ASSESSING *IN VITRO* METHODOLOGIES FOR THE DETERMINATION OF PROTEIN DIGESTIBILITY, AMINO ACID DIGESTIBILITY, AND PROTEIN QUALITY

By

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ABSTRACT

This study examined the potential of two *in vitro* static digestion models, namely the pH-drop and INFOGEST 2.0, to determine *in vitro* protein digestibility and amino acid digestibility in assessing protein quality. The pH-drop model directly measured *in vitro* protein digestibility for the subsequent calculation of the *in vitro* Protein Digestibility Corrected Amino Acid Score (IV-PDCAAS) value. However, the INFOGEST model digestion products were analyzed by three methods: i) OPA derivatization, ii) total nitrogen via Kjeldahl, and iii) individual amino acid analysis to determine *in vitro* protein digestibility and IV-PDCAAS. The latter analysis was additionally used to assess *in vitro* amino acid digestibility, subsequently used to calculate *in vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS). Among the four assessment methods, the OPA assay and total amino acid analysis from the INFOGEST digestion products demonstrated closer associations with *in vivo* PDCAAS compared to the pH-drop model and the Kjeldahl analysis. However, the pH-drop model, with a straightforward and simple approach, exhibited better repeatability across measurements. Using three popular assays, the PDCAAS, the DIAAS, and the Protein Efficiency Ratio (PER), to evaluate protein quality and permitted protein content claims of samples, it was observed that there was no difference in terms of protein content claims determined when using the *in vivo* or four *in vitro* methodologies. The results indicated that the PDCAAS generally offered higher protein permitted claims than the DIAAS and the PER methods and their respective protein content claims, highlighting that the selection of assessment methods impacts the limiting amino acid and protein quality and extends to subsequent claims for the sample.

In conclusion, the pH-drop model is straightforward and highly repeatable; however, the INFOGEST model showed a closer correlation with *in vivo* data. Additionally, the *in vitro* static

INFOGEST digesta proved versatile in evaluating various aspects of protein quality in both *in vitro* protein and amino acid digestibility through amino acid analysis. Both models can be valuable screening tools for protein quality assessment. Further development of these *in vitro* methodologies can offer effective and sustainable non-animal testing alternatives for protein quality assessment and protein content claims on product packaging.

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DEDICATION

Con xin gửi tặng cho ba mẹ và cả nhà luận văn này. Cảm ơn ba mẹ và cả nhà đã luôn yêu thương và tin tưởng vào con.

To my beloved Nathaniel Dollin-Goodall! You are always there to hold me up when times are tough, to reassure me when I am insecure, and to celebrate with me in the good times. Thank you for being my rock!

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LIST OF ABBREVIATIONS

AA	Amino acid
AAA	Aromatic amino acids
AAD	Amino acid digestibility
AAS	Amino acid score
ANF	Anti-nutrition factor
AOAC	Association of Official Agricultural Chemists
DIAAS	Digestible Indispensable Amino Acid Score
FAO	Food and Agriculture Organization
HIS	Histidine
IAA	Indispensable amino acid
ILE	Isoleucine
ISO	International Organizations for Standardization
LEU	Leucine
LYS	Lysine
MET	Methionine
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PER	Protein Efficiency Ratio
PHE	Phenylalanine
PQ	Protein quality
SAA	Sulphur amino acids
SCP	Single-cell protein
THR	Threonine

TIM	TNO Gastro-Intestinal Model
TRP	Tryptophan
UNU	United Nations University
USA	The United States of America
VAL	Valine
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

"To eat is a necessity, but to eat intelligently is an art" (Rochefoucauld, n.d.). Dietary protein is recognized as one of the essential nutrients that can provide energy, “building blocks”, and components for various biochemical reactions in living organisms. Thus, establishing accurate amino acid (AA) requirements is vital for public health and regulatory purposes. Besides the nutritional perspective, we must consider sustainability when choosing our proteins. One of the emerging solutions is shifting to sustainable diets using more plant-based foods since meat and dairy products are typically resource-intensive (Searchinger et al., 2014). One challenge for plant-based proteins is their limitations in one or some indispensable amino acids (IAAs) compared to animal-based options (Herreman et al., 2020; Marinangeli & House, 2017). To overcome this problem, it is suggested that processing to improve general AA profiles or mixing ingredients to create compensation between plant-based proteins (Herreman et al., 2020; Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Nosworthy, Neufeld, et al., 2017).

When selecting suitable proteins, protein quality (PQ) is a valuable tool for the industry and regular consumers, expressing the capacity to provide IAAs to meet the requirements (Kurpad, 2013). The Canadian government currently assesses PQ using a growth-based method called the Protein Efficiency Ratio (PER), which measures the efficiency of weight gain relative to protein consumption (Health Canada, 1981). Meanwhile, the United States of America (USA) uses the Protein Digestibility-Corrected Amino Acid Score (PDCAAS) method for protein quality evaluation based on amino acid scoring and true fecal nitrogen digestibility (USA Food and Drug Administration, 2021). In 2013, the Food and Agriculture Organization (FAO) introduced the Digestible Indispensable Amino Acid Score (DIAAS) method as a suitable assay for determining PQ using the ileal digestibility of individual IAAs (FAO, 2013). Depending on the method, foods

can be labelled to different protein content claims. Hence, it potentially impacts public health and consumer choices (Marinangeli & House, 2017; Mathai et al., 2017; Rutherfurd et al., 2015; Sá et al., 2023, 2024).

Despite using different ways to calculate PQ, all three aforementioned methods are currently conducted on animals, including rats and swine. Bioassays are straightforward ways to study nutrient digestion and bioavailability. Nonetheless, using animals creates challenges for researchers and industries concerning ethical issues, prolonged time, unavoidable budget, and technical requirements for animal care. As alternatives to bioassays, *in vitro* models offer the ability to study the digestibility and bioavailability of nutrients without living organisms. These models allow food researchers to lower expenses, cut personnel, and shorten the time for analyzing performance. Moreover, many consumers follow non-animal diets due to ethical constraints, religious beliefs, health, and environmental reasons. Therefore, employing this alternative technology for testing nutritive value is critical while maintaining the products' specialty.

Until now, there has been little discussion about the standardization of methodologies for measuring *in vitro* protein digestibility (PD) and the PDCAAS, especially amino acid digestibility (AAD) and the DIAAS values. The following literature review provides a fundamental overview of protein digestion and nutritional value and outlines the advantages and limitations of PQ assays. In addition, this work evaluated the potential of *in vitro* digestion models to select suitable approaches to measure *in vitro* digestibility and PQ. Overall, this study aimed to examine two *in vitro* static digestion models named pH-dop and INFOGEST to determine *in vitro* PD, AAD, and PQ as the PDCAAS and the DIAAS values on different protein sources.

CHAPTER 2: LITERATURE REVIEW

2.1. PROTEIN DIGESTION AND AMINO ACIDS REQUIREMENT

2.1.1. Protein and amino acid role in human health

Protein is an essential nutrient that contributes energy, nitrogen, and AAs to humans. They are present throughout the body, from skeletal muscles to other structures and tissues such as skin, blood, liver, kidney, and brain (Institute of Medicine, 2005). Proteins are constantly being synthesized and degraded inside the body, known as the protein turnover process. These reactions combine with other factors, including metabolic demands for growth/maintenance, diet supplements, *de novo* formation, and excretion process, determining the whole-body protein homeostasis (Figure 1) (Millward, 2012b). In order to keep the nitrogen equilibrium while maintaining the body's protein mass, a daily minimum dietary intake of nitrogen is required and is defined as the protein requirements (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012; Millward, 2012a). This amount covers daily metabolic demand from maintenance and growth with modest physical activity and other special conditions in infants, children, pregnancy, and lactation. The Recommended Dietary Allowance of adults for protein is 0.83 g protein per kg body weight for adults with a PDCAAS value of 1.0 (EFSA Panel on Dietetic Products Nutrition and Allergies, 2012; WHO & FAO, 2007).

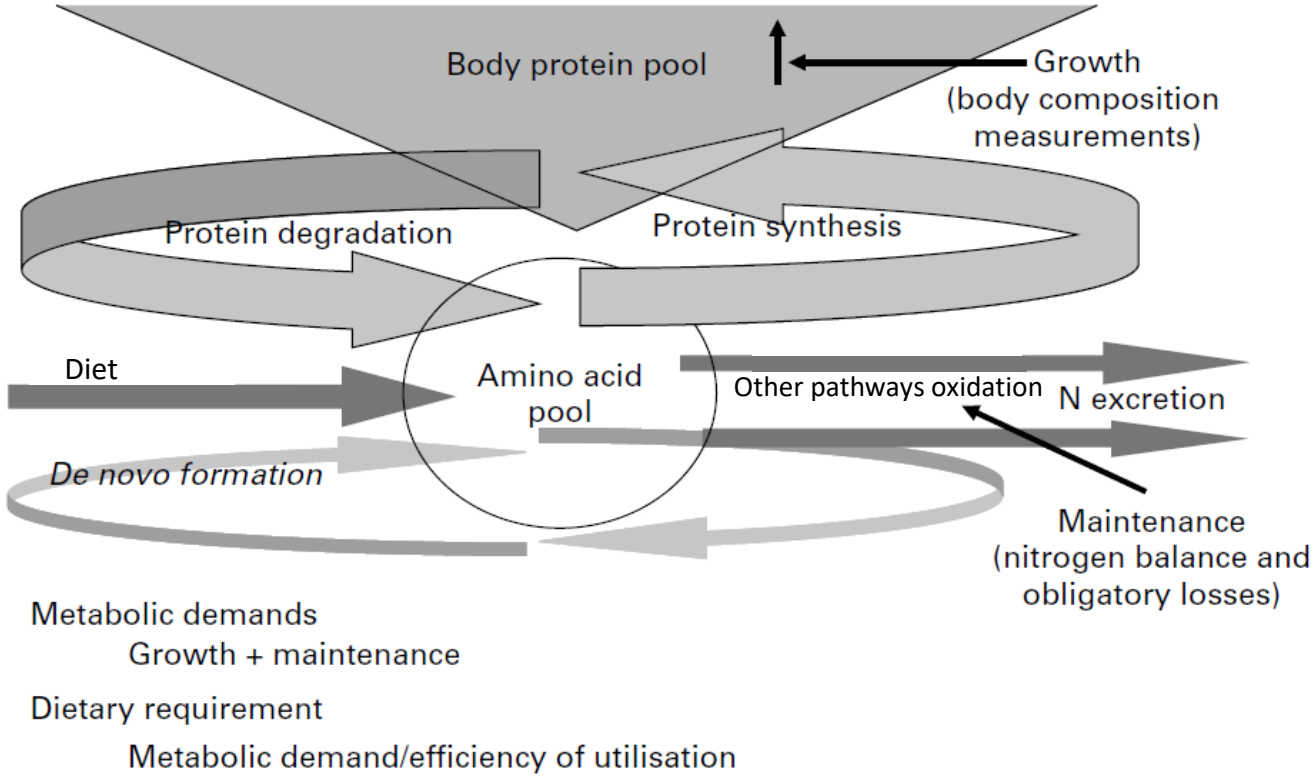


Figure 1. Protein homeostatic pathway and protein requirement assessment (Millward, 2012a)

Hundreds of AAs occur naturally, but only twenty are involved in human protein construction. All these AAs have L-configuration and belong to the α-AA group, except for glycine. Selenocysteine and pyrrolysine have occasionally been referred to as nonstandard proteinogenic amino acids. The reason is their unique roles in protein synthesis since their information was not universally genetically encoded (Driscoll & Copeland, 2003; Krzycki, 2005). The standard proteinogenic AAs can be categorized based on polarity, such as positive/negative charged, polar uncharged, hydrophobic, or based on side chain group type, such as branched chain, sulphur, or aromatic, to name a few. In addition, nine among 20 standard AAs which are histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP), and valine (VAL) are called indispensable amino acids (IAAs) (or referred to as essential AAs in some documents) because they cannot be synthesized at all by the human body or they can be synthesized, just not at rates need to meet metabolic demand.

Looking into the structure, AA comprise an amino (NH_3) group, which can provide nitrogen from dietary proteins to living organisms. Since the 19^{th} century, it has been widely assumed that nitrogen contributes about 16% of the weight of all proteins. With that assumption, the crude protein content can be calculated by measuring the nitrogen content of the protein and then multiplying it by the conversion factor (referred to as the Jones factor) of 6.25 (Breese Jones, 1931; Mæhre et al., 2018). It is argued that this conversion factor should not apply to all proteins because nitrogen does not always hold 16% of protein weight (Mariotti et al., 2008). Additionally, foods contain other nitrogenous compounds unrelated to protein, called non-α-amino-nitrogen. Hence, using the 6.25 Jones factor may lead to overestimations for dairy-based proteins or underestimation with plant-based proteins, leading to inaccurate PQ evaluations (Rutherfurd et al., 2015).

2.1.2. Protein digestion process

In contrast to water, salts, or micronutrients such as vitamins, all intact proteins must be digested to AAs and small peptides before they can be absorbed (Bhutta & Sadiq, 2012). The digestion of dietary proteins follows three gastrointestinal tract digestion phases, including mouth, stomach, and intestine, where protein is hydrolyzed by different enzymes at different levels (Blanco & Blanco, 2017; Boland, 2016).

Inside the mouth, mastication initially breaks down food into smaller particles. The oral phase is relatively quick compared to the whole digestion time, usually requiring some hours. Regarding digestion, this phase mainly involves carbohydrate hydrolysis with amylase at a neutral pH level. However, oral sensory perception plays an important role in the whole "eating experience" and signals the decision for chewing and swallowing (Fiszman & Tarrega, 2020). The oral phase is considered to have little to no effect on protein digestion other than to increase surface area before entering the next stage.

Protein digestion primarily starts within the stomach, where proteins in the oral bolus interact with gastric proteases (Bhutta & Sadiq, 2012). The gastric mucosa main cells secrete gastric juice consisting of water, hydrochloric acid, enzymes, and mucoproteins. Pepsinogen is a zymogen that is activated by H^+ ions from the acid in the stomach and then transforms into the active form of pepsin. After being activated, pepsin continues to act autocatalytically on other pepsinogen molecules. The main force for the digestive action of gastric juice is pepsin by hydrolysis of inner-peptide bonds to produce polypeptides with N-terminal AAs, oligopeptides, and a small number of free AAs (Freeman et al., 1979). Protein digestion in the stomach depends on gastric emptying rate, pH conditions, and the ingested food characteristics, including its compositions and buffering capacity (Bhutta & Sadiq, 2012). The hydrochloric acid maintains the

optimum pH for pepsin activity of 1.0–2.0 in the stomach. The enzyme will be inactivated once the stomach pH reaches 5.0. With pepsin's sensitivity to pH, researchers considered that the gastric phase may not be the main digestion stage for protein (Bhutta & Sadiq, 2012). Proteins, peptides, and AAs have high buffering capacities, which can resist pH change during digestion in the gastric phase (Mennah-Govela et al., 2019). Unfortunately, the relationship between food properties and the impact of food buffering on gastric juice secretions and the rate of gastric emptying needs further investigation (Mennah-Govela & Bornhorst, 2021).

In the following phase, proteins, peptides, and AAs from gastric chyme are further hydrolyzed inside the small intestine by the action of enzymes in pancreatic juice. The small intestine can be divided into three main parts: the duodenum, jejunum, and ileum. Upon entering the duodenum, sodium bicarbonate excreted from the pancreas will neutralize the gastric chyme. Except for amylase and lipase, all pancreatic enzymes are secreted as zymogens and activated in the intestinal lumen (Blanco & Blanco, 2017). Proteolytic enzymes include three endopeptidases named trypsin, chymotrypsin, and elastase, and two exopeptidases named carboxypeptidases A and B. The pancreas secretes trypsinogen, which then is activated to trypsin in the brush border membrane from removing a hexapeptide from the N-terminal end by the enzyme enterokinase (or referred to as enteropeptidase) (Bhutta & Sadiq, 2012). Then, trypsin further activates all trypsinogen by autocatalysis activities. Other peptidases, named chymotrypsin, elastase, and carboxypeptidases, are secreted as chymotrypsinogen, proelastase, and procarboxypeptidases, respectively. These three zymogens are activated by the presence of trypsin in the intestinal lumen. All activated enzymes are responsible for the main hydrolysis of peptide bonds, thus creating free AAs and short-chain polypeptides.

The final stages of protein digestion are associated with intestinal mucosal cells. The brush-border membrane peptidases play essential roles in further hydrolysis of free AAs, di- and tripeptides prior to absorption (Holmes & Lobley, 1989; Miner-Williams et al., 2014). At least six exooligopeptidases have been identified, which can catalyze the cleavage of the peptide bonds adjacent to the oligopeptide N-terminus and release the chain's first AA. Along with the free AAs, small peptides can also be absorbed into enterocytes. Some of these peptides, called bioactive peptides, can benefit human bodies (Udenigwe & Aluko, 2012). The digested AAs and small peptides can be absorbed mainly by selective transport systems or diffusion to transport particles from the intestine lumen into the bloodstream. The absorption process for protein reaches a peak within the jejunum and finalizes within the ileum (FAO, 2013).

In the end part of the gastrointestinal tract, the undigested materials will continue going into the large intestine, which consists of complex microorganism activities. The gram-positive anaerobic rods and cocci predominate inside the colon compared to gram-negative bacteria, yeast, or fungi (Macfarlane & Macfarlane, 2011). Despite the occurrence of AA fermentation, the majority of bacteria operate through carbohydrate metabolisms. The presence and activities of these microbial bodies contribute to changes in nitrogenous compounds in excreted materials at the end of the digestion process (Mariotti et al., 2008; Schaafsma, 2012). Studies proved that fecal nitrogen is a pool of ileal effluent, sloughed-away cells and mucins from the colon, and inner systemic circulation compounds such as urea and creatine (FAO, 2013).

2.1.3. Amino acid requirements

Among 9 IAAs, MET is the only one to donate the sulphur atom in various reactions in human metabolic pathways. In humans, MET is the precursor of homocysteine and cysteine (CYS) via transmethylation and transulphuration pathways (Figure 2) (Elango, 2020). CYS can be utilized for glutathione synthesis and producing the glutamate-CYS-glycine tripeptide, one of the primary antioxidants in human cells. Homocysteine can undergo the re-methylation process to converse back to MET with betaine. Because of the close relationship with MET and its essential role for humans, CYS is usually grouped with MET under the sulphur AA (SAA) group in nutritional assessment. Animal proteins usually contain less CYS; thus, MET is likely to contribute more than half of the total SAA amount in mixed diets with animal proteins (Joint FAO/WHO, 1991). On the other hand, vegetable proteins such as legumes usually have more CYS than MET. Hence, using the total SAA in nutritional assessments is recommended rather than individual MET or CYS amounts.

Similarly, TYR is also classified as a conditional IAA and is usually considered under one group named the aromatic AA group (AAA) with PHE (Institute of Medicine, 2005). TYR is synthesized via the hydroxylase reaction of PHE in the liver and kidney (Figure 3) (Bhagavan & Ha, 2015). The reaction requires oxygen molecules, phenylalanine hydroxylase as an enzyme, and tetrahydrobiopterin as a coenzyme. TYR then follows a degradation pathway to create fumarate and acetoacetate. Additionally, TYR is utilized in neurotransmitters and hormone synthesis. In addition, an in-born metabolic disease is reported named hyperphenylalaninemia, caused by the deficiency of phenylalanine hydroxylase leading to the accumulation of PHE in tissues and plasma (Bhagavan & Ha, 2015). This disease presents mental impairment, and treatment consists of a low PHE diet or supplement of enzymes for patients.

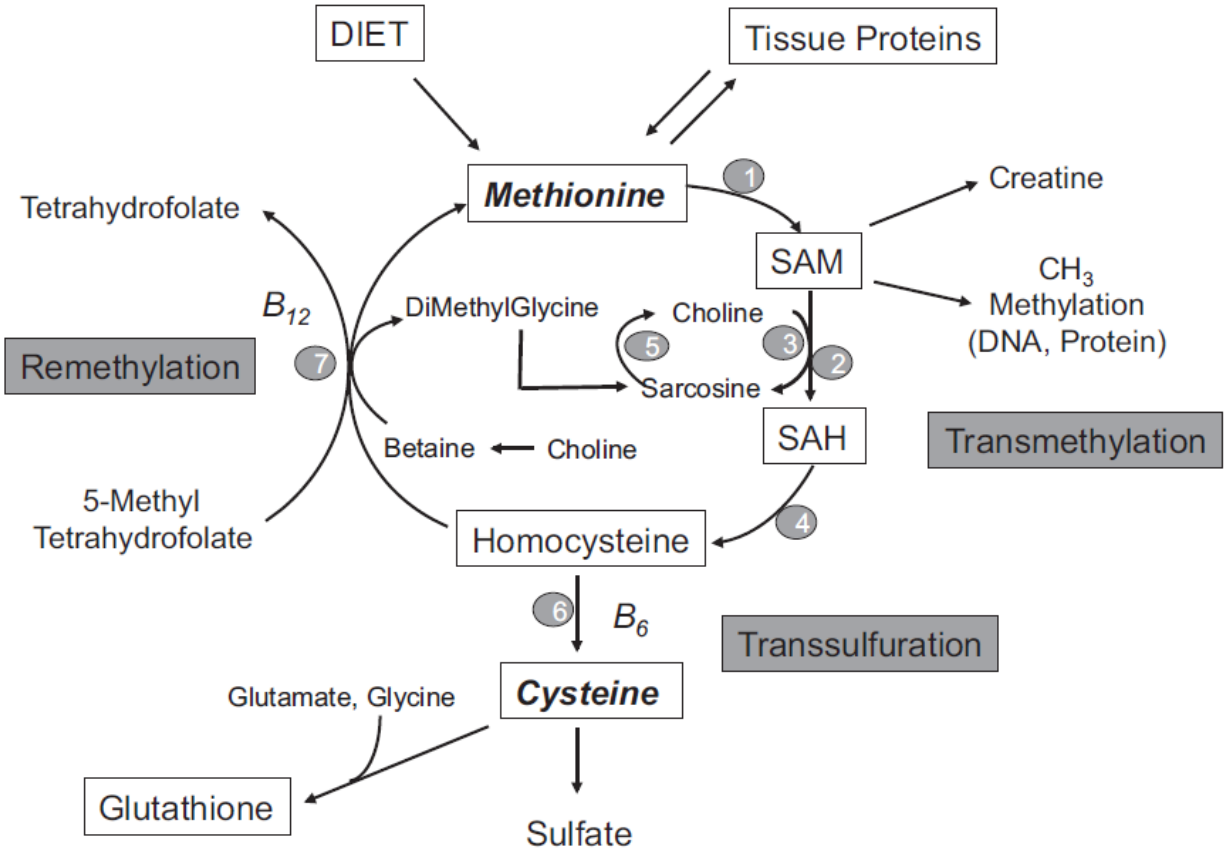


Figure 2. Methionine (MET) and cysteine (CYS) metabolism pathways (Elango, 2020).

Enzymes: (1) Mat1a/Mat2a, methionine adenosyltransferase; (2) SAM-dependent methylase; (3) glycine N-methyltransferase; (4) SAH hydrolase; (5) sarcosine dehydrogenase; (6) cystathionine β-synthase; (7) methionine synthase. B6, vitamin B-6; B12, vitamin B-12; SAM, S-adenosylmethionine, SAH, S-adenosylhomocysteine.

