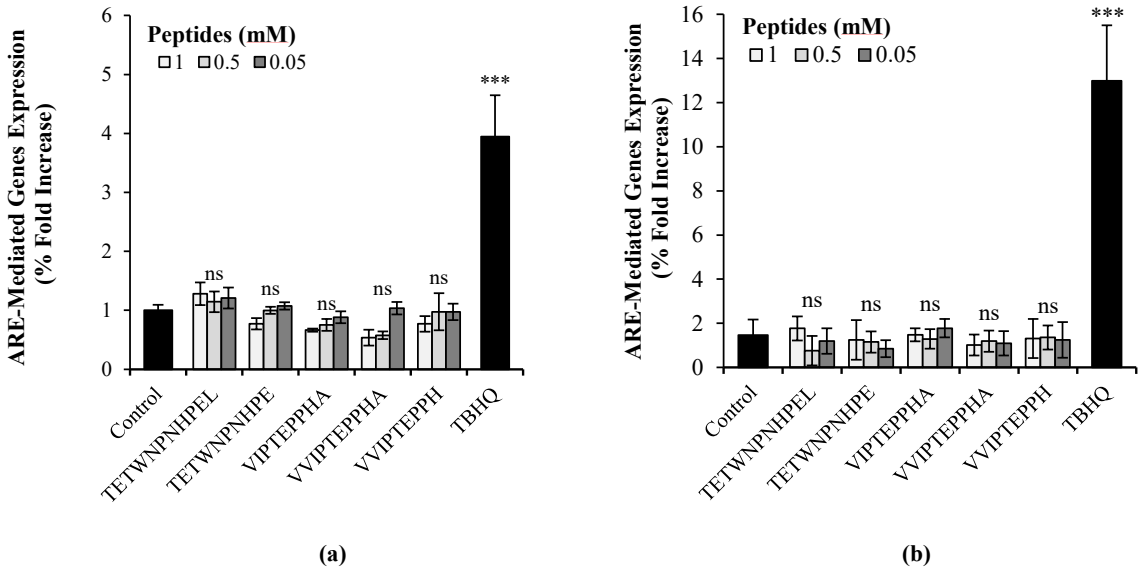


Figure 6-4. Modulation of the Nrf2-ARE pathway by faba bean-derived antioxidant peptides in: **(a)** basal conditions; **(b)** the presence of oxidative stress (H_2O_2 0.25 mM). The different peptide treatments were compared to their respective controls (untreated cells and cells treated with 0.25 mM H_2O_2) by one-way Anova and the Dunnett's posthoc test (***, $p < 0.001$; ns, not significant $p > 0.05$). Tert-butylhydroquinone (TBHQ) was used as a positive control.

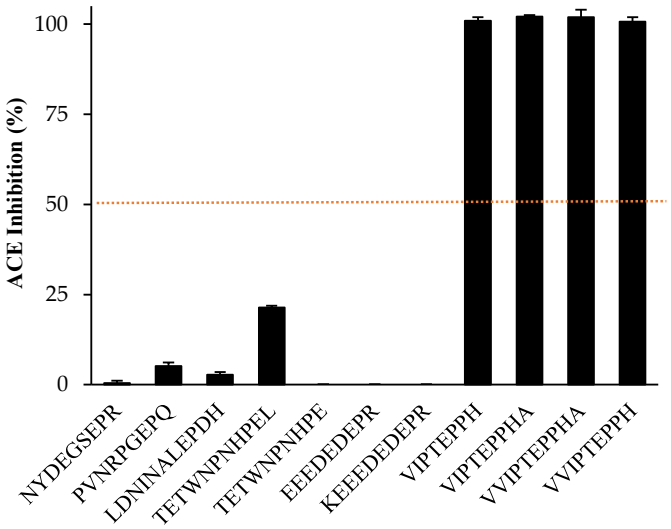
6.4.2 Mechanism of ACE inhibition by Faba Bean-Derived Peptides

6.4.2.1 ACE Inhibition Activity

The 11 faba bean-derived peptides were screened for ACE inhibition activity. Four peptides, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH, demonstrated a strong inhibition activity (Figure 6-5), where 100% inhibition was obtained at peptide concentration of 10 mM. In comparison, the nine other peptides had negligible inhibitory activity. The half-maximal ACE inhibitory concentration (IC_{50}) of the 4 faba bean peptides ranged from 43 to 123 µM (Figure 6-5). These values are comparable to other peptides identified in the gastrointestinal digestate of various food products (Chen et al., 2022; Heo et al., 2017; Yin et al., 2022). Moreover, these four peptides are likely responsible for the ACE inhibitory effect of the complete 3 kDa of faba bean gastrointestinal digestate, since their IC_{50} when expressed in µg/mL (80, 118, 53 and 43 µg/mL respectively) are importantly lower than the digestate permeate (1348 µg/mL) (Martineau-Côté, Achouri, Wanasundara, et al., 2022). Although the inhibitory activity of these peptides is important, it remains 100 to 300 times lower than that of captopril on a molar basis, the latter being a commercialized ACE inhibitor for hypertension treatment.

Despite similarity in their sequence, the four faba bean peptides were significantly different in their ACE inhibitory potency. For instance, **V**VIPTEPPHA and **V**VIPTEPPH had significantly lower IC_{50} (p <0.05) compared to VIPTEPPHA and VIPTEPPH, respectively, suggesting that the presence of an additional valine residue at the N-terminal extremity of the peptide may play a key role in the ACE inhibitory activity. On the contrary, the presence of an alanine residue at the C-terminal extremity of VIPTEPPH**A** and VVIPTEPPH**A** seems to increase the IC_{50} compared to VIPTEPPH and VVIPTEPPH, respectively.

Noteworthy, the four ACE inhibitor peptides were revealed to be potent antioxidants in the ORAC, ABTS and/or the DPPH assay, suggesting multifunctionality. This trait is an additional benefit that can serve the management of hypertension.



(a)

	ACE Inhibition	
	(IC_{50} µM)	(IC_{50} µg/mL)
NYDEGSEPR	-	-
PVNRPGEPQ	-	-
LDNINALEPDH	-	-
TETWNPNHPEL	-	-
TETWNPNHPE	-	-
EEEDEDEPR	-	-
KEEEDEDEPR	-	-
VIPTEPPH	90 ± 10^{a}	81 ± 9^{a}
VIPTEPPHA	123 ± 5^{b}	118 ± 4^{b}
VVIPTEPPHA	50 ± 5^{c}	53 ± 6^{c}
VVIPTEPPH	43 ± 1^{c}	43 ± 1^{c}
Captopril	*0.37 ±0.06*	*0.08 ±0.01*

(b)

Figure 6-5. ACE inhibitory activity (mean ± standard deviation) of faba bean-derived peptides after in vitro gastrointestinal digestion. Means without a common letter differ (p < 0.05), as analyzed by one-way Anova and the Tukey's test. (a) ACE inhibition (%) was determined at 10 mM for all peptides as a first screening. (b) The IC50 of the four most potent ACE inhibitory peptides was determined.

6.4.2.2 ACE Inhibition Pattern of Faba Bean-Derived Peptides

The ACE inhibition pattern of VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH was assessed through kinetic experiments. The initial velocity of reaction was measured at different

substrate (0.5-2 mM) and peptide concentrations and Lineweaver-Burk double reciprocal plots were built to identify the inhibition pattern. As shown in Figure 6-6, the four peptides exhibited a noncompetitive inhibition pattern. Indeed, in the four cases, the Lineweaver-Burk curves are converging on the X-axis, indicating that the apparent K_m is unchanged and the apparent V_{max} is decreased with the addition of the inhibitory peptides. This result means that the peptides can bind both the free enzyme and the enzyme-substrate complex with a similar affinity. The inhibitor binding site is therefore located outside the enzyme active site. The loss of ACE activity in the presence of peptides can therefore be explained by conformational changes caused by peptides binding rather than competition for the active site.

The inhibition constant (K_i) was calculated from secondary plots for each peptide. The secondary plots were constructed in plotting the Lineweaver-Burk curve slope against the peptide concentration. The K_i value was calculated from the negative intercept on the X-axis of the secondary plot. The K_i value was 87 µM for VIPTEPPH, 107 µM for VIPTEPPHA, 45 µM for VVIPTEPPH and 54 µM for VVIPTEPPHA. These K_i values are nearly identical to the IC_{50}, confirming the noncompetitive inhibition pattern. The minor differences in the IC_{50} and the K_i values can be explained by experimental imprecision.

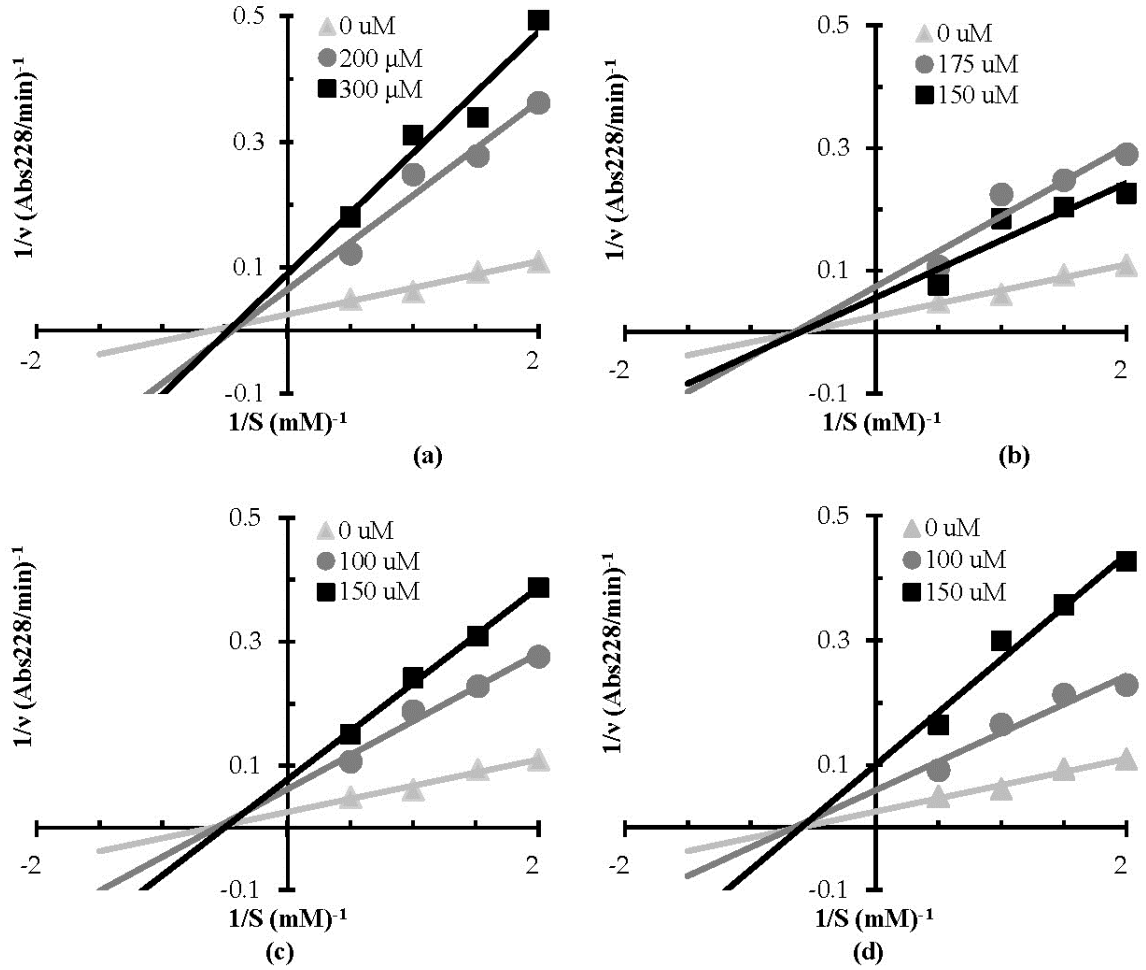


Figure 6-6. Double reciprocal (Lineweaver-Burk) plots of ACE inhibition by faba bean-derived-inhibitory peptides. Each point represents the mean of three experiments: **(a)** VIPTEPPH; **(b)** VIPTEPPHA; **(c)** VVIPTEPPHA; and **(d)** VVIPTEPPH.

6.4.2.3 Investigation of the Potential Binding Mode between Faba Bean-Derived Peptides and ACE by Molecular Docking

Molecular docking was used to investigate and compare the potential binding mode of faba bean peptides to ACE. The ACE active site is composed of three substrate binding pockets namely S1, S2 and S1'. S1 is composed of Ala 354, Glu 384 and Tyr 523, S2 is composed of Gln 281, His 353, Lys 511, His 513 and Tyr 520 whereas S1' is composed of Glu162 (Pina & Roque, 2009). The catalytic mechanism of ACE implies a zinc (II) coordination motif (HEXXH), composed of two histidine (His 383 and His 387) and a glutamic acid (Glu411) residue. Commercialized ACE inhibitors, such as captopril and linosipril are competitive inhibitors (Natesh et al., 2004; Natesh et al., 2003) of ACE,

meaning that they inhibit ACE activity in competing for the active site. Their mechanism of action is well understood and imply direct interaction with the ACE catalytic site composed of a zinc coordination motif in the active site.

For noncompetitive inhibitors, the inhibition mechanism is still not well characterized. Only a few recent studies attempted to elucidate it (Duan et al., 2014; Heo et al., 2017; Lan et al., 2018; Vy et al., 2020; Xie et al., 2022). Since the kinetic study revealed that the four faba bean peptides (VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH) act as noncompetitive inhibitors that bind ACE outside the active site, global docking was performed on the whole ACE molecular structure to predict the most probable binding site. HPEPDOCK (Zhou et al., 2018) was used, which is a docking software that can perform blind flexible protein peptide docking. In this software, peptide flexibility is considered using an ensemble of peptides conformation (Zhou et al., 2018).

Before performing docking with the four faba bean-derived peptides, the docking procedure was validated with two ACE ligand, the peptide bradykinin-potentiating peptide b (BPPb) (pEGLPPRPKIPP, where pE is a pyroglutamic acid residue) and Angiotensin II (DRVYIHPF), for which co-crystallized structure with ACE are available. The docked peptides were aligned to the peptide structure as-found in the ACE-BPPb and ACE-Angiotensin II co-crystallized structures (PDB 4APJ and 4APH, respectively) to calculate the root mean square deviation (RMSD) (Table 6-2). In both cases, the model with the lowest HPEPDOCK docking energy score (model 1) had the lowest RMSD, which was within the generally accepted range of 0–2 Å (Xiao et al., 2018). The lowest docking energy score lead therefore to the best docking pose in both cases with the correct orientation. The molecular interactions between the two peptides and ACE were analyzed with LigPlot+ to evaluate if the principal molecular interactions stabilizing the peptide and ACE complexes were correctly identified. For Angiotensin II, the principal hydrogen bonds with ACE residue, namely, Gln 281, Tyr 520, Lys 511, His 513, His 383, His 387, and Ala 356 were identified, which is in good agreement with the literature (Masuyer et al., 2012). For BPPb, the principal hydrogen bonds with Lys 118, Asp 121, Tyr 520, Ser 516, Ser 517, Ala 356, Tyr 360 and Gln 281 were identified which again is in good agreement with the literature (Masuyer et al., 2012). The small variations between the interactions found experimentally by co-crystallization (Masuyer et al., 2012) and with the docking simulation can be explained by small variation of the docked ligand orientation and software imprecision. Since the RMSD value was in the expected range for the top prediction and that the

important molecular interactions were successfully identified, the docking protocol was considered reliable and applied to the four faba bean peptides.

Table 6-2. Docking energy scores and root-mean-square deviation (RMSD) obtained for BPPb and Angiotensin II in the docking validation.

	BPPb[1]		**Angiotensin II**	
	Docking Score	RMSD (Å)	Docking Score	RMSD (Å)
Model 1	-347.323	0.000	-288.495	0.000
Model 2	-242.122	1.995	-280.668	3.199
Model 3	-239.320	2.004	-271.124	5.026
Model 4	-235.063	1.664	-270.934	4.234
Model 5	-227.442	3.746	-269.242	5.353
Model 6	-226.858	4.147	-266.581	4.295
Model 7	-226.715	3.685	-262.468	3.553
Model 8	-220.964	3.850	-261.550	2.901
Model 9	-219.574	5.991	-255.798	4.485
Model 10	-218.664	3.196	-255.775	3.200

[1] Bradykinin-potentiating peptide b.

The docking simulation with the 4 faba bean-derived peptides revealed that the most probable binding site of the four faba bean-derived peptides is located at the entrance of the active site cavity (Figure 6-7). The docked peptides were mostly stabilized by hydrogen bonds, hydrophobic interactions, and salt bridges. The ACE active site cavity is "protected" by a "lid" composed of three alpha helix of the N-terminal region of ACE α1, α2 and α3, consisting of the residues 40–71, 74–107 and 109–120 respectively (Figure 6-7). These three helix possess several charged amino acid residue that prevent the entry of large substrates to the ACE active site cavity (Natesh et al., 2003). Therefore, the binding of inhibitory peptides in this region is likely to limit the substrate entry and/or product exit from the active site cavity and thus decrease ACE activity.

The four faba bean peptides formed hydrogen bonds with ACE residue in this region during the docking simulation, namely Trp 59, Tyr 62, Asn 85, Thr 92 (Table 6-3). This binding site is in good agreement with the noncompetitive inhibition mode observed. This mechanism of action was recently proposed for three noncompetitive casein-derived peptides GVSLPEW, GYGGVSLPEW and VGINYW (Xie et al., 2022) and a Spirulina-derived peptide TMEPGKP (Heo et al., 2017).