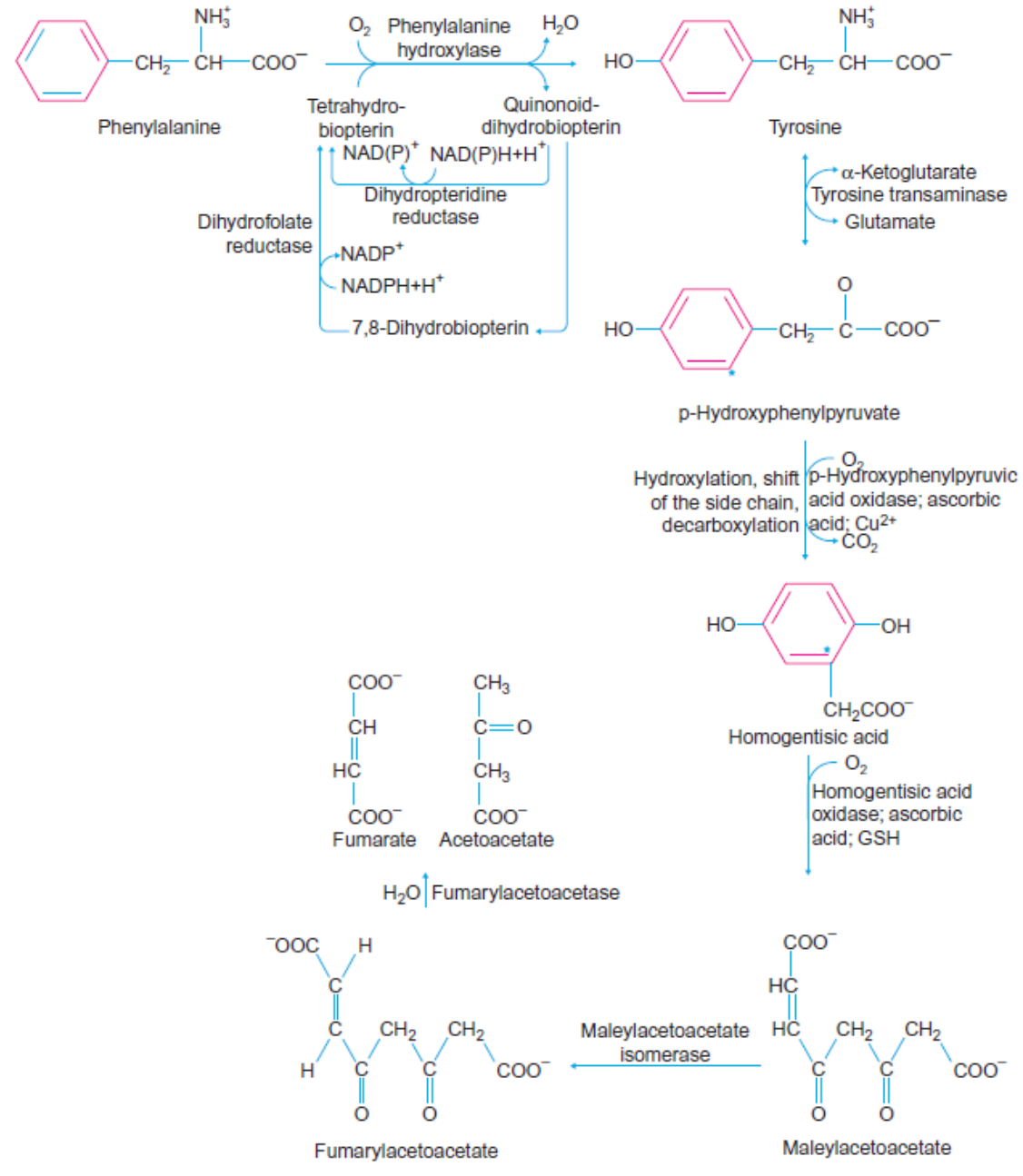


Figure 3. Conversion of phenylalanine to tyrosine and the oxidative pathway of tyrosine (Bhagavan & Ha, 2015).

As humans obtain the bulk of IAAs through daily diets, the term AA requirement defines the quantitaty of AA to maintain the nitrogen turnover. This requirement is personal-oriented, considering the consumers' age, physiological condition, and state of health (Joint FAO/WHO, 1991). The AA scoring (AAS) system is defined as the ratio of the AA content of food with human AA requirements expressed as mg AA per g of protein. Hence, the AA requirement is essential to evaluate PQ. However, the method to identify AA requirements is still an ongoing debate among researchers about precision and practicality.

In 1973, FAO/World Health Organization (WHO) suggested using a single reference pattern for all age populations as a safety margin in estimating protein needs for older age groups (Joint FAO/WHO, 1991). Since 1985, FAO/WHO/United Nations University (UNU) has reported different AAS patterns to establish the minimal requirement of IAAs for separate age groups (Joint FAO/WHO/UNU Expert Consultation, 1985). This proposal supported the idea that PQ depends on age and divided the AAS into four patterns for infants, preschool, older children, and adults. The available data for IAA requirements was divided by the recommended safe level of protein intake for each age group to achieve the updated corresponding AAS pattern. Only the AAS pattern for infants was developed based on the AA pattern of human milk. Additionally, it is concluded that a protein meeting young children's requirements was qualified for older age groups but not vice versa.

The IAAs' requirement in the 1985 report was later criticized because they were assessed by the nitrogen balance technique as inadequate criteria (Joint FAO/WHO, 1991). This method is based on the differences between nitrogen intake and excretion (Institute of Medicine, 2005). While this is the only method with sufficient data to determine the total protein requirement, it potentially underestimates the AA requirements (Hudson et al., 2021; Joint FAO/WHO, 1991).

Other methods include the stable isotope carbon or labeled nitrogen AA with higher sensitivity with rapid changes and suitable for various populations (Institute of Medicine, 2005). The AAS for preschool children was adopted for all ages in PQ evaluation except infants as an interim measure in the 1991 report. After one year old, IAA requirements for growth approach insignificance and almost contributed entirely to maintenance. In contrast, AA needs are almost entirely for tissue growth in young animals. Maintenance needs for humans, who have a longer lifespan than many animals, become more important and will take the majority of dietary protein (Institute of Medicine, 2005). The WHO/FAO/UNU expert consultations 2007 provided updated information about the AA requirements (WHO & FAO, 2007). First, the AA requirements for infants and children were suggested to develop with a factorial approach for maintenance, which was expressed as mg per kg of body weight at all ages and growth, which reflected the AA pattern of human tissue protein. The FAO experts also adopted the methodology, which calculated AAS by dividing the total AA requirement by the mean protein requirement at 0.66g/kg.

In the 2013 reports, FAO experts agreed on human milk's AA profile as the basic scoring pattern for infants and the temporary use of preschool children's pattern for all other age groups until further revision (FAO, 2013). The same report proposed four new scoring patterns for infants, preschool children, older children and adolescents, and adults, emphasizing treating each AA as an individual nutrient.

In conclusion, with the importance of human health, many efforts have been put into studying the protein digestion process and identifying an adequate AA requirement for humans. As many AAS patterns were developed with different methodologies, selecting a suitable pattern will strongly impact how a protein is evaluated. Furthermore, the quality of a protein is not constant but varies depending on target populations.

2.2. ANIMAL-BASED vs. PLANT-BASED PROTEINS

2.2.1. The future roles of proteins in a sustainable food system

The world population is predicted to reach 9.7 billion in 2050 (United Nations, 2019), creating significant gaps equivalent to 593 million hectares of land for agricultural purposes and a 56% increase in the requirement for crop calories for sustainable foods (Searchinger et al., 2014). As a result, the globe faces a reduction in natural resources available per capita, leading to more demand for a sustainable food system. One of the emerging solutions is shifting to sustainable diets using more plant-based foods since meat and dairy products typically demand more resources, including land and water.

Simultaneously, socio-economic growth contributes more to the growing demand for protein in the market (Henchion et al., 2017). One of the most important driving forces is the increasing consumer awareness of the value of protein nutrition and its health benefits compared to other nutrients. The global protein ingredients market was estimated to be more than 72 billion USD in 2021 and is expected to grow significantly at an exponentially compounded rate of 5.9% annually until 2030 (Statista, 2022).

Within the global market, animal-based protein contributed about 71% of revenue (Precedence Research, 2022). It is estimated that plant-based protein is expected to have a significant growth demand. This increase links to the fact that animal-based protein production typically has a significant environmental impact and is resource-intensive. In 2021, FAO reported that the livestock industry uses almost 80% of all agricultural land (FAO, 2022) while only providing 37% of the protein demand (Poore & Nemecek, 2018). In terms of water-use efficiency, it is shown that the total water-use efficiency of litter per g of animal-based protein is high, 47.64 for beef and 22.6 for pork, except for chicken, which is at the low 14.49. Meanwhile, plant-based

proteins require much less water, such as 11.17 for soybeans and 13.32 for peas (Damerau et al., 2019). Plant-based proteins also performed better on both energy input in general and protein delivery efficiency (González et al., 2011). Plant-based protein contents positively correlate with energy efficiency, while animal-based proteins have the opposite trend. From an environmental perspective, animal-based foods create two-thirds of global agricultural greenhouse gas emissions and 18% of the total emissions (Karwacka et al., 2020). The GlobAgri-WRR model estimated that meat from ruminants could produce 20 more times emissions than pulses per gram of protein (Searchinger et al., 2014). In the same study, exchanging 30% of global animal-based proteins with legumes will decrease the food gap to 49% and reduce the greenhouse mitigation gap by 4.8%. In general, plant-based proteins proved that they create much less environmental food prints compared to animal-based counterparts.

The ability for nitrogen fixation of the legume family (*Fabaceae* or *Leguminosae*) also contributed to their important roles in the future sustainable protein industry. Nitrogen is usually inaccessible to plants because it stays in the atmosphere as nitrogen gas (N_2). Hence, farmers have to supplement nitrogen for most of the plants via fertilizers. The legume family hosts bacteria inside their root nodules, which have the unique ability to convert atmospheric nitrogen gas (N_2) to ammonia (NH_3) or ammonium (NH_4^+) through the nitrogen fixation process (Beijerinck, 1901). This ability benefits the legumes, reduces the need for fertilizer, and enhances the soil quality (Postgate, 1998). Crop rotation between legumes and other plants is recommended to increase production and lower environmental impacts (Reckling et al., 2016). Another way to utilize this unique ability is intercropping instead of the common monocropping practice. Research showed positive results when growing cabbage or carrots with fava beans (Lepse et al., 2017) or maize with peanuts (Feng et al., 2021).

2.2.2. Nutritional value comparison

Regarding proteins, it is common to believe that animal-based products are superior because they contain an adequate amount of all IAAs. In comparison, plant-based proteins are usually ranked lower due to limitations in AAs profile. Soybeans are one of the few exceptions among plant-based proteins that offer high crude protein content but also a complete AA profile; thus, soybean and soybean meals are generally recognized as ideal plant-based proteins. Meanwhile, the legume has an unbalanced AA profile with a low content of SAA or TRP. One possible explanation is that the predominant protein in legume seeds is globulins, which are relatively low in SAAs (Baudoin & Maquet, 1999). Cereal is a stable food in human diets around the world. They mainly contribute to daily energy with their carbohydrates. However, cereal grains are typically LYS deficient. To overcome these limitations, combining plant-based proteins is recommended to compensate for the respective limitations in each source (Hall et al., 2017). A study shows that the AA score increased from 0.8 for extruded pinto beans and 0.73 for extruded buckwheat to 0.93 for extruded blended of both ingredients (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017).

For human and monogastric animals, plant-based foods contain some secondary metabolites compounds named anti-nutritional factors (ANFs), which can interfere with the bioavailability of other nutrients and human health (Fekadu Gemede, 2014; Samtiya et al., 2020). One ANFs, phytate, can reduce mineral bioavailability by forming complexes with metal ions, which might lead to zinc deficiency. The regurgitation ability of ruminant animals allows enzymes in the first stomach chamber to separate phytate before chewing and digestion the second time. Unlike cows, humans can reduce phytate contents in plants by other methods such as polishing, soaking, or heat treatments (Sarwar Gilani et al., 2012). Other well-known ANFs are enzyme

inhibitors, which reduce the activities of many digestive enzymes, such as amylase and protease. Research in rats indicates that high levels of dietary trypsin inhibitors from legumes may cause up to 50% substantial reductions in PD and AAD (Gilani et al., 2012). Saponins, primarily found in plants, also show amylase, protease, and lipase inhibitory activities. Low levels of these plant-derived secondary compounds are not considered harmful, but high levels in the diet are toxic. Other ANFs, such as tannins, lectins, and haemagglutinins, cause precipitation and agglutination with other nutrients. Phenolic compounds will reduce the bioavailability of AAs; thus, they might cause weight loss, growth delay, and health problems (Sarwar Gilani et al., 2012). Treatments such as soaking, autoclaving, fermentation, roasting, and cooking can effectively reduce the ANFs in plant-based proteins (Revilla, 2015; Shi et al., 2018; Vagadia et al., 2017; Zhang et al., 2017). In contrast, previous studies showed that certain ANF compounds could have positive effects, including anti-obesity, anti-inflammatory, hypocholesterolemia, and anticarcinogenic activities (de Mejia et al., 2003; Fekadu Gemede, 2014; Friedman & Brandon, 2001; Marrelli et al., 2016). While the debate about ANFs continues from both nutrition and health perspectives, it is necessary to quantify and study the digestion of these ANFs in the meantime.

On the other hand, foods contain protein-derived bioactive peptides, which have one or several impacts on body functions via affecting biological processes (Bouglé & Bouhallab, 2017). The structure of these peptide sequences plays the most critical role in their health-promoting benefits. Proteins contain these inactive AA sequences and release them via enzyme proteolysis during digestion, food processing, and fermentation (Udenigwe & Aluko, 2012). Angiotensin I-converting enzymes are one of the primary mechanisms that increase blood pressure regulation. Several short bioactive peptides show inhibitory properties for these enzymes, thus lowering hypertension and being used as antihypertensive agents, such as VAL-PRO-PRO or ILE-PRO-

PRO from milk or pea protein hydrolysates (Bouglé & Bouhallab, 2017; Humiski & Aluko, 2007; H. Li et al., 2011). Supplements of α-lactalbumin support improving cognitive functioning due to high TRP contents and the ability to pass the blood-brain barrier (Booij et al., 2006). The ability to scavenge free radicals of pea protein hydrolysates, quinoa, and flaxseed were studied and proved that they have antioxidant properties, benefitting consumers (Aluko & Monu, 2003; Humiski & Aluko, 2007; Udenigwe & Aluko, 2010). Another well-known bioactive peptide is lunasin, which is found in soybean and some grains and has anti-inflammation and anticancer properties (de Mejia & Dia, 2009).

2.2.3. Protein processing methods

Among living organisms on the Earth, only some species have the ability to prepare food, but humans are the only species that can process ingredients and cook a meal. Most of our foods are processed, such as harvested produce or animal products, and cooking raw ingredients to increase food safety, sensory characteristics, and nutritional profile.

Standard practices such as rice polishing or wheat bran removal for cereals can effectively remove phytate contents (Sarwar Gilani et al., 2012). Milling is a traditional method to separate the bran layer from the grains, grinding grains into flour. Unlike in-depth studies exploring starch digestion, there is much less information about milling or particle size effects on protein digestion kinetics. *In vivo* work in weanling pigs shows that the AAD of IAAs in soy protein concentrate might peak after grinding to approximately 180 μm (Casas et al., 2017). For broilers, small particle size positively impacts the AAD of soybean meal but is negatively related to maize's values (Siegert et al., 2018). The *in vitro* PD of hammer-milled cowpeas is independent of the particle size while dependent on the cryo-mill (Tinus et al., 2012). For milling brown rice, the optimal particle size recommended for nutrient digestibility is 600 μm for young pigs and 800 μm for adult

sows (E. Li et al., 2019). Also, a study on walnuts and peanuts demonstrated that the type of nuts and particle size affected *in vitro* digestibility and bio-accessibility of both protein and lipids (Paz-Yépez et al., 2019), emphasizing the relationship between proteolysis and particle size. Since size distribution does not have a general linear relationship with AAD, future studies should focus on formulations for humans by specifying the optimal size for specific materials.

Even though it is widely accepted that cooking will help humans digest food better and increase the nutritional value of ingredients, some authors reported a decrease in the digestibility of cooked foods (Wen et al., 2015). During thermal treatment, the dominant Maillard reaction produces aroma, brown color, and taste compounds, as well as leads to the formation of toxic substances such as acrylamide, heterocyclic amines, and advanced glycation end products (Carpenter, 1960; Hurrell & Carpenter, 1976; Moughan & Rutherfurd, 2008). The Maillard reactions also modify LYS once the ε-amino group reacts with reducing sugars. This process results in a biologically unavailable Schiff base of LYS to produce brown pigments. The Schiff base products preclude utilization for protein synthesis among all LYS contents in heat-treated foods. However, the conventional analysis method for AA profile with strong acid hydrolysis can not differentiate the normal and the inactive derivatives, leading to overestimating the bioavailable LYS amount (Moughan & Rutherfurd, 2008). Despite being time-consuming, the guanidination reaction method was studied extensively among several chemical and biological assays developed. This method measures reactive LYS content by the reaction of the ε -amino group with o-methylisourea to produce acid stable homoarginine.

In addition, the ANFs in specific plant-based proteins may decline to some extent through treatments by heat or fermentation (Alonso et al., 2000; Rakić et al., 2007; Samtiya et al., 2020; Sarwar Gilani et al., 2012; Zhang et al., 2017). Another study has indicated that legumes'

nutritional value is enhanced after cooking due to reduced lectin and saponin content (Singh et al., 2017). Extrusion is a well-known industry scale method using a screw system to process foods under specific moisture, temperature, and pressure conditions. It is reported that digestibility can be improved after extrusion, possibly because ANFs factor activity in trypsin/chymotrypsin inhibitors and hemagglutinin is reduced (Alonso et al., 2000; Nosworthy et al., 2018; Nosworthy, Franczyk, Medina, et al., 2017). Extrusion is being applied to make meat analogs from soy protein concentrations, which can improve protein quality/digestibility, thus introducing more applications for sustainable plant-based proteins (Day & Swanson, 2013). Although these processing techniques have shown the ability to reduce ANFs and alter protein digestibility, a direct impact on AAD has not yet been investigated and conducted in unifying methods.

Overall, plant-based proteins play important roles in the sustainable food system with less environmental impacts and the ability to supply the increasing population. From a nutritional point of view, animal-based protein is considered superior to plant-based proteins with an incomplete IAAs profile. Other factors, such as ANFs and bioactive peptides, also contribute to the nutritional profiles of proteins. Various processing and cooking methods can eliminate unwanted ANFs and improve PQ and digestibility; however, they can create unavailable compounds for digestion. Sustainability and nutritional profiles are some of the critical factors that industry and consumers take into consideration when selecting proteins. To support this process, regulatory organizations need reliable tools to measure these factors.

2.3. PROTEIN QUALITY EVALUATION METHOD

Public health and the industry need a suitable tool to evaluate and select suitable proteins from a nutritional standpoint. Currently, dietary PQ is assessed in various ways. The PQ of foods is not a constant number, but it can be altered depending on the physiological requirements of the target population and, most importantly, how it is measured (Mansilla et al., 2020). For regulatory purposes, the three approaches that are of current interest include the PER, the PDCAAS, and the DIAAS methods.

2.2.1. The Protein Efficiency Ratio

After its introduction in 1919, the PER became one of the first PQ assessment methods to be adopted (Gilani & Lee, 2003; Osborne et al., 1919). Recognized for its simple techniques, the PER determines PQ by measuring the weight gain associated with grams of protein ingested by rats over a defined period, thus considered a growth-based method (Mansilla et al., 2020). The USA also uses the PER as the official method to assess PQ for foods with a sole protein source, such as infant formulas (USA Food and Drug Administration, 2021). It is required that the PER in infant formula should not be less than 85% of casein if determined (Codex Alimentarius - FAO, 1987).

In Canada, the rat is the official model in the PER method (Health Canada, 1981). Besides, chickens can be used as the digestion model for the PER method (Buamah & Singsen, 1975). However, the animals must be in the rapid growing stages to maximize the effect of protein consumption in a short period, such as weanling rats or 1-week-old chickens (Mansilla et al., 2020). Using older animals provides variable results due to significant differences in AA requirements and digestive capacity. The PER method involves the provision of test diets and water for *ad libitum* consumption over 28 days. For study purposes, diets are required to be

isocaloric, and formulations must provide the minimum protein requirement for animals at 10% protein of the dry weight of the diet. With the low protein level, protein is utilized for synthesis purposes rather than for energetic metabolic pathways. Other nutrients such as fat, vitamins, minerals, and cellulose are coordinated to meet the total requirements of laboratory rats. After 28 days of feeding, the test diet intake (calculated by subtracting the feed offered to feed weight back) and body weight measurement are collected. The PER value is then calculated as follows:

$$PER = \frac{Weight\ gain\ (g)}{Protein\ intake\ (g)}$$

High-quality proteins are included as controls for variability among facilities and trials. Casein is one of the control references with a standardized PER value of 2.5. After calculating, the "real" PER value of each tested ingredient/diet will be adjusted compared to casein's PER value. Even though this allows the comparison between tests, it is recognized that the PER method ranking is not proportional.

The PER is implemented in Canada to determine food protein ratings (Government of Canada - Canadian Food Inspection Agency, 2020). The protein rating of the testing food is determined as the product of its PER value and the protein contained in a reasonable daily intake. The latter value is calculated by the product of the crude protein value and the recommended daily intake amount of tested foods. Products can be claimed as "a source of protein" or "an excellent source of protein" when their protein rating values are from 20-39.9 or ≥ 40, respectively.

The PER is a direct method of measuring PQ and is considered straightforward in applications (Marinangeli & House, 2017). However, previous studies have identified certain disadvantages of this approach. In the first place, the PER solely takes into account a summative biological response to protein intake, while it does not properly consider the credit for maintenance requirements. A protein-free diet group can be included in the experiment to estimate the

requirement for body maintenance. As a result, the PER value only takes growth value into account. However, a protein might be considered unsupportive for growth purposes but can have adequate quality for maintenance purposes (Joint FAO/WHO, 1991). Secondly, as the PER method requires a 28-day trial, giving young small animals a protein-free diet for a long time will inevitably create more challenges in animal care and ethical concerns. Next, there are inevitable differences when conducting bioassays on humans and other animals. The discrepancies result in inaccurate evaluation, leading to underestimating some vegetable proteins while overestimating some animal proteins (Joint FAO/WHO, 1991; Mansilla et al., 2020). Fourthly, the PER ranking can lead to misunderstandings since assigning the value of 2.5 to casein makes the value unproportional. Compared to foods with a PER of 1, foods with a PER value of 2 do not offer double the quality (Gilani & Lee, 2003). Finally, PER values are not additive. In other words, producers and researchers cannot calculate the PER values of new foods/diets based on the PER data of individual ingredients included. They must conduct a new bioassay for every new food or product or fomula (Mansilla et al., 2020).

2.2.2. The Protein Digestibility Corrected Amino Acid Score

In 1991, the Joint FAO/WHO Expert Consultation officially introduced and recommended using the PDCAAS method to assess PQ through the AAS and the true fecal nitrogen digestibility measurements (Joint FAO/WHO, 1991). The PDCAAS method is officially applied in the USA to measure PQ and is used to declare product protein claims (USA Food and Drug Administration, 2021).

The digested nutrients available for physiological functions are also defined as "bioavailable" (Fernández-García et al., 2009). This term includes "bioaccessibility" representing the volume of a compound released from a food matrix in the gastrointestinal tract, and

"bioactivity" meaning the amount that target tissues will uptake for physiological responses. With the assumption that nutrient disappearance relates to absorption during digestion, the measure of the non-recovery of nutrients at various locations along the gastrointestinal tract is defined as nutrient digestibility. Hence, PD is often used to estimate the bioavailable protein/AAs. The digestibility in the PDCAAs method is measured based on fecal nitrogen digestibility. Like the PER method, rats are commonly required as the digestion model in the PDCAAS assessment. Rats' feces will be collected during the feeding trial. The apparent nitrogen digestibility is determined first as the difference between nitrogen intake and excretion in the feces. Next, the PDCAAS method requires correction from the apparent into the true fecal digestibility value by considering the endogenous nitrogen loss. The latter is measured by determining nitrogen losses from a control group that will be fed a nitrogen-free diet.

The AA profile of testing foods will be compared to the requirement reference patterns of IAAs by the FAO/WHO in 1991 to calculate the AAS value, expressing how much the testing protein meets the metabolic needs of the consumer (Table 1). To avoid potential underestimation problems, FAO/WHO recommended using the AAs requirement pattern of preschool children (2-5 years) when evaluating PQ for school children (10-12 years) and adult populations.

$$Amino\ acid\ score\ for\ each\ IAA\ = \frac{mg\ of\ AA\ per\ g\ of\ protein\ (test\ protein)}{mg\ of\ AA\ per\ g\ of\ protein\ (reference\ pattern)}$$

The lowest score among all IAAs is the AAS, and the respective IAAs will be the first limiting AA of the test protein. Finally, the PDCAAS value is then calculated by multiplying the AAS value by the true fecal nitrogen digestibility value as follows:

$$\text{PDCAAS} = \text{AAS x true fecal nitrogen digestibility}$$

Table 1. The amino acid scoring patterns for infants, pre-schoolchild, schoolchild, and adults (Joint FAO/WHO, 1991)

Age group	**Daily amino acid requirement (mg/ g crude protein)**								
	HIS	ILE	LEU	LYS	SAA	AAA	THR	TRP	VAL
Infant (birth to 6 months)	26	46	93	66	42	72	43	17	55
Pre-school child (2-5 years)	19	28	66	58	25	63	34	11	35
School child (10-12 years)	19	28	44	44	22	22	28	9	25
Adult	16	13	19	16	17	19	9	5	13

His, histidine; Ile, isoleucine; Leu, leucine; SAA, sulphur amino acids (Methionine + Cysteine); AAA, aromatic amino acids (Phenylalanine + Tyrosine); Thr, threonine; Trp, tryptophan; Val, valine;

The PDCAAS has been adopted as the official method to evaluate PQ in the USA (USA Food and Drug Administration, 2021). The authorities also allow the implementation of the PDCAAS value to determine protein claims on ready-to-eat products. First, the corrected protein level of the test food is calculated as the product of the PDCAAS with the amount of protein (g) in the reference amount customarily consumed. Then, based on the ratio between corrected protein levels concerning the daily reference amount for protein (50 g), a "good source" product can provide 10-19.9% of the daily value of protein, whereas an "excellent source" means that products contain >20% of the daily value of protein.