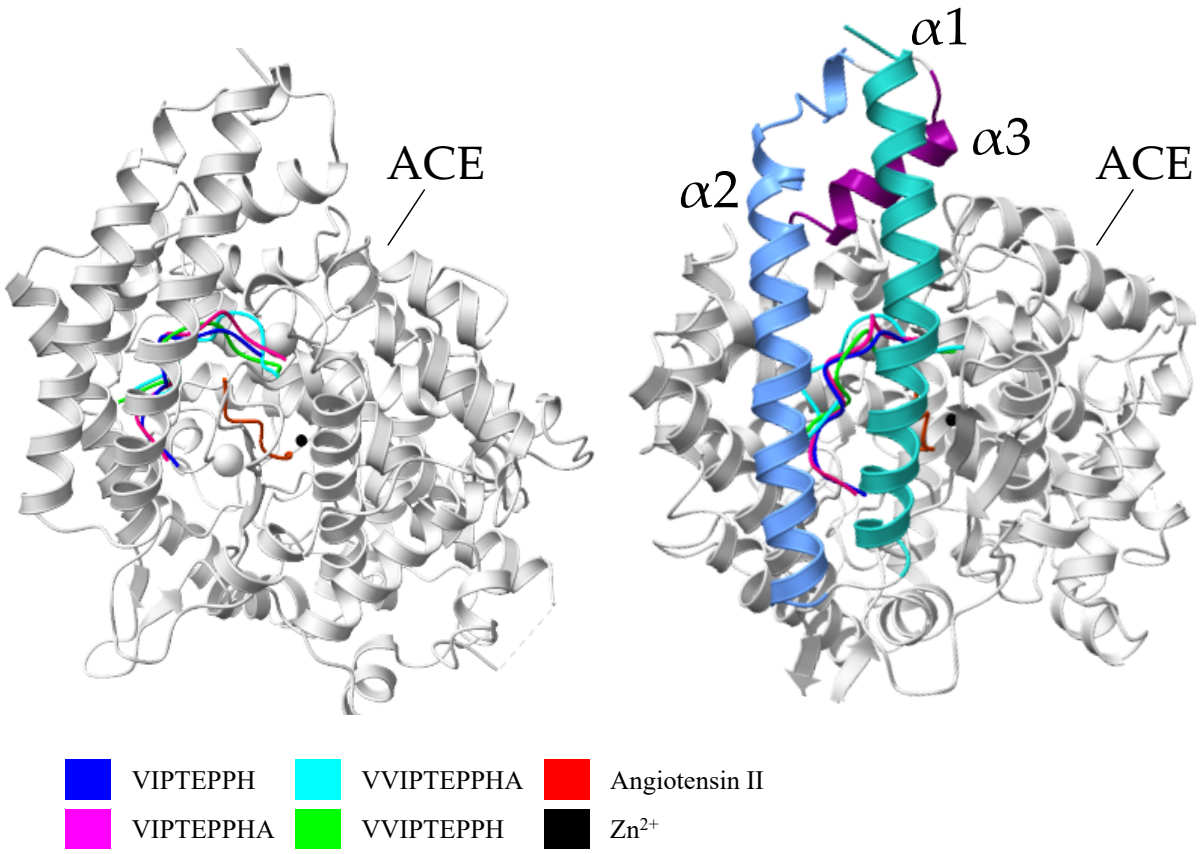


Figure 6-7. Global views of the best docked poses of faba bean ACE inhibitory peptides with the C-domain of human somatic ACE. Molecular docking simulations were performed using HPEPDOCK (Zhou et al., 2018) and data visualization was performed using UCSF ChimeraX (Pettersen et al., 2021).

Hydrogen bonds are important molecular interactions that stabilize molecular complexes. During the docking simulations, there were significant differences in the number of hydrogen bonds formed with ACE residue, which can explain the different inhibitory potency among the four faba bean peptides (Table 6-3). The peptides VVIPTEPPH and VVIPTEPPHA were stabilized by a higher number of hydrogen bonds (9 and 7, respectively) compared to VIPTEPPH and VIPTEPPHA (3 and 2, respectively), which is in good agreement with their inhibitory activity potency. Moreover, VVIPTEPPH and VVIPTEPPHA formed hydrogen bonds with ACE residues that are closer to the active site pockets, namely His 410, Arg 522, Gly 404, Arg 402 and Tyr 394, which again could explain their higher activities. More specifically, the first valine residue in VVIPTEPPH and VVIPTEPPHA formed 4 and 2 hydrogen bonds respectively with ACE residue, confirming the importance of this residue in the stabilization of the inhibitory peptide and ACE complexes. The valine

at the N-terminal extremity of VVIPTEPPH and VVIPTEPPHA formed a hydrogen bond with His 410, which is right next to Glu 411, an important residue of the ACE catalytic center. Therefore, the results of the docking simulation are in good agreement with the noncompetitive inhibition pattern or the four peptides and their inhibitory activity potency.

Table 6-3. Molecular interactions (hydrogen bonds and salt bridges) identified between faba bean peptides (VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH) and the ACE residue from molecular docking simulations.

ACE (PDB 4APH)[1]	**Faba bean-derived peptides**		
	VIPTEPPH		
Atom name (Residue)	Atom name (Residue)	Interaction Type	Distance (Å)
NH1 (Arg 124)	O (Pro 3)	Hydrogen bond	3.26
NE (Arg 124)	OE2 (Glu 5)	Hydrogen bond	3.13
OH (Tyr 62)	O (Pro 3)	Hydrogen bond	2.85
	VIPTEPPHA		
Atom name (Residue)	Atom name (Residue)	Interaction Type	Distance (Å)
NH2 (Arg 124)	O (Pro 3)	Hydrogen bond	2.28
OH (Tyr 62)	O (Ile 2)	Hydrogen bond	3.20
	VVIPTEPPHA		
Atom name (Residue)	Atom name (Residue)	Interaction Type	Distance (Å)
NE2 (His 410)	O (Val 1)	Hydrogen bond	2.85
OH (Tyr 360)	N (Val 1)	Hydrogen bond	3.18
OH (Tyr 135)	OE1 (Glu 6)	Hydrogen bond	2.71
NH2 (Arg 124)	O (Pro 7)	Hydrogen bond	3.16
NH2 (Arg 124)	O (Glu 6)	Hydrogen bond	2.71
ND2 (Asn 85)	ND1 (His 9)	Hydrogen bond	2.63
NE (Arg 124)	OE1 (Glu 6)	Salt bridge	3.79
	VVIPTEPPH		
Atom name (Residue)	Atom name (Residue)	Interaction Type	Distance (Å)
NH2 (Arg 522)	O (Val 2)	Hydrogen bond	2.81
NE2 (His 410)	N (Val 1)	Hydrogen bond	2.94
N Gly 404)	N (Val 1)	Hydrogen bond	3.18
O (Arg 402)	N (Val 1)	Hydrogen bond	2.96
OH (Tyr 394)	N (Val 1)	Hydrogen bond	1.97
O (Asn 136)	ND1 (His 9)	Hydrogen bond	2.56
OG1 (Thr 92)	OE2 (Glu 6)	Hydrogen bond	3.07
OH (Tyr 62)	O (Pro 7)	Hydrogen bond	3.04
NE1 (Trp 59)	O61 (Thr 5)	Hydrogen bond	2.52

[1] Angiotensin-converting enzyme (ACE) in complex with angiotensin-II.

6.5 Conclusions

This study reported for the first time a comprehensive investigation of the mechanism of action of faba bean-derived bioactive peptides after *in vitro* gastrointestinal digestion. The mechanism of action of 7 novel bioactive peptides-derived from faba bean flour gastrointestinal digestate was ascertained. NYDEGSEPR, TETWNPNHPEL, TETWNPNHPE, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH were revealed to be potent antioxidant peptides, through free radical scavenging, principally through a HAT-based mechanism. Combinations of faba bean peptides lead mostly to an additive and/or synergistic antioxidant effect, which indicates the importance of consuming faba bean proteins as a whole ingredient. Four peptides, namely VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH were also potent ACE inhibitory peptides, making them multifunctional, which is of great interest in the management of noncommunicable diseases. The four peptides are noncompetitive inhibitor of ACE and their most probable binding sites are located near to the entrance of the active site cavity. From these results, it can be concluded that the antioxidant and ACE inhibitory activity of faba bean flour after *in vitro* gastrointestinal digestion can be associated with the release of bioactive peptides with synergistic and multifunctional activities. Future research will be needed to investigate the bioavailability of these peptides to confirm their bioactive potential. *In vivo* assays will also need to be performed to confirm the bioactive properties of faba bean-derived peptides after gastrointestinal digestion.

6.6 References

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CONNECTING STATEMENT V

Chapter V and VI revealed that faba bean flours have a strong potential to be used as a functional food ingredients due to their bioactive properties after gastrointestinal digestion, particularly in terms of antioxidant and antihypertensive activity. These health-promoting properties are associated with the release of bioactive peptides after gastrointestinal digestion. However, to further evaluate their real health-beneficial potential *in vivo*, faba bean-derived peptides need to be bioavailable, meaning that they have to cross the intestinal barrier to reach their targeted sites of actions.

In Chapter VII, transepithelial transport of faba bean peptides in comparison to pea and soy after gastrointestinal digestion was therefore assessed using a cellular model mimicking the intestinal barrier. The peptides with the capacity to cross the intestinal barrier were identified by tandem mass spectrometry and the residual antioxidant activity after peptide transportation was assessed has an indicator of bioactive property preservation.

The results from this research were presented at the 2023 AOCS Annual Meeting & Expo:

- Martineau-Côté, D. Achouri, A., Karboune S. & L'Hocine, L. (2023, April 30–May 3). Transepithelial transport of faba bean peptides across a Caco-2 and HT29-MTX co-culture monolayer after gastrointestinal digestion [Oral Presentation]. 2023 AOCS Annual Meeting & Expo, Denver, Colorado, USA.

CHAPTER VII. TRANSEPITHELIAL TRANSPORT ACROSS A CACO-2 AND HT29-MTX-E12 CO-CULTURE MONOLAYER OF FABA BEAN BIOACTIVE PEPTIDES, IN COMPARISON TO PEA AND SOY, AFTER *IN VITRO* GASTROINTESTINAL DIGESTION AND ASSESSMENT OF THEIR ANTIOXIDANT ACTIVITY

7.1 Abstract

In this study, the transepithelial transport of bioactive peptides derived from faba bean flour gastrointestinal digestate was investigated *in vitro* using a Caco-2 and HT29-MTX-E12 co-culture monolayer. Pea and soy were used as control legumes. The profile of transported peptides was determined by mass spectrometry. For all legumes, the ORAC value significantly decreased ($p < 0.05$) after transepithelial transport (24-36% reduction), but a different trend was observed for the ABTS assay, where the transported peptides had a significantly higher activity ($p < 0.05$). The results revealed that 9 faba bean peptides crossed the intestinal cellular monolayer and 5 of them were antioxidants (TETWNPNHPEL, TETWNPNHPE, VIPTEPPHA, VVIPTEPPH and VVIPTEPPHA). Interestingly, the most potent antioxidant peptides, TETWNPNHPEL and TETWNPNHPE, were further hydrolyzed to smaller fragments (TETWNPNHP and TWNPNHPE) after cellular uptake. The faba bean metabolized peptides were synthesized and both were potent antioxidants when assessed with the ABTS (EC_{50} of 1.2 ± 0.2 and 0.4 ± 0.1 mM) and the ORAC assay (2.5 ± 0.1 and 3.4 ± 0.2 mM of Trolox equivalent/mM), explaining the preservation of the antioxidant activity after transepithelial transport. These results demonstrate for the first time the *in vitro* bioavailability of faba bean peptides produced after *in vitro* gastrointestinal digestion.

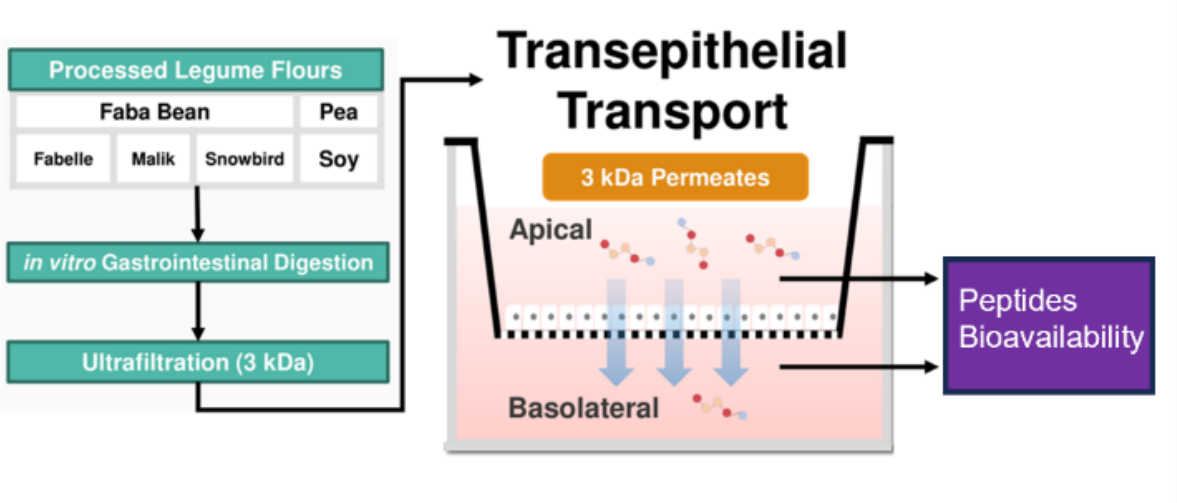


Figure 7-1. Graphical abstract

7.2 Introduction

Faba bean (*Vicia faba* L.) is a promising high-quality and sustainable protein source. Its high protein content (30%) and well-balanced amino acid composition (Millar et al., 2019) makes it a suitable protein source to include in various food products (Coda et al., 2017; Millar et al., 2017; Rosa-Sibakov et al., 2016; Tazrart et al., 2016). Besides its high nutritional value, faba bean also has a high bioactive potential, being a rich source of bioactive compounds, such as polyphenols, L-DOPA, γ-aminobutyric acid, resistant starch, fibres, and bioactive peptides (Martineau-Côté, Achouri, Karboune, et al., 2022) that have reported health-enhancing properties. Food-derived bioactive peptides are promising health-promoting agents in the management of non-communicable diseases (Duffuler et al., 2022). Countless highly active peptides have been identified in food protein hydrolysates of diverse sources (Manzoor et al., 2022) and faba bean is no exception. Faba bean protein hydrolysates with antioxidant (Ashraf et al., 2020; Felix et al., 2019; Jakubczyk et al., 2019; Karkouch et al., 2017; León-Espinosa et al., 2016; Martineau-Côté, Achouri, Karboune, et al., 2024; Martineau-Côté, Achouri, Wanasundara, et al., 2022), antidiabetic (Felix et al., 2019; Martineau-Côté, Achouri, Wanasundara, et al., 2022), antihypertensive (Felix et al., 2019; Martineau-Côté, Achouri, Karboune, et al., 2024; Martineau-Côté, Achouri, Wanasundara, et al., 2022), cholesterol-lowering (Ashraf et al., 2020; León-Espinosa et al., 2016), anticancer (León-Espinosa et al., 2016), anti-inflammatory (Jakubczyk et al., 2019), immune-modulating (Asledottir et al., 2023) and food intake regulation (Dugardin et al., 2020) properties have been identified to date. In a previous study (Martineau-Côté, Achouri, Wanasundara, et al., 2022), we have demonstrated that faba bean peptides generated after *in vitro* gastrointestinal digestion have a high bioactive potential, particularly in terms of antihypertensive and antioxidant activity. These bioactive properties revealed to be associated with the release of bioactive peptides after in vitro gastrointestinal digestion (Martineau-Côté, Achouri, Karboune, et al., 2024). Among these, 7 novel faba bean derived peptides (NYDEGSEPR, TETWNPNHPEL, TETWNPNHPE, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA and VVIPTEPPH) with antioxidant activity were identified and their mechanism of action investigated (Martineau-Côté, Achouri, Karboune, et al., 2024) . However, the bioactive properties of protein hydrolysate produced in non-physiological conditions and measured with *in vitro* assays do not always translate in to a high activity *in vivo* (Duffuler et al., 2022). Indeed, for bioactive peptides to reach their targeted site of action (Bouglé & Bouhallab, 2017; Sun et al., 2020), they need to resist gastrointestinal digestion and be transported across the intestinal barrier. Peptide transportation across the intestinal barrier

depends on many factors including molecular weight, charge, polarity, food matrix composition and physical form, among others (Sun et al., 2020). It is now accepted that food derived di and tri peptides can be absorbed intact in the small intestine (Miner-Williams et al., 2014). Some of them were shown to exert health benefits in humans. As an example, it was demonstrated in clinical trials that the antihypertensive milk-derived tri-peptide IPP was able to reach the blood stream (Foltz et al., 2007) and exert pressure-lowering effects (Cicero et al., 2013). Clinical evidences of food-derived oligopeptides reaching the blood circulation in humans is not a scientific consensus (Miner-Williams et al., 2014). Research is still at its early stage. Yet, it was demonstrated that lunasin, a soy-derived peptide of 43 amino acids, can reach the bloodstream in human subjects after soy intake (Dia et al., 2009).

While human and animal models are the most significant way to assess bioactive peptide absorption and bioavailability, they pose ethical and economical limitations. Cellular models present a viable alternative, as they mimic the intestinal barrier without these drawbacks. To achieve this, intestinal cells are cultivated on a permeable support to mimic the intestinal barrier. The peptides of interest are added to the apical (AP) side, representing the intestinal lumen and the *in vitro* bioavailable peptides are recovered in the basolateral (BL) side, representing the intestinal circulation.

Although numerous enterocyte cell lines are available, Caco-2 cells are the preferred and most widely used to model the human intestinal barrier (Langerholc et al., 2011). Initially used to study drug absorption, stability and metabolization (Hubatsch et al., 2007), Caco-2 cells usage has been extended to study dietary-derived bioactive peptide absorption (Lammi et al., 2016; L. Wang et al., 2019). These are colorectal adenocarcinoma cells that differentiate spontaneously into enterocyte-like cells when they reach confluence (Lea, 2015). The cells form a polarized intestinal monolayer, which expresses tight junctions, microvilli, brush border digestive enzymes (Lea, 2015) and peptides transporters such as Peptide transporter 1 (PepT1) (Jochems et al., 2018). These properties enable the study of the main mode of peptide transport (carrier-mediated, passive diffusion, paracellular and transcytosis (Sun et al., 2020). To mimic more closely the intestinal barrier, the Caco-2 model is further improved by the co-cultivation of HT29-MTX-E12 cells (Lozoya-Agullo et al., 2017). HT29-MTX-E12 cells are an adenoma carcinoma cell line that was differentiated into mucin-producing cells in presence of methotrexate (MTX) (Lesuffleur et al., 1990). Mucins are highly glycosylated proteins that form a gel in the presence of water and act as a physical barrier in the intestine (Johansson et al.,

2011). This mucus layer limits particle diffusion and is therefore an essential element to consider when studying peptide transport (Boegh et al., 2015).

Data on the bioavailability of faba bean peptides after gastrointestinal digestion is lacking and therefore need to be assessed to clearly anticipate it's bioactive potential *in vivo*. Therefore, in the present study, we applied the co-culture of Caco-2 and HT29-MTX-E12 cell lines to model faba bean peptides transportation across the intestinal barrier with the objectives to investigate whether faba bean bioactive peptides released after gastrointestinal digestion can be transported intact across the intestinal barrier. To that end, *in vitro* gastrointestinal digestates of flours from three Canadian faba bean varieties were investigated (Fabelle, Malik and Snowbird) in comparison to those of two conventional legumes (pea and soy). The transported peptides were identified by mass spectrometry, and the residual antioxidant activity after peptides transportation was assessed as an indicator of bioactive property preservation.