Conveniently, the Canadian government has allowed the calculation of PER values of a product from established PDCAAS values, according to the following equation (Government of Canada - Canadian Food Inspection Agency, 2021):

$$\text{Estimated PER of food} = \text{PDCAAS of food} \times 2.5$$

Compared to previous methods, the PDCAAS is more effective in terms of time, requiring only 5 days versus 28 days of feeding compared to the PER method (Mansilla et al., 2020). The PDCAAS method also considers the digestibility of protein/nitrogen instead of just measuring the effect of test foods on animal growing effects. Another strong advantage of this assessment is that the PDCAAS values are additive, thus allowing the calculation of the mixed foods without the need to carry on a trial for each formula. As many fecal nitrogen digestibility values are available, the PDCAAS method became more applicable for mixed products (Marinangeli & House, 2017). It is also noted that the PDCAAS method requires specific attention due to coprophagy characteristics in rats. Thus, wire bottom cages for these animals are highly recommended.

Several limitations of PDCAAS have been reported (FAO, 2013). First, similar to the PER method, the PDCAAS assay can overestimate or underestimate the quality of some proteins, given the differences in AA requirements and dietary behaviors between rats and humans. The selected animal model can affect the final PQ results. For instance, SAA is in higher demand in weaning rats than in humans, chickens, or swine across all development stages (Table 2). Thus, proteins with limitations in SAA, such as pulses, may be underestimated when tested using the rat model. Next, when values exceed 1.0 (or 100%), the PDCAAS value is truncated to a limit of 1.0. Some scientists argued that IAAs supplied in excess cannot provide additional nutritional benefits. However, humans usually have mixed diets with various ingredients. Thus, truncation will eliminate the extra benefit of high-quality protein and erase the compensation ability of proteins to each other (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Staniak et al., 2014). Previous studies also showed that getting enough protein during different meals might be possible because the human body breaks down proteins and then stores them for later use (Searchinger et al., 2014; Young & Pellett, 1994). Experts recommend that mixed foods should be allowed to have PDCAAS values higher than 1 (FAO, 2013).

Table 2. Total AAs requirements (mg/g CP) of rats, chickens, and swine at different age/ developmental levels (Mansilla et al., 2020; National Research Council, 1994, 1995)

Species	**Age/development**	**Amino acids requirement (mg/g crude protein)**								
		HIS	**ILE**	**LEU**	**LYS**	**SAA**	**AAA**	**THR**	**TRP**	**VAL**
Rat	4 weeks – 6 months	18.7	41.3	71.9	61.3	65.3	68	41.3	13.3	49.3
Rat	Adult	16	62	36	22	46	38	36	10	46
Layer chicken	0-6 weeks	14.4	33.3	61.1	47.2	34.4	55.6	37.8	9.4	34.4
Broiler chicken	0-3 weeks	15.2	34.8	52.2	47.8	39.1	39.1	34.8	8.7	39.1
Swine	5-7 kg	22.3	33.8	65.8	65.4	36.9	61.5	40.4	10.8	42.3
Swine	7-11 kg	22.4	33.3	65	64.6	36.7	60.8	40.1	10.5	42.2
Swine	11-25 kg	23.0	34.9	67.5	67.0	37.8	63.2	41.6	11.0	43.5

His, histidine; Ile, isoleucine; Leu, leucine; SAA, sulphur amino acids (Methionine + Cysteine); AAA, aromatic amino acids (Phenylalanine + Tyrosine); Thr, threonine; Trp, tryptophan; Val, valine;

High ANF contents can enhance endogenous nitrogen losses, thus decreasing the respective protein value (Schaafsma, 2012). Therefore, The PDCAAS will likely overestimate the PQ of products containing ANFs, focusing on plant-based proteins. Next, the PDCAAS method determines PQ based on nitrogen measurements instead of individual IAAs. As a result, this method does not adequately take into account the bioavailability of AAs. Finally, fecal digestibility values cannot accurately reflect the digestibility. Since the ileum is the last site of AA absorption, ileal digestibility is more suitable for assessing the true protein value of a food (Rowan et al., 1994; Schaafsma, 2012). There are a lot of microbes present in the large intestine, which also make a significant impact on fecal nitrogen content. Research demonstrates that a bacterial residue largely contributes to rat fecal nitrogen (Mansilla et al., 2020; Mason & Palmer, 1973). As a result, the PDCAAS method can overestimate the digestibility of test protein due to bacterial assimilation in the large intestine, which can falsely enhance the bioavailability of AAs (FAO, 2013; Marinangeli & House, 2017).

2.2.3. The Digestible Indispensable Amino Acid Score

Recognizing the limitations of the PDCAAS method, the FAO has recommended the DIAAS as a potential replacement (FAO, 2013). In principle, the DIAAS assesses PQ via the ileal digestibility of individual IAA instead of the true fecal nitrogen digestibility of the PDCAAS method. One of the key findings is recognizing dietary AAs as individual nutrients with individual AAD that accurately expresses the amount of AAs the intestine can absorb from food.

Similarly to the PDCAAS, the DIAAS method requires analysis of the IAAs in the test protein. In a food mixture containing multiple proteins, the AA profiles of each ingredient will be calculated and summed up together for the total input AAs amount. The animal digesta will be collected and analyzed for the IAA contents. Comparison between the ingested and remaining

IAAs contents from the digesta allows the researcher to calculate the ileal digestibility coefficient (%). The digestible IAA (DIAA) content is defined as mg of each IAA in 1 g protein of food multiplied by the ileal digestibility coefficient for the same dietary IAA. FAO experts published new reference patterns for AA requirements for different age populations in the 2013 report (FAO, 2013). The DIAA ratio (DIAAR) value is calculated by comparing the DIAA value to respective IAAs in the reference patterns (Table 3) (FAO, 2013). The smallest DIAAR will be known as the DIAAS value, and the respective IAAs will be the limiting AAs for the test foods.

$$\text{DIAAR (\%)} = \frac{\text{mg digestible IAA in 1g of the dietary protein}}{\text{mg of the same digestible IAA in 1g of the reference protein}} \times 100$$

$$DIAAS = the\ lowest\ DIAAR\ (\%)$$

The ileal digestibility is more accurate for measuring AA bioavailability and digestibility than total fecal nitrogen digestibility (Lee et al., 2016; Mathai et al., 2017). It is categorized into apparent ileal digestibility, standardized ileal digestibility, or true ileal digestibility depending on consideration of endogenous loss and how accurate in description digestibility and bioavailability (Stein et al., 2007). The apparent ileal digestibility is determined by directly subtracting the AA amount between the ingested and the digesta. Researchers can collect whole ileal digesta (ileorectal anastomosis) or a portion of ileal digesta (T-cannula technique), depending on the procedures in use. With the T-canula approach, an indigestible marker is usually included in the diet to allow digestibility calculation even though only a portion of the ileal outflow is collected. In this case, the apparent ileal digestibility value is calculated as follows (Stein et al., 2007):

$$Apparent\ ileal\ digestibility\ (\%) = \left[1 - \left(\frac{AA_{digesta}}{AA_{diet}} \times \frac{Marker_{diet}}{Marker_{digesta}}\right)\right] \times 100$$

Table 3. The amino acid scoring patterns for infants, children, adolescents, and adults (FAO, 2013)

Age group	**Daily amino acid requirement (mg/kg)**								
	HIS	ILE	LEU	LYS	SAA	AAA	THR	TRP	VAL
Infant (birth to 6 months)	21	55	96	69	33	94	44	17	55
Child (6 months to 3 years)	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult	16	30	61	48	23	41	25	6.6	40

His, histidine; Ile, isoleucine; Leu, leucine; SAA, sulphur amino acids (Methionine + Cysteine); AAA, aromatic amino acids (Phenylalanine + Tyrosine); Thr, threonine; Trp, tryptophan; Val, valine;

However, the apparent ileal digestibility still contains ileal endogenous AA losses, including AA from endogenous proteins such as mucoproteins, sloughed cells, digestive enzymes, amides, and ingested hair (Nyachoti et al., 1997; Stein et al., 2007). These AAs are secreted into the intestinal lumen but will not be reabsorbed before reaching the distal ileum. The ileal endogenous AA losses have two parts. First, regardless of the diet, animals will inevitably lose a certain amount of dietary AAs, called basal endogenous losses amount. A nitrogen-free diet is included in the experiment to quantify these basal losses as follows:

$$Basal\ endogenous\ AA\ losses\ =\ AA_{digesta}\ \times \frac{Marker_{diet}}{Marker_{digesta}}$$

Next, the apparent ileal digestibility value is corrected to establish the standardized ileal digestibility value (Stein et al., 2007) with the following equation:

$$Standardized\ ileal\ digestibility\ (\%)$$
$$= Apparent\ ileal\ digestibility\ (\%) + \left[\left(\frac{Basal\ endogenouse\ AA\ losses}{AA_{diet}}\right) \times 100\right]$$

Secondly, animals also lose a certain amount of AA specified in their diets, like fiber or ANFs. If researchers know the specific endogenous losses, the standardized ileal digestibility value can be further corrected into the true ileal digestibility (or the "real" ileal digestibility), meaning the digested AA amount excluding basal and specific endogenous losses. The standardized ileal digestibility value is recommended for determining the PQ of protein devoid of ANFs or plant fibers. In contrast, true ileal digestibility is more suitable for the digestibility assay of plant proteins (Butts et al., 2012). The true ileal digestibility value reflects only the undigested dietary AA partition and allows estimation of the metabolic costs associated with synthesizing and recycling endogenous gut AA losses. Despite higher accuracy, the true ileal digestibility and total ileal endogenous AA loss values are insufficiently available; thus, the intermediate standardized ileal digestibility value is used more commonly.

Based on the FAO definition, the corrected protein level of food is calculated by the product of the DIAAS value and the protein in grams per serving of food. Foods can be claimed as an "excellent source" or "good source" of protein when they have the correct protein level value ≥ 100 or between 75 and 99, respectively (FAO, 2013). A food item with a DIAAS value less than 75 cannot have a claim made for protein. Consequently, most legumes and cereal grains are not considered high-quality protein sources because their DIAAS values are less than 75 in general, except soy and oat proteins (Bailey et al., 2020; Bailey & Stein, 2020). This ranking creates misunderstanding among consumers, as many organizations and governments recommend increasing plant-based protein in diets (Wiggins et al., 2019).

The DIAAS method addresses methodological concerns about the PDCAAS method by introducing individual ileal AAD and untruncated scores. One significant advantage of this method is that ingredients can have DIAAS values greater than 100. Compared to the PDCAAS method, the untruncated values enable a more accurate assessment of the PQ of mixed products or complementary diets (FAO, 2018). Additionally, the DIAAS emphasizes the digestibility of individual AAs rather than calculating protein/nitrogen digestion as a whole total number. In 2011, FAO published an updated reference pattern for the DIAAS method compared to the 1991 version for the PDCAAS.

The FAO has recommended the DIAAS method since 2013. Currently, ileal digestibility has been applied by the poultry and swine industry for feed formulation. However, the DIAAS approach is still novel and has not yet been adopted as the official method for humans to evaluate PQ in any country. The DIAAS method may be limited by the number of specialized analytical procedures, mainly by collecting ileal digesta (FAO, 2013, 2018; Mansilla et al., 2020; Wiggins et al., 2019; Wolfe et al., 2016). In humans, collecting ileal digesta is limited, such as for ileostomy

patients (Mansilla et al., 2020). However, with their special physicological conditions, consuming and digesting foods of these individuals creates specific differences compared to healthy populations. The digesta collecting procedure is highly invasive for animals, either sacrificing the animal or placing a T-cannula via surgery. Another methodological challenge is that the DIAAS method requires further calculation steps such as indigestible marker, basal, and ingredient-specific endogenous AA losses to determine from the apparent ileal digestibility to the standardized ileal digestibility and the true ileal digestibility.

Despite advocating as a superior tool to replace the PDCAAS method, the DIAAS faces many methodological challenges. The PDCAAS remains the more popular method with sufficient data and allows interchange with other methods, such as the PER. Implementing the DIAAS method requires more investment in scientific and regulatory frameworks.

2.2.4. Impacts of protein assessment methods on public health

From a human dietary perspective, the PQ is a tool to measure how much each food/protein can fit the requirements of consumers and can facilitate guidance on protein choice among consumers (Mansilla et al., 2020). As many methodologies exist for measuring PQ, the differences in quality value and protein claims of the same proteins by different assays are unavoidable. Moreover, it is recognized that one protein is ranked differently depending on the selected IAA reference patterns (Sá et al., 2023). From a regulatory standpoint, adopting or changing the official method to evaluate PQ and how protein content claims are permitted required not only nutritional evidence but also a thorough assessment of potential public health impacts (FAO, 2013; Marinangeli & House, 2017; Wiggins et al., 2019)

Little work has been done to compare the impact of the implementation of the PER, the PDCAAS, and the DIAAS on public health. A study showed meaningful differences between their PDCAAS and DIAAS values between 14 protein sources against one requirement pattern (Rutherfurd et al., 2015). The result showed that PDCAAS values were inflated from 3% to 574% compared to the respective DIAAS values among 12 proteins. It is concluded that the significant differences between the PDCAAS and the DIAAS values of poor-quality proteins are crucial for populations with marginal dietary protein intake.

Another study with a comprehensive comparison between PQ measurement and regulation showed a good comparison between protein sources (Table 4) (Marinangeli & House, 2017)**.** While animal-based proteins can secure excellent to good claims despite methods of choice, plant-based proteins face more challenges due to lower PQ value. The DIAAS method has also proven to be a disadvantage to meat alternatives because of its cut-off mark of 75 for protein claims. Thus, it will undoubtedly create more difficulties in promoting the use of sustainable plant-based protein among industries and consumers.

In conclusion, there is no universal standard assay for PQ evaluation. The PER and the PDCAAS are the two official methods currently implemented in North America for measuring PQ and permitting protein claims on food products. The FAO/WHO experts also proposed the DIAAS method that considered IAAs as separate nutrients with individual ileal digestibility and new reference patterns. Implementing the DIAAS methods faces many challenges, from sample collecting to analytical methodology. Furthermore, the impact on public health from using different PQ evaluation methods requires more attention for regulatory purposes.

Table 4. Comparison of the PQ value and permitted protein content claims (Marinangeli & House, 2017)

Food	**Canadian regulatory system**		**USA regulatory system**		**FAO proposal system**	
	PER (adjusted) value	Claim	PDCAAS (truncated) value	Claim	DIAAS value	Claim
Whole milk	2.5	Excellent source	1.0	Good source	1.14	Good source
Hard-boiled egg	3.1	Good source	1.0	Good source	1.13	Good source
While bread	1.0	No claim	0.28	No claim	0.29	No claim
Baked beans	1.51	No claim	0.6	No claim	0.56	No claim
Boiled chickpea	2.32	Good source	0.74	Good source	0.83	Good source
Soy-based tofu	2.3	Good source	0.56	Good source	0.52	No claim

2.4. MODELS FOR THE STUDY OF PROTEIN DIGESTION

In addition to updated information about AA requirements and proposing the novel DIAAS method for PQ evaluation, FAO/WHO experts suggested a new suitable *in vitro* or biological assay for the routine prediction of PQ of the entire range of foods (FAO, 2013). Following this suggestion, the *in vitro* static models offer the alternative to study digestion without the restrictions of a living body while maintaining the simulated biological conditions, helping researchers effectively screen the PQ for various foods and overcome many challenges.

2.3.1. The *in vivo* protein digestion models

The digestive system breaks down foods and absorbs nutrients into the body for maintenance, recovery, and growth. The gastrointestinal tract is the main digestive system, including several components from the mouth, the esophagus, to the stomach, then the small intestine, the large intestine, and ended by the anus. Even though all dietary foods follow the same pathway, individual nutrients will have a specific process for digestion and absorption. The digestion processes of specific nutrients occur in different regions of the GI tract and require specific physiological conditions such as enzymes for hydrolysis, pH of the environment, time of transition, and transporters for digested nutrients. As mentioned in "Section 2.1.2, Protein Digestion Process", dietary protein is digested mainly in the stomach and small intestine with the participants of pepsin, pancreatin secretion/ zymogen activation, bile acids, and transporters in the intestinal membrane.

For various models to study protein digestion, i*n vivo* models reflect those conducted in living organisms. *Ex vivo* methods also use tissues directly taken from an animal to mimic specific physiological conditions. In considering studies related to human protein nutrition, experimenting in the human body is the ideal option, giving the most accurate results, but there are concerns like

ethics, efficiency, and cost (FAO, 2013). As such, alternative animal models have been used to understand human digestive processes, including the use of rats, pigs, dogs, and chickens. Depending on the study's purposes, it is essential to select suitable subjects because differences in physiological conditions, eating habits, development stages, and nutritional requirements can impact results (Table 2) (Mansilla et al., 2020).

Regarding understanding PD and utilization, as mentioned above, the PER and PDCAAS methods are official bioassays in North America to assess the PQ of food proteins (Government of Canada - Canadian Food Inspection Agency, 2020; USA Food and Drug Administration, 2021). Rats are the required model to collect data like food consumption, weight changes, and fecal analysis. Thus, researchers can experiment without sacrificing the subjects. Animals require special attention during experiments, including water, feed, and other facilities. Moreover, experimental subjects require a specific adjustment time before and after tests.

In the case of DIAAS, growing pigs are the recommended model for determining PQ for human foods (FAO, 2013). The ileal digesta of subjects is collected by dissecting the ileum or a cannulated pig (Fuller & Tomé, 2005). The “Slaughter Method” is typically used in smaller animals, such as rats and chickens fed with a test diet with a marker compound. Digesta is collected at specific periods after eating to mimic physiological digestive conditions (Butts et al., 2002). This approach allows collecting digesta at different endpoints as required and analyzing with indigestible markers. However, only one digesta sample can be obtained per animal, so the animal cannot be used as the control (Donkoh et al., 1994). On the other hand, the cannulation technique in pigs is widely used to collect ileal digesta. Compared to the previous method, cannulation allows for studying digestion without sacrificing the animals. Pigs are fitted with one or two cannulate so the digesta are collected from the lumen of the distal ileum in an exteriorized vessel.

Despite offering the most accurate results, scientists have been paying more interest in other methods because *in vivo* methods have limitations in cost, time, technique, and ethical issues. Additionally, *in vitro* models are suitable alternative options for testing PQ while maintain the non-animal specialty of plant-based products. While studying outside of living bodies eliminates biological differences between subjects, the *in vitro* models allow researchers to experiment with various conditions, such as diet formulations and unique components.

2.3.2. The *in vitro* dynamic digestion models

In recent years, *in vitro* digestion models have been developed to provide alternative systems mimicking the physiological conditions of a digestive system. These *in vitro* models can be applied to study the structural changes, digestibility, and nutrient release under simulated conditions related to humans and other animals (Bryan & Classen, 2020; Butts et al., 2012; Hur et al., 2011). *In vitro* digestion methods usually require a shorter time and lower cost in the long term than *in vivo* bioassays. However, due to the complex physiology of the gastrointestinal tracts, more studies are required to improve the setting to establish the conditions of the desired systems and generate strong correlations between *in vivo* and *in vitro* models. However, researchers should consider several factors, such as enzymes, temperatures, pH, and digestion times to mimic targets. Elderly, infants, pregnant women, and adults have different physiological conditions and nutrition requirements. Moreover, researchers might consider dietary habits like meals, diet patterns, and a reasonable amount of food consumed.

In vitro systems are either mono-compartmental, simulating one compartment of the gastrointestinal tract, or multi-compartment based on designs. Depending on their complexity, the *in vitro* models can be classified as static and dynamic. The *in vitro* dynamic systems allow the movement of foods between different compartments and different dilutions of pH and enzyme.

These models can replicate the complex digestion process in various populations and be applied for in-depth studies about food digestion and pharmaceutical products. However, dynamic models are relatively complex, creating difficulties in terms of cost to set up and maintain, and may not be available to most researchers (Didier Dupont & Mackie, 2015).

Some popular mono-compartment systems include the Dynamic Gastric Model (UK), the Human Gastric Stimulator (USA), and the Artificial Colon (France) (Cordonnier et al., 2015; Kong & Singh, 2010; Wickham et al., 2012). However, these mono-compartment models usually study digestion at specific points (Didier Dupont & Mackie, 2015).

The TNO Gastro-Intestinal Model (TIM) (Zeist, Netherlands) is one of the most in-depth studied systems, especially the TIM-1, which involves gastric and small-intestinal phases (Mans Minekus et al., 1995). Researchers are continuously optimizing and applying this model, including the development of the large-intestinal model (TIM-2) (M. Minekus et al., 1999; Rebello et al., 2014), infant GI conditions (Havenaar et al., 2013), or elderly (Mans Minekus, 2015). TIM models offer high accuracy, reproducibility, and correlation with *in vivo* data; hence, they are suitable for examining bio-accessibility or phytochemicals stability. These systems stand out with relevant human physiological conditions such as digestion time, pH, enzyme secretions, peristaltic movement, and absorption of digested products and water. Even though this model features a dialysis compartment that allows the assessment of absorbable products, the *in vivo* absorption processes may not be fully simulated, carried out by BBM enzymes and transportations (Mackie, 2020).

2.3.3. The *in vitro* static pH-drop digestion models

Static models have been applied for digestibility measurement with a ratio of food to enzymes and electrolytes at a constant pH. With their simplicity, these methods have been widely used for food and pharmaceutical studies (Brodkorb et al., 2019).

The multienzyme pH-drop model is a simple *in vitro* static mono-compartmental system that is a standard system with efficiency. Samples are hydrolyzed by trypsin, chymotrypsin, and protease; then, digestibility will be measured by pH changes of solution after 10 minutes (Hsu et al., 1977). When the protein hydrolysis occurs, the number of carboxylic groups from free AAs increases, leading to more free protons in the solutions. As a result, the change of pH in the reaction chamber is related to the digestibility of the protein by all the enzymes (Franczyk, 2018; Hsu et al., 1977). The *in vitro* PD from the pH-drop model is calculated as following equations:

$$In\ vitro \text{ Apparent Protein digestibility } (\%) = 65.66 + 18.10 \times \Delta pH(10\ minutes)$$

$$In\ vitro \text{ True Protein digestibility } (\%) = 97.1887 \times (1 - e^{-3.1245} \times \Delta pH(10\ minutes)$$

The pH-drop model is more commonly applied with advantages in terms of time and cost. Every reaction includes 62.5 mg of crude protein with the same amount of enzymes, ensuring the consistency of the digestion model. Casein or high-quality protein can be included as the control between repeats. This model provides a moderate correlation to *in vivo* fecal nitrogen digestibility with $R^2 = 0.64$ and an excellent correlation with *in vivo* PDCAAS values with $R^2 = 0.97$ with processed split peas (Nosworthy, Franczyk, Medina, et al., 2017).

However, there are other factors contributing to the changes in pH solution, such as the presence of ANFs in plant-based proteins or the buffering capacity of testing foods (Franczyk, 2018; Hsu et al., 1977; Mennah-Govela et al., 2019). Additionally, with the developed regression data, all tested proteins will have a minimum PD of 65.7% even if the pH solution remains unchanged after 10 minutes (Tinus et al., 2012). Given the fact that all proteins will be digested

differently in human bodies with a wide range of physiological conditions, the pH-drop model can overestimate the PD of some proteins. Finally, the pH-drop digestion model can measure *in vitro* fecal nitrogen digestibility but not individual AAD or ileal digestibility. Thus, this model can be applied to determine *in vitro* PDCAAS and not for *in vitro* DIAAS value. As Canadian authorities allow, the *in vitro* PER value can be calculated from the PDCAAS measure.