7.3 Materials and Methods

7.3.1 Chemicals

Three dehulled faba bean cultivars (Fabelle Malik and Snowbird), one dehulled pea cultivar (Amarillo) and one dehulled soy cultivar (AAC-26-15) were used in this study. Faba bean cultivars Fabelle and Malik were provided by AGT Foods and Ingredients (Saskatoon, SK, CA), and Snowbird by W.A. Grain & Pulse Solutions (Innisfail, AB, CA). Certified yellow pea (CDC Amarillo) and soybean (Cdn #1, Variety AAC 26-25, Non-GMO & IP, Lot 261510504AT) were provided by Greenleaf Seeds (Tisdale, SK, CA) and Huron seeds (Clinton, ON, CA), respectively. Faba bean and pea samples were supplied as milled flours, and soybean as whole seeds. Soy flour was prepared as previously described in Martineau-Côté, Achouri, Wanasundara, et al. (2022). For cell culture, Dulbecco's Modification of Eagle's Medium (DMEM) containing 4.5g/L glucose, with phenol-red and without sodium pyruvate, 200 mM L-glutamine, Dulbecco's Phosphate-Buffered-Saline (D-PBS) without Ca^{2+} and Mg^{2+}, Dulbecco's Phosphate-Buffered-Saline (D-PBS) with Ca^{2+} and Mg^{2+}, nonessential amino acid solution 100x, heat-inactivated fetal bovine serum (FBS), 5000 IU penicillin and 5000 µg/ml streptomycin solution, trypsin solution (0.05%) containing 0.53 mM EDTA in HBSS were purchased from Wisent Bioproducts (Saint-Jean-Baptiste, QC, Canada). Hank's Balanced Salt Solution (HBSS) was purchased from Gibco (Thermo Fisher Scientific, San Jose, CA, USA). Caco- 2 cells (ATCC® HTB- 37™, passage 18 when purchased) were procured from ATCC

(Manassas, Virginia, USA). HT29-MTX-E12 cells (passage 50 when purchased) were obtained from European Collection of Authenticated Cell Cultures (ECACC). 12 mm Transwell® with 0.4 µm pore polyester membrane insert (Corning 3460), alcian blue 8GX, 4-nitrophenol solution 10 mM, p-nitrophenyl phosphate tablets and lucifer yellow CH dipotassium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The peptides derived from faba bean flour gastrointestinal digestate, TETWNPNHPEL and TETWNPNHPE and their metabolites, TETWNPNHP and TWNPNHPE, were synthesized by Biomatik (Kitchener, Ontario, Canada). Their purity (>98%) and quality was checked by reverse-phase HPLC and mass spectrometry analysis.

The Pierce BCA Assay kit was purchased from Thermo Fisher Scientific (San Jose, CA, USA) and the Cell Titer-Glo 2.0 kit was purchased from Promega (Fitchburg, Wi, USA). All chemicals and reagents used were of analytical grade. Deionized water was used in all experiments.

7.3.2 Processing and *in vitro* Gastrointestinal Digestion of Faba Bean, Pea and Soy Flours

Before *in vitro* gastrointestinal digestion, the legume flours were thermally treated through boiling as previously described (Martineau-Côté, Achouri, Wanasundara, et al., 2022). Legume flours were then digested *in vitro* using the standardized INFOGEST protocol (Brodkorb et al., 2019) with modifications as previously described (Martineau-Côté, Achouri, Pitre, et al., 2024; Martineau-Côté, Achouri, Wanasundara, et al., 2022). After digestion, bioaccessible peptides were recovered through centrifugation and ultrafiltration on a 3 kilo dalton (kDa) molecular weight cut-off membrane to remove residual proteases from the mixture (Martineau-Côté, Achouri, Wanasundara, et al., 2022). The permeates of three independent digestions of legume flours were pooled together for cell transport study. Pooled permeates osmolality was measured using a Micro-Osmometer (Model 3320, Advanced Instruments INC., Norwood, Massachusetts) and permeates were diluted in water to reach a physiological osmolality of 285–300 mOsm/kg, and their pH adjusted to a physiological value of pH 7.3. Protein content in the diluted permeates was determined with the Pierce BCA protein assay kit using bovine serum albumin as standard. The 3 kDa permeates were frozen at -80 °C until the transport experiment.

7.3.3 Cell Culture

Caco-2 and HT29-MTX-E12 cells were cultivated separately in growth medium, which was composed of DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 1% non-essential amino acids and 2 mM L-glutamine and incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were sub-cultivated once a week at 80–90% confluence using a trypsin-EDTA solution and culture medium was changed every 2-3 days. Three passages were completed prior to the transport studies to allow cell phenotype stabilization (Hubatsch et al., 2007). Caco-2 cells between passage 35 and 38 and HT29-MTX-E12 cells between passage 56 and 59 were used in this study.

7.3.4 Characterization of the Caco-2 and HT29-MTX-E12 Cell Monolayer

Caco-2 cell differentiation was evaluated by *in situ* measurement of alkaline phosphatase (ALP) activity following the procedure of Ferruzza et al. (2012). Mucus production by HT29-MTX-E12 cells was confirmed by alcian blue staining following the procedure of Pan et al. (2015). Detailed procedure for these two assays can be found in supplementary material.

7.3.5 Evaluation of Cell Viability After Incubation with 3 kDa Permeates of Faba Bean, Pea and Soy *in vitro* Gastrointestinal Digestates

To assess the impact of the 3 kDa permeates of the legume digestates on the viability of the Caco-2 and HT29-MTX-E12 co-culture, two different concentrations of peptides (1120 and 2240 µg peptide/mL of 3 kDa digestate permeate) were incubated with the Caco-2 and HT29-MTX-E12 co-culture (ratio 9:1), which were seeded in growth medium in 96 wells black plate with clear bottom at a density of 1×10^5 cells/cm^2 for 21 days. These peptide concentrations were selected to mimic potential realistic small intestinal exposure as described previously by Mahler et al. (2012) and R. Zhang et al. (2020), considering a realistic serving of 50 up to 100 g of dried pulse (Richter et al., 2019). More details can be found in supplementary materials. On the day of the assay, growth media was discarded, cells were washed with 100 µL of D-PBS and then incubated with 100 µL of 3 kDa permeate of legume digestates diluted in Hanks' Balanced Salt Solution (HBSS) for 2 hours at 37 °C. Cells treated with HBSS were used as control of viable cells. After the incubation period, cell viability was measured using the Cell Titer-Glo 2.0 kit (Promega, Fitchburg, Wisconsin) (Martineau-Côté, Achouri, Wanasundara, et al., 2022).

7.3.6 Transepithelial Transport of Faba Bean, Pea and Soy Peptides across a Caco-2 and HT29-MTX-E12 Co-Culture Monolayer

The transport study was conducted following the procedure of Pan et al. (2015) with minor modifications. Caco-2 and HT29-MTX-E12 (ratio 9:1) cells were seeded at the density of 1×10^5 cells/cm^2 in growth medium on 12 mm polyester transwell inserts with 0.4 µm pore size and cultivated for 21 days. The 9:1 ratio was selected based on enterocyte and goblet cells proportion in the human small intestine (Chen et al., 2010; Dawson, 1983; Kleiveland, 2015; Pan et al., 2015). The growth medium was changed every 2–3 days. Monolayer integrity was evaluated by transepithelial electrical resistance measurement (TEER) with a Millicell ERS-2 Voltohmmeter (EMD Millipore, Billerica, MA) at 37 °C. TEER values were expressed as Ω× cm^2 and calculated as follows:

$$TEER = (R - R_0) \times A \quad \text{(eq 1)}$$

where R is the total measured resistance (Ω), R_0 is the blank resistance (insert without cells), and A is the insert effective surface (cm^2). Growth medium was changed one last time 24 hours before the transport experiment. On the day of the assay, growth medium was discarded, and cells were washed twice with HBSS. Only the wells with a TEER>160 Ω× cm^2 in HBSS were used for the experiment (Hubatsch et al., 2007). 0.5 mL of faba bean, pea, and soy 3 kDa permeates diluted in HBSS to a final concentration of 2240 µg of peptides/mL were added to the apical (AP) side and 1.5 mL of HBSS was added to the basolateral side (BL). The cells were incubated for 2 hours at 37 °C. At the end of the incubation period, the apical and basolateral compartment fractions were collected and frozen at -80 °C. The basolateral fractions were freeze-dried and suspended in 0.5 mL of water to reach the same volume as the apical side. The protein content in the apical and basolateral fractions after the transport experiment was determined by the pierce BCA protein assay kit using bovine serum albumin as standard.

Cell monolayer integrity was verified again at the end of the transport experiment by TEER measurement and lucifer yellow permeability assay. The later was measured by adding 0.5 mL of 100 µg/mL lucifer yellow solution prepared in HBSS to the apical side and 1.5 mL of HBSS to the basolateral side. The plate was incubated for 1 hour at 37 °C and 150 µL on the basolateral side was collected and added to a 96 well black plate with clear bottom. Fluorescence was measured ($\lambda_{excitation}$=485 nm, $\lambda_{emission}$=528 nm) using a Synergy HTX microplate reader (Bio-Tek, Winooski, VT) to quantify lucifer yellow permeability.

7.3.7 Peptide Sequencing by Mass Spectrometry

The peptides present in each fraction (3 kDa permeate of legume digestate, apical and basolateral compartments) were identified by mass spectrometry as described in Martineau-Côté, Achouri, Wanasundara, et al. (2022). All MS/MS spectrum were analyzed using PEAKS Studio (Bioinformatics Solutions, Waterloo, ON Canada; version 10.6) using database searching. PEAKS Studio was set up to search a *Vicia faba* (faba bean)*, Glycine max* (soy) and *Pisum sativum* (pea) database (UniProt/SwissProt). The probability of the identified peptides to be bioactive was predicted *in silico* using Peptide Ranker (Mooney et al., 2012). The physicochemical properties of the identified peptides (isoelectric point, net charge, and hydrophobicity) were calculated using PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/).

7.3.8 Antioxidant Activity of 3 kDa Permates of Legume Digestates and Faba Bean derived Peptides After Transepithelial Transport

The antioxidant activity of the 3 kDa permeate of legume digestate before and after transepithelial transport was evaluated by means of the 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and Oxygen Radical Absorbance Capacity (ORAC) assays. The two antioxidant assays were performed as previously described in Martineau-Côté, Achouri, Wanasundara, et al. (2022). These assays were also used to measure the antioxidant activity of faba bean transported peptides metabolites. The results were expressed as the effective peptide concentration required to scavenge 50% of free radicals (EC_{50}) for the ABTS assays and as µmol of Trolox equivalent per mg of peptides for ORAC.

7.3.9 Statistical Analysis

Each analysis was performed in triplicate and results were expressed as mean ± standard deviation (SD). The transepithelial transport experiments were performed on three different days with a different passage of cells. Data were analysed through analysis of variance (ANOVA) ($p < 0.05$) followed by the Tukey's honest significant difference (HSD) or the Dunnett's post-hoc test ($p < 0.05$) using the XLSTAT software (Addinsoft, NY) to determine significant differences.

7.4 Results and Discussion

7.4.1 Characterization of the Caco-2 and HT29-MTX-E12 Cell Monolayer

Caco-2 and HT29-MTX-E12 cells were co-cultivated on a permeable support for 21 days to form a polarized intestinal monolayer mimicking the intestinal barrier. Before conducting the transport experiment, the cell monolayer was characterized through measurement of transepithelial electrical resistance (TEER), alkaline phosphatase activity and alcian blue staining to confirm that the Caco-2 and HT29-MTX-E12 cells adopted a differentiated enterocyte and a goblet cell-like phenotype, respectively (Supplementary Figure 1). Transepithelial electrical resistance (TEER) was measured during the differentiation period to monitor the monolayer integrity and the formation of tight junctions. As expected, the TEER increased exponentially until the cells reached confluence (7 days). Then, the TEER plateaued for the remaining differentiation period, demonstrating the presence of an intact monolayer. The TEER value after 21 days of culture was in the same range as other studies (Chen et al., 2010; Hubatsch et al., 2007; Pan et al., 2015; Vieira et al., 2016). Alkaline phosphatase (ALP) activity was measured as a marker of Caco-2 cells differentiation (Matsumoto et al., 1990). Alkaline phosphatase activity was measured at 7, 14 and 21 days post seeding and as expected, the activity increased significantly by 2.7 fold after 14 days ($p = 0.013$) and by 5.2 fold after 21 days ($p < 0.001$), which is in good agreement with other studies (Matsumoto et al., 1990). Intestinal mucus production by HT29-MTX-E12 cells was confirmed by alcian blue staining (Supplementary Figure 1). HT29-MTX-E12 were effectively producing mucins as indicated by the blue coloration. The blue coloration was absent in Caco-2 cells, confirming the absence of mucins. These results demonstrated that the Caco-2 and HT29-MTX-E12 cells adopted a differentiated enterocyte and a goblet-cell like phenotype, indicating that the formed cell monolayer was representative of the intestinal barrier and was a suitable model for the peptide transport experiments.

7.4.2 Cell Viability and Cell Monolayer Integrity After Incubation with the 3 kDa Permeates of Faba Bean, Pea and Soy Digestates

Cell viability after a 2-hours treatment with the 3 kDa permeates of legume digestate (2240 and 1120 µg peptides/mL) was measured to ensure that the selected peptide doses had no cytotoxic effect. As shown in Figure 7-2, none of the selected peptide concentration had a significant impact on cell viability ($p > 0.05$). Since both concentrations were representative of a realistic legume serving, the highest dose was selected to perform the transport experiments. After the transport experiment, the

cell monolayer integrity was evaluated to validate that the incubation with the samples did not alter the cell monolayer. To that end, transepithelial electrical resistance and lucifer yellow permeability was measured. Lucifer yellow is a small hydrophilic dye that is principally transported across the cellular monolayer via the paracellular route and is therefore used to demonstrate impermeability of the tight junctions (Lea, 2015). As shown in Figure 7-2, the TEER value was unaffected by the 2-hours sample exposure ($p > 0.05$) and Lucifer yellow permeability was below 10% and not significantly different from untreated cells ($p > 0.05$), stating that the cell monolayer remained intact after exposure with the legume's digestate samples (Chen et al., 2010).

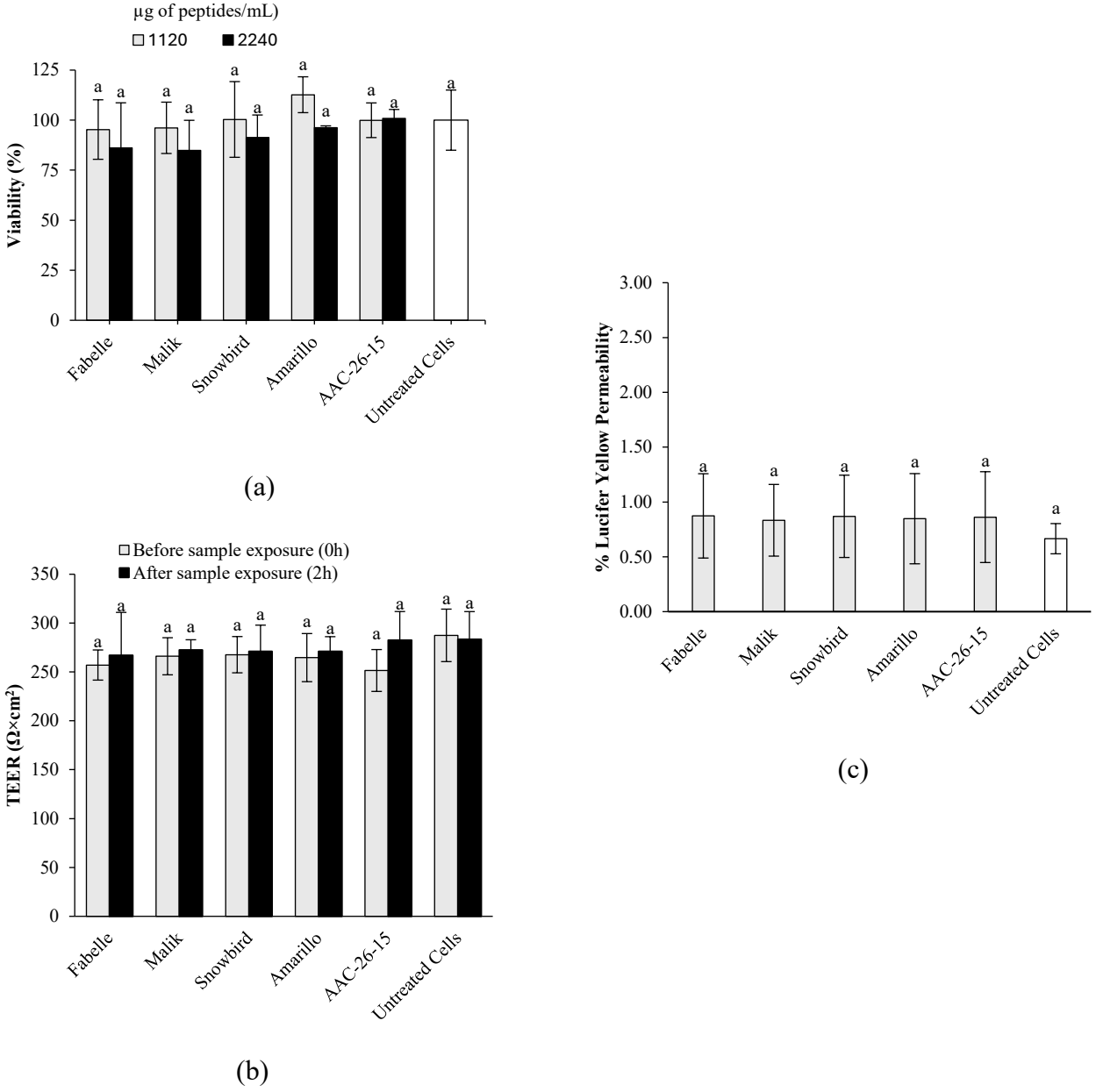


Figure 7-2. Cell viability and monolayer integrity after incubation with the 3 kDa permeate of legume digestates. Data are means ± standard deviation of three experiments performed on three different days. Means with a common letter are not significantly different ($p > 0.05$) as analyzed by ANOVA and the Tukey's test; (a) Cell viability is expressed as a percentage of untreated cells; (b) TEER was measured in HBSS before the addition of the 3 kDa permeates of legume digestate (T=0) and after the 2-hours incubation period with the 3 kDa permeates of legume digestate (2240 µg/mL); (c) Lucifer yellow permeability was measured after the 2-hours incubation with the 3 kDa permeates of legume digestate (2240 µg/mL).

7.4.3 Transepithelial Transport of Faba Bean, Pea and Soy Peptides

The 3 kDa permeates of *in vitro* gastrointestinal digestate of faba bean, pea, and soy at a final concentration of 2240 µg of peptides/mL were added to a differentiated Caco-2 and HT29-MTX-E12 co-culture monolayer to simulate small intestinal absorption. The peptide recovery (%) in the apical (AP) and basolateral (BL) compartment at the end of the transport experiment was evaluated using the BCA assay to estimate the quantity of transported peptides (Table 7-1). A total peptide recovery ranging from 84–90% was obtained for faba bean, pea, and soy, which was in the expected range for this type of experiment (Hubatsch et al., 2007). The unrecovered peptides may have been internalized by the cells and/or further hydrolysed to free amino acids. No significant difference in the amount of transported peptides at the end of the experiment was found between faba bean, pea, and soy ($p > 0.05$), reaching 0.6-1.2% of the peptides added to the apical side.. This finding suggest that the peptides had a similar permeability for the three legumes on a quantitative level. The obtained peptide recovery in the basolateral side was in the same range as other studies, although this characteristic is highly dependent on the peptide properties (Karaś, 2019). For instance, Aiello et al. (2018) reported a recovery in the basolateral side of 0.05, 0.02 and 0.009% for three soy-derived hypocholesterolemic peptides. For casein derived peptides, the peptide recovery in the basolateral compartment was reported to range from 0.018% (Quirós et al., 2008) up to 44.81% (Wang & Li, 2017).

Table 7-1. Peptides recovery (%) in the apical (AP) and basolateral (BL) compartments after cellular transepithelial transport experiment as assessed with the BCA assay.

	Peptides Recovery (%)		
	Apical Side[a]	**Basolateral Side[b]**	**Total[c]**
Faba Bean			
Fabelle	86.2 ± 7.5[a]	1.2 ±0.9[a]	87.4 ± 7.3[a]
Malik	86.6 ± 4.7[a]	1.1 ±0.3[a]	87.7 ± 4.5[a]
Snowbird	86.0 ± 7.4[a]	0.6 ±0.2[a]	86.6 ± 7.3[a]
Pea			
Amarillo	89.3 ± 7.8[a]	0.8 ±0.4[a]	90.1 ± 8.1[a]
Soy			
AAC-26-15	82.9 ± 4.9[a]	0.6 ±0.1[a]	83.5 ± 4.9[a]
***p*-value**	0.832	0.346	0.808

Values are means ± standard deviation of three experiments. Means in a column with a common letter are not significantly different ($p > 0.05$) as analysed by one-way ANOVA and the Tukey's test. Peptides recovery (%) was calculated as follows:

[a] Peptides recovery in the AP compartment = 100 x (V_{AP} x C_{AP})/ (V_{Ctrl} x C_{Ctrl})

[b] Peptides recovery in the BL compartment = 100 x (V_{BL} x C_{BL})/ (V_{Ctrl} x C_{Ctrl})

[c] Total peptide recovery = 100 x [(V_{AP} x C_{AP}) + (V_{BL} x C_{BL})]/ (V_{Ctrl} x C_{Ctrl})

Where V_{Ctrl} is the volume added to the AP side at the start of the experiment (t=0h) and V_{AP} and V_{BL} are the volume recovered on each side at the end of the experiment (t=2h). C_{ctrl} is the peptide concentration added to the apical side at the beginning of the experiment (t=0h) and C_{AP} and C_{BL} are the peptide concentration measured in the AP and BL side at the end of the experiment (t=2h). All peptide concentrations were measured with the use of the BCA assay and the peptide content in the AP and BL compartment of cells treated with HBSS were subtracted from the readings.

Although peptide transportation was identical on a quantitative level for the three legumes, the nature of the transported peptide might still be different. To evaluate peptide transportation on a qualitative level, the peptides present in the control, the apical and the basolateral fractions were sequenced par Q-exactive MS/MS to identify peptides that are resistant to intestinal brush border peptidases and that are absorbable. The control being the 3 kDa permeate of legume digestate before incubation with the intestinal cell monolayer. The complete list of identified peptides in the control, the apical and the basolateral fractions for all legumes and their assigned parent proteins are presented in Supplementary Table 1. As expected, the majority of identified peptides are fragments of globulins, the most abundant storage protein found in faba bean, pea, and soy (Din et al., 2021; Mertens et al., 2012; Warsame et al., 2020). More specifically, 11S globulins, which are legumins for faba bean and pea and glycinin (for soy) were the most frequent parent proteins. The data of Supplementary Table 1 are summarized in Figure 7-3 to illustrate the number of identified peptides in the control, the apical and the basolateral fractions for the three legumes.

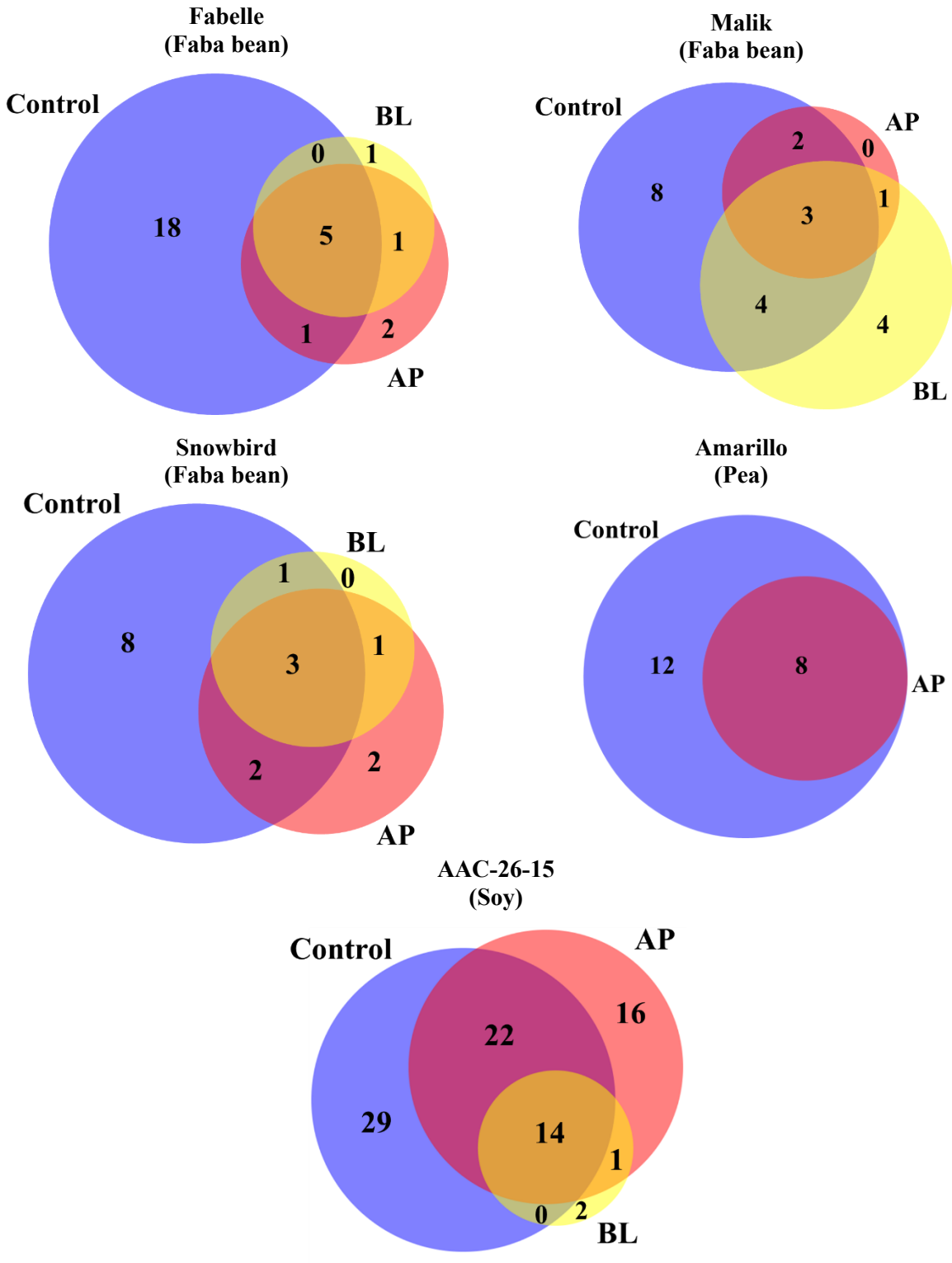


Figure 7-3. Number of unique peptides identified in the 3 kDa permeates of legumes digestates before incubation with the Caco-2 and HT29-MTX-E12 cell monolayer (control) and in the apical (AP) and in the basolateral (BL) fractions after 2-hours of incubation with the intestinal cell monolayer. The blue, pink and yellow circle represents the control, the apical (AP), and the basolateral (BL) compartment, respectively. Only the peptides found in 3 independent

transport experiments are reported. The proportional Venn diagrams were created using DeepVenn (https://www.deepvenn.com/).

For Fabelle, 5 peptides (TETWNPNHPEL, TETWNPNHPE, EEEDEDEPR, KEEEDEDEPR, and VVIPTEPPH) were found in common in the control, the apical and the basolateral fractions. The presence of these peptides in the three fractions means that they are resistant to brush border peptidases and can be transported intact across the cellular intestinal monolayer. For Malik and Snowbird, three peptides were found in common in each of the three fractions. For the three faba bean varieties, one peptide (TETWNPNHP) was found in common in both the apical and basolateral fractions that was absent in the control. This peptide was probably generated after further hydrolysis by brush border peptidases. For Fabelle, 18 peptides were only found in the control and 8 peptides were only found in the control for Malik and Snowbird. Since these peptides from Fabelle, Malik and Snowbird were only found in the control and absent from the apical and basolateral side, it means that they were neither resistant to the brush border peptidases of the intestinal cells nor transported. These peptides were possibly hydrolysed by brush border peptidases to lower molecular weight peptides and/or free amino acids. The number of transported peptides identified for faba bean (6 for Fabelle, 9 for Malik and 5 for Snowbird (Figure 7-6)) is in good accordance with other work conducted on plant-based protein hydrolysates. For instance, Bollati et al. (2022) identified 5 transported peptides in a hempseed peptic hydrolysate and Lammi et al. (2016) identified 11 transported peptides in a tryptic lupin hydrolysate and 8 peptides in a peptic lupin hydrolysate. In the case of soy, 14 peptides were found in common in the control, the apical and the basolateral fractions. Twenty-nine peptides were present only in the control, meaning that these peptides were not resistant to brush border peptidases. Twenty-two peptides were found in common in the control and in the apical fraction but not in the basolateral fraction. These peptides are therefore resistant to brush border peptidases but were not transported across the intestinal epithelium. The fact that a higher number of peptides were identified for soy compared to faba bean can be explained by the more extensive soy protein database (Rathi et al., 2016), facilitating peptide identification.

In the case of pea, 12 peptides were found only in the control and 8 peptides were found in common in the control and the apical side. None of them were identified in the basolateral fraction. Still, the data on recovery suggest that a similar quantity of peptides were transported to the basolateral side for pea, faba bean and soy. A possible explanation for this is that the pea and faba bean proteome is fragmented, which makes peptide identification challenging. Moreover, smaller peptides were

possibly not identifiable in the MS experimental conditions used, which represent a limitation of this study. Other authors have reported comparable situations. For instance, Corrochano et al. (2019) did not identify any peptides in the basolateral fraction when performing transport experiment with an *in vitro* gastrointestinal digestate of lactoferrin and α-lactalbumin, whereas they identified 14-31 peptides in the basolateral fractions with those of whey protein isolate, bovine serum albumin, β-lactoglobulin and a milk protein sport product.

7.4.4 Peptides Structure and Transportability Relationship

The relationship between peptide structure and transportability is still not well understood (Wang et al., 2019a). In the same vein, our data are demonstrating that faba bean and soy absorbable peptides are highly heterogenous in terms of length, molecular weight, polarity and N and C terminal residues (Table 7-2), making the establishment of a link between peptide structure and transportability challenging. For faba bean, the transported peptides had a molecular weight ranging from 960 to 1337 Da and were composed of 8 to 11 residues. For soy, the transported peptides were even more heterogeneous in terms of length and molecular weight. They were composed of 8 to 19 residues with a molecular weight ranging from 985 to 2250 Da. These two properties for faba bean and soy peptides were in the same range as other absorbable peptides identified in soy (814-1983 Da) (Zhang et al., 2018), hempseed (869-1292 Da) (Bollati et al., 2022), lupin (789-2719 Da) (Lammi et al., 2016) and casein (<500-1600 Da) (Wang et al., 2016) protein hydrolysates.

Faba bean and soy transported peptides had also a wide range of hydrophobicity, fluctuating from +12.49 to +38.08 kcal/mol for faba bean and from +7.62 to +56.56 kcal/mol for soy, based on the Wimley and White scale (Wimley & White, 1996), which is in a comparable range as other studies. Bollati et al. (2022) identified 5 hempseed derived transported peptides with hydrophobicity ranging from +0.18 to +13.58 kcal/mol. The N and C-terminal residues are highly heterogeneous, and no patterns can be established, contrary to a defined pattern as reported by Wang et al. (2019a). This finding can be explained by the high diversity of digestive proteases and peptidases used to mimic gastrointestinal digestion in the present study, leading to a highly diverse peptide profile.

Although the transepithelial transported peptides profile is highly heterogenous in terms of size, length, polarity and N and C-terminal residue, some similarities can still be observed between them. Ninety-six percent of the absorbable peptides identified for faba bean and soy were negatively charged at physiological pH. For faba bean, the net peptide charge ranged from -6 and -1 whereas it ranged

from -11 to 1 for soy peptides. This finding is in good agreement with other studies. For instance, Wang et al. (2016) found that a negatively charged peptide fraction of casein hydrolysate had a higher permeability in Caco-2 cells compared to a positively charged fraction. Picariello et al. (2010) also found that negatively charged peptides had a better resistance to gastrointestinal digestion.

Table 7-2. Physicochemical properties and *in silico* predicted bioactive fragments of transported faba bean and soy peptides.

Transported Peptides	Physicochemical Properties[a]					Occurrence Frequency of Bioactive Fragments (*in silico* Predicted)[b]									
Sequence[c]	Length	MW (Da)	PI	Net charge	Hydrophobicity (Kcal× mol^{-1})	1	2	3	4	5	6	7	8	9	10
Faba Bean															
EEEDEDEPR	9	1147	3.27	-6	+35.28	0.11	0.11	0.11	-	-	-	0.33	-	-	-
KEEEDEDEPR	10	1275	3.64	-5	+38.08	0.20	0.20	0.10	-	-	-	0.30	-	-	-
TETWNPNHP	9	1095	5.06	-1	+14.25	0.89	0.22	0.11	0.11	-	-	-	-	-	-
TETWNPNHPE	10	1224	4.07	-2	+17.88	0.80	0.20	0.20	0.10	-	-	-	-	0.10	-
TETWNPNHPEL	11	1337	4.07	-2	+16.63	0.73	0.18	0.18	0.36	-	-	-	-	0.09	-
TWNPNHPE	8	994	5.06	-1	+14.00	0.75	0.13	0.25	0.13	-	-	-	-	0.13	-
VIPTEPPHA	9	960	5.06	-1	+13.45	0.89	0.56	-	0.11	-	-	-	-	0.11	-
VVIPTEPPH	9	988	5.06	-1	+12.49	0.89	0.56	-	-	-	-	-	-	0.11	-
VVIPTEPPHA	10	1059	5.06	-1	+12.99	0.90	0.50	-	0.10	-	-	-	-	0.10	-
Soy															
DEDDEDEQIPSHPP	14	1623	3.22	-7	+36.21	0.43	0.29	0.07	-	-	-	-	-	0.07	-
DEDEDEDEDKPRPS	14	1676	3.44	-7	+45.97	0.21	0.21	0.07	0.07	-	-	-	-	-	-
DEDEDEDEDQPR	12	1491	3.11	-8	+43.34	0.17	0.17	0.08	-	-	-	-	-	-	-
DQDEDEDEDEDQPR	14	1735	3.05	-9	+47.75	0.29	0.14	0.07	-	-	-	-	-	-	-
DQDQDEDEDEDEDQPR	16	1978	3.00	-10	+52.16	0.29	0.14	0.07	-	-	-	-	-	-	-
EEDEDEQPRPI	11	1356	3.34	-5	+31.44	0.27	0.27	0.09	-	-	-	0.09	-	-	-
EEDEGEQPRP	10	1185	3.46	-4	+30.07	0.40	0.50	0.20	-	-	-	0.10	-	-	-
EPQQPGEKEEDEDEQPRPI	19	2250	3.56	-6	+44.47	0.58	0.47	0.11	-	0.05	0.05	0.05	0.05	-	-
EQDEDEDEDEDKPR	14	1749	3.40	-8	+49.77	0.25	0.14	0.06	0.06	-	-	-	-	-	-
EQDEDEDEDEDKPRPS	16	1933	3.40	-8	+50.37	0.25	0.19	0.06	0.06	-	-	-	-	-	-
EQDQDQDEDEDEDEDQPR	18	2235	2.98	-11	+56.56	0.39	0.11	0.06	-	-	-	-	-	-	-
GEKEEDEGEQPRP	13	1500	3.76	-4	+37.65	0.54	0.62	0.23	-	-	-	0.08	-	-	-
HEDDEDEDEEEDQPRPD	17	2099	3.29	-11	+56.71	0.24	0.18	0.06	-	-	0.06	0.18	-	-	-
KEEDEDEQPRPI	12	1485	3.72	-4	+34.24	0.33	0.33	0.08	-	-	-	0.08	-	-	-
KEEDEGEQPRP	11	1313	3.87	-3	+32.87	0.45	0.55	0.18	-	-	-	0.09	-	-	-
QFPFPRPP	8	985	11.6	1	+7.62	0.75	0.88	0.25	-	-	-	-	-	0.13	0.13

Transported peptides found in common in the three faba bean varieties (Fabelle, Malik and Snowbird) are underlined. [a]Physicochemical properties of the transported peptides were predicted using the PepDraw software (http://www.tulane.edu/~biochem/WW/PepDraw/) (PI : Isoelectric point) [b]The occurrence frequency of bioactive fragments in the bioavailable faba bean and soy peptide sequences were predicted *in silico* with the BIOPEP-UWM database (Minkiewicz et al., 2019) (1: DPP-IV inhibitor, 2: ACE inhibitor, 3: DPP-III inhibitor, 4: Antioxidant, 5: Antiamnestic, 6: Antithrombotic, 7: Stimulating vasoactive substance release, 8: Regulating the stomach mucosal membrane activity, 9: α-glucosidase inhibitor, 10: Renin inhibitor). The frequency of occurrence data are shaded proportionally (a darker color means a higher frequency or occurrence). [c]Amino acid sequences are abbreviated with one letter code.

Another common trait in the faba bean and soy absorbable peptide is the presence of proline residues in their sequence. All the transported identified peptide possessed at least one proline residue in their sequence and 64% of them possessed multiple proline residues. This is in good agreement with previous report that have demonstrated that proline containing peptides are more resistant to gastrointestinal digestion (Boutrou et al., 2013; Hausch et al., 2002; Tagliazucchi et al., 2016). However, numerous peptides identified in the control and the apical fractions of faba bean, pea and soy had a negative net charge and a proline residue but were not transported across the intestinal cell monolayer (Supplementary Table 1), suggesting the presence of other factors, possibly influencing peptide permeability.

The physicochemical properties of peptides can also influence the transport route taken across the intestinal barrier. Based on the current body of knowledge, there are 4 possible transport routes for peptides, namely paracellular transport through tight junctions, passive diffusion, transcytosis and/or intestinal active transporters such as PepT1 (Sun et al., 2020). The paracellular route is mostly used by hydrophilic peptides since the tight junction are a water filled extracellular route (Wang & Li, 2018; Wang et al., 2019b), whereas passive diffusion and transcytosis are preferred by lipophilic peptides since they need to interact with the membrane lipid bilayer to be absorbed (Karaś, 2019; Sun et al., 2020). PepT1 is mostly involved in the transport of small di and tripeptides (Karaś, 2019; Wang et al., 2017). Based on this body of knowledge, the paracellular route is the more probable mode of transport for the bioavailable peptides identified in soy, except for QP-8 that is less polar (+7.62 kcal/mol). In the case of faba bean peptides, the paracellular route is more probable for ER-9 and KR-10, which have multiple negative charge and are more polar. However, transcytosis and passive diffusion are possible for the seven remaining faba bean peptides since they are less polar. Further research will be needed to understand the respective mode of transport of the faba bean and soy identified peptides.