2.3.4. The *in vitro* static INFOGEST digestion model

The COST ACTION FA1005 group has developed a static *in vitro* digestion model that simulates physiological conditions of the upper gastrointestinal tract, including the gastric and small intestinal stages called the INFOGEST model (D. Dupont et al., 2019; M. Minekus et al., 2014). A more recent version of INFOGEST 2.0 included the oral phase and gastric lipase, improved the simulation of the whole digestion process (Brodkorb et al., 2019)

The digestion procedure is estimated to take 7 working days, the longest to determine enzyme activities with standardized assays (Figure 2). This preparation step is considered the most important because the result will determine the amount of enzymes/bile to be used and ultimately impact the whole digestion process (Brodkorb et al., 2019). Simulated digestive fluids for all digestive stages will be prepared in x1.25 concentrated stocks and can be stored in aliquots at -20^{o}C for a year. It is also recommended to perform a preliminary digestion run to estimate the acid/based requirement for pH adjustment between each step. The preparation can take up to 5 days at least, and it is suggested to test enzyme activities after prolonged storage or using a new bottle. This step will ensure the enzymes/bile amount used for every INFOGEST digestion and the repeatability of the procedure. The actual digestion for each sample will be in 5 hours, including three main digestion steps. Thus, the INFOGEST can assess nutrient digestion or release from the food matrix at various endpoints.

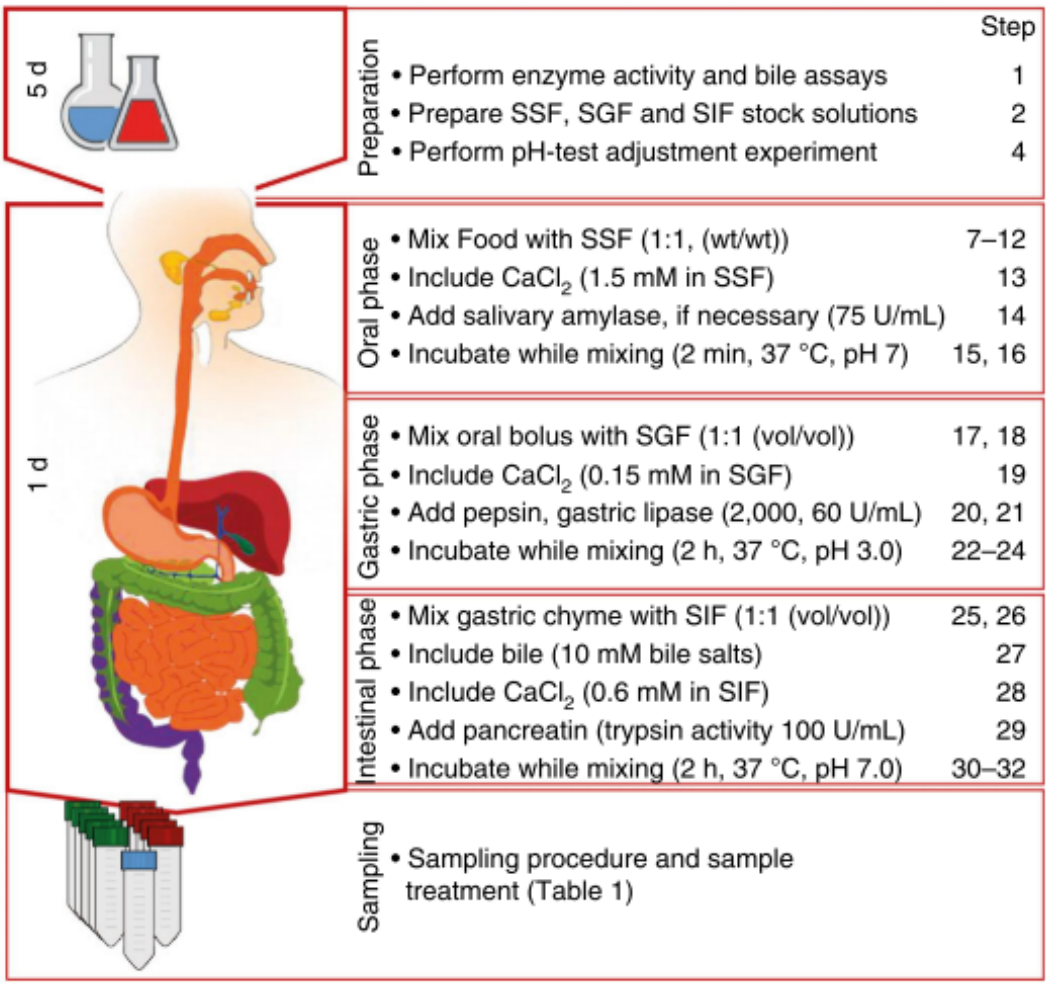


Figure 4. The flow diagram of the INFOGEST 2.0 digestion method (Brodkorb et al., 2019).

SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid

Table 5. Average parameters in static *in vitro* digestion models (Mackie et al., 2020; Ménard et al., 2018).

Digestion	Condition	Infant	Adult (fed)	Adult (fasted)	Elderly
Oral	pH	-	7	-	6.8
	Meal : SSF (w/v)	-	50:50	-	50:50
	α-Amylase (U mL^{-1})	-	75	-	150
	Time (min)	-	2	-	3 s
Gastric	pH	5.3	3	1.2	4
	Oral bolus : SGF (v/v)	63:37	50:50	5:95	40:60
	Pepsin (U mL^{-1})	268	2000	2500 (10000 U mg^{-1} test protein)	450
	Gastric lipase (U mL^{-1})	19	60	-	153
	Time (min)	60	120	60	180
Intestinal	pH	6.6	7	-	6.5
	Gastric chyme: SIF (v/v	62:38	50:50	-	-
	Trypsin (U mL^{-1})	16	100	-	46
	Pancreatic lipase (U mL^{-1})	90	2000	-	-
	Bile acids (mM)	3.1	10	-	5.34
	Time (min)	60	120	-	180

SSF: simulated salivary fluid; SGF: simulated gastric fluid; SIF: simulated intestinal fluid.

For the standard model, foods will be digested with amylase in the oral phase, followed by pepsin and gastric lipase in the gastric phase, and bile and pancreatin in the intestinal phase. It is suggested that researchers can stimulate the desired digestion model using slightly different physiological conditions and enzyme assays (Table 5). A modified model for infants born at term and aged 28 days was also developed to simulate infant digestion (Ménard et al., 2018).

In the original paper, the authors listed one extra day for the last step, which included sample treatment, sampling, and storage for subsequent analysis (Brodkorb et al., 2019). However, depending on the study goals, it might vary significantly and should be planned.

As a static system, the INFOGEST model has limitations for simulating the gastric or intestinal phase as it is treated as one phase. Thus, for each digestion stage, the pH adjustment and all gastric fluid/enzyme additions will only happen once in the beginning. Additionally, the *in vitro* digestion INFOGEST model does not include degradation with peptidases from intestinal brush border membranes in contrast to human protein digestive processes. Hydrolyses by the brush border membrane enzymes help degrade the oligopeptides within intestinal chime into tri-, di-peptides, and AAs, ready for absorption (Freeman et al., 1979). Hence, fewer oligopeptides can be broken down and prepared for absorption without these peptidases compared to *in vivo* models. Studies have demonstrated that this inconsistency can lead to differences in protein hydrolysis from 55 to 77%, and they recommend adding peptidases to the model (Ozorio et al., 2020; Picariello et al., 2015).

Despite all recognized limitations, the INFOGEST model shows potential in protein digestion studies compared to *in vivo* data. Using SDS-PAGE and HPLC-MS/MS methods to measure the amount of free AAs and small peptides, protein hydrolysis of several isolated foods with the INFOGEST model demonstrated correlation with *in vivo* porcine samples (Egger et al.,

2017). Compared to human jejunal digesta, the *in vitro* digesta of casein and whey milk protein powder from the INFOGEST model have similar protein degradation and peptide release (Sanchón et al., 2018). Limited data also show the potential of using the INFOGEST model in measuring PQ via the DIAAS method. The *in vitro* AAD, DIAAR, and DIAAS were measured using the INFOGEST digestion model with good agreement with *in vivo* data (Sousa et al., 2020, 2023). Authors suggested more improvements related to standardizing the methodologies of measuring *in vitro* AAD and DIAAS value, such as using the sample weight for 0.04g of protein each digestion, the inclusion of nitrogen-free cookies instead of water-blank, separation methods for digestible fractions as well as pancreatin solution preparation. However, more comparability data is needed to validate further and standardize this method, emphasizing heated foods (Sousa et al., 2023). Apart from validation, it requires further testing for a suitable methodology for analyzing nitrogen and AAs contents of the INFOGEST digesta, contributing to the standardized workflow of determining *in vitro* AAD and DIAAS values.

Overall, the official digestion models for dominant PQ evaluation methods are animals with biological conditions suitable for humans. As these bioassays have been addressed with many challenges, more *in vitro* digestion models have been developed and improved to be the alternative option. The *in vitro* static models show the potential of a quick and effective tool for studying protein digestion. Limited data about standardized workflows have been published and compared for measuring PD, AAD, and PQ based on these *in vitro* static digestion models.

2.5. RESEARCH GAPS

The *in vitro* digestion models can simulate physiological human digestion with numerous advantages; however, they have not been validated as a standard method to determine PQ. This thesis aims to assess the potentials of two *in vitro* static models, the pH-drop and the INFOGEST 2.0 model, to determine the *in vitro* PD and AAD and use the acquired data to calculate *in vitro* PQ values in terms of PDCAAS and DIAAS. The suitability of the two digestion models was validated by comparing the *in vivo* PD and PDCAAS values and the *in vitro* results. Moreover, as a newly developed model, there is no established procedure to analyze the INFOGEST digesta after the digestion process. Hence, in this study, the INFOGEST digestion product was measured using three different methods, including the OPA method, the Kjeldahl method, and the AA analysis method for digestibility, aiming to select the most compatible workflow for this digestion model. Heat-treated plant-based samples were digested and analyzed for *in vitro* digestibility and PQ data to evaluate the two models further. The impact of PQ assessment methods was also studied by comparing the *in vitro* PER, PDCAAS, DIAAS, and their respective permitted claims on plant-based samples included in this thesis.

CHAPTER 3: HYPOTHESES AND OBJECTIVES

3.1. HYPOTHESES

- Hypothesis 1: The *in vitro* PD (IV-PD) and IV-PDCAAS using the INFOGEST 2.0 digestion have a higher correlation to *in vivo* data than the pH-drop model.
- Hypothesis 2: The IV-PD, IV-AAD, IV-PDCAAS, and IV-DIAAS results determined from the INFOGEST model correlate to the *in vivo* data.
- Hypothesis 3: Thermal treatment will have a significant impact on IV-PD, IV-AAD, IV-PDCAAS, and IV-DIAAS measured with the *in vitro* static pH-drop and the INFOGEST digestion models.
- Hypothesis 4: The PQ assessment methods will significantly influence the *in vitro* PQ value and the permitted protein contents claims.

3.2. OBJECTIVES

- Determine IV-PD and IV-PDCAAS using the *in vitro* static pH-drop digestion model.
- Compare methodologies to determine IV-PD, IV-AAD, IV-PDCAAS, and IV-DIAAS using the *in vitro* static INFOGEST 2.0 digestion model.
- Evaluate the correlation of IV-PD and IV-PDCAAS from the *in vitro* static pH-drop and the INFOGEST 2.0 digestion models with in-house *in vivo* data.
- Assess the effect of thermal treatment on IV-PD, IV-AAD, IV-PDCAAS, and IV-DIAAS using the *in vitro* static pH-drop and the INFOGEST digestion models.
- Compare the impact of the PQ assessment method among *in vitro* PER, PDCAAS, and DIAAS on their permitted protein content claims.

CHAPTER 4: THE POTENTIAL OF *IN VITRO* DIGESTION MODELS IN THE DETERMINATION OF PROTEIN DIGESTIBILITY, AMINO ACID DIGESTIBILITY, AND PROTEIN QUALITY

4.1. ABSTRACT

From previous in-house *in vivo* protein quality studies, eight samples were selected and digested using the *in vitro* pH-drop and the INFOGEST digestion model to investigate the ability of two models to determine digestibility and protein quality. While the pH-drop model can determine *in vitro* protein digestibility directly, the INFOGEST digestion products were analyzed by three different methods, including the OPA method, the Kjeldahl method and the AA analysis method. All samples displayed high digestibility results with low variation across all four measurement methods. The limiting AAs for the selected samples were TRP, SAA, and LYS. Nut, oilseed A, and single-cell protein (SCP) A had the highest IV-PQ values, while oilseed B was ranked the lowest in all three IV-PDCAAS, IV-DIAAS, and IV-PER. SCP A and SCP B samples showed relatively higher variation between measurements than other samples. There were strong correlations between in-house PDCAAS data and acquired IV-PDCAAS results. Between the four assessment methods, the OPA (R^2 = 0.942) and the total AA analysis (R^2=0.944) from the INFOGEST model had stronger relationships to the *in vivo* PDCAAS than the pH-drop model (R^2=0.912) and the Kjeldahl analysis (R^2=0.904) from the INFOGEST model. However, the pH-drop model demonstrated a higher repeatability between measurements. Only the INFOGEST digesta can be analyzed for the IV-AAD and IV-DIAAS results. The IV-PQ results demonstrated that eight samples were ranked differently with different protein quality evaluation assays. The PDCAAS generally offered higher protein permitted claims than the DIAAS and the PER methods. The present data demonstrated the potential of *in vitro* digestion models in determining

digestibility and protein quality, which could be applied as a screening tool for researchers, industries, and consumers.

Keywords: amino acid digestibility, DIAAS, *in vitro* digestion models, PDCAAS, protein digestibility, protein quality.

4.2. INTRODUCTION

From a nutritional perspective, animal-based proteins are usually superior to plant-based proteins with incomplete IAAs profiles. However, plant-based proteins are a suitable solution to sustainable food security in the future with the rapidly growing population (Searchinger et al., 2014). The quality of proteins can change depending on the processing methods to increase nutritional characteristics, remove undesirable compounds, or alter the availability of AAs (Moughan & Rutherfurd, 2008; Nosworthy, Neufeld, et al., 2017; Sarwar Gilani et al., 2012; Udenigwe & Aluko, 2012). The industry and consumers need reliable tools to select suitable proteins that satisfy sustainability and nutrition requirements.

Based on its contribution to growth, the PER assay is a direct protein assessment method currently implemented in Canada for protein rating (Health Canada, 1981). From a dietary perspective, the AA composition, especially for IAAs, can significantly impact the quality of any protein. Experts from FAO/WHO published updated information about IAA requirements patterns for various age populations (FAO, 2013; Joint FAO/WHO/UNU Expert Consultation, 1985; Joint FAO/WHO, 1991). Even though the method to determine these references is still an ongoing debate, it is agreed that the quality of a protein depends on target consumers with different physiobiological conditions. Furthermore, digestibility is an important factor for PQ, assuming protein disappearance relates to absorption during digestion (Joint FAO/WHO, 1991). Considering both factors of the AAs' composition and digestibility, the PDCAAS method was developed based on the IAAs' reference patterns published by FAO/WHO in 1991 and the true fecal protein digestibility. This method became widely used and has been applied for protein content claims in the USA (USA Food and Drug Administration, 2021). Over a decade ago, the FAO/WHO recommended a replacement for the PDCAAS method, the DIAAS method, which is based on the

true ileal digestibility of individual IAAs (FAO, 2013). Implementation of the DIAAS method is facing many challenges due to sample collection difficulties, lack of data availability, and limited understanding of their impact on protein content claims and public health (Craddock et al., 2021; Mansilla et al., 2020; Marinangeli & House, 2017).

All three methods mentioned above for PQ evaluation use rats, chicken, or swine for growth measurement or fecal or ileal digesta collections (FAO, 2013; Health Canada, 1981; Joint FAO/WHO, 1991). Bioassays are dominant in nutrient digestion and quality evaluation studies because of their availability and high relation with human studies data. However, some disadvantages have been addressed with *in vivo/ex vivo* digestion models, such as differences in IAAs requirements, eating habits, and dietary restrictions (Joint FAO/WHO, 1991; Mansilla et al., 2020). Additionally, animals require attention to all aspects, including food, water, cages/housing, and other facilities before, during, and even after the experiments. These contribute to more limitations in time, cost, technique requirement, and personnel of bioassays. Ethical concerns are often mentioned when using animals, despite scientists' efforts to avoid unnecessary sacrifices. To address the need for substantiating PQ claims, researchers in Canada and the USA explore alternative options, such as focusing on amino acid score alone or employing *in vitro* methods to measure digestibility, encouraging more ethical approaches in the evaluation of PQ without relying on animal testing (House et al., 2023). *In vitro* digestion models offer an alternative option to overcome the abovementioned challenges for studying protein digestion and assessing PQ. Additionally, using the *in vitro* digestion model can allow a wide range of experimental conditions while showing a close relationship to *in vivo* data from humans, rats, and swine (FAO, 2013; Mans Minekus, 2015; Nosworthy, Franczyk, Medina, et al., 2017; Sousa et al., 2023). The *in vitro* static digestion models, such as the pH-drop and the INFOGEST models, show great potential to be a

standard method to measure PQ (Brodkorb et al., 2019; Franczyk, 2018; Hsu et al., 1977; Sousa et al., 2020; Tinus et al., 2012). However, more supporting data is required, especially in developing standardized workflows for measuring PD, AAD, and PQ.

Therefore, this study aims to assess the potential of two *in vitro* static models, the pH-drop and the INFOGEST, in measuring PD, AAD, and PQ. The INFOGEST digesta were analyzed using three different methods, including a) the OPA method, b) the Kjeldahl method, and c) the AA analysis method; and then all the *in vitro* results was compared with *in vivo* data for the suitable workflow in determining IV-PD, IV-AAD, IV-PDCCAS, and IV-DIAAS. Protein samples were selected from the same bath from previous in-house *in vivo* PD and PDCAAS trials. Thus, the potential of the pH-drop and the INFOGEST digestion model was validated by comparing the correlation between *in vivo* data and *in vitro* results. In addition, this study intended to investigate the impact of all *in vitro* PQ data and their permitted protein content claims.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals

All chemicals were purchased from Sigma (Oakville, Ontario, Canada) except for rabbit gastric enzymes RGE15 from Lipotech (Marseille, France) and bile extract CAS 8008-63-7 from Santa Cruz Biotechnology (Dallas, Texas, USA). The AccQ·Tag^{TM} Ultra Derivatization Kit was purchased from Waters Inc. (Mississauga, Ontario, Canada).

The AA analysis runs used Milli-Q water, while all remaining tests and digestions used reverse osmosis (RO) water.

4.3.2. Sample

This research aimed to focus on the samples with established *in vivo* digestibility and protein quality databases. This step allowed us to verify the reliability of the test *in vitro* methodologies by assessing the *in vitro* digestibility and protein quality value and subsequently analyzing the correlation between *in vitro* and corresponding *in vivo* data of the exact same samples. The primary objective was to investigate the relationship between the data determined by new methods and those known values determined by established official methods, regardless of the samples utilized.

From previous *in vivo* PDCAAS studies, eight samples were secured with unpublished in-house results for PD and PDCAAS from rodent model bioassays. Procedures for each *in vivo* study were approved by the Institutional Animal Care Committee following the Canadian Council on Animal Care guidelines. In the scope of this study, all *in vivo* data were used only for comparison purposes with *in vitro* results statistically. Eight selected samples were casein, one nut, two legumes (noted legume A and legume B), two oilseeds (noted oilseed A and oilseed B), and two single-cell proteins (noted SCP A and SCP B).

These samples were selected based on available databases for comparison with acquired *in vitro* results to validate the potential of two digestion models. Additionally, as all samples present alternative solutions for sustainable protein markets, this study provided more information about these novel food choices regarding *in vitro* PD, AAD, PQ, and permitted protein content claims. All samples were analyzed and digested as-is received forms from commercial suppliers from 2020 to 2023. Samples were refrigerated at 4ºC and later stored at -20ºC.

4.3.3. Experimental design

The experiment design is described in Figure 6. As mentioned in section 4.3.2, eight samples were digested by two different *in vitro* static digestion models. The crude protein was analyzed with the Dumas method before digestion.

First, the PD results by the pH-drop model were calculated (noted by PH-PD). The PD-PDCAAS and the permitted protein contents claims were determined with the AAS of the raw samples. Following the conversion equation in section 2.2.2, the PH-PDCAAS were used to calculate for the PH-PER with other claims for protein content as Canadian regulatory (see "Section 4.3.10. *In vitro* protein quality calculations and determination of protein claims).

On the other hand, the digesta from the INFOGEST model allowed the calculation for both protein and AAD. The micro-Kjeldahl method, the o-phthaldialdehyde method, and the total AA analysis were applied to determine *in vitro* protein digestibility, noted as KD-PD, OPA-PD, and IFG-PD, respectively. In the same way, the PDCAAS, the PER, and the protein claims were assessed for individual respective methods. Additionally, the AA analysis from the INFOGEST digesta allowed the calculation of individual AAD, hence applying for the IFG-DIAAS and the protein claims (see "Section 4.3.10. *In vitro* protein quality calculations and determination of protein claims).

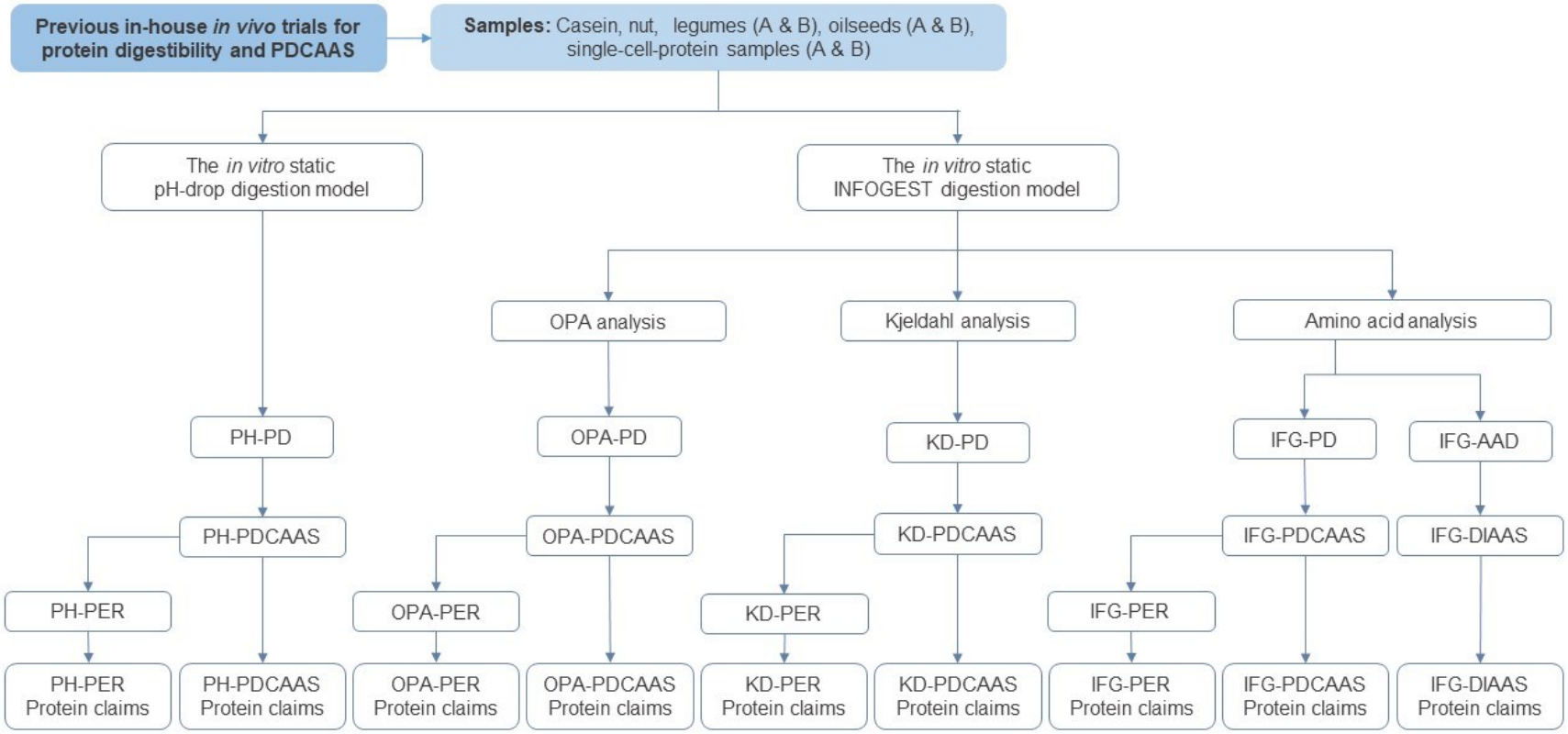


Figure 5. Study 4 experimental design

4.3.4. Crude protein analysis

The nitrogen analyses were run in singlet. For all samples, nitrogen contents were determined by Central Testing Labs (Winnipeg, Manitoba, Canada) using the Dumas Combustion Method. The crude protein contents were calculated from acquired nitrogen data with the 6.25 factor, except for raw pistachios with the factor of 5.3 (Joint FAO/WHO, 1991; Mariotti et al., 2008; U.S. Department of Agriculture, 2019a).