7.4.5 Antioxidant Activity of Faba Bean, Pea and Soy *in vitro* Gastrointestinal Digestate After Transepithelial Transport

To assess whether the antioxidant activity of the 3 kDa permeates of the legume digestates was maintained after transepithelial transport, the antioxidant activity of the control, the apical and the basolateral fractions was measured and compared. The antioxidant activity was measured with the 2.2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and the oxygen radical absorbance

capacity (ORAC) assays (Figure 7-4). These two antioxidant assays are complementary since they are based on two different free radical scavenging mechanisms, the former measuring free radical scavenging through single electron transfer (SET) and the latter measuring free radical scavenging through hydrogen atom transfer (HAT).

For the ORAC assay (Figure 7-4), there were no significant differences between the control and the apical fractions in all legume varieties, stating that exposure to intestinal brush border peptidases did not alter the antioxidant activity potency of the 3 kDa permeates of legume digestate. However, the ORAC value of the basolateral fraction significantly decreased comparatively to the control for all three legumes, indicating a decrease in the antioxidant activity after transepithelial transportation. The lower ORAC after transportation may be explained by several factors, such as the non-transportation of highly active peptides, the partial hydrolysis of highly active peptides to fragments with a lower activity or the transportation of peptides with no activity. It is also important to consider that the 3 kDa permeate of legume digestate is a legume flour hydrolysate that contains a mixture of bioactive molecules such as polyphenols, oligosaccharides, and free amino acids (Martineau-Côté, Achouri, Wanasundara, et al., 2022). Particularly, tryptophan, tyrosine, histidine, methionine, cysteine are important free radical scavengers (Xu et al., 2017). These other bioactive molecules may have contributed to some extent to the total antioxidant activity measured. As demonstrated in Supplementary Table 1, several peptides with a peptide ranker score close to one (suggesting a high probability of bioactivity) were not transported, which could explain the decrease of antioxidant activity. Although the basolateral fractions of the three legumes have a lower ORAC values than the initial 3 kDa permeate of legume digestate, they still maintained a high residual antioxidant activity. The residual antioxidant activity was 64% for Fabelle, 69% for Malik, 72% for Snowbird, 76% for pea and 75% for soy, meaning that faba bean after gastrointestinal digestion have a high potential to exert an antioxidant effect *in vivo*.

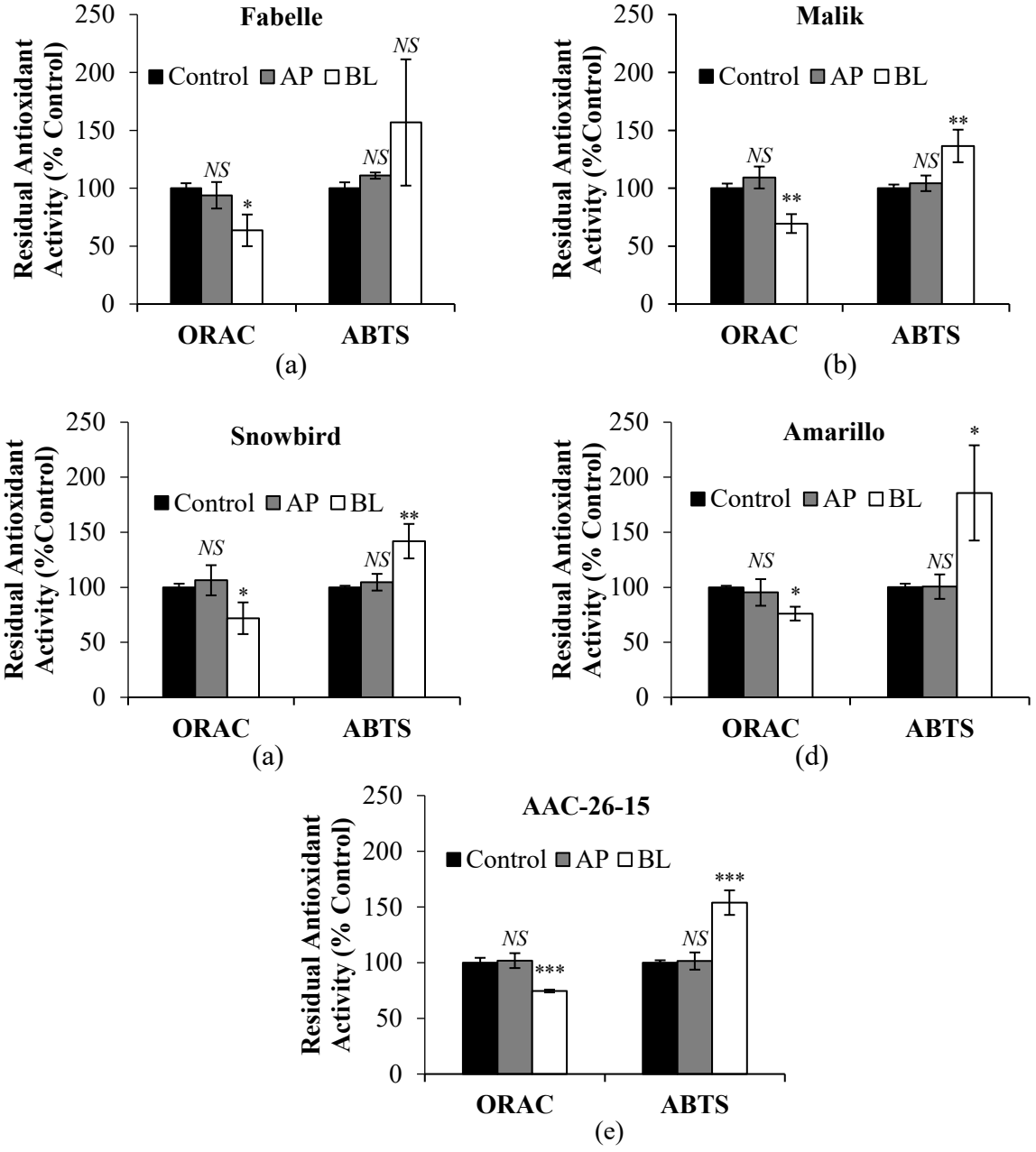
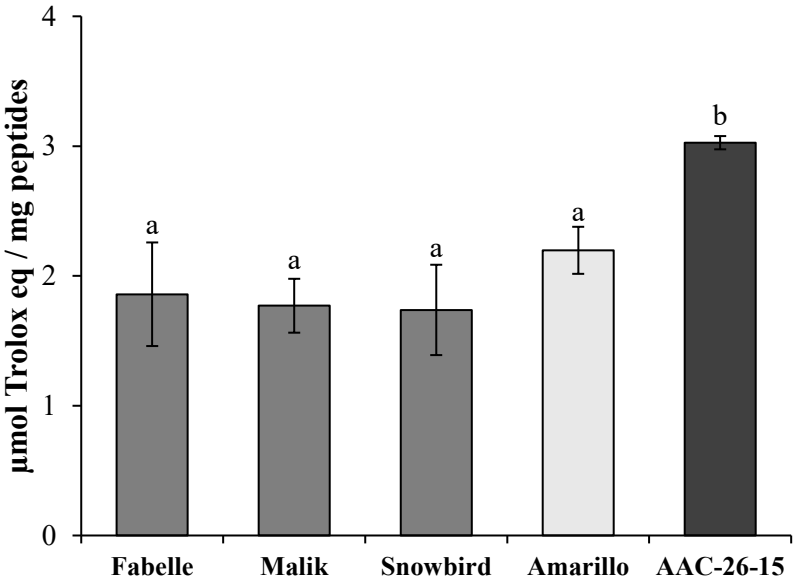


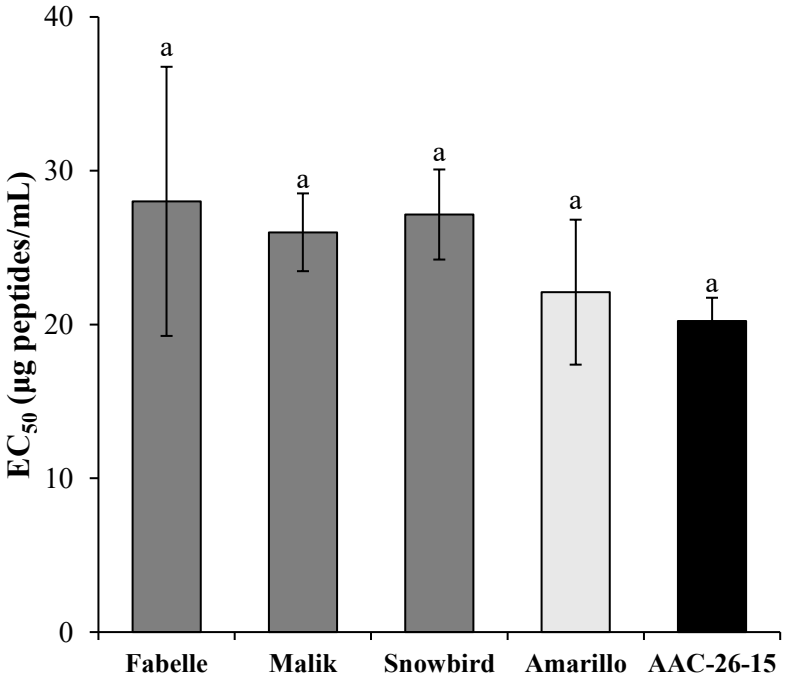
Figure 7-4. Residual antioxidant activity of the apical (AP) and basolateral (BL) fractions at the end of the transport experiment comparatively to the initial antioxidant activity of the 3 kDa permeate of legume digestate (control) as determined by the ORAC and the ABTS assay. For the ORAC assay, the residual antioxidant activity is defined as the ORAC value of the AP and BL fractions (expressed as µmol of Trolox equivalent per mg of peptides) divided by the ORAC value of the control. For the ABTS assay, the residual antioxidant activity is the EC_{50} of the control divided by the EC_{50} of the apical and basolateral fractions respectively. The EC_{50} is the effective peptide concentration required to scavenge 50% of ABTS free radicals. Data are expressed as Mean ± standard deviation of three experiment. Residual antioxidant activity of the AP and BL fractions for the two-antioxidant assays were compared to their respective control by ANOVA and the Dunnett's post hoc test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant); (a) Fabelle; (b) Malik; (c) Snowbird; (d) Pea (Amarillo); (e) Soy (AAC-26-15).

In the case of the ABTS assay (Figure 7-4), a different trend was observed. The basolateral fraction had a higher antioxidant activity compared to the control for Malik, Snowbird, Pea, and Soy, meaning that the transported fractions (BL) have a higher antioxidant activity than the control at an equivalent concentration. For Fabelle, the antioxidant activity tends also to increase, but the difference was not statistically significant ($p = 0.121$). The increase of antioxidant activity after transportation can be explained by several factors, such as the transportation of highly active peptides, the non-transportation of inactive peptides and further hydrolysis of peptides to smaller fragments with a higher activity. Again, this result indicates that the antioxidant activity of faba bean, pea and soy are maintained after transportation, suggesting a high bioactive potential *in vivo*. The different trend observed in the ORAC and ABTS assay can be explained by their respective mechanism of action.

If we compare the antioxidant activity of pea, faba bean and soy digestate after transepithelial transport (Figure 7-5), we can observe that the ORAC value of the basolateral fraction of soy is significantly higher than faba bean and pea. This same trend was observed previously (Martineau-Côté, Achouri, Wanasundara, et al., 2022) in the 3 kDa permeates of legume digestate before transepithelial transport, indicating that the same antioxidant activity pattern is observed before and after transepithelial transport. Interestingly, in the case of the ABTS assay, there are no significant differences in the EC_{50} for soy, pea and faba beans after transepithelial transport, although a lower EC_{50} for Malik, Snowbird and Soy compared to Fabelle and Amarillo was found in the 3 kDa permeate of legume digestate. This result demonstrates that transepithelial transport may have an impact on bioactivity potency and reiterate the importance to investigate its effect further.



(a)



(b)

Figure 7-5. Comparison of the antioxidant activity of faba bean (Fabelle, Malik, Snowbird), pea (Amarillo) and soy (AAC-26-15) peptides after transepithelial transport (basolateral fractions). For the ORAC assay the results are expressed as µmol of Trolox equivalent per mg of peptides. For the ABTS assay, the results are expressed as the effective peptide concentration required to scavenge 50% of ABTS free radicals (EC_{50}). Data are expressed as Mean ± standard deviation of three experiments. Means with a common letter are not significantly different ($p > 0.05$) as analysed by one-way ANOVA and the Tukey's test; (a) ORAC assay; (b) ABTS assay.

7.4.6 Antioxidant Activity of Peptides Derived From Faba Bean *in vitro* Gastrointestinal Digestion and Transepithelial Transport

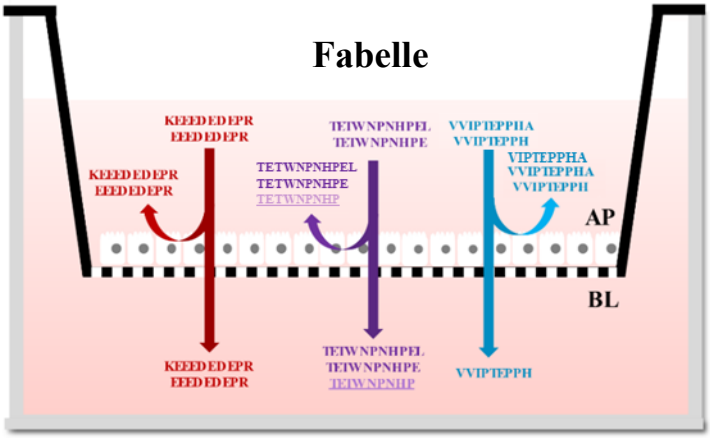
In a previous study (Martineau-Côté, Achouri, Wanasundara, et al., 2022), we have demonstrated that faba bean peptides generated after *in vitro* gastrointestinal digestion have a high bioactive potential, particularly in terms of antihypertensive and antioxidant activity. These bioactive properties revealed to be associated with the release of bioactive peptides after in vitro gastrointestinal digestion (Martineau-Côté, Achouri, Karboune, et al., 2024). Seven potent antioxidant peptides were identified, namely NYDEGSEPR, TETWNPNHPEL, TETWNPNHPE, VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH (Table 7-3). These peptides had a strong antioxidant activity, particularly in the ABTS and ORAC assay (Table 7-3). Among them, the peptides TETWNPNHPEL and TETWNPNHPE had the highest antioxidant activity. The peptides VIPTEPPH, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH, in addition to being antioxidant, were angiotensin-converting enzyme inhibitors (Martineau-Côté, Achouri, Karboune, et al., 2024).

Table 7-3. Antioxidant Activity of Peptides Derived from Faba Bean Flour *in vitro* Gastrointestinal Digestion and Transepithelial Transport

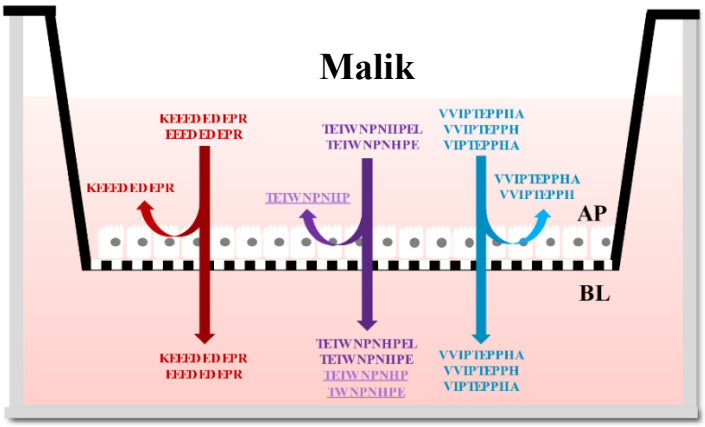
	Antioxidant Activity (Martineau-Côté, Achouri, Karboune, et al., 2024)		Transported Peptides		
	ORAC (µM TE eq / mM)	ABTS EC_{50} (mM)	Fabelle	Malik	Snowbird
NYDEGSEPR	557 ± 5	2.8 ± 0.5	-	-	-
PVNRPGEPQ	6.0 ± 0.1	-	-	-	-
LDNINALEPDH	57 ± 1	-	-	-	-
TETWNPNHPEL	2482 ± 39	0.5 ± 0.2	+	+	-
TETWNPNHPE	2838 ± 78	4.2 ± 0.2	+	+	+
EEEDEDEPR	2.0 ± 0.1	-	+	+	+
KEEEDEDEPR	3.0 ± 0.3	-	+	+	+
VIPTEPPH	56 ± 2	5.7 ± 0.3	-	-	-
VIPTEPPHA	47 ± 3	3.7 ± 0.1	-	+	-
VVIPTEPPHA	49 ± 2	2.9 ± 0.1	-	+	-
VVIPTEPPH	59 ± 1	2.8 ± 0.2	+	+	+

From the transport experiment results, 5 of these faba bean bioactive peptides were transported to the basolateral compartments, namely TETWNPNHPEL, TETWNPNHPE, VIPTEPPHA, VVIPTEPPHA, and VVIPTEPPH, suggesting that these peptides have the potential to reach the blood stream and exert their bioactive properties (Figure 7-6 and Table 7-3). The peptide NYDEGSEPR and VIPTEPPH were not transported, meaning that their bioactive potential *in vivo*

are limited. The peptides EEEDEDEPR and KEEEDEDEPR were also transported in all faba bean varieties, but these peptides showed only very minor antioxidant activity (Table 7-3). The absence of NYDEGSEPR and the transportation of EEEDEDEPR and KEEEDEDEPR could have contributed to the decrease of the ORAC activity after transepithelial of the 3 kDa permeate of gastrointestinal digestate of the three faba bean varieties.