4.3.5. The *in vitro* static pH-drop digestion model

The *in vitro* static pH-drop digestion model was followed by the original method developed in 1977 with modifications from Tinus in 2012 and Franczyk in 2018 (Franczyk, 2018; Hsu et al., 1977; Tinus et al., 2012). Casein was included as the control for the digestion. Samples and control were digested in triplicate. A multi-enzymes cocktail was prepared with 1.6 mg/ml of trypsin, 3.1 mg/ml of chymotrypsin, 1.3 mg/ml of peptidase, and water. The cocktail was stirred until all enzymes dissolved while heated to 37°C. The pH of the enzyme solution was adjusted with 0.1M NaOH or HCl solution to stable at 8.0 ± 0.05. Then, the cocktail was stored in an ice bath, keeping the temperature at 0-4°C during the experiments. Samples were weighed to achieve 62.5 ± 1 mg of protein in individual beakers. Then, samples were stirred until homogenous with 10 ml water and heated to 37°C. 0.1M NaOH and HCl solutions were used to bring the pH of the solution to 8.0 ± 0.05, recording as the $pH_{initial}$. Immediately, 1 mL of enzyme cocktail was added to the sample solution. The reaction started, and the temperature was kept stable at 37°C. The pH_{final} was recorded after 10 minutes, and the IV-PD of the pH-drop digestion model was calculated as follows:

$$\Delta pH_{10minutes} = pH_{initial} - pH_{final}$$

$$\text{True fecal } \textit{in vitro} \text{ PD: PH-PD(\%)} = 97.1887x\ [1 - (e^{-3.1245} \times \Delta pH10min)]$$

4.3.6. The *in vitro* static INFOGEST digestion model

The *in vitro* static INFOGEST digestion model was developed under the COST action INFOGEST network framework as a standardized protocol to study nutrient digestion (Minekus et al., 2014). All samples were digested with the INFOGEST 2.0 method, with additional improvements in the oral phase and usage of gastric lipase (Brodkorb et al., 2019). All related resources, including videos and spreadsheets, are available for the public on the INFOGEST website https://www.cost-infogest.eu/ and Youtube channel https://youtube.com/@infogestinvitroandinvivofo9373?si=xU09d2gh0MV5K3rb. In addition, other modifications followed the published articles by Moughan and Sousa to improve the standardized digestion protocol's repeatability and accuracy (Egger et al., 2017; Moughan et al., 2005; Sousa et al., 2020, 2023). All digestions were run in triplicate with casein as the control protein. Required chemicals and reagents were followed with the INFOGEST 2.0 protocol. Required enzymes and chemicals which are not available to purchase in Canada were replaced with equivalent options. The INFOGEST included three main steps: i) preparation, ii) digestion, and iii) post-digestion digesta treatment.

i) PREPARATION STEP: This step involved testing the activities of all digestive enzymes and the concentrations of bile extract, preparing the stock solutions for simulated digestive fluids, and testing the pH adjustment for the samples. The preparation is the most critical step for the *in vitro* INFOGEST digestion model because inaccurate enzyme activities will lead to an incorrect digestion rate of the whole protocol (Brodkorb et al., 2019). Five enzyme activity assays and one bile concentration assay were carried out. Detailed protocols for all enzyme activity tests are mentioned in **APPENDIX A - D**. Bile concentrates were determined with the bile acid assay kit purchased from Sigma (Oakville, Ontario, Canada). All enzyme activities and bile

concentration were tested when opening new bottles or after a pro-long storage time, as recommended by the authors.

Amylase activity assay: One amylase activity unit was defined as the amount that can release 1 mg of maltose equivalent from starch in 3 minutes at pH 6.9 and 20°C. Amylase solution was prepared with water with an estimated 1 unit activity/ml and stored in an ice bath. The substrate solution was 1% (wt/vol) potato starch in sodium phosphate buffer with 6.7 mM NaCl. The starch solution was adjusted to pH 6.9 and incubated at 20°C. The substrate was reacted with the enzyme solution for 3 minutes. Then, the colour reagent 96 mM 3,5-dinitrosalicyclic acid with 5.3 M sodium potassium tartrate was added to stop the reactions. Solutions were boiled for 15 minutes, creating colour gradients. Additional water was added to the tube before the absorbance was read at 540 nm by a spectrophotometer (Evolution One Spectrophotometer with Insight™ Pro Software, Thermo Fisher Scientific Inc.).

Gastric lipase activity assay: Tributyrin reacts with water under lipase at 37 °C to produce sn-2 monobutyrin and butyric acid. Thus, the gastric lipase activity was measured by the μmol of butyric produced from changes in the pH solution in 5 minutes. A 0.5 ml tributyrin was dissolved in a 14.5 ml assay solution of 2 mM sodium taurodeoxycholate, 150 mM NaCl, and 1 μM BSA. The substrate solution was stirred until it reached a fine oil-in-water and heated to 37°C. Lipase solution was prepared at 1 mg/ml with water and then stored on ice. The enzyme solution was added to the reaction chamber, and 0.1M NaOH solution was added to maintain the pH of 5.5 for 5 minutes.

Pepsin activity assay: The pepsin enzyme activity was measured based on the TCA-soluble TYR-containing products from hemoglobin, hence changing the absorbance at 280 nm of 0.001 per minute at pH 2.0 and 37°C. As disclosed by the manufacturer, gastric lipase contains specific

amounts of pepsin. Thus, the pepsin and gastric lipase enzymes were tested for the pepsin activity assay before determining the suitable amount for digestion. Enzyme solutions were prepared with 10 mM Tris buffer, 150 mM NaCl, pH 6.5; then, further diluted into serial concentrations ranging from 5-50 μg/mL in 10 mM HCl. 500 μL of hemoglobin solution was mixed with 100 μL of each enzyme solution and incubated for 10 min at 37 °C. Next, 1 ml of 5% (wt/vol) TCA solution was added to stop the reaction. Finally, the solutions were centrifuged at 6,000g for 30 minutes at room temperature before the supernatant absorbances were read at 280 nm in quartz cuvettes.

Trypsin activity assay: The pancreatin enzyme in the intestinal digestion phase was determined based on the trypsin activity assay. The activity unit was defined as the amount of enzyme that hydrolyze 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at pH 8.1 and 25°C. The pancreatin suspension was prepared with modifications to improve solubility and the repeatability of the measurements (Sousa et al., 2023). For both enzyme activity measure and intestinal digestion, the pancreatin was diluted with simulated intestinal fluid and ten vortexed for 10 seconds. The solution was treated with ultrasound (45 Hz, 130W) for 5 minutes before centrifuged at 2000 g for 5 minutes. The final supernatant was separated into a new tube and then stored on ice. The pancreatin supernatant was further diluted to a series of concentrations for the enzyme activity assay. The reaction solution included 2.6 ml of 46 mM Tris–HCl buffer (pH 8.1) with 300 μL of the substrate 10 mM TAME in water at 25°C. A 100 μL of each pancreatin concentration was added. The absorbance was recorded at 247 nm in 10 minutes until leveling off.

Preparation of simulated digestive fluids: Three simulated digestion fluids (SDF) were prepared, including the simulated salivary fluid (SSF), the simulated gastric fluid (SGF), and the simulated intestinal fluid (SIF) for oral, gastric, and intestinal digestion phage, respectively. All SDFs were prepared in 1.25x concentrations and stored in aliquots at -20°C for one year. The

volume of all stock solutions for three SDFs is listed in Table 6 (Brodkorb et al., 2019). All salts were mixed at the required volume except for $CaCl_2(H_2O)_2$, which was added immediately before digestion. Before use, all SDFs were pre-warmed to 37°C in the water bath and diluted to the 1x concentration with water and enzyme solutions.

Preliminary pH-adjustment run: Before digestion, one preliminary run is recommended to measure and record the required HCl and NaOH amounts for pH adjustment in each digestion step. This pH-adjustment test can help the digestion run smoother because some samples, especially solid ones, require a long time for pH stability.

ii) DIGESTION STEP:

Samples were digested in triplicates. Casein and a nitrogen-free cookie sample were included in each digestion run as control and blank samples. The nitrogen-free cookies were used as the background to calculate the protein/AAs originating from the enzymes/bile. The cookies were made from 40.8 g of purified corn flour, 15.7 g of sucrose, 0.7 g of baking powder, 0.5 g of ground ginger, and 36.9 g of margarine, then divided into portions of 35 g and, baked for 30 minutes at 175°C (Moughan et al., 2005; Sousa et al., 2020). All SDFs were pre-warmed at 37°C, and all enzymes/bile solutions prepared and stored on ice before digestion.

The digestion followed the *in vitro* static INFOGEST 2.0 digestion model (Brodkorb et al., 2019; Egger et al., 2017; Sousa et al., 2020). Briefly, the sample was weighed to the equivalent of 40 mg of crude protein and then dissolved in 1 ml water for each digestion. For the oral phase, the sample solution was diluted with 1 ml of SSF pH 7 that contained 300 U amylase/ml of total oral bolus. The oral digestion solution was gently rotated at 37°C for 2 min. Then, 2 ml of the SGF pH 3 containing 2,000 U pepsin and 60 U gastric lipase per ml of the final volume of the gastric phase were added. The gastric chyme solution was incubated for 2 hours at 37°C on a rotator. For the

intestinal digestion phase, 4 ml of SIF pH 7 containing 100 U trypsin of pancreatin and 10 mmol bile per ml of the final digesta volume was added to the gastric chyme solution. The digesta was mixed by the rotator for another 2 hours at 37°C. Digestion was stopped by heating the tubes to 100°C for 5 minutes to inactivate all enzyme activities. Detailed instructions for the INFOGEST digestion model was listed in **APPENDIX E**.

iii) DIGESTA SAMPLE TREATMENT STEP:

Post-digestion treatments aimed to separate the digesta into digestible and undigestible fractions by MeOH precipitation. These steps followed the framework published in 2023 (Sousa et al., 2023). MeOH was added to digesta to reach the final concentration of 80% (vol/vol). Then, digesta tubes were incubated at -20°C for 1 hour, allowing the precipitation of large molecules. Digesta was centrifuged at 2,000 g at 4°C for 15 minutes. The supernatants were collected into new tubes without taking the interface. The pellets were washed twice by adding MeOH (100%) and centrifuged 2,000 g at 4°C for 5 minutes. Finally, the pellets were put into a Thermo Savant DNA120 SpeedVac Concentrator and Savant™ UVS400 Universal SpeedVac™ Vacuum System (Thermo Fisher Scientific) in moderated heat for 8 hours until completely dried. The end products acquired after the INFOGEST digestion were described in Figure 6. The added MeOH volume, the total collected supernatant, and the dried pellet weights are all recorded for later calculation. Both supernatants and pellets were stored at -20°C for other analysis.

Table 6. Volume and final concentration of salt solutions to prepare simulated digestion fluids at 1.25x concentration (Brodkorb et al., 2019)

Salt solution	**Stock concentration (M)**	**SSF (pH 7)**		**SGF (pH 3)**		**SIF (pH 7)**	
		Required volume to prepare 0.4 L of 1.25× concentration (ml)	Final concentration	Required volume to prepare 0.4 L of 1.25× concentration (ml)	Final concentration	Required volume to prepare 0.4 L of 1.25× concentration (ml)	Final concentration
KCl	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	1	6.8	13.6	12.5	25	42.5	85
NaCl	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)CO_3$	0.5	0.06	0.06	0.5	0.5	-	-
HCl	6	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$	0.3	0.025	1.5	0.05	0.15	0.04	0.6

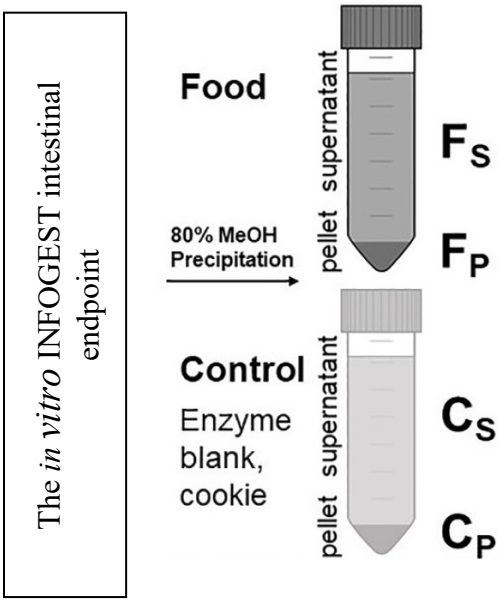


Figure 6. The ending products from the *in vitro* INFOGEST digestion model (Sousa et al., 2023)

4.3.7. The o-phthaldialdehyde assay

The INFOGEST digestible and undigestable fractions were analyzed for the protein content using the o-phthaldialdehyde (OPA) assay (Church et al., 1983). All pellets and supernatant samples were hydrolyzed by acid (AOAC, 1995) before reacting to the OPA solution and measured by spectrophotometer. All samples were measured in singlets. Detailed instructions for the OPA assay were provided in **APPENDIX F.**

For liquid samples, 1 ml of sample was transferred to a centrifuge tube with TCA solution to 7.5% wt/vol of the final concentration. Then, the tubes were vortexed and centrifuged at 17,000 g for 60 minutes. The supernatant was transferred to a new cryogenic vial, followed by storage at -20ºC. 0.2 ml of liquid or 2 mg of solid samples was transferred to an 11 Pyrex ml glass tube. 4 mL of 6.3 N HCl and two drops (approximately 20 μL) of 2-octanol were added to the tubes. All tubes were purged with nitrogen gas and tightly capped, then incubated at 110ºC. After 24 hours, the tubes were removed and cooled in the fume hood, followed by adding 3.9 ml of 25% NaOH. The contents of the tubes were vortexed and transferred to a 50 ml glass beaker. Each tube was washed three times with 4 ml water and transferred to all solutions into the respective beakers. The solution was mixed well before filtered through a 0.22 μm syringe to a cryogenic vial.

All samples were analyzed within the same day of the hydrolysis by measuring the absorbance at 340nm after reacting with the OPA solution. The OPA reagent was prepared on the day of analysis by combining 25 ml 0.1M sodium tetraborate, 2.5 ml 5M SDS, 0.1 ml β-mercaptoethanol, 1 ml OPA in methanol, and 21.4 ml Milli-Q water in an amber flask (Church et al., 1983). The solution was stirred at room temperature for at least one hour before use. Into a cuvette, 0.2 ml of the samples were reacted to 1 ml of the OPA solution.

The absorbance was read at 340nm after 2 minutes and compared through the regression with the L-leucine standard curve **(APPENDIX J)** for α-amino nitrogen content estimation.

4.3.8. The Kjeldahl method

The *in vitro* INFOGEST pellets and supernatants were subjected to the micro-Kjeldahl test to determine their protein contents. Samples were digested with 3 ml of concentrated H_2SO_4 in a high-temperature block heater (Velp DK Digester System). A JP recirculating water vacuum pump and SMS Scrubber systems were attached to the digester in order to remove and neutralize all corrosive vapours. The final solutions were distilled with NaOH (40%) by the FOSS Kjeltec KT200 system for trapping ammonia in saturated boric acid solutions. Finally, the nitrogen contents of each sample were determined by direct titration of the received solutions with 0.05N HCl. The nitrogen and protein content were calculated as follows:

$$\%\ total\ organic\ nitrogen = \frac{(S - B) \times 0.05\ \times 14.0074\ \times 100}{Sample\ weight\ in\ mg}$$

With S = ml of 0.05N HCl for the sample

B = ml of 0.05N HCl for the blank

$$\%\ crude\ protein = \%\ total\ organic\ nitrogen\ \times conversion\ factor \quad (6.25)$$

4.3.9. The amino acids analysis

The AA profiles of samples before digestion, the pellets, and the supernatant from both digestible and undigestible fractions of the *in vitro* INFOGEST were determined as follows. In total, each sample was analyzed in a singlet with three protocols, including i) the regular AA hydrolysis (AOAC official method 982.30), ii) the performic acid oxidized hydrolysis (AOAC official method 45.4.05), and iii) the alkaline hydrolysis (ISO 13904:2005) (AOAC, 1995; ISO, 2016). Each hydrolysis run included an internal reference standard, including High Nitrogen Casein (80 mesh) Material 400601 (15-20 mg), organic pea protein isolate (15-20 mg), soy flour

NIST® Standard Reference Material 3234 (20-30 mg), or Bovine Serum Albumin A7030-10G (10-15 mg). For pre-digestion samples, approximately 45-55 mg of sample were weighed for each AA hydrolysis. For post-digestion samples, dried pellet samples were weighted from 1-3 mg for each hydrolysis due to the low amount of pellets obtained. 1 ml of supernatant fraction samples were dried in hydrolysis tubes under nitrogen flow. The dried weight from 1 ml supernatant was recorded for later calculation. The hydrated molecular weights were used to quantify all AAs.

i) REGULAR AA HYDROLYSIS:

A total of 14 AAs, including alanine (ALA), ARG, ASP, GLU, GLY, ILE, LEU, lysine (LYS), PHE, PRO, serine (SER), THR, tyrosine (Tyr), and VAL were digested hy the regular AA hydrolysis. Weighted samples were mixed with 4mL of 6N HCl containing 1% phenol and norvaline as an internal standard in an 11 ml Pyrex glass tube. Approximately 20 ul of 2-octanol (two drops) was added to each hydrolysis tube to avoid bumping. The tubes were purged with nitrogen gas, sealed tight, and incubated for 24 hours at 110ºC. After hydrolysis, samples were allowed to cool down to room temperature and then neutralized with 4 mL of 25% NaOH. Samples were adjusted to a pH of 5.5-6.0 and then brought to a volume of 50 ml with water. The hydrolysis was completed by filtering 1 ml sample into cryovials via a 0.22µm Nylon luer lock filter. The hydrolysates were then stored at -20ºC.

The samples were subjected to pre-column derivatization with the AccQ·Tag™ Ultra Derivatizationon Kit (Waters Inc.) to convert polar and nonvolatile compounds, improve the target thermal stability, and incorporate functional groups for better chromatography detector and separation performances (He, 2012). In a polypropylene vial, the sample was reacted with 70 ul of AccQ-Tag borate buffer, 10 ul of calibration standard or hydrolysate sample, and 20 ul of AccQ-

Tag reagent and vortexed. The vials were rested for 1 minute, followed by 10 minutes of incubation on a heating block at 55ºC. After that, each vial was inspected to ensure no bubbles inside.

Ultra-high performance liquid chromatography (UPLC) was performed with the Shimadzu Nexera UPLC system (Kyoto, Japan) with a Waters AccQ C18 column (100 mm x 2.1 mm, 1.7 μm) and a SIL-30AC autosampler. Mobile phase A was prepared by diluting the AccQ-Tag Ultra Eluent A solution 20 times, while mobile phase B was 2% formic acid in acetonitrile. The column temperature was set to 60°C from starting to 30 minutes, 70°C from 30 to 52 minutes, and 60°C from 52 to 217 minutes. The autosampler and the reaction equipment were maintained at 4°C and 130°C, respectively. The flow rates were set at 0.45 ml/minute for the mobile phase and 0.25 ml/minute for the derivatizing reagent. The absorbance was measured at 260 nm, and the run time was 17 minutes. The Lab Solutions software (Shimadzu, Kyoto, Japan) was used to process data from the UPLC.

ii) PERFORMIC ACID OXIDIZED AA HYDROLYSIS:

The contents of HIS, MET, and CYS for each sample were determined by the official AOAC method 985.28 with hydrolysis by the performic acid (AOAC, 1995). Briefly, samples were weighed into a 30 ml glass tube. The performic acid was prepared freshly by combining 9 parts of 0.1% phenolic 88% formic acid with 1 part 30% hydrogen peroxide, then stored for at least 1 hour at 4°C before use. The oxidation started by adding 2 ml of performic acid and 20 ul of 2-octanol (two drops) to each hydrolysis tube. All tubes were sealed by glass stoppers and incubated at 4°C overnight, converting CYS and MET into cysteic acid and methionine sulfone. Post oxidation, 0.35 mg sodium metabisulfite was added to each hydrolysis tube to stop the reaction. The tubes were swirled after 5 minutes, 30 minutes, and 2 hours marks. Then, all samples were hydrolyzed with 2ml of 2.5mM norvaline in concentrated HCl in the heating block at 110°C

for 18 hours. The finished steps for the hydrolysis included neutralizing the solutions to a pH 5.5-6.0, bringing them to 50 ml volume with water, filtering with 0.22µm Nylon luer lock, and storing the hydrolysates at -20ºC. Post hydrolysis, the samples were derivatized and analyzed similarly to the regular AA analysis.

iii) ALKALINE AA HYDROLYSIS:

The alkaline hydrolysis was applied to measure the TRP content by ISO protocol (ISO 13904:2005) (ISO, 2016). 8.4g of barium hydroxide and 14 ml of Milli-Q water were added into a polypropylene flask containing weighted samples. All flasks were loosely capped and autoclaved for 20 hours at 110ºC. After hydrolysis, 30 mL Milli-Q water was added, followed by 5 ml of 0.5M orthophosphoric acid and 8.4 ml 6N HCl. The pH of the solutions was adjusted to 2.95-3.20. After that, 20 mL of methanol and water were added to a final volume of 100 mL. The samples were filtered through a 0.22 µm Nylon filter into an amber glass GC vial and stored at -20ºC. The hydrolysates were analyzed on the UPLC system with a Phenomenex Luna C18 column (250 mm x 4.6 mm, 3 µm) at a 1 ml/minute flow rate and a total run time of approximately 34 minutes. The excitation and emission parameters were set to 280 nm and 356 nm, respectively. The mobile buffer was prepared with 0.3% glacial acetic acid and 0.05% 1,1,1-trichloro-2-methyl-2-propanol in Milli-Q water, adjusting to pH 5.0 with ethanolamine.

4.3.10. *In vitro* protein quality calculations and determination of protein claims

This section describes the calculation for three target protein evaluations, including the PDCAAS, the DIAAS, and the PER, from two *in vitro* static digestion models, the pH-drop and the INFOGEST (Figure 5). The protein content claims permitted from each PQ assay were included.