(a)



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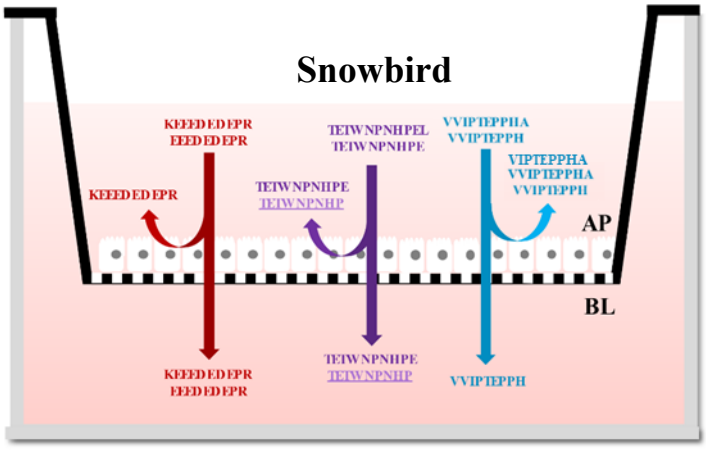
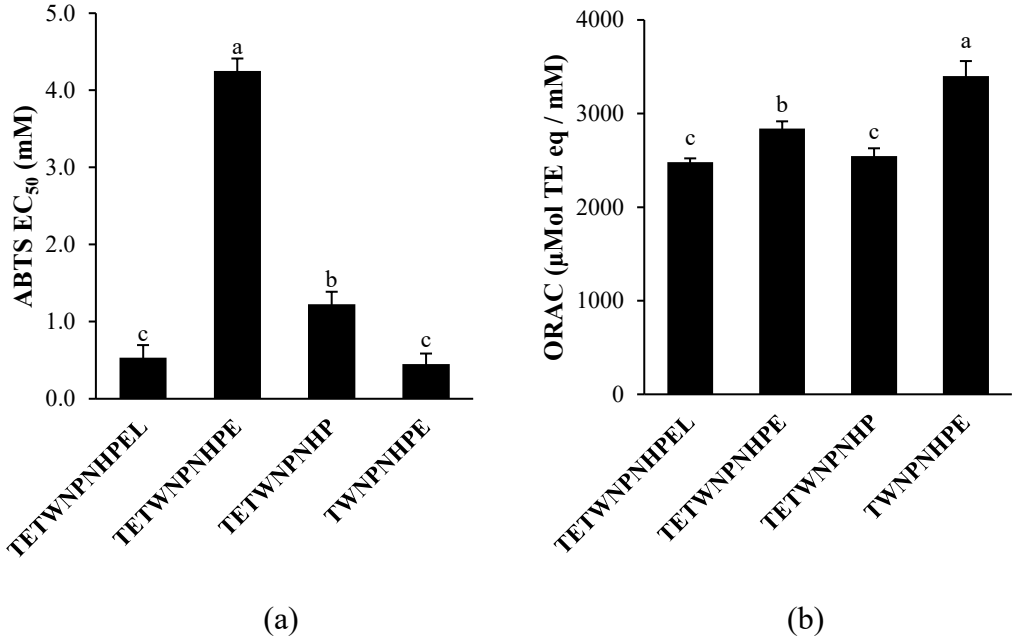


(c)

Figure 7-6. Graphical representation of transported faba bean derived peptides. Peptide fragments that are coming from the same location on the same parent protein are grouped and in the same color. Peptides generated after exposition to the cell monolayer (metabolites) are underlined and in a lighter font color; (a) Fabelle; (b) Malik; (c) Snowbird.

Interestingly, the peptides TETWNPNHPEL and TETWNPNHPE were partly metabolized after incubation with the intestinal cell monolayer to smaller fragments, TETWNPNHP and TWNPNHPE (Figure 7-6). The fragment TETWNPNHP was formed for all three faba bean varieties and the fragment TWNPNHPE was only found in Malik (Figure 7-6 and Table 7-3). The formation of these peptides metabolites may have an impact on the antioxidant activity of the basolateral fractions since TETWNPNHPEL and TETWNPNHPE were major contributor of the overall antioxidant activity (Martineau-Côté, Achouri, Karboune, et al., 2024). Based on that, the peptides metabolites TETWNPNHP and TWNPNHPE were synthesized to measure their antioxidant activity in comparison to the parent fragments TETWNPNHPEL and TETWNPNHPE (Figure 7-7). As shown in Figure 7-7, the metabolites TETWNPNHP and TWNPNHPE had a significantly higher ($p < 0.05$) activity in the ABTS assay compared to TETWNPNHPE, as shown by their lower EC_{50}. The activity of TETWNPNHP was more than 3 times higher than TETWNPNHPE, which could have contributed to the increased activity of the 3 kDa permeate of faba bean digestate after transepithelial transport in the ABTS assay. In the ORAC assay, TWNPNHPE had a significantly higher activity compared to both TETWNPNHPEL and TETWNPNHPE. However, the metabolite TETWNPNHP had a lower activity compared to TETWNPNHPE ($p < 0.05$) but was not significantly different than TETWNPNHPEL ($p > 0.05$). The formation of TETWNPNHP could therefore explain to a certain extent the decreased activity of the 3 kDa permeate of faba bean digestate after transepithelial transport in the ORAC assay.



(a) (b)

Figure 7-7. Antioxidant activity of synthesised faba bean peptides derived from *in vitro* gastrointestinal and transepithelial transport. Data are expressed as Mean ± standard deviation of three experiments. Means with a common letter are not significantly different (p >0.05) as analysed by one-way ANOVA and the Tukey's test; (a) ABTS assay; (b) ORAC assay.

7.5 Conclusions

This study has demonstrated for the first time that faba bean peptides generated from a simulated human gastrointestinal digestion model are bioavailable in a cellular model. These peptides can be transported intact across the intestinal barrier while maintaining their antioxidant activity. There were no differences in peptide transportability on a quantitative level between faba bean, pea, and soy, but there were differences in terms of peptide profile and activity potency. Nine faba bean peptides crossed the intestinal cells to the basolateral compartment. Three of them were antihypertensive agents (VIPTEPPHA, VVIPTEPPH and VVIPTEPPHA) and 5 of them were antioxidants (TETWNPNHPEL, TETWNPNHPE, VIPTEPPHA, VVIPTEPPH and VVIPTEPPHA). Interestingly, the most potent antioxidant peptides, TETWNPNHPEL and TETWNPNHPE, were partly hydrolyzed to smaller fragments (TETWNPNHP and TWNPNHPE). Both metabolites revealed to be potent antioxidants, explaining the preservation of the antioxidant activity after transepithelial transport. Based on the results of this study, it can be inferred that the benefits of faba

bean protein consumption is likely to go beyond fulfilling nutritional needs. The integration of faba bean proteins in the diet could be helpful in the management of non-communicable disease and be used as functional and health promoting food ingredient. Further investigation with *in vivo* models will be suitable to confirm the present findings.

7.6 References

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7.7 Supplementary Materials

7.7.1 Alkaline Phosphatase (ALP) Activity

Caco-2 and HT29-MTX-E12 (ratio 9:1) cells were seeded at a density of 1×10^5 cells/cm^2 in growth medium on transwell inserts. On the day of the assay, growth medium was discarded and cells were washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+) on the apical and basolateral sides. All solutions were pre-warmed to 37 °C. Reaction buffer was prepared in mixing a 2.5 mg/mL p-nitrophenyl phosphate (p-NPP) solution containing 100 mM diethanolamine, 150 mM NaCl and 2 mM $MgCl_2$ with a 10 mM Tris-HCl buffer at pH 8.0 containing 150 mM NaCl (ratio 3:1). 1.0 mL of the reaction buffer was added to the apical side and 1.0 mL of PBS+ to the basolateral side and the plate was incubated at 37 °C. 100 µL of the reaction buffer in the apical side was collected after 0, 2, 4, 6 and 8 minutes of reaction and added to a 96 well microplate plate kept on ice. The wells of the microplate contained 50 µL of 0.5 N NaOH to stop the reaction. Absorbance was recorded at λ=405 nm with an Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT, USA) and a standard curve was built with 2-nitrophenol (p-NP). Absorbance was converted to concentration and p-NP concentration in the test sample was plotted as a function of time. The slope of the linear curve was used to calculate ALP activity. ALP activity was expressed as mU, where 1 mU represents the release of 1 nmol of p-NP per minute at 37 °C. Alkaline phosphatase activity was normalized to cellular protein content. To do so, cells were scrapped on ice in RIPA buffer to provoke cell lysis and transferred to a micro-centrifuge tube. The tube was incubated at 4 °C with shaking for 30 minutes and then centrifuged at 16,000 x g for 20 minutes at 4 °C. Protein content in the supernatant was determined with the Pierce BCA protein assay kit using bovine serum albumin as standard.

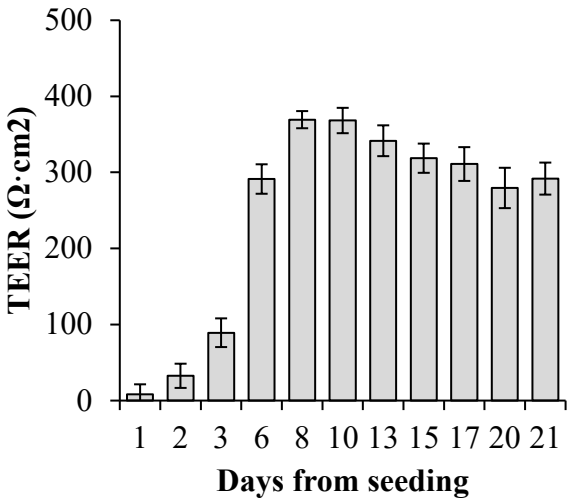
7.7.2 Alcian Blue Staining

Briefly, Caco-2 (negative control), Caco-2 and HT29-MTX-E12 co-culture (ratio 9:1) and HT29-MTX-E12 cells were seeded at a final density of 1×10^5 cells/cm^2 in growth medium on transwell inserts and cultivated for 21 days. After 21 days, growth medium was discarded and cells were washed twice with PBS. Cells were fixed with a 4% formaldehyde solution prepared in PBS for 30 minutes at room temperature. Cells were washed again twice with PBS and once with 1% acetic acid. Cells were stained for 30 minutes at room temperature with a 1% alcian blue solution prepared

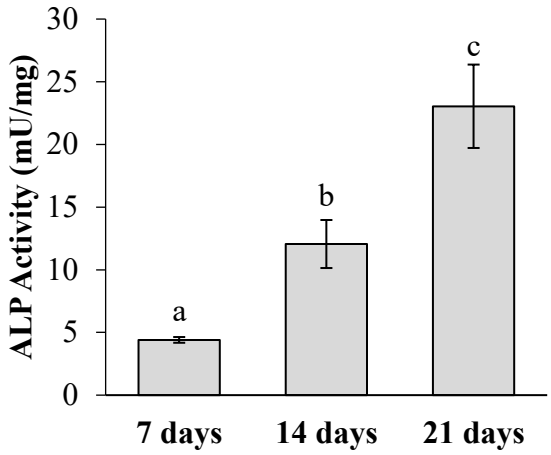
in 3% acetic acid. Cells were washed twice with PBS and visualized with a ZEISS Primovert inverted microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

7.7.3 Selection of the Peptide Concentration From Faba Bean, Pea and Soy 3 kDa Permeate of *in vitro* Gastrointestinal Digestate for Transepithelial Transport Studies

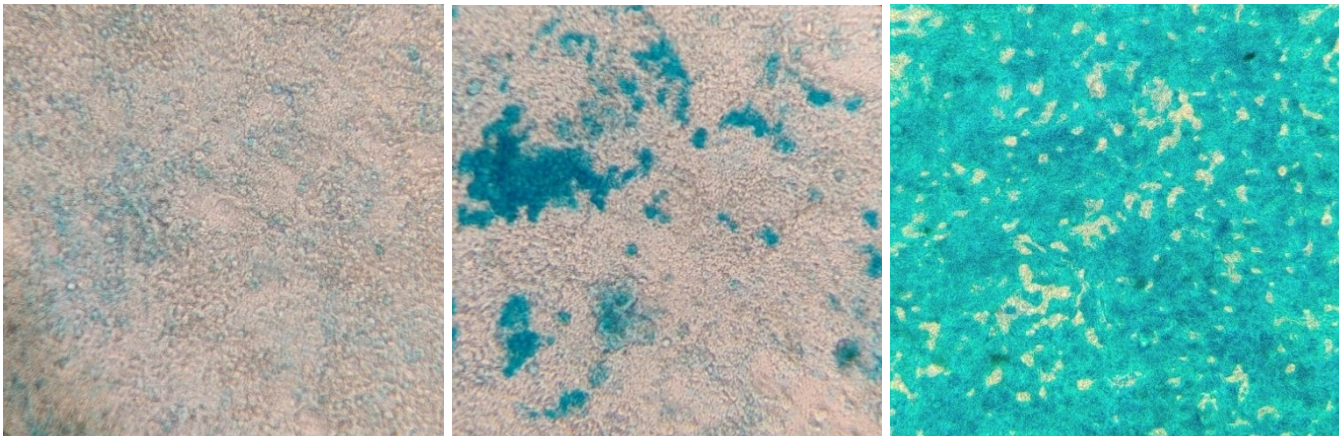
Peptide concentration used for the transepithelial transport experiment was selected to mimic potential intestinal exposure in a realistic manner. The approach describe below was used previously by Mahler et al. (2012) and Zhang, Zhang, Ma, and Cui (2020). The protein nutritional need for an adult is 0.8 g per kg of body weight per day (Richter et al., 2019). The consumption of 50 up to 100 g of dried pulse is accepted as a realistic serving in a healthy diet (Willett et al., 2019). This serving size represents on average 15–30 g of proteins for faba bean, assuming a 30% protein content. The small intestine has a surface area of ~200 m^2 (DeSesso & Jacobson, 2001). Most of dietary peptide absorption takes place in the jejunum (Picariello, Ferranti, & Addeo, 2016), which as a surface of ~60 m^2 (Kararli, 1995). Therefore, the consumption of 50–100 g of dried faba bean will lead to a potential exposure of ~25–50 µg protein/cm^2 in the jejunum. The transwell insert used in the experiment (12 wells) has a growth surface of 1.12 cm^2. However, the microvilli structure increase the surface area by a factor 20 (Campbell, Berry, & Liang, 2019). The real absorption surface was therefore 22.4 cm^2 per insert. Thus, the addition of 0.5 mL of a solution of 1120–2240 µg of peptides/mL per wells leads to the targeted concentration.



(a)



(b)



Caco-2 Caco2 and HT29-MTX (ratio 9:1) HT29-MTX

(c)

Supplementary Figure 7-1. Characterization of the Caco-2 and HT29-MTX co-culture monolayer; (a) Transepithelial electrical resistance (TEER) was measured over time to evaluate cell monolayers integrity. Data are means ± standard deviation of twelve monolayers of one representative experiment; (b) Caco-2 cell differentiation was evaluated by in situ measurement of alkaline phosphatase activity (ALP). Data are expressed as Mean ± standard deviation of three experiments and means without a common letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the Tukey's test; (c) mucus production by HT29-MTX cells was confirmed by means of alcian blue staining and visualization was performed with an inverted phase microscope (20x magnification). The presence of a blue coloration indicates the presence of intestinal mucins.

Supplementary Table 7-1. Faba bean, pea and soy peptides identified in the control (3 kDa permeate of legume digestate before the transport experiment) and in the apical (AP) and the basolateral (BL) fractions at the end of the transepithelial transport experiment

Fabelle (Faba bean)

Peptides[1]	Control	AP	BL	MH+ [Da]	Accession Number	Fragment Location	Precursor Protein	Organism	Peptide Ranker Score[2]
EDEDEDEKEEQ	+	-	-	1394.5080	CAA81262.1	*306–316*	Legumin	*Vicia faba*	0.04
EDEDEDEKEEQEQ	+	-	-	1651.6092	CAA81262.1	*306-318*	Legumin	*Vicia faba*	0.03
EEEDEDEPR	+	+	+	**1147.4387**	**CAA81262.1**	***327-335***	**Legumin**	***Vicia faba***	**0.08**
ETWNPNHP	-	+	-	994.4377	CAA81262.1	*53-60*	Legumin	*Vicia faba*	0.37
KEEEDEDEPR	+	+	+	**1275.5337**	**CAA81262.1**	***326-335***	**Legumin**	***Vicia faba***	**0.07**
SEKEDEDEDEKEEQ	+	-	-	1738.6776	CAA81262.1	*303-316*	Legumin	*Vicia faba*	0.04
SQGEEEEEEER	+	-	-	1350.5292	CAA81262.1	*283-293*	Legumin	*Vicia faba*	0.05
TETWNPNHP	-	+	+	**1095.4854**	**CAA81262.1**	***52-60***	**Legumin**	***Vicia faba***	**0.22**
TETWNPNHPE	+	+	+	**1224.5280**	**CAA81262.1**	***52-61***	**Legumin**	***Vicia faba***	**0.15**
TETWNPNHPEL	+	+	+	**1337.6121**	**CAA81262.1**	***52-62***	**Legumin**	***Vicia faba***	**0.27**
QEEDEDEDEDEKEE	+	-	-	1767.6202	CAA38757.1	*262-275*	Legumin A1 pre-pro-polypeptide	*Vicia faba*	0.04
QEEDEDEDEDEKEER	+	-	-	1923.7213	CAA38757.1	*262-276*	Legumin A1 pre-pro-polypeptide	*Vicia faba*	0.05
QEEDEDEDEKE	+	-	-	1394.5080	CAA38758.1	*271-281*	Legumin A2 primary translation product	*Vicia faba*	0.05
QEEDEDEDEKEE	+	-	-	1523.5506	CAA38758.1	*271-282*	Legumin A2 primary translation product	*Vicia faba*	0.05
QEEDEDEDEKEER	+	-	-	1679.6517	CAA38758.1	*271-283*	Legumin A2 primary translation product	*Vicia faba*	0.07
QEEEEEEEEEK	+	-	-	1436.5548	CAA27313.1	*285-295*	Legumin B	*Vicia faba*	0.04
QQQPDSHQ	+	-	-	967.4229	CAA27313.1	*127-134*	Legumin B	*Vicia faba*	0.13
VIPTEPPHA	-	+	-	960.5150	CDQ12453.1	*155-163*	Tonoplast intrinsic protein 32	*Vicia faba*	0.25
VVIPTEPPH	+	+	+	**988.5463**	**CDQ12453.1**	***154-162***	**Tonoplast intrinsic protein 32**	***Vicia faba***	**0.19**
VVIPTEPPHA	+	+		1059.5834	CDQ12453.1	*154-163*	Tonoplast intrinsic protein 32	*Vicia faba*	0.19
NYDEGSEPR	+	-	-	1066.4436	AAA33660.1	*30-38*	Convicilin	*Pisum sativum*	0.14
NQLDSTPR	+	-	-	930.4640	CAA47809.1	*173-180*	Legumin	*Pisum sativum*	0.24
EDVPNHGT	+	-	-	868.3796	CAA34906.1	*141-148*	Lipoxygenase-2	*Pisum sativum*	0.20
GGSSTHPYP	+	-	-	902.4003	CAA34906.1	*233-241*	Lipoxygenase-2	*Pisum sativum*	0.52
NDLGNPDHGEH	+	-	-	1204.4979	CAA34906.1	*216-226*	Lipoxygenase-2	*Pisum sativum*	0.22
LGNPDSGENH	+	-	-	1039.4440	CAA30666.1	*213-222*	Lipoxygenase-3	*Pisum sativum*	0.19
NDLGNPDSGENH	-	-	+	1268.5139	CAA30666.1	*211–222*	Lipoxygenase-3	*Pisum sativum*	0.16
KEEDEEDEPR	+	-	-	1275.5337	CAW45393.1	*13–22*	Unnamed protein product	*Glycine max*	0.07

Supplementary Table 7-1. (continued).

Malik (Faba bean)									
Peptides[1]	**Control**	**AP**	**BL**	**MH+ [Da]**	**Accession Number**	**Fragment Location**	**Precursor Protein**	**Organism**	**Peptide Ranker Score[2]**
EDEDEDEKEEQ	+	+	-	1394.5080	CAA81262.1	*306–316*	Legumin	*Vicia faba*	0.04
KEEEDEDEPR	**+**	**+**	**+**	**1275.5337**	**CAA81262.1**	***326-335***	**Legumin**	***Vicia faba***	**0.07**
EEEDEDEPR	**+**	**-**	**+**	**1147.4387**	**CAA81262.1**	***327-335***	**Legumin**	***Vicia faba***	**0.08**
QEEDEDEDEKEE	-	-	+	1523.5506	CAA38758.1	*271-282*	Legumin A2 primary translation product	*Vicia faba*	0.05
QEEDEDEDEKEER	+	-	-	1679.6517	CAA38758.1	*271-283*	Legumin A2 primary translation product	*Vicia faba*	0.07
QEEEEEEEEEK	+	+	-	1436.5548	CAA27313.1	*285-295*	Legumin B	*Vicia faba*	0.04
SEKEDEDEDEKEEQ	+	-	-	1738.6776	CAA81262.1	*303-316*	Legumin	*Vicia faba*	0.04
SQGEEEEEEER	+	-	-	1350.5292	CAA81262.1	*283-293*	Legumin	*Vicia faba*	0.05
TETWNPNHP	**-**	**+**	**+**	**1095.4854**	**CAA81262.1**	***52-60***	**Legumin**	***Vicia faba***	**0.22**
TETWNPNHPE	**+**	**-**	**+**	**1225.5121**	**CAA81262.1**	***52-61***	**Legumin**	***Vicia faba***	**0.15**
TETWNPNHPEL	**+**	**-**	**+**	**1337.6121**	**CAA81262.1**	***52-62***	**Legumin**	***Vicia faba***	**0.27**
TWNPNHPE	**-**	**-**	**+**	**994.4377**	**CAA81262.1**	***54-61***	**Legumin**	***Vicia faba***	**0.31**
VIPTEPPHA	**+**	**-**	**+**	**960.5150**	**CDQ12453.1**	***155-163***	**Tonoplast intrinsic protein 32**	***Vicia faba***	**0.25**
VVIPTEPPH	**+**	**+**	**+**	**988.5463**	**CDQ12453.1**	***154-162***	**Tonoplast intrinsic protein 32**	***Vicia faba***	**0.19**
VVIPTEPPHA	**+**	**+**	**+**	**1059.5834**	**CDQ12453.1**	***154-163***	**Tonoplast intrinsic protein 32**	***Vicia faba***	**0.19**
IGANEPSEH	+	-	-	953.4323	CAA61947.1	*150-158*	Fructose-1,6-bisphosphate aldolase	*Pisum sativum*	0.11
LGNPDSGENH	+	-	-	1039.4440	CAA30666.1	*213-222*	Lipoxygenase-3	*Pisum sativum*	0.19
NDLGNPDHGEH	+	-	-	1204.4979	CAA34906.1	*216-226*	Lipoxygenase-2	*Pisum sativum*	0.22
NYDEGSEPR	-	-	+	1067.4277	AAA33660.1	*30-38*	Convicilin	*Pisum sativum*	0.14
YDEGSEPR	-	-	+	952.4007	AAA33660.1	*31–38*	Convicilin	*Pisum sativum*	0.16
KEEDEEDEPR	+	-	-	1275.5337	CAW45393.1	*13–22*	Unnamed protein product	*Glycine max*	0.07
QFPFPRPP	+	-	-	985.5255	BAA23360.2	*152–159*	Alpha subunit of beta conglycinin	*Glycine max*	0.97

Supplementary Table 7-1. (continued).

Snowbird (Faba bean)									
Peptides[1]	**Control**	**AP**	**BL**	**MH+ [Da]**	**Accession Number**	**Fragment Location**	**Precursor Protein**	**Organism**	**Peptide Ranker Score[2]**
EDEDEDEKEEQ	-	+	-	1,394,508	CAA81262.1	306–316	Legumin	*Vicia faba*	0.04
EEEDEDEPR	**+**	**-**	**+**	**1147.4387**	**CAA81262.1**	**327-335**	**Legumin**	***Vicia faba***	**0.08**
KEEEDEDEPR	**+**	**+**	**+**	**1275.5337**	**CAA81262.1**	**326-335**	**Legumin**	***Vicia faba***	**0.07**
QEEDEDEDEDEKEE	+	-	-	1767.6202	CAA38757.1	262-275	Legumin A1 pre-pro-polypeptide	*Vicia faba*	0.04
QEEDEDEDEKEE	+	-	-	1523.5506	CAA38758.1	271-282	Legumin A2 primary translation product	*Vicia faba*	0.05
QEEDEDEDEKEER	+	-	-	1679.6517	CAA38758.1	271-283	Legumin A2 primary translation product	*Vicia faba*	0.07
QEEEEEEEEEK	+	+	-	1436.5548	CAA27313.1	285-295	Legumin B	*Vicia faba*	0.04
QQQPDSHQ	+	-	-	967.4229	CAA27313.1	127-134	Legumin B	*Vicia faba*	0.13
SEKEDEDEDEKEEQ	+	-	-	1738.6776	CAA81262.1	303-316	Legumin	*Vicia faba*	0.04
TETWNPNHP	**-**	**+**	**+**	**1095.4854**	**CAA81262.1**	**52-60**	**Legumin**	***Vicia faba***	**0.22**
TETWNPNHPE	**+**	**+**	**+**	**1224.528**	**CAA81262.1**	**52-61**	**Legumin**	***Vicia faba***	**0.15**
TETWNPNHPEL	+	-	-	1337.6121	CAA81262.1	52-62	Legumin	*Vicia faba*	0.27
VIPTEPPHA	-	+	-	960.515	CDQ12453.1	155-163	Tonoplast intrinsic protein 32	*Vicia faba*	0.25
VVIPTEPPH	**+**	**+**	**+**	**988.5463**	**CDQ12453.1**	**154-162**	**Tonoplast intrinsic protein 32**	***Vicia faba***	**0.19**
VVIPTEPPHA	+	+	-	1059.5834	CDQ12453.1	154-163	Tonoplast intrinsic protein 32	*Vicia faba*	0.19
NDLGNPDHGEH	+	-	-	1204.4979	CAA34906.1	216–226	Lipoxygenase-2	*Pisum sativum*	0.22
KEEDEEDEPR	+	-	-	1275.5337	CAW45393.1	13–22	Unnamed protein product	*Glycine max*	0.07

Supplementary Table 7-1. (continued).

Amarillo (Pea)									
Peptides[1]	**Control**	**AP**	**BL**	**MH+ [Da]**	**Accession Number**	**Fragment Location**	**Precursor Protein**	**Organism**	**Peptide Ranker Score[2]**
SDDEDTAPPR	+	-	-	1102.4649	BAB32793.1	973–982	110 kDa 4SNc-Tudor domain	*Pisum sativum*	0.35
NYDEGSEPR	+	-	-	1066.4436	AAA33660.1	30–38	Convicilin	*Pisum sativum*	0.14
GDGMPGGGSNGSGPGPK	+	+	-	1444.6126	AAA82975.1	627–643	Heat shock protein hsp70	*Pisum sativum*	0.73
QEEDEDEEK	+	-	-	1150.4384	CAA10722.1	250-258	LegA class precursor	*Pisum sativum*	0.04
QEEDEDEEKQPR	+	-	-	1531.6509	CAA10722.1	250-261	LegA class precursor	*Pisum sativum*	0.09
QEEEEDEDEER	+	+	-	1436.5297	CAA10722.1	268-278	LegA class precursor	*Pisum sativum*	0.05
RGEEEEEDKKE	+	-	-	1377.613	CAA10722.1	286-296	LegA class precursor	*Pisum sativum*	0.04
KEDEDEDEEEE	+	-	-	1395.492	CAA47809.1	308-318	Legumin	*Pisum sativum*	0.04
KEDEDEDEEEEE	+	-	-	1524.5346	CAA47809.1	308-319	Legumin	*Pisum sativum*	0.04
KEDEDEDEEEEEE	+	+	-	1653.5772	CAA47809.1	308-320	Legumin	*Pisum sativum*	0.04
KEDEDEDEEEEEER	+	-	-	1809.6783	CAA47809.1	308-321	Legumin	*Pisum sativum*	0.05
HSEKEEEDEDEPR	+	-	-	1628.6672	CAA47809.1	328-340	Legumin	*Pisum sativum*	0.05
SEKEEEDEDEPR	+	-	-	1491.6083	CAA47809.1	329–340	Legumin	*Pisum sativum*	0.06
EEEDEDEPR	+	+	-	1147.4387	CAA47809.1	332–340	Legumin	*Pisum sativum*	0.08
REEEEEEEEDEEK	+	+	-	1708.6669	S26688	299–311	Legumin K	*Pisum sativum*	0.03
EEEEEEEEDEEK	+	-	-	1552.5658	S26688	300-311	Legumin K	*Pisum sativum*	0.03
NDLGNPDHGEH	+	+	-	1204.4979	CAA34906.1	216-226	Lipoxygenase-2	*Pisum sativum*	0.22
NDLGNPDSGENH	+	+	-	1268.5139	CAA30666.1	211-222	Lipoxygenase-3	*Pisum sativum*	0.16
VVIPTEPPHA	+	-	-	1059.5834	CDQ12453.1	154-163	Tonoplast intrinsic protein 32	*Vicia faba*	0.19
VVIPTEPPH	+	+	-	988.5463	ACU23484.1	237–245	Unknown	*Glycine max*	0.19

Supplementary Table 7-1. (continued).

AAC-26-15 (Soy)

Peptides[1]	Control	AP	BL	MH+ [Da]	Accession Number	Fragment Location	Precursor Protein	Organism	Peptide Ranker Score[2]
EGKDEDEEEEGH	+	+	-	1402.5243	AAB71140.1	*82-93*	2S albumin pre-pro-peptide	*Glycine max*	0.08
DEDEDEEQDER	+	-	-	1408.4985	BAA23360.2	*141-151*	Alpha subunit of β-conglycinin	*Glycine max*	0.06
EEDEDEQPRP	+	+	-	1243.5075	BAA23360.2	*94-103*	Alpha subunit of β-conglycinin	*Glycine max*	0.15
EEDEDEQPRPI	**+**	**+**	**+**	**1356.5916**	**BAA23360.2**	***94-104***	**Alpha subunit of β-conglycinin**	***Glycine max***	**0.14**
EEDEDEQPRPIP	+	+	-	1453.6444	BAA23360.2	*94-105*	Alpha subunit of β-conglycinin	*Glycine max*	0.15
EPQQPGEKEEDEDEQPR	+	+	-	2039.8792	BAA23360.2	*86-102*	Alpha subunit of β-conglycinin	*Glycine max*	0.11
EPQQPGEKEEDEDEQPRPI	**+**	**+**	**+**	**2250.0161**	**BAA23360.2**	***86-104***	**Alpha subunit of β-conglycinin**	***Glycine max***	**0.12**
GSEEEDEDEDEEQDER	+	+	-	1939.6798	BAA23360.2	*136-151*	Alpha subunit of β-conglycinin	*Glycine max*	0.05
KEEDEDEQPRPI	**-**	**+**	**+**	**1484.6866**	**BAA23360.2**	***93-104***	**Alpha subunit of β-conglycinin**	***Glycine max***	**0.11**
KQEEDEDEEQQ	+	+	-	1406.5556	BAA23360.2	*166-176*	Alpha subunit of β-conglycinin	*Glycine max*	0.03
QFPFPRPP	**+**	**+**	**+**	**985.5255**	**BAA23360.2**	***152-159***	**Alpha subunit of β-conglycinin**	***Glycine max***	**0.97**
QFPFPRPPH	+	-	-	1122.5844	BAA23360.2	*152-160*	Alpha subunit of β-conglycinin	*Glycine max*	0.92
GVMNGGMQPR	+	-	-	1047.4712	BAA03681.1	*371-380*	Basic 7S globulin	*Glycine max*	0.32
MNGGMQPR	+	-	-	891.3813	BAA03681.1	*373-380*	Basic 7S globulin	*Glycine max*	0.48
VMDKPNGPV	+	+	-	956.4871	BAA03681.1	*343-351*	Basic 7S globulin	*Glycine max*	0.28
EDEGEQPRP	+	-	-	1056.4594	ADD38965.1	*94-102*	β-conglycinin alpha' subunit	*Glycine max*	0.21
EDQDEDEEQDKE	+	-	-	1508.551	ADD38965.1	*179-190*	β-conglycinin alpha' subunit	*Glycine max*	0.05
EDQDEDEEQDKESQ	+	-	-	1723.6416	ADD38965.1	*179-192*	β-conglycinin alpha' subunit	*Glycine max*	0.04
EEDEGEQPRP	**+**	**+**	**+**	**1185.502**	**ADD38965.1**	***93-102***	**β-conglycinin alpha' subunit**	***Glycine max***	**0.15**
EEDEGEQPRPFP	**-**	**-**	**+**	**1429.6232**	**ADD38965.1**	***93-104***	**β-conglycinin alpha' subunit**	***Glycine max***	**0.29**
EEEDQDEDEEQDKE	-	+	-	1766.6362	ADD38965.1	*177-190*	β-conglycinin alpha' subunit	*Glycine max*	0.05
ESEEEEEDQDEDEEQDKE	+	-	-	2240.796	ADD38965.1	*173-190*	β-conglycinin alpha' subunit	*Glycine max*	0.03
GEKEEDEGEQPRP	**+**	**+**	**+**	**1499.6611**	**ADD38965.1**	***90-102***	**β-conglycinin alpha' subunit**	***Glycine max***	**0.13**
KEEDEGEQPRP	**-**	**-**	**+**	**1313.597**	**ADD38965.1**	***92-102***	**β-conglycinin alpha' subunit**	***Glycine max***	**0.13**
TEVGPDDDEK	+	-	-	1104.4694	AAB03894.1	*329-338*	Glucose binding protein	*Glycine max*	0.07
AGNPDIEHPET	+	+	-	1179.5278	BAA19058.1	*166-176*	Glycinin	*Glycine max*	0.28
DEDDEDEQIPSHPP	**+**	**+**	**+**	**1622.6456**	**BAA74953.1**	***287-300***	**Glycinin**	***Glycine max***	**0.16**
DEDDEDEQIPSHPPR	-	+	-	1778.7467	BAA74953.1	*287-301*	Glycinin	*Glycine max*	0.23
DEDEDDEDEQIPSHPP	-	+	-	1866.7152	BAA74953.1	*285-300*	Glycinin	*Glycine max*	0.12
DEDEDEDEDDEDEQIPSHPP	+	+	-	2354.8544	BAA74953.1	*281-300*	Glycinin	*Glycine max*	0.07
DEDEDEDEDKPRPS	**+**	**+**	**+**	**1675.6569**	**BAA74953.1**	***311-324***	**Glycinin**	***Glycine max***	**0.11**

Supplementary Table 7-1. (continued).

AAC-26-15 (Soy)

Peptides[1]	Control	AP	BL	MH+ [Da]	Accession Number	Fragment Location	Precursor Protein	Organism	Peptide Ranker Score[2]
DEDEDEDEDQPR	+	+	+	**1491.5357**	**BAA74953.1**	***338-349***	**Glycinin**	***Glycine max***	**0.08**
DEDEDEDKPRPS	+	-	-	1431.5873	BAA74953.1	*313-324*	Glycinin	*Glycine max*	0.16
DEDEDEDQPR	+	-	-	1247.4661	BAA74953.1	*340-349*	Glycinin	*Glycine max*	0.13
DEDEQIPSHPP	+	+	-	1263.549	BAA74953.1	*290-300*	Glycinin	*Glycine max*	0.27
DEDEQIPSHPPR	+	+	-	1419.6501	BAA74953.1	*290-301*	Glycinin	*Glycine max*	0.39
DQDEDEDEDEDQPR	+	+	+	**1734.6213**	**BAA74953.1**	***336-349***	**Glycinin**	***Glycine max***	**0.08**
DQDQDEDEDEDEDQPR	+	+	+	**1977.7069**	**BAA74953.1**	***334-349***	**Glycinin**	***Glycine max***	**0.07**
EDDEDEDEEEDQPRPD	-	+	-	1961.7007	BAA19058.1	*282-297*	Glycinin	*Glycine max*	0.06
EDDEDEDEEEDQPRPDHPPQRPS	+	+	-	2761.1097	BAA19058.1,	*282-304*	Glycinin	*Glycine max*	0.90
EDDEDEQIPSHPP	-	+	-	1507.6186	BAA74953.1	*288-300*	Glycinin	*Glycine max*	0.18
EDEDEDEDKPRPS	+	-	-	1560.6299	BAA74953.1	*312-324*	Glycinin	*Glycine max*	0.13
EDEDEDEDQPR	+	-	-	1376.5087	BAA74953.1	*339-349*	Glycinin	*Glycine max*	0.10
EDQPRPDHPPQRPSRPEQ	-	+	-	2166.0438	BAA19058.1	*291-308*	Glycinin	*Glycine max*	0.24
EEDQPRPDHPPQ	+	-	-	1444.6454	BAA19058.1	*290-301*	Glycinin	*Glycine max*	0.26
EEDQPRPDHPPQRPSRPEQ	+	-	-	2295.0864	BAA19058.1	*290-308*	Glycinin	*Glycine max*	0.20
EEEDQPRPDHPPQRPSRPEQ	+	-	-	2424.129	BAA19058.1	*289-308*	Glycinin	*Glycine max*	0.17
EQDEDEDEDEDKPR	+	+	+	**1748.6733**	**BAA74953.1**	***309-322***	**Glycinin**	***Glycine max***	**0.07**
EQDEDEDEDEDKPRPS	+	+	+	**1932.7581**	**BAA74953.1**	***309-324***	**Glycinin**	***Glycine max***	**0.09**
EQDQDQDEDEDEDEDQPR	+	+	+	**2234.8081**	**BAA74953.1**	***332-349***	**Glycinin**	***Glycine max***	**0.05**
EQTPSYPPR	+	+	-	1074.5215	BAA19058.1	*266-274*	Glycinin	*Glycine max*	0.49
GNPDIEHPET	-	+	-	1108.4907	BAA19058.1	*167-176*	Glycinin	*Glycine max*	0.31
HEDDEDEDEEEDQPR	+	+	-	1886.6798	BAA19058.1	*281-295*	Glycinin	*Glycine max*	0.05
HEDDEDEDEEEDQPRPD	+	+	+	**2098.7596**	**BAA19058.1**	***281-297***	**Glycinin**	***Glycine max***	**0.05**
HEDDEDEDEEEDQPRPDHPPQRPS	-	+	-	2898.1686	BAA19058.1	*281-304*	Glycinin	*Glycine max*	0.90
HEDDEDEDEEEDQPRPDHPPQRPSRPE	+	-	-	3280.3651	BAA19058.1	*281-307*	Glycinin	*Glycine max*	0.92
HEDDEDEDEEEDQPRPDHPPQRPSRPEQ	-	+	-	3408.4237	BAA19058.1	*281-308*	Glycinin	*Glycine max*	0.92
LAGNPDIEHPET	+	+	-	1292.6119	BAA19058.1	*165-176*	Glycinin	*Glycine max*	0.26
NNQLDQNPR	+	-	-	1099.5128	BAA19058.1	*153-161*	Glycinin	*Glycine max*	0.25
QDEDEDEDEDQPR	+	-	-	1619.5943	BAA74953.1	*337-349*	Glycinin	*Glycine max*	0.08
REQDEDEDEDEDKPR	+	-	-	1904.7744	BAA74953.1	*308-322*	Glycinin	*Glycine max*	0.06
REQDEDEDEDEDKPRPS	+	-	-	2088.8592	BAA74953.1	*308-324*	Glycinin	*Glycine max*	0.07
EEEEEDEKPQ	+	+	-	1261.5068	AAA33966.1	*280-289*	Glycinin G1	*Glycine max*	0.04
EEEEEEDEKPQ	+	-	-	1390.5494	AAA33966.1	*279-289*	Glycinin G1	*Glycine max*	0.04

Supplementary Table 7-1. (continued).