***In vitro* PDCAAS and the USA protein claims**

The PDCAAS data were calculated following the FAO/WHO guidelines (Joint FAO/WHO, 1991). At first, the AA profiles of all samples were determined and expressed in mg of AA per gram of test protein. The contents of each IAA in the samples were compared against the preschool child's reference pattern (2-5 years) (Table 1). The AAS for the PDCAAS method (PDCAAS-AAS) was the smallest ratio among all IAAs, and the respective AA was the limited AA of that sample.

$$PDCAAS - AAS = \frac{mg\ of\ AA\ per\ g\ of\ protein\ (test\ protein)}{mg\ of\ AA\ per\ g\ of\ protein\ (reference\ pattern)}$$

The true fecal PH-PD% from the pH-Drop digestion model was mentioned in section 4.3.5. On the other hand, multiple fractions were collected from the INDOGEST digesta. Each digestion included foods, control (casein), and blank (nitrogen-free cookies). The digesta from all three types were separated into digestible and undigestible fractions, each fraction including pellets and supernatants (Figure 6). The nitrogen/total amino acid was determined for individual pellets and supernatants from 3 methods (the OPA – section 4.3.7, the micro-Kjeldahl – section 4.3.8, and the total AA – section 4.3.9). The digestibility was determined following Sousa et al. (2023), assuming AAs from the cookie blank originated from the enzymes/bile background; hence, any value below this point was set to zero.

$$OPA - PD(\%) \,/\, KD - PD(\%) \,/\, IFG - PD(\%) \;=\; \frac{F_S - C_S}{(F_S - C_S) + \max(0; F_P - C_P)} \times 100$$

with F_S: the nitrogen/total AA content from the supernatant fraction of the food sample

F_P: the nitrogen/total AA content from the pellet fraction of the food sample

C_S: the nitrogen/total AA content from the supernatant fraction of the cookie sample

C_P: the nitrogen/total AA content from the pellet fraction of the cookie sample

The IV-PDCAAS results of each method were calculated as the product of the PDCAAS-AAS of the raw ingredients and the IV-PD (%) of the respective method.

$$\mathrm{PH-PDCAAS} = \mathrm{AAS\ x\ PH-PD\ (\%)}$$

$$\mathrm{OPA-PDCAAS} = \mathrm{AAS\ x\ OPA-PD\ (\%)}$$

$$\mathrm{KD-PDCAAS} = \mathrm{AAS\ x\ KD-PD\ (\%)}$$

$$\mathrm{IFG-PDCAAS} = \mathrm{AAS\ x\ IFG-PD\ (\%)}$$

The protein claims from the PDCAAS was determined according to the USA regulations (USA Food and Drug Administration, 2021). Briefly, the protein level of the food per consumption was calculated based on the published Reference Amount Customarily Consumed (RACC) and the crude protein. Then, the corrected protein level in the food was determined as the product of the PDCAAS and the protein level per consumption of the same food. Finally, the results were derived from 50 g as the daily reference value for protein for the percentage contributing to the daily value.

$$Level\ of\ protein\ in\ the\ food\ per\ RACC\ (g) = RACC\ (g) \times crude\ protein\ (\%)$$

$$Corrected\ protein\ level\ (g) = PDCAAS \times Level\ of\ protein\ in\ the\ food\ per\ RACC\ (g)$$

$$\%\ Daily\ value = \frac{Corrected\ protein\ level\ (g)}{Daily\ value\ of\ protein - 50\ g}$$

A food is categorized as "a good source of protein" when it can provide 10-19.9% of the daily value. The “an excellent source of protein" claim is allowed when the food contains >20% of the daily value.

***In vitro* PER and the Canadian protein claims**

The IV-PER value of each sample was obtained by multiplying their IV-PDCAAS value by 2.5 as the Canadian government has allowed (Government of Canada - Canadian Food Inspection Agency, 2021):

$$\mathrm{IV-PER} = \mathrm{IV-PDCAAS} \times 2.5$$

Following Canadian regulations, the protein rating was determined based on the PER value and the protein contained according to the “Reasonable daily intake” amount (Government of Canada - Canadian Food Inspection Agency, 2020). If a protein rating value is from 20-39.9 or ≥ 40, the protein is qualified as “a good source of protein” or “an excellent source of protein”, respectively.

$$Level\ of\ protein\ in\ a\ reasonable\ daily\ intake\ (g) = Reasonable\ daily\ intake\ (g) \times crude\ protein\ (\%)$$

$$Protein\ rating = PER \times Level\ of\ protein\ in\ a\ reasonable\ daily\ intake\ o(g)$$

***In vitro* DIAAS and the FAO/WHO proposing protein claims**

Considering each IAA as an individual nutrient, the DIAAS values were calculated from the *in vitro* INFOGEST digesta following the FAO/WHO guidelines in 2013 with modifications from Sousa et al. (2023). The AA contents of all pellets and supernatants were determined with three hydrolyses and the UPLC system in section 4.3.9. The digestibilities of individual IAAs were determined as follows:

$$IFG-AAD(\%) = \frac{F_S - C_S}{(F_S - C_S) + \max(0; F_P - C_P)} \times 100$$

with F_S: the nitrogen/total AA content from the supernatant fraction of the food sample

F_P: the nitrogen/total AA content from the pellet fraction of the food sample

C_S: the nitrogen/total AA content from the supernatant fraction of the cookie sample

C_P: the nitrogen/total AA content from the pellet fraction of the cookie sample

The DIAA content was calculated as mg of each IAA in 1 g protein of food multiplied by the IFG-AAD of the same dietary IAA. The DIAAR value was defined by comparing the DIAA value to respective IAAs in the reference patterns (Table 3) (FAO, 2013). The smallest DIAAR was the DIAAS value, while the respective IAA was the limiting AA for the testing food.

$$DIAA\ (mg)\ of\ individual\ IAA = IAA\ (mg) \times IFG - AAD(\%)\ of\ the\ same\ IAA$$

$$\text{DIAAR (\%)} = \frac{\text{DIAA (mg) in 1g of the dietary protein}}{\text{mg of the same digestible IAA in 1g of the reference protein}} \times 100$$

$$DIAAS = the\ lowest\ DIAAR\ (\%)$$

The FAO proposed that if the DIAAS value of a food is lower than 75, no protein claim is permitted. In case the DIAAS value is >75 or >100, the food was permitted to be “a good source of protein” or “an excellent source of protein”, respectively.

4.3.11. Statistical analysis

Results from replicates were expressed into means ± standard error values and coefficient of variation (CV%). Results were compared via one-way ANOVA (p-values <0.05) with post-hoc analysis using Tukey’s multiple comparison test. The relationship between in-house *in vivo* and *in vitro* data of PD (PH-PD, OPA-PD, KD-PD, IFG-PD) and PDCAAS (PH-PDCAAS, OPA-PDCAAS, KD-PDCAAS, IFG-PDCAAS) was compared via regression analysis, Bland Altman plots, and Pearson correlation coefficient. All statistical works were analyzed with Microsoft Excel 365 (Microsoft Corporation, USA) and GraphPad Prism 10.0.1 (GraphPad Software Inc., USA).

4.4. RESULT AND DISCUSSION

4.4.1. Amino acid profile and scores in the PDCAAS method

The crude protein and the AA composition (% as-is basis) of all samples are listed in Table 7. The nut sample had the lowest crude protein content (40.7%). Casein had 84.9% of crude protein, the highest among all samples. All other samples were processed protein concentrates and isolates, having 60.8 to 79.8% of crude protein. In terms of AAs, GLU contributed the highest percentage of all samples. Next, ASP, LEU, and ARG contents were high among all AAs. However, these data could not fully express the quality of each protein.

Table 8 showed the AAS for all samples, calculated with the PDCAAS method. All IAAs of casein scored more than 1, with TRP taking the lowest score at 1.14. LYS was limited in nut, oilseed A, and SCP A; however, their scores were close to the perfect 1.0 ratio compared to the reference pattern. Nut had a balanced AA profile despite a low crude protein content with a high AAS of 0.93. SCP B also displayed a great AA profile with an AAS of 0.94, which is limited in SAA. As a pulse, legume A was limited in SAA and TRP, scoring at 0.61 and 0.78, respectively. TRP was the limited AA in legume B, with the AAS at 0.71. The sample with the lowest AAS was oilseed B at 0.49, with a limitation in LYS.

The AA content and AAS displayed the first glimpse of the samples' PQ. Casein had a high crude protein percentage and a balanced AA profile compared to the reference pattern. Despite lowest crude protein content, the nut offered a high-quality AA profile with AAS at 0.93 with LYS limitation. In contrast, oilseed B had the lowest AAS at 0.49 with LYS while owning high crude protein. Most samples were limited with LYS and SAA, except legume B with TRP limitations.

Table 7. Amino acid content g/100g as received of samples

Sample	Crude protein (%)	HIS	SER	ARG	GLY	ASP	GLU	THR	ALA	PRO	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
Casein	84.9	2.0	5.2	3.1	1.7	6.6	20.8	3.8	2.8	9.9	0.4	7.4	4.4	2.3	6.0	4.7	8.7	4.5	1.1
Nut	40.7	2.0	2.6	4.2	1.9	4.1	10.0	1.4	1.9	1.7	1.8	2.2	1.2	1.7	2.5	1.9	3.1	2.1	0.7
Legume A	60.8	1.3	2.7	5.1	2.2	6.1	9.6	1.9	2.2	2.3	0.5	3.6	1.7	0.4	2.5	2.3	4.2	2.2	0.5
Legume B	72.1	2.0	4.4	3.9	2.4	9.2	13.2	2.1	2.4	3.7	0.6	5.9	2.5	0.8	3.6	2.7	7.3	5.0	0.6
Oilseed A	79.9	1.6	3.3	5.1	4.0	6.3	17.3	3.1	3.5	4.9	1.9	4.4	1.8	1.6	4.0	3.3	5.9	3.2	1.3
Oilseed B	79.8	2.0	3.5	6.4	3.5	8.7	17.5	2.5	3.5	3.3	0.7	2.2	2.0	1.6	4.1	3.6	5.2	4.2	1.3
SCP A	73.2	1.7	3.8	4.7	3.1	8.8	13.8	2.8	3.2	3.8	0.8	4.2	2.5	1.0	3.6	3.5	5.8	3.7	1.0
SCP B	60.9	1.1	2.7	2.7	2.6	6.0	8.9	2.8	3.8	2.2	0.5	4.4	1.8	0.9	3.2	2.8	4.1	2.4	0.8

ASP = Asparagine; THR = Threonine; SER = Serine; GLU = Glutamine; PRO = Proline; GLY = Glycine; ALA = Alanine; CYS = Cysteine; VAL = Valine; MET =Methionine; ILE = Isoleucine; LEU = Leucine; TYR = Tyrosine; PHE = Phenylalanine; HIS = Histidine; LYS = Lysine; ARG = Arginine; TRP = Tryptophan; SCP = single-cell protein.

Table 8. Amino acid score of all samples

Sample	THR	VAL	SAA	ILE	LEU	AAA	HIS	LYS	TRP	AAS	Limiting AA
Casein	1.32	2.01	1.27	1.98	1.56	1.67	1.26	1.50	1.14	1.14	TRP
Nut	0.98	1.77	3.45	1.65	1.17	1.29	2.63	0.93	1.49	0.93	LYS
Legume A	0.90	1.19	0.61	1.37	1.04	1.01	1.10	1.02	0.78	0.61	SAA
Legume B	0.84	1.43	0.78	1.33	1.52	1.65	1.43	1.42	0.71	0.71	TRP
Oilseed A	1.12	1.43	1.76	1.46	1.11	1.00	1.07	0.95	1.46	0.95	LYS
Oilseed B	0.94	1.46	1.15	1.60	0.98	1.24	1.30	0.49	1.49	0.49	LYS
SCP A	1.13	1.40	1.03	1.72	1.21	1.34	1.20	0.98	1.28	0.98	LYS
SCP B	1.35	1.50	0.94	1.62	1.01	1.09	0.95	1.26	1.17	0.94	SAA

THR = Threonine; VAL = Valine; SAA = Methionine and Cysteine; ILE = Isoleucine; LEU = Leucine; AAA = Phenylalanine and Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan; AAS = amino acid score; SCP = single-cell protein.

4.4.2. The *in vitro* protein digestibility

The summary of PD from an in-house database and acquired from 4 methods were exhibited in Table 9. From the bioassays, all samples were generally highly digestible; the highest TPD was casein (99.3%), and the lowest TPD was SCP A (86.9%). Casein displayed consistent results across all *in vitro* methodologies and relatively low variation within measurements (CV: 0.2-1.4%). Using casein as a control showed good reproducibility and low potential variability between each digestion day. Casein demonstrated the highest digestibility result from the INFOGEST digesta with the OPA method at 99.9%. The digesta measured with the Kjeldahl method and AA analysis had similar digestibility at 98.9% and 98.7%, respectively. The pH-drop model had 93.6% digestibility, lower than the results from the INFOGEST model. Apart from the control, nut and SCP A were the most (95%) and the least digestible (89.3%) samples, according to the pH-drop model. Similar to the true protein digestibility, all samples showed relatively high digestibility and slight variation between replication (CV: 0.4-0.1%). Regarding the OPA method, SCP A had a significantly lower digestibility at 87.6%, while all other samples were reportedly digested more than 91%. Both oilseed A (98%) and oilseed B (98.1%) were highly digestible. The variation between replications in the OPA method was higher than in the pH-drop digestion model, in which most proteins have a coefficient of variations of less than 5% except for SCP B (CV: 5.9%). Similar to the OPA method, the Kjeldahl method measured all samples with relatively high repeatability. The only sample with >5% variation was SCP A at 7.3%. The highest and lowest digestible samples measured by the Kjeldahl method were alighted with the OPA methods but with a closer difference from oilseed B 97.9% to SCP A 93.5%. The AA analysis demonstrated the highest digestibility by oilseed A (100%), nut, and legume A (97.6%), while SCP A ranked lowest at 85.4%. Between replications, most samples varied slightly. SCP A and SCP B displayed

higher variations, at 6.6% and 7.1%, respectively. Overall, the *in vitro* methodologies demonstrated relatively high digestibility for all samples. The OPA method reported the highest IV-PD at 100% (oilseed A) and the lowest at 85.4%, owning the most significant digestibility differences among samples. The pH-drop digestion model usually measured lower results than the other three *in vitro* methodologies from the INFOGEST digestion, except nut, SCP A, and SCP B. SCP A got the lowest digestibility among all samples across all assays. Additionally, most proteins exhibited less than 5% of the coefficient of variation, except for SCP A digestibility (the Kjeldahl method - CV: 7.3%, the AA analysis - CV: 6.6%), and SCP B digestibility (the OPA method – CV: 5.9%, and the AA analysis - CV: 7.1%). The pH-drop method generally showed lower variation than the other three methods.

The correlations between the in-house true protein digestibility and four assessing *in vitro* methodologies were displayed in Figure 7A-7D. Between two *in vitro* digestion models, the pH-drop method had a smaller square of correlation (R^2=0.084) than other analyses from the INFOGEST digesta. The protein digestibility equation for the OPA method (R^2= 0.691) had the strongest relationship with the true protein digestibility, the Kjeldahl method R^2 = 0.203 and the AA analysis R^2 = 0.529.

The Bland-Altman analysis in Figure 8A-8D showed more information about the agreement between the methods. In comparison between the two digestion models, the pH drop exhibited a more substantial agreement with the true protein digestibility with no point out of the 95% agreement. The AA analysis and the Kjeldahl method had two points out of the 95% agreement limitation. Meanwhile, the OPA method only displayed one point out of the limitation. Regarding percentage differences compared to the average, the protein digestibility measured with the OPA method had the smallest range, while the AA analysis data got the most extensive range.

The relationship between all methodologies to determine protein digestibility was expressed in the matrix form in Figure 9. With the Pearson correlation coefficient analysis, the highest agreement across all the methods was between the OPA data and the true protein digestibility with r = 0.83. The second strongest agreement was between the protein digestibility measured with the AA analysis and the in-house rat assays (r = 0.73). The pH-drop had the lowest agreement with other methodologies, with r = 0.09 with the Kjeldahl method and r = 0.14 with the OPA method.

In conclusion, all samples were highly digested with *in vitro* digestion models, ranging from 87.6% (SCP A with OPA method) to 100% (oilseed A with AA analysis). Most measures expressed low variability (less than 5%), except for the AA analysis with SCP A and SCP B samples. All the IV-PD results showed a relatively low coefficient of determinations with the TPD. Between the two digestion models, the pH-drop had a lower R^2 (0.084) than the INFOGEST ones (Kjeldahl method = 0.203; the AA analysis = 0.529; and the OPA method = 0.691). Most of the predicted points lay within the 95% agreement limit. Across the *in vitro* methodologies, the AA analysis had the highest number of points (2) outside the agreement and the most significant percentage differences compared to the average. Finally, according to the Pearson correlation coefficient analysis, the pH-drop method was not in agreement with other methodologies, especially with the Kjeldahl method with r = 0.09.

Table 9. Summary of digestibility for all proteins, including the in-house true protein digestibility, pH-drop *in vitro* protein digestibility, and INFOGEST *in vitro* protein digestibility measured with the OPA method, Kjeldahl method, and AA analysis.

Sample	TPD	PH-PD	STD	CV	OPA-PD	STD	CV	KD-PD	STD	CV	IFG-PD	STD	CV
Casein	99.3	93.6	0.4	0.4	99.9	0.2	0.2	98.9	1.0	1.0	98.7	1.4	1.4
Nut	92.3	95.0	0.0	0.0	91.5	2.5	4.8	95.4	3.3	3.5	97.6	2.2	2.2
Legume A	96.1	90.2	0.2	0.1	94.5	1.0	1.1	95.5	4.5	1.7	97.6	0.0	3.8
Legume B	92.3	89.6	0.1	0.1	92.7	1.9	2.1	95.8	4.5	4.7	93.0	3.2	3.4
Oilseed A	96.5	90.3	0.2	0.3	98.0	1.0	1.0	97.4	1.7	4.6	100.0	1.6	0.0
Oilseed B	95.7	89.6	0.1	0.2	98.1	5.4	1.0	97.9	2.7	1.7	96.4	6.4	1.7
SCP A	86.9	89.3	0.4	0.4	87.6	3.9	4.5	93.5	6.8	7.3	85.4	5.6	6.6
SCP B	93.2	90.6	0.1	0.1	91.9	1.0	5.9	97.3	1.7	2.8	90.5	3.7	7.1

TPD = True protein digestibility; PH-PD = pH-drop in vitro protein digestibility; OPA-PD = OPA in vitro protein digestibility; KD-PD = Kjeldahl in vitro protein digestibility; IFG-PD = total amino acid in vitro protein digestibility; STD = Standard deviation; CV% = Coefficient of variation; SCP = single-cell protein.

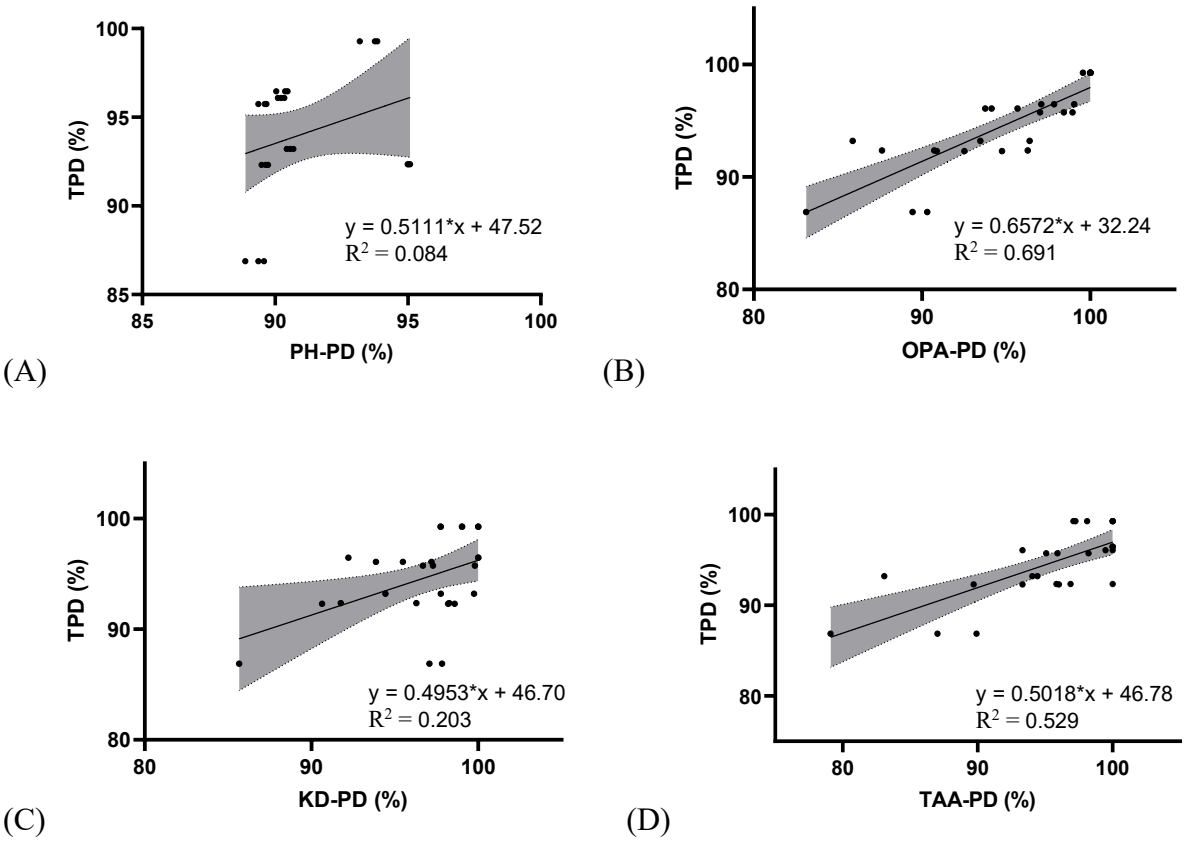


Figure 7. Relationship between in-house *in vivo* true protein digestibility (TPD %) data and *in vitro* protein digestibility by (A) pH-drop digestion model (PH-PD %), (B) OPA method (OPA-PD %), (C) Kjeldahl method (KD-PD %), (D) total amino acid analysis (IFG-PD %) from INFOGEST digestion model, and including the regression line (black line), equation and coefficient of determination (R^2) in addition to the confidence (dotted line – gray area).

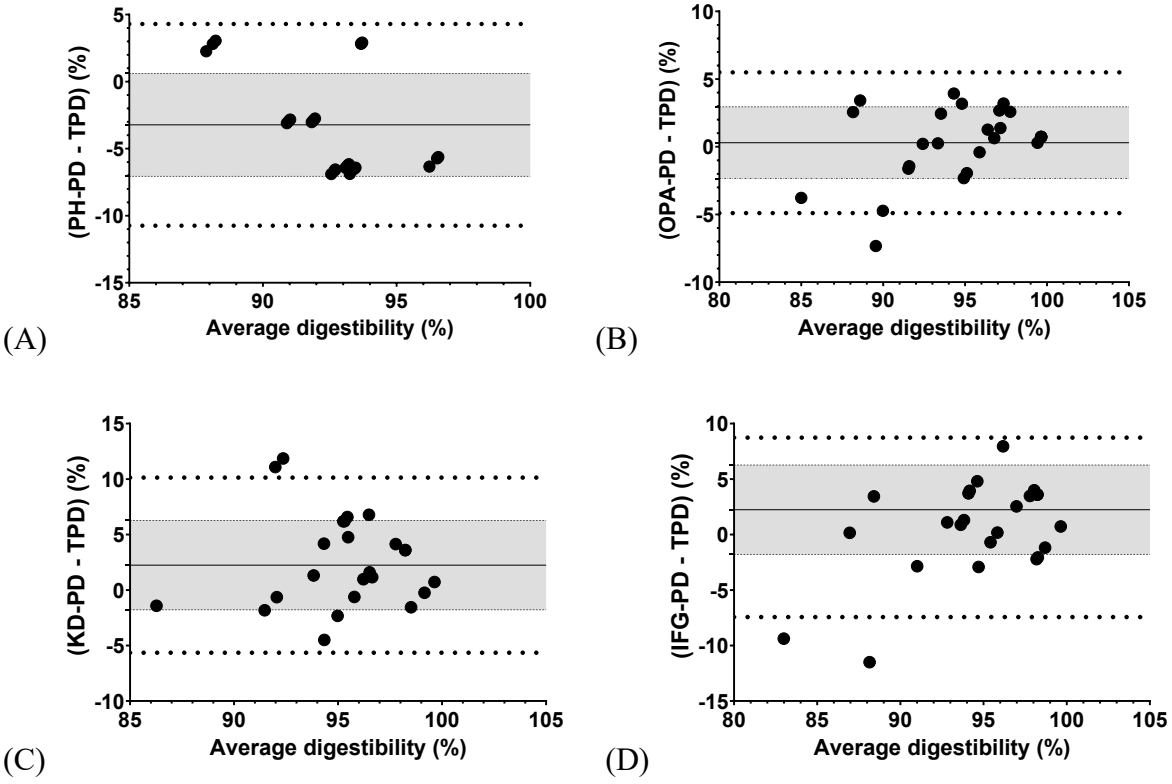


Figure 8. Bland-Altman for digestibility measures between the average and the percentage difference of the in-house true protein digestibility and (A) pH-drop digestion model (PH-PD %), (B) OPA method (OPA-PD %), (C) Kjeldahl method (KD-PD %), (D) total amino acid analysis (IFG-PD %) from INFOGEST digestion model, with upper and lower limits of 95% agreement (dotted line), confidence line (dashed line), confidence area (gray area) in addition to bias (solid line).

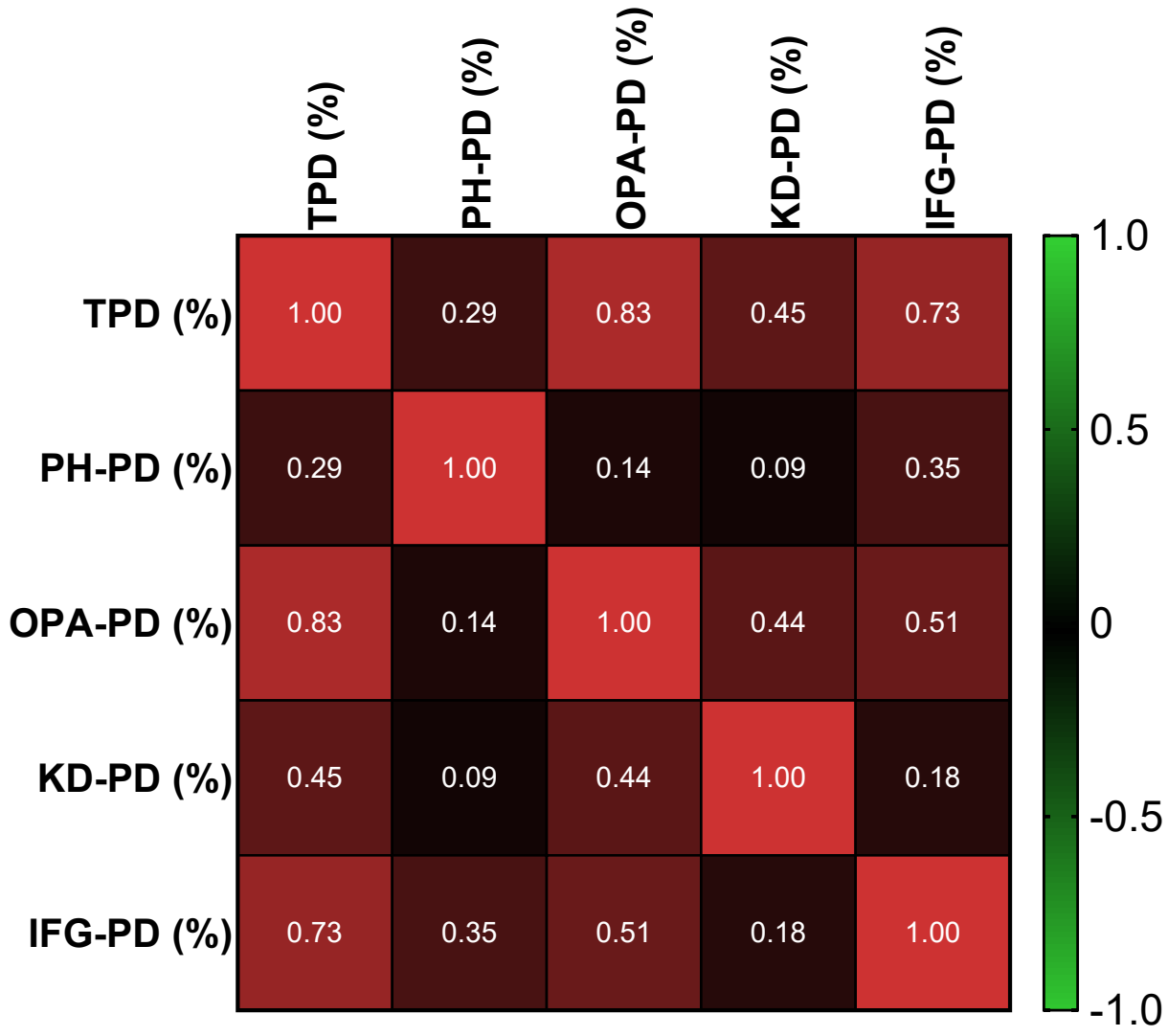


Figure 9. Correlation matrix between in-house true protein digestibility (TPD %) and *in vitro* protein digestibility by pH-drop digestion model (PH-PD %), OPA method (OPA-PD %), Kjeldahl method (KD-PD %), and total amino acid analysis (IFG-PD %) from INFOGEST digestion model

4.4.3. The *in vitro* Protein Digestibility Corrected Amino Acid Score

The amino acid score contents measured for the *in vitro* tests were discussed in section 4.4.1. Amino acid profile and scores in the PDCAAS method. Table 10 shows the summary of the protein quality with the PDCAAS method. Comparing *in vivo* and *in vitro* tests, the limited AA and the AAS of all samples were similar except for the AAS of legume A. Casein exhibited an excellent AA balance to reference pattern with the AAS higher than 1. Nut, oilseed A, SCP A, and SCP B showed great AAS values at 0.93, 0.95, 0.98, and 0.94, respectively. Oilseed B had the lowest AAS (0.49) with LYS limitation. Nut, oilseed A, and SCP A were limited to LYS. Pongamia had low TRP compared to the daily reference requirement. The last two samples, legume A and SCP B, were reported with SAA limitations. The PDCAAS values were presented in Table 10, including the untruncated data for casein. With truncation, casein was evaluated with the PDCAAS at 100 and CV at 0% by all *in vitro* methodologies. The differences with casein showed without truncation. The pH-drop model reported lower PDCAAS value than the *in vivo* and all INFOGEST results. In general, all methods' repeatability of casein measurements was high, with CV% from 0.18 to 1.44. Among all samples, the lowest and highest data with the pH-drop method were oilseed B (43.45) and nut (88.11). The PH-PDCAAS values for SCP A (87.29), oilseed A (85.92), and SCP B (84.8) were relatively higher than pongamia (63.92) and legume A (55.2). The coefficient of variations among the pH-drop was low, less than 0.41%. The data acquired from the OPA method ranged from 93.24 (oilseed A) to 47.62 (oilseed B). Despite a more comprehensive PDCAAS value range, this method was reported with higher variations than the pH drop, especially the CV for SCP B, which was at 5.9%. The oilseed A and B were also the highest (92.68) and lowest (47.52) PDCAAS samples measured by the Kjeldahl method. Besides control samples, all the KD-PDCAAS values were higher than the OPA-PDCAAS ones. Most

proteins were reported with less than 5% variation with the Kjeldahl method, except for SCP A (7.3%). Similarly, the AA analysis showed a low variation between measurements. The two samples with relatively high CV were SCP A (6.56%) and SCP B (7.12%). In terms of the IFG-PDCAAS value, oilseed A and oilseed B were ranked highest and lowest in protein quality, at 95.15 and 46.78, respectively. Overall, the lowest protein quality sample measured by all methodologies was oilseed B. At the same time, oilseed A was evaluated with the highest quality protein with the *in vivo* assay and all *in vitro* methodologies except for the pH-drop method. Regarding the variation, the pH-drop digestion model displayed the lowest CV% among all tests. All *in vitro* methods had less than 5% of the variation, except for SCP B OPA-PDCAAS (5.9%), SCP A KD-PDCAAS (7.3%), SCP A IFG-PDCAAS (6.56%), and SCP B IFG-PDCAAS (7.12%).

Figure 10 mentioned the linear regression relationship between the *in vivo* and the *in vitro* PDCAAS data. Overall, all correlations were strong, with R^2>0.9. In more detail, the Kjeldahl and the pH-drop method had the lower R^2 at 0.904 and 0.912, respectively. The other two *in vitro* assays, the OPA method (R^2 = 0.942) and the total AA analysis (R^2 = 0.944), had strong relationships with the PDCAAS data.

The relationships between *in vivo* and *in vitro* analysis are presented within the Bland-Altman analysis in Figure 11. While the pH-drop and the OPA method had all data points within the 95% agreement limitations with the *in vivo* PDCAAS, the Kjeldahl and the AA analysis methodologies each had one point out of this range. Comparing the percentage differences with the average, the OPA, the Kjeldahl, and the AA analysis methods had a smaller range (0-15%) than the pH-drop method (0-20%).

The correlation matrix in Figure 12 explains the relationship between the methodologies. In terms of PDCAAS, all methods strongly agreed with each other, with all the r >0.94. The pH-

drop model had the highest r value at 0.98, with the OPA and the Kjeldahl method. Despite the smallest r value in the matrix (0.95), the relationship between *in vivo* PDCAAS and the KD-PDCAAS was relatively strong.

The IV-PDCAAS results strongly agreed with the *in vivo* PDCAAS data with all samples. Across *in vitro* tests, oilseed Bs had the lowest IV-PDCAAS (43.45 - 47.62), while oilseed A had the highest quality value (85.92 - 95.15). The CV% was less than 5% for most measurements. Similar to the IV-PD results, the variation of IV-PDCAAS for SCP A and SCP B was higher than in other samples (5-7%). The *in vitro* results were strongly correlated with the *in vivo* database with all R^2>0.9 with linear regression, and most of the data points were within the 95% agreement limits with the Bland-Altman analysis. All the analyses with INFOGEST data had higher R^2 than the pH drop. The OPA method and the AA analysis strongly correlated with the *in vivo* PDCAAS. Between *in vitro* methods, the correlation matrix displayed strong agreements with each other.

Table 10. Summary of the amino acid score, limiting AA, and PDCAAS (%) for all proteins measured from in-house bioassays and four *in vitro* methodologies from the pH-drop digestion model, and INFOGEST digestion model with the OPA method, Kjeldahl method, and AA analysis.

Sample	Measured with *in vivo* test			Measured with *in vitro* test												
	AAS	Limiting AA	PDCAAS	AAS	Limiting AA	PH -PDCAAS	STD	CV (%)	OPA -PDCAAS	STD	CV (%)	KD -PDCAAS	STD	CV (%)	IFG -PDCAAS	STD	CV (%)
Casein	1.11	TRP	100.0	1.11	TRP	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00
Casein *untruncated*	1.11	TRP	110.48	1.11	TRP	106.70	0.41	0.38	113.90	0.20	0.18	112.70	1.15	1.02	112.50	1.62	1.44
Nut	0.93	LYS	86.19	0.93	LYS	88.11	0.04	0.05	84.88	4.07	4.80	88.49	3.09	3.50	90.47	2.01	2.22
Legume A	0.70	SAA	67.15	0.61	SAA	55.20	0.08	0.14	57.83	0.62	1.08	58.44	1.02	1.74	59.71	2.27	3.81
Legume B	0.66	TRP	60.64	0.71	TRP	63.92	0.09	0.14	66.12	1.39	2.10	68.33	3.20	4.68	66.34	2.26	3.40
Oilseed A	0.99	LYS	95.15	0.95	LYS	85.92	0.22	0.25	93.24	0.94	1.00	92.68	4.28	4.61	95.15	0.00	0.00
Oilseed B	0.51	LYS	48.80	0.49	LYS	43.45	0.08	0.18	47.62	0.49	1.02	47.52	0.80	1.69	46.78	0.79	1.68
SCP A	0.97	LYS	83.99	0.98	LYS	87.29	0.35	0.40	85.67	3.84	4.49	91.44	6.67	7.30	83.45	5.48	6.56
SCP B	0.91	SAA	85.20	0.94	SAA	84.80	0.11	0.14	86.06	5.08	5.90	91.12	2.51	2.76	84.76	6.04	7.12

AAS = Amino acid score; Limited AA = Limited amino acid; PDCAAS = Protein Digestible Corrected Amino Acid Score; PH-PDCAAS = PDCAAS value measured with the pH-drop model; OPA-PDCAAS = PDCAAS value measured with the OPA method; KD-PDCAAS = PDCAAS value measured with the Kjeldahl method; IFG-PDCAAS = PDCAAS value measured with the total amino acid analysis; STD = Standard deviation; CV% = Coefficient of variation; TRP = Tryptophan; LYS = Lysine; SAA = Methionine and Cysteine; SCP = single-cell protein.

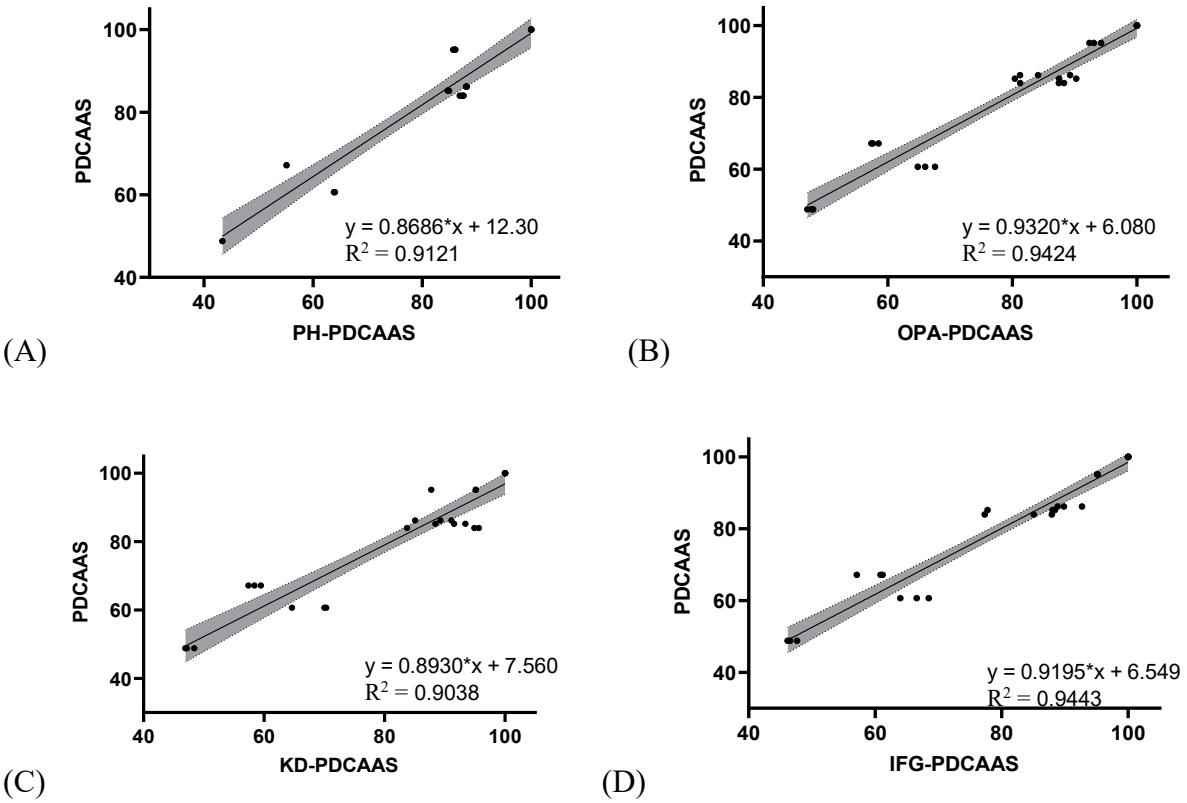


Figure 10. Relationship between in-house *in vivo* PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model, and including the regression line (black line), equation and coefficient of determination (R^2) in addition to the confidence limits (dotted line –gray area).

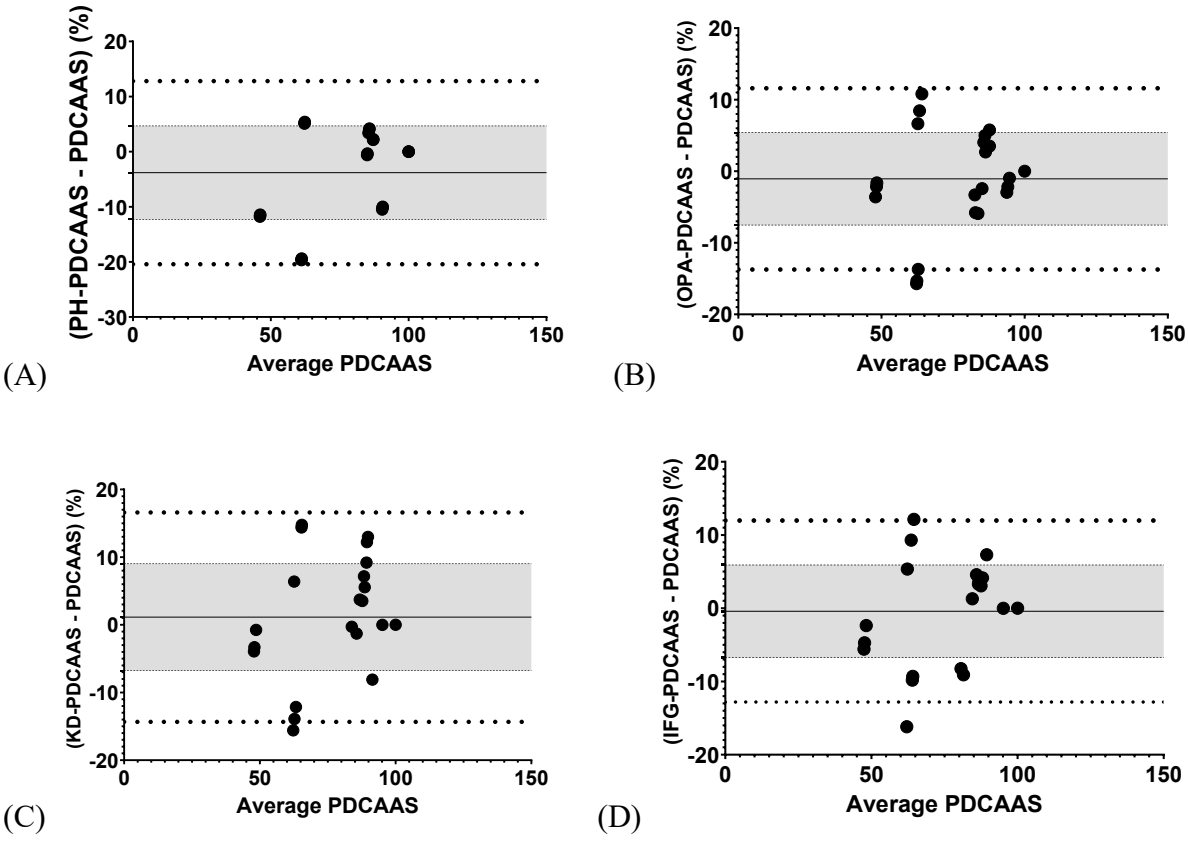


Figure 11. Bland-Altman for digestibility measures between the average and the percentage difference of in-house *in vivo* PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model, with upper and lower limits of 95% agreement (dotted line), confidence line (dashed line), confidence area (gray area) in addition to bias (solid line).

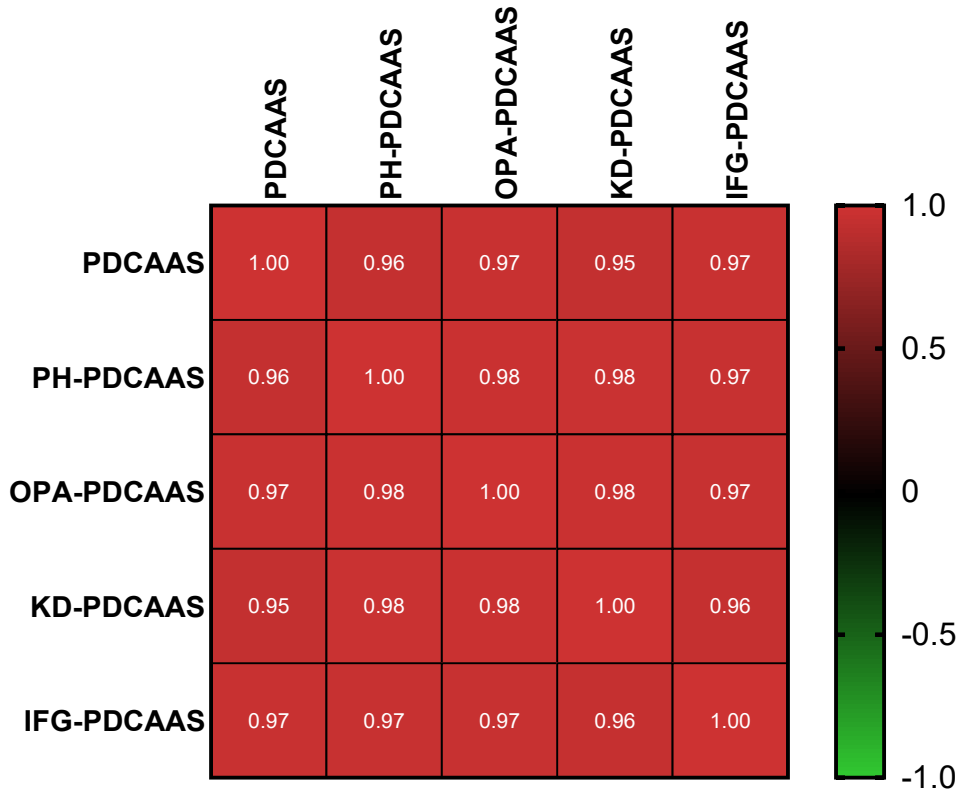


Figure 12. Correlation matrix between in-house PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model.

4.4.4. The *in vitro* amino acid digestibility and Digestible Indispensable Amino Acid Score

As the experimental design, the INFOGEST digesta was analyzed for the AA profile, aiming to determine each IAA *in vitro* AAD and calculation of the *in vitro* DIAAS according to FAO/WHO guidelines using the reference pattern of the preschool children age group (6-month – 3 (FAO, 2013).

Table 11 summarized the digestibility of individual IAAs, standard deviation, and coefficient of variation. Casein exhibited high digestibility percentages for all IAAS for the control, from the highest 99.9% (AAA), to the lowest 94.3% (ILE). The variation between measurements was less than 5% in all IAAs of casein. Nut had the most significant digestibility difference (22.8%), with HIS at 77.2% and AAA (aromatic amino acids: phenylalanine and tyrosine) at 100%. Four IAAs of nut were measured with relatively high variation, especially at 17% of HIS. The lowest digestibility observed in Legume A and oilseed A in the respective order was LYS at 92.4% and TRP at 87.3%. Legume A and oilseed A were highly digested with the INFOGEST model with many IAAS with 100% digestibility, such as HIS and SAA. On the contrary, SAA digested the poorest among IAAs in oilseed B, at 88.2 %, with a high variation (15.8%). AAA ranked the highest digestible IAAs for oilseed B, legume B, and SCP A. TRP had the lowest digestibility in legume B, SCP A, and SCP B, at 84.7%, 73.2%, and 76.9%, respectively. The variation for legume B, SCP A, and SCP B samples was higher than others. Overall, all samples displayed high digestibility results yet high coefficients of variations. This might be because of the small amount of dried pellet samples obtained by the ends of the INFOGEST digestion model. HIS, SAA, and AAA were highly digested, while TRP had the least digestibility in 4 out of 7 samples.

The summary results for the DIAAR and DIAAS values are shown in Table 12. As a high-quality protein, casein exhibited high IV-DIAAR higher than 1 for all IAAs. The final DIAAS value was 1.153, with SAA being the limiting AA. With these results, casein was classified as "an excellent source of protein". Compared to the IV-PDCAAS values (Table 10), the limiting AA of casein changed from TRP to LYS. As per FAO/WHO guidelines, the casein's IV-DIAAS (115.3) was higher than the truncated IV-PDCAAS values (100 for all methodologies) and the untruncated values (106.7-113.90). Despite differences with a study about casein in humans (DIAAS value at 145 with SAA limitations), both results demonstrated that casein was an excellent protein with all DIAAR higher than 100 (Guillin et al., 2022). The "Nut" sample also displayed great IV-DIAAR values, all higher than 100, except for LYS. As a result, "Nut" was concluded as "a high-quality source" of protein and limited in LYS with IV-DIAAS of 87. This data agreed with a study on pigs with raw nut, reporting DIAAS at 86 with LYS as the limited AA (Bailey & Stein, 2020). Similarly, oilseed A had limitations with LYS and the highest IV-DIAAS among all samples at 95.7, hence qualified as "a high-quality source of protein". In an overview study in 2020, oilseed A had a DIAAS value 72 with LYS limitation with pigs studies (Herreman et al., 2020). Despite the similarities in limited AAs, the data in this study reported oilseed A as a high-quality protein with a higher IV-DIAAS value of 95.7. The lowest IV-DIAAS (LYS: 44.0) belonged to the oilseed B with no permitted protein claim. A study on pigs reported that oilseed B meal was limited in SAA with a DIAAS of 58.5 and did not qualify for protein content claims (Gonzalez-Vega & Stein, 2012). Both legume A and legume B were also not qualified for protein quality claims due to SAA limitation with IV-DIAAS values at 57 and 66.3, respectively. Another study on pigs also agreed that the legume A was not qualified for protein claim by the DIAAS method with SAA limitation and PQ at 55 (Herreman et al., 2020). Until today, no DIAAS values for legume B, SCP B, and

SCP A were reported in the literature. In this study, SCP A and SCP B samples were "high quality" proteins with SAA limitations, with the IV-DIAAS values at 83.7 and 78.3, respectively. Eight samples were in agreement about the limited AA between the IV-PDCAAS and IV-DIAAS methods (Table 11 and Table 12). While the PDCAAS method results showed that legume B and SCP A lacked TRP and LYS, respectively, these two samples were limited in SAA by the DIAAS methods. There were differences in the IV-PQ values between the two methods; hence, the permitted protein claims were discussed further in the upcoming section. Additionally, the IV-DIAAS results were higher than the available DIAAS data in the literature.