AAC-26-15 (Soy)

Peptides[1]	Control	AP	BL	MH+ [Da]	Accession Number	Fragment Location	Precursor Protein	Organism	Peptide Ranker Score[2]
EEEEEEEDEKPQ	+	+	-	1519.592	AAA33966.1	*278-289*	Glycinin G1	*Glycine max*	0.04
VIKPPTDEQQQRPQ	-	+	-	1663.8765	AAA33966.1	*264-277*	Glycinin G1	*Glycine max*	0.10
KEEEEEEESKKEEEE	+	-	-	1909.8034	KAG4386526.1	*421-435*	Hypothetical protein	*Glycine max*	0.03
GDDDMPGAGGAGSGAGPK	-	+	-	1516.6337	KAG5063899.1	*630-647*	Hypothetical protein	*Glycine max*	0.76
GVANPEETHPK	+	-	-	1178.5801	KAG4944389.1	*91-101*	Hypothetical protein	*Glycine max*	0.17
NSWDPPNPH	-	+	-	1063.4592	AAA33983.1	*130–138*	Lectin pre-peptide	*Glycine max*	0.77
SWDPPNPH	-	+	-	949	AAA33983.1	*131-138*	Lectin pre-peptide	*Glycine max*	0.86
NDLGDPDKGENH	+	-	-	1310.561	AAB41272.1	*208-219*	Lipoxygenase-3	*Glycine max*	0.19
ADTGGGDAVRPV	+	+	-	1156.5595	AAB71226.1	*2-13*	Metallothionein-II protein	*Glycine max*	0.17
ADTSGGDAVRPV	-	+	-	1186.57	AAB65792.1	*2-13*	Metallothionein-II protein	*Glycine max*	0.16
SGGDAVRPV	+	-	-	857.4476	AAB65792.1	*5-13*	Metallothionein-II protein	*Glycine max*	0.37
EHAMNPVPQ	+	-	-	1038.4674	ACU18271.1	*20–28*	Unknown	*Glycine max*	0.24
FVDLEPTVIDEVRTGT	-	+	-	1790.9173	ACU19580.1	*67–82*	Unknown	*Glycine max*	0.12
KGPDPTPGKPM	+	+	-	1124.5772	ACU13726.1	*14–24*	Unknown	*Glycine max*	0.85
SGDVWFPQPAPK	+	+	-	1328.6635	ACU18647.1	*309–320*	Unknown	*Glycine max*	0.83
VIPTEPPHQ	+	+	-	1017.5365	ACU23484.1	*238–246*	Unknown	*Glycine max*	0.18
VVIPTEPPH	+	+	-	988.5463	ACU23484.1	*237–245*	Unknown	*Glycine max*	0.19
VVIPTEPPHQ	+	-	-	1116.6049	ACU23484.1	*237–246*	Unknown	*Glycine max*	0.13
SADDFEPPLIPPK	+	-	-	1425.7263	CAW94712.1	*216–228*	Unnamed protein product	*Glycine max*	0.72
NALEPDHRVE	-	+	-	1179.5753	CAA47809.1	*39–48*	Legumin	*Pisum sativum*	0.15

Peptides identified by Q-Exactive MS/MS in the 3 kDa permeate of legumes digestate before the exposition to the cell monolayer (control) and in the apical (AP) and basolateral (BL) fractions at the end of the transport experiment. Only the peptides found in the apical and basolateral fractions of three independent transport experiments are reported. (+) indicates that the peptide was detected and (-) indicates that the peptide was not detected. [1] Amino acid sequence is abbreviated with one letter code. [2]The probability scores of peptides to be bioactive (peptide ranker score) was computed using peptide ranker (Mooney et al., 2012). A score close to 0 indicate that the peptide has a low probability to be bioactive whereas a score close to 1 indicate a high probability.

CHAPTER VIII. GENERAL DISCUSSION AND CONCLUSIONS

This research has contributed to close a knowledge gap on the nutritional and the bioactive value of Canadian faba bean varieties (Fabelle, Malik and Snowbird) in comparison to pea and soy.

Firstly, the nutritional quality of faba beans was ascertained with an original *in vitro* gastrointestinal digestion work flow, enabling the evaluation of the ileal digestibility of amino acids and the nutritional quality following the most recent FAO recommendations (FAO, 2013). This is the first study reporting a varietal comparison of newly developed faba bean varieties with the use of ileal amino acid digestibility and the IV-DIAAS. This *in vitro* digestion model was used to assess the impact of a conventional food processing (i.e. boiling) on the nutritional value, thereby giving a realistic portrait of the nutritional value of faba bean proteins. The first limiting amino acids of faba bean varieties were either sulfur-containing amino acids or tryptophan, as reported for other pulses (Boye, Zare, & Pletch, 2010). The variety Snowbird had the highest protein content compared to Malik and Fabelle. Yet, based on the IV-DIAAS value, the faba bean variety Malik had the highest nutritional value, followed by Fabelle and then Snowbird. The variety Malik had the lowest content of total phenolics and phytic acid compared to the two other faba bean varieties, which may have contributed to the higher amino acid digestibility and protein quality. After boiling, there were no significant differences between the three faba bean varieties. The IV-DIAAS of faba beans were generally higher than pea, but lower than soy. Boiling had a negative impact on amino acid digestibility, probably as a result of increased protein aggregation and reactivity with the food matrix, since the legumes were boiled as flours and not as seeds. This study has demonstrated the suitability of the INFOGEST *in vitro* gastrointestinal digestion workflow to study the impact of food processing on the nutritional value of faba beans and sets a benchmark for future investigations regarding emerging food processing techniques and bioprocessing. Compared to *in vivo* data, the obtained IV-DIAAS for faba beans, pea and soy were underestimated. It was hypothesised that these lower scores might be explained by the lack of a jejunal-ileal digestion phase in the standard INFOGEST gastrointestinal digestion protocol.

In an original approach, the digestion protocol was, therefore, modified to include a last digestion phase mimicking brush border digestion. This improved INFOGEST protocol was applied to investigate the nutritional quality of diverse protein ingredients, including flours from the three studied faba bean varieties, pea and soy, and two dairy proteins (casein and whey protein isolate) to evaluate the physiological significance of the newly developed model. This research is the first reporting the addition of a jejunal-ileal digestion phase to the INFOGEST protocol in order to evaluate the IV-DIAAS. There is currently a strong interest in the scientific community to find a way to mimic the protein digestion that occurs in the small intestine brush border (Brodkorb et al., 2019; Mackie, Mulet-Cabero, & Torcello-Gómez, 2020; Vivanco-Maroto, Santos-Hernández, Sanchón, Picariello, Recio, & Miralles, 2022). The proposed alternative is usually the use of non-commercially available extract of the porcine brush border membrane (Mamone & Picariello, 2023). However, the preparation of this extract is time consuming and requires purification steps, thereby not readily accessible, not to mention the lack of standardization of this biological product. In this study, we proposed the use of a commercially available aminopeptidase as an alternative to porcine BBM. The modified protocol showed better correlations with published *in vivo* results compared to the standardized INFOGEST protocol. This improved digestion protocol could be used as a valuable cost-effective, and easily standardizable tool for the evaluation of protein digestibility *in vitro* in a more physiologically relevant manner. However, there is still room for improvement of this model. Despite the significant increase of ileal amino acids digestibility and IV-DIAAS with the modified digestion protocol, the values remained underestimated compared to *in vivo* data. This is possibly due to an overestimation of the digestive enzyme contribution to the amino acid content. Indeed, the contribution of the digestive enzyme was evaluated in performing blank digestion with water instead of food, as recommended in the INFOGEST protocol. Through this approach, the digestive enzymes are more prone to autolysis as there is no protein substrate (Ménard et al., 2023). Some authors have recently suggested various modifications to the INFOGEST protocol to limit enzyme autolysis in the blank digestion, and which are still subject to debate. One of the modifications consists of using a food matrix containing no proteins to perform the blank digestions (Sousa et al., 2023) or performing the blank digestion with inactivated enzymes (Kondrashina et al.). Others authors have suggested decreasing by a factor 10 trypsin activity

required for the duodenal phase in order to decrease the amount of pancreatin to be added in the digestate and thus decrease the blank digestive enzyme background (Ariëns et al., 2021). With the food ingredient tested in their study, there was no significant impact on the degree of hydrolysis of proteins (Ariëns et al., 2021). These proposed modifications still deserve further research and validation to further improve the assessment of protein quality *in vitro*. Nonetheless, although the herein obtained IV-DIAAS values were slightly underestimated compared to *in vivo* data, the improved INFOGEST protocol proved to be a very valuable and relevant tool for a primary evaluation of new protein for comparative purposes. Indeed, strong positive correlations were obtained between the *in vitro* and the *vivo* data. As an example, the two dairy proteins, whey protein isolate and caseins have higher IV-DIAAS score, followed by soy and pulses, in concordance to *in vivo* data. The proposed *in vitro* approach enables a rapid screening of the nutritional value of various protein sources and ingredients and will contribute to limit animal testing, while still considering the most recent recommendations for protein quality assessment. This is more needed as new proteins are entering the market at a fast-growing rate.

Secondly, the bioactive properties of the three faba bean varieties were compared to the ones of pea and soy through the screening of their released bioactive peptides in the gastrointestinal digestate. This is the first study reporting a varietal comparison of newly developed Canadian varieties in terms of bioactive properties. Dietary protein sources have the potential to procure health benefits which extends beyond fulfilling nutritional need. Therefore, a complete protein quality assessment must take these potential health benefits into account. Currently, the health benefits related to bioactive peptides remain controversial since the peptides produced through food processing and non-physiological hydrolysis procedure often failed to maintain their alleged bioactive activities *in vivo*, being non-resistant to gastrointestinal digestion and showing poor permeability across the intestinal barrier. This study has, therefore, taken this into account by studying the bioactive properties in the physiological relevant context of gastrointestinal digestion. Legume flours were used as the starting material to perform *in vitro* gastrointestinal digestion to mimic protein digestion in a way that is representative of how these protein sources are usually consumed (i.e. as whole food). After digestion, small bioactive compounds were recovered by

ultrafiltration on a 3 kDa cut-off membrane. A combination of various *in vitro* and cellular models were used to assess the antioxidant, antihypertensive and antidiabetic activities of the digestates 3 kDa permeates. The antihypertensive properties of two faba bean varieties, Fabelle and Malik varieties were significantly higher than peas but lower than soy. The *in vitro* antidiabetic activity was higher for soy, but no differences were found at the cellular level. For the antioxidant activity, the *in vitro* activities of faba beans were generally similar or higher than pea and similar or lower than soy. Interestingly, the antioxidant activity, of faba bean Fabelle and Snowbird varieties were higher than both pea and soy with cellular models. Other bioactive compounds present in the legume flour digestates, such as polyphenols and oligosaccharides, could also contribute to the observed antioxidant effect. Considering this, a correlative analysis between the measured antioxidant effect and the carbohydrate and polyphenols contents in the digestates 3 kDa permeates were carried out, revealing no significant positive correlations. To confirm that peptides were largely responsible for the measured bioactive effects, the digestate 3 kDa permeates of the faba bean variety Fabelle, which showed the most promising bioactivities, was further fractionated and the peptides were identified by mass spectrometry. Eleven identified peptides with promising bioactive potential were selected for further investigation. These peptides were chemically synthesized to ascertain their bioactivities. Seven peptides among these were revealed to be potent antioxidants. Among them, 4 were also potent antihypertensive agents, making them multifunctional peptides. The mechanisms of actions of these antioxidant and antihypertensive peptides were studied in more details with a combination of *in vitro*, computational and cellular models. These results demonstrated that faba bean peptides after *in vitro* gastrointestinal have the potential to contribute to the prevention of noncommunicable diseases in a healthy diet, since chronic oxidative stress and high blood pressure are important risk factors of non-communicable diseases. Chronic inflammation is another important risk factor of numerous noncommunicable diseases. The anti-inflammatory properties of faba bean peptides after *in vitro* gastrointestinal digestion would be, therefore, a pertinent topic to explore in the future. For instance, their impact on the modulation of the mitogen-activated protein kinase (MAPK), the nuclear factor kappa B (NF-κB), and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathways could be explored (Liu et al., 2022).

Finally, the *in vitro* bioavailability of faba bean peptides released during gastrointestinal digestion was investigated in comparison to pea and soy through the use of a cellular model mimicking the intestinal barrier. It was demonstrated through this investigation that faba bean peptides were maintaining their antioxidant activity after intestinal uptake. It was also demonstrated that 7 of the peptides identified in the faba bean digestate were transported across the intestinal barrier. Among these, 5 peptides were potent antioxidants, 3 peptides were potent antihypertensive agents, and 3 had both activities, making them multifunctional. Moreover, two antioxidant peptides were further metabolized during intestinal uptake to fragments with high antioxidant activities. These findings show that faba bean peptides released after gastrointestinal digestion have a strong potential to be absorbed intact and maintain their bioactive effect *in vivo*. Future investigations using *in vivo* models and clinical human studies are needed to ascertain the health benefits of faba bean consumption. These are required since the 2D cell model used remain an approximation of the human small intestine, as it contained only two cell types. Although enterocytes and goblet cells are the most abundant cell type composing the brush border membrane, other cells are present, such as endocrine cells, M-cells and immune cells. The present model could therefore be further improved by including more cell type, to mimic more closely the brush border membrane. The addition of Raji B cells for instance, has been proposed to mimic M-cells (Antunes et al., 2013). The addition of THP-1 and MUTZ-3 cells were also proposed to mimic immune cells (Paul et al., 2023). Still, these more complex models would need a throughout validation. Moreover, after absorption, the peptides will undergo first pass hepatic metabolism before being systemically bioavailable (Bruno et al., 2013). It would be possible to include hepatocytes (HepG2 cells) in the basolateral side to mimic hepatic metabolism (Lammi et al., 2016). Other approaches could be the use to mimic more closely the human small intestinal brush border, such as the Ussing chamber (Ozorio et al., 2020), which is a tissue-based 3D model, but this requires animal tissue sampling, which is an important drawback of this approach.

Peptide and amino acids absorption ends in the ileum, however, unabsorbed peptides continue their route throughout the large intestine and the colon. These peptides have the potential to modulate gut microbial metabolism and by that, contribute to global health. In future research, the impact of faba bean peptides after gastrointestinal digestion on

modulations of gut microbiota could be considered, as this research area remains largely unexplored.

In conclusion, as hypothesized, there were differences in terms of bioactive and nutritional properties for the three Canadian faba bean varieties studied. The variety Malik stood out for its nutritional value, while the variety Fabelle stood out for its bioactive potential. These results are highly valuable to the Canadian faba bean producers and breeders to assist with the development of faba bean varieties with the highest nutritional and health promoting attributes. Currently, the variety Snowbird is the most cultivated in Canada (Khazaei et al., 2021), but our result shows that the varieties Malik and Fabelle might be more promising varieties to consider in the future. Other properties, such as the sensory attributes and the techno-functional properties would also need to be taken in consideration. Lastly, this research has demonstrated that Canadian faba beans have nutritional and bioactive properties comparable to pea and soy and is therefore an excellent sustainable alternative to these two protein sources in the food industry.

CHAPTER IX . CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

9.1 Main contributions to knowledge

1. This is the first study reporting a varietal comparison and the impact of a conventional processing (i.e. boiling) on the nutritional quality of three newly developed Canadian Faba Bean varieties (Fabelle, Malik and Snowbird) in comparison to pea and soy with the use of most recent recommendations for protein nutritional quality assessment (i.e. ileal amino aid digestibility and the DIAAS).
2. It is also the first study that considered faba bean protein quality as a whole processed food and not as purified proteins, thereby taking into account the impact of processing and food matrix interactions during the gastro-intestinal digestion.
3. This is the first study reporting the addition of a jejunal-ileal digestion phase to the INFOGEST protocol for the determination of amino acid digestibility and the IV-DIAAS. This will contribute to further improvements of this *in vitro* gastrointestinal digestion system to mimic protein digestion in a more physiologically relevant manner.
4. This is the first study reporting a varietal comparison of the bioactive properties of three newly developed Canadian Faba Bean varieties (Fabelle, Malik and Snowbird) in comparison to pea and soy after gastrointestinal digestion with a combination of *in silico*, *in vitro* and cellular models.
5. This study has demonstrated for the first time that faba bean peptides generated after gastrointestinal digestion have the potential to be bioavailable, since they can cross the intestinal barrier while maintaining their bioactive properties.
6. This study has demonstrated for the first time that certain faba bean peptides are metabolized during intestinal uptake, leading to the formation of peptides with a higher bioactive activity.
7. Nine new bioactive oligopeptides with excellent antioxidant and antihypertensive properties were discovered from a simulation of human gastrointestinal digestion and transepithelial transport of faba bean flours. The mode of actions of these peptides were elucidated for the first time with the use of *in vitro*, cellular and computational studies.

9.2 Recommendations for future research

1. Investigate the impact of conventional and emerging food processing techniques and bioprocessing (such as ultrasound, high pressure processing, germination, fermentation, etc.) on the nutritional value and the bioactive properties of Canadian Faba bean varieties.
2. Investigate the nutritional quality and bioactive properties of food products formulated with Canadian faba bean flours.
3. Compare the performance of the developed jejunal-ileal digestion phase with a single aminopeptidase to brush border membrane extract.
4. Investigate other potential bioactive properties of faba bean flours after gastrointestinal digestion that can complement its antidiabetic, antihypertensive and antioxidant properties (renin inhibitor, alpha-glucosidase inhibitor, anti-inflammatory properties).
5. Investigate the health-modulating effects of unabsorbed faba bean peptides on the gut microbiota.
6. Investigate further the mode of transport of faba bean peptides after gastrointestinal digestion (transporter mediated, paracellular route via tight junctions, transcytosis route, and passive transcellular diffusion).
7. Investigate the bioavailability of other bioactive compounds after gastrointestinal digestion of faba bean flours (i.e. polyphenols and oligosaccharides).
8. Confirm the health-promoting activities (antioxidant and antihypertensive) of faba bean flours (variety Fabelle) after gastrointestinal digestion with the use of *in vivo* models.

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