Table 11. Summary of amino acid digestibility [value ± standard deviation (CV%)] from INFOGEST digesta

Sample	Amino acid digestibility (%)								
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA
Casein	97 ± 4.1 (4.2)	97.5 ± 2.7 (2.7)	96.5 ± 4.7 (4.9)	99 ± 1.4 (1.4)	94.3 ± 2.2 (2.4)	99.6 ± 1.1 (1.1)	98.4 ± 4.2 (4.3)	96.8 ± 4.2 (4.3)	99.9 ± 0.5 (0.5)
Nut	77.2 ± 13.1 (17)	97.9 ± 3.8 (3.8)	92.3 ± 6.9 (7.4)	98.8 ± 2.2 (2.2)	98.8 ± 2.2 (2.2)	98.2 ± 1.7 (1.7)	96.4 ± 6.4 (6.7)	92.4 ± 6.9 (7.5)	100 ± 0 (0)
Legume A	100 ± 0 (0)	95 ± 5.4 (5.7)	92.4 ± 4.7 (5.1)	95.7 ± 5.9 (6.1)	94.4 ± 5.6 (6)	96.4 ± 3.4 (3.5)	98.7 ± 2.4 (2.4)	100 ± 0 (0)	99.5 ± 1 (1)
Legume B	89.5 ± 16.1 (18)	91.5 ± 8.2 (8.9)	91 ± 3.7 (4.1)	93.7 ± 3.1 (3.3)	92.7 ± 6 (6.4)	93 ± 3.3 (3.6)	84.7 ± 8.7 (10.3)	91 ± 7.9 (8.6)	96.6 ± 3.3 (3.4)
Oilseed A	100 ± 0 (0)	98.9 ± 2 (2)	98.8 ± 1.7 (1.7)	98.5 ± 2.3 (2.4)	98.6 ± 2.5 (2.6)	100 ± 0 (0)	87.3 ± 8 (9.2)	100 ± 0 (0)	94.4 ± 3.1 (3.3)
Oilseed B	97.8 ± 3.9 (4)	94.4 ± 3 (3.2)	89.2 ± 4.3 (4.8)	97.7 ± 2.3 (2.4)	99.2 ± 1.4 (1.4)	99 ± 1 (1.1)	94.9 ± 4.6 (4.8)	88.2 ± 13.9 (15.8)	99.3 ± 1.3 (1.3)
SCP A	84.3 ± 13.9 (16.5)	82.5 ± 8 (9.7)	85.5 ± 5.5 (6.4)	78.3 ± 5.2 (6.6)	80.6 ± 5.9 (7.4)	80 ± 5.9 (7.4)	73.2 ± 12.1 (16.6)	87.2 ± 11.2 (12.9)	93.1 ± 2.5 (2.7)
SCP B	100 ± 0 (0)	79.8 ± 9.6 (12.1)	88 ± 8.1 (9.2)	86.6 ± 10.1 (11.6)	93.9 ± 7 (7.4)	93.5 ± 7.3 (7.9)	76.9 ± 9.1 (11.8)	90.2 ± 10.5 (11.6)	98.8 ± 0.7 (0.8)

HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine; SCP = single-cell protein.

Table 12. Summary of DIAAR (value ± standard deviation), limiting AA, DIAAS, and protein classification from INFOGEST digesta.

Sample	DIAAR									DIAAS		Classification
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA		Limiting AA	
Casein	117.6 ± 2.4	141.6 ± 3.2	150.0 ± 3.5	163.0 ± 1.3	162.8 ± 3.9	172.0 ± 0.0	147.0 ± 0.0	115.3 ± 3.9	202.0 ± 0.0	115.3	SAA	Excellent quality
Nut	192.9 ± 32.6	107.1 ± 0.0	87.0 ± 6.2	144.0 ± 0.0	144.0 ± 0.0	125.0 ± 0.0	193.0 ± 0.0	295.0 ± 21.6	156.0 ± 0.0	87.0	LYS	High quality
Legume A	105.0 ± 0.0	93.5 ± 5.3	95.7 ± 5.0	92.7 ± 5.9	113.0 ± 7.0	110.7 ± 4.0	98.7 ± 2.3	57.0 ± 0.0	121.7 ± 0.6	57.0	SAA	No claim
Legume B	121.6 ± 21.9	84.5 ± 7.5	131.0 ± 5.0	108.7 ± 3.5	108.3 ± 7.1	155.7 ± 5.5	78.0 ± 7.9	66.3 ± 5.8	193.3 ± 6.2	66.3	SAA	No claim
Oilseed A	101.6 ± 0.0	121.9 ± 2.4	95.7 ± 1.5	115.0 ± 2.6	126.0 ± 3.5	122.0 ± 0.0	165.3 ± 15.0	163.0 ± 0.0	114.3 ± 3.8	95.7	LYS	High quality
Oilseed B	120.7 ± 4.7	97.3 ± 3.1	44.0 ± 1.7	116.3 ± 2.5	139.0 ± 1.7	107.0 ± 1.0	182.7 ± 8.3	94.0 ± 14.4	149.0 ± 1.7	44.0	LYS	No claim
SCP A	96.1 ± 15.8	102.5 ± 9.8	85.0 ± 5.6	89.0 ± 5.6	121.0 ± 9.0	106.3 ± 7.5	121.3 ± 20.0	83.7 ± 10.8	150.7 ± 4.2	83.7	SAA	High quality
SCP B	90.5 ± 0.0	117.7 ± 14.2	112.7 ± 10.2	105.7 ± 11.9	133.0 ± 9.5	103.7 ± 8.4	116.3 ± 13.4	78.3 ± 9.0	130.7 ± 0.6	78.3	SAA	High quality

HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine; DIAAS = Digestible Indispensible Amino Acid Score; Limited AA = Limited amino acid; SCP = single-cell protein

4.4.5. Comparison between protein quality and protein claim indications

Based on the in-house PDCAAS and IV-PDCAAS values shown in section 4.4.2, the protein content claim according to the USA was determined in Table 13. The reference amounts customarily consumed (RACC) for nut and legume A were based on the FDA guidelines (USA Food and Drug Administration, 2018). If the RACC information is unavailable for other samples, the reference amount of the item or similar items in Canadian regulations was applied (Government of Canada, 2022; Marinangeli & House, 2017). In the cases of casein, oilseed A, legume B , oilseed B, and SCP A, as both the RACC and the reference amount are not published, the reference amount of "Dried texturized soy protein and soy protein isolate" was utilized. In general, all samples were rated as excellent and good protein sources. The permitted protein claims were similar to all methodologies despite differences in PDCAAS values. As casein PDCAAS values were high and truncated to 100, the corrected protein content values and the permitted protein claim were similar between all *in vivo* and *in vitro* methodologies. Nut, legume A, oilseed A, oilseed B, and SCP A were classified as "an excellent source of protein". Legume B, providing around 17.5-20% daily protein value, oilseed B, providing around 13.86-16% daily protein value, and SCP B, providing around 14.46-16% daily protein value, were ranked as "a good source of protein".

Table 14 showed the adjusted PER values calculated from the PDCAAS results and the permitted protein claims according to Canadian regulations. The reference amount (Schedule M) was used if a reasonable daily intake was not available (Schedule K) (Government of Canada, 2016, 2022; Marinangeli & House, 2017). Similar to Table 12, the amount for casein, legume B, oilseed B, and SCP A was the reference amount of the "Dried texturized soy protein and soy protein isolate" because there was no available information. Among all samples, SCP B and

oilseed B were the only samples that could not obtain any permitted claim for protein content with protein rating values < 20 across all methodologies. According to the PER method, casein and legume A were the only two excellent protein sources. Most of the samples were ranked as a good source of protein, including nut, oilseed A, legume B, and SCP A.

Figure 13 demonstrated the differences between protein contents claims that all samples were able to achieve based on IV-PDCAAS, IV-PER, and IV-DIAAS results. It is observed that the PDCAAS methods allowed samples to get higher claims (excellent or high) while the DIAAS assays ranked samples lower than the PDCAAS. Between 3 methods, the PER showed more similar results for protein content claims with the DIAAS than the PDCAAS.

Regarding the permitted protein claims, there are differences between all samples, with casein being the only exception as the "excellent protein source" among all three IV-PQ assays. Both the DIAAS and PER methods rated the nut as a high-quality protein, while the PDCAAS method classified the nut as an excellent protein. Legume A, oilseed B, and legume B were not qualified for any claim with the DIAAS proposals, yet according to the PDCAAS and PER methods, these are classified as excellent or good proteins. SCP B was a high-quality/good source of protein by the DIAAS and the PDCAAS methods, but it is not allowed for any claims with Canadian regulations.

Table 13. Summary of reference amounts customarily consumed (RACC), PDCAAS, corrected protein content, and protein content claim statements on samples by the USA regulations

Sample	RACC	PDCAAS	Corrected protein content (g)	Claim	PH-PDCAAS	Corrected protein content (g)	Claim	OPA-PDCAAS	Corrected protein content (g)	Claim	KD-PDCAAS	Corrected protein content (g)	Claim	IFG-PDCAAS	Corrected protein content (g)	Claim
Casein	20[a]	100.00	16.98	Excellent source	100	16.98	Excellent source	100	16.98	Excellent source	100	16.98	Excellent source	100	16.98	Excellent source
Nut	30	86.19	10.52	Excellent source	88.11	10.76	Excellent source	84.88	10.36	Excellent source	88.49	10.81	Excellent source	90.47	11.05	Excellent source
Legume A	35	67.15	14.29	Excellent source	55.2	11.75	Excellent source	57.83	12.31	Excellent source	58.44	12.44	Excellent source	59.71	12.71	Excellent source
Legume B	20[a]	60.64	8.74	Good source	63.92	9.21	Good source	66.12	9.53	Good source	68.33	9.85	Good source	66.34	9.56	Good source
Oilseed A	20[a]	95.15	15.21	Excellent source	85.92	13.74	Excellent source	93.24	14.91	Excellent source	92.68	14.82	Excellent source	95.15	15.21	Excellent source
Oilseed B	20[a]	48.80	7.78	Good source	43.45	6.93	Good source	47.62	7.6	Good source	47.52	7.58	Good source	46.78	7.46	Good source
SCP A	20[a]	83.99	12.30	Excellent source	87.29	12.78	Excellent source	85.67	12.55	Excellent source	91.44	13.39	Excellent source	83.45	12.22	Excellent source
SCP B	14[b]	85.20	7.27	Good source	84.8	7.23	Good source	86.06	7.34	Good source	91.12	7.77	Good source	84.76	7.23	Good source

[a] Use Reference amount of "Dried texturized soy protein and soy protein isolate"; [b] Using Reference amount data.

Table 14. Protein content claim statements on sampled based on the PER method and Canadian regulations

Food	Reasonable daily intake or reference amount	Adjusted PER	Protein rating	Claim	Adjusted PH-PER	Protein rating	Claim	Adjusted OPA-PER	Protein rating	Claim	Adjusted KD-PER	Protein rating	Claim	Adjusted TAA-PER	Protein rating	Claim
Casein	20[a]	2.50	42.44	Excellent source	2.50	42.44	Excellent source	2.50	42.44	Excellent source	2.50	42.44	Excellent source	2.50	42.44	Excellent source
Nut	28	2.15	24.56	Good source	2.20	25.11	Good source	2.12	24.18	Good source	2.21	25.21	Good source	2.26	25.78	Good source
Legume A	100	1.68	102.08	Excellent source	1.38	83.92	Excellent source	1.45	87.92	Excellent source	1.46	88.84	Excellent source	1.49	90.77	Excellent source
Legume B	20[a]	1.52	21.85	Good source	1.60	23.03	Good source	1.65	23.82	Good source	1.71	24.62	Good source	1.66	23.90	Good source
Oilseed A	20	2.38	38.03	Good source	2.15	34.34	Good source	2.33	37.27	Good source	2.32	37.04	Good source	2.38	38.03	Good source
Oilseed B	20[a]	1.22	19.46	No claim	1.09	17.33	No claim	1.19	18.99	No claim	1.19	18.95	No claim	1.17	18.65	No claim
SCP A	20[a]	2.10	30.75	Good source	2.18	31.96	Good source	2.14	31.37	Good source	2.29	33.48	Good source	2.09	30.56	Good source
SCP B	14	2.13	18.17	No claim	2.12	18.09	No claim	2.15	18.35	No claim	2.28	19.43	No claim	2.12	18.08	No claim

[a] *Use Reference amount of "Dried texturized soy protein and soy protein isolate".*

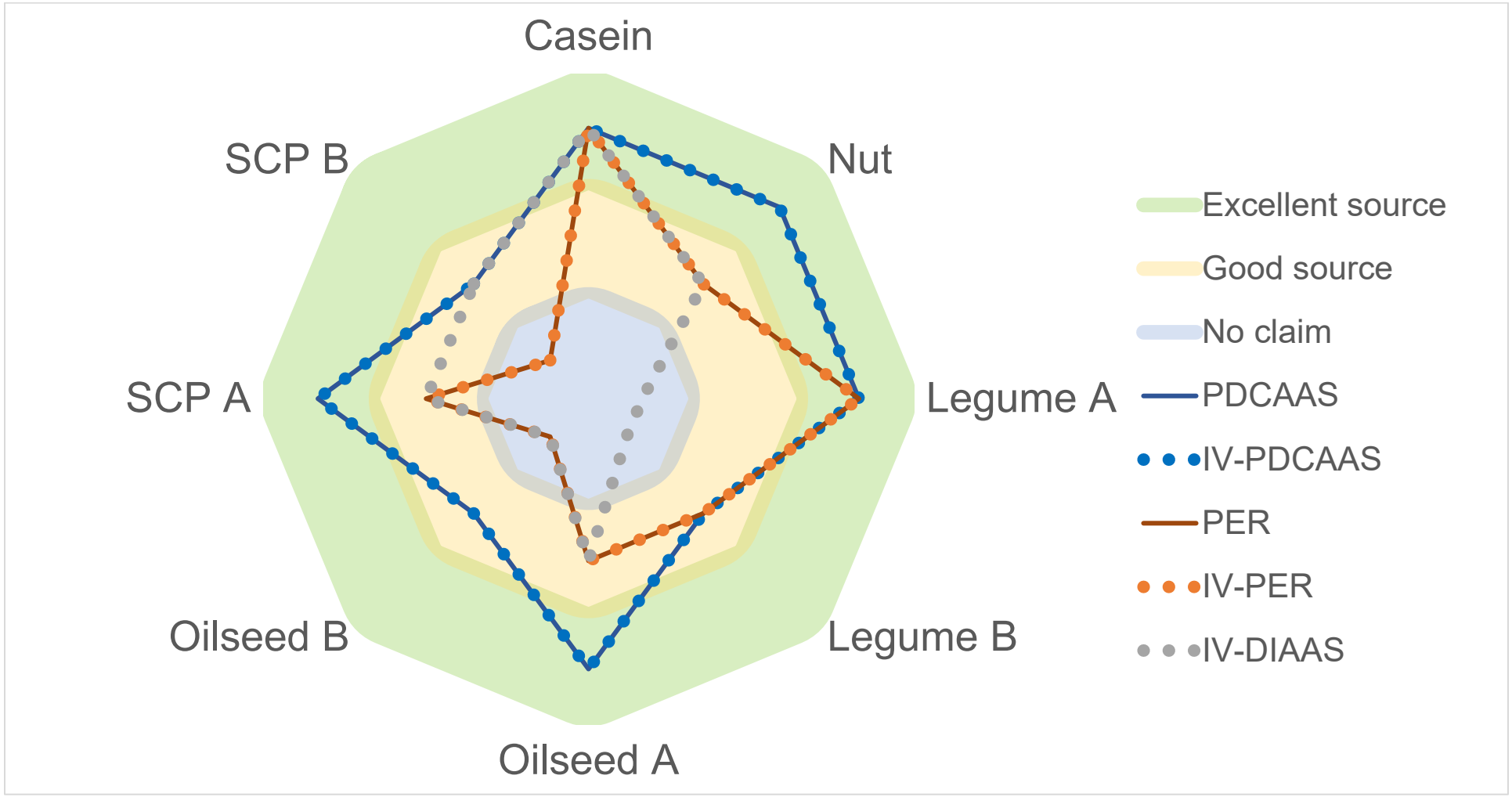


Figure 13. Summary chart of protein contents claims of all samples from *in vivo* and *in vitro* PDCAAS, PER, and DIAAS

4.5. CONCLUSION

Firstly, both models exhibited the ability to determine IV-PD and IV-PDCAAS. The IV-PD results ranged from 87.6 - 100% with <5% variation between replications. Despite a weaker relationship between in-house *in vivo* and *in vitro* results for PD, all *in vitro* tests show strong agreement with PDCAAS results. Comparing the methods, the OPA and the total AA analysis from the INFOGEST model exhibited a stronger relationship to the *in vivo* data for TPD and PDCAAS than the pH-drop model and the Kjeldahl analysis from the INFOGEST model. However, the pH-drop model demonstrated better repeatability between measurements. This might be because of the less complex nature of the pH-drop method than the INFOGEST model. However, the INFOGEST digestion model offered the possibility to analyze for IV-AAD and determine IV-DIAAS of samples. TRP, SAA, and LYS were limiting for the selected samples in this study. There are differences in terms of limiting AA between IV-PDCAAS and IV-DIAAS methods for casein, legume B, and SCP A. Compared to available *in vivo* DIAAS values from the literature, there are certain agreements with the acquired limited AA, IFG-DIAAS, and permitted protein claims.

The current study also provided more information about the digestibility and PQ of proteinaceous samples. Casein showed good characteristics as the control for *in vitro* digestion studies for PQ, with high IV-PD, IV-AAD, and IV-PQ with <5% variation across all testing methods. All samples displayed great IV-PD and IV-AAD. Oilseed B had the lowest IV-PDCAAS, IV-DIAAS and IV-PER values. Among all samples, SCP B and SCP A usually had the highest variation between measurements.

Additionally, this study aimed to compare the differences between permitted protein claims with different PQ assessments. The PDCAAS methods usually ranked tested samples higher than

the DIAAS and the PER assays. Following the proposed DIAAS method, many samples, including nut, legume A, legume B, oilseed A, oilseed B, and SCP A, only obtained lower protein content claims than when using the implementing assays in the USA and Canada. Thus, the DIAAS had the advantage of measuring PQ more accurately, but its cut-off mark of 75 for protein claims creates more challenges for applying sustainable proteins in industries and for general public food choices.

The pH-drop and the INFOGEST digestion models presented potential tools for determining IV-PD, IV-AAD, and IV-PQ. The INFOGEST model offered more options to measure the IV-PD with close relationships with the *in vivo* data. Conversely, the pH-drop model is faster with simple steps and highly repeatable measurements. More *in vivo* and *in vitro* data comparisons will further validate the *in vitro* methodologies, such as processed samples with heat or interactions with other nutrients during digestion. Moreover, selecting the suitable PQ evaluation assay can affect the final protein permitted claims. Therefore, more comparisons between protein claims by each PQ method in future studies can help us understand their impacts on public food choices.

CHAPTER 5: COMPARISON OF THE EFFECTS OF PROCESSING IN PLANT-BASED SAMPLES ON PROTEIN DIGESTIBILITY, AMINO ACID DIGESTIBILITY, AND PROTEIN QUALITY USING *IN VITRO* METHODOLOGIES

5.1. ABSTRACT

This study assessed the ability to determine *in vitro* protein and amino acid digestibility in assessing the protein quality of two *in vitro* static digestion models, pH-drop and INFOGEST 2.0. Both methods were applied in the digestion of samples with low protein quality - gelatin isolate, low digestibility - All-bran® wheat bran cereal, as well as raw and heat-treated wheat, rice, yellow pea, and chickpea. While the pH-drop model can directly determine *in vitro* protein digestibility (IV-PD) for IV-PDCAAS, the INFOGEST digestion products were analyzed by three different methods, including OPA derivatization, Kjeldahl, and individual amino acid analysis, to determine IV-PD. Only the amino acid analysis method offered the additional ability to determine *in vitro* amino acid digestibility and *in vitro* Digestible Indispensable Amino Acid Score (IV-DIAAS), the recommended method by the FAO/WHO. Assessing by the PDCAAS method, all four *in vitro* methods determined a wide range of IV-PD from 65.2% to 100% and IV-PDCAAS from 0.3 (gelatin) to 1.12 (untruncated casein). Even though there are changes after heat treatment, the improvement of IV-PDCAAS by boiling and roasting was only observed in the analysis of the INFOGEST digestion products. All IV-PDCAAS strongly correlated with *in vivo* data, with a high R^2 value from 0.92 (AA analysis method) to 0.96 (Kjeldahl method). The AA analysis of the INFOGEST digestion products added the ability to determine the IV-AAD and IV-DIAAS from 118 to 0.1. Moreover, in this study, all four *in vitro* concluded similar claims for most of the samples by the PDCAAS, the PER, and the DIAAS methods, which agreed to *in vivo* reported data. Finally, it is observed that the PDCAAS assay can give samples with higher protein content

claims than the DIAAS and the PER methods. Overall, the present data demonstrated the potential of *in vitro* digestion models in determining digestibility and protein quality, which could be applied as a screening tool for researchers, industries, and consumers.

Keywords: amino acid digestibility, DIAAS, *in vitro* digestion models, PDCAAS, protein digestibility, protein quality

5.2. INTRODUCTION

The quality of protein is defined as the ability to fulfill the amino acid requirements of consumers, influenced by factors such as the amino acid composition and the digestibility in the gastrointestinal tract (FAO, 2013). There is more than one way to measure protein quality. While the PER and the PDCAAS methods are popular in North America, the FAO/WHO has proposed the DIAAS assay, which can overcome the disadvantages of previous methods (FAO, 2013; Government of Canada - Canadian Food Inspection Agency, 2020; Joint FAO/WHO, 1991). A protein is assessed differently for each method and categorized with different protein content claims. Despite using different approaches, the protein quality is measured by animals, such as rats or swine, in all three methods. However, these bioassay studies are facing challenges in costs, time, technical issues, and ethical problems. Recognizing the importance of an alternative way to study protein quality, tremendous efforts have been invested in developing *in vitro* digestion models and suitable methodologies to measure *in vitro* protein quality.

The pH-drop model is a simple static system that digests the protein with multiple enzymes in one compartment, and allows the measurement of the digestibility based on pH changes over 10 minutes (Hsu et al., 1977). The pH-drop has been reported with the ability to predict true fecal protein digestibility (R^2=0.56-0.67) and PDCAAS (R^2 = 0.75-0.97) for various samples (Nosworthy et al., 2018; Nosworthy, Franczyk, Medina, et al., 2017; Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017). The COST Action group also developed and standardized another *in vitro* static three-stage digestion named INGOEST (Brodkorb et al., 2019; M. Minekus et al., 2014). It has been reported that the INFOGEST model can effectively digest casein, whey, and skim milk powder with comparable results from *in vivo* models (Egger et al., 2017; Sanchón et al., 2018). Although the INFOGEST has been observed as an adequate tool with similar digestion

conditions to human gastrointestinal tracts, the model must be combined with analytical methods to create a suitable workflow for quantifying protein digestibility and quality. A recent publication from Sousa et al. suggested a workflow for the INFOGEST application to determine the *in vitro* amino acid digestibility and the DIAAS value (Sousa et al., 2023). As a newly developed method, this workflow needs more contributed data for various samples and comparison with other *in vitro* digestion models.

In the scope of this thesis, chapter 4 has provided more information about the ability of both the pH-drop and the INFOGEST models to determine *in vitro* digestibility and protein quality of high-concentration protein with high digestibility samples. The correlation between in-house *in vivo* and *in vitro* protein digestibility and the PDCAAS values was significant. Continually, this study aims to assess the ability of the pH-drop and the INFOGEST models to measure the *in vitro* protein digestibility, amino acid digestibility, and protein quality of low protein concentrations and low digestible samples such as All-Bran® wheat bran cereal and gelatin. Additionally, wheat, rice, yellow peas, and chickpeas are staple foods globally, each holding significant nutritional and culinary importance across regions and cultures. Typically, these foods are not consumed in their raw state due to their tough textures, potential ANFs, and low digestibility. Instead, they are commonly subjected to heat processing methods such as boiling, roasting, or steaming before consumption. (Alonso et al., 2000; Carmody & Wrangham, 2009). Within the scope of this study, we aimed to investigate the changes in digestibility and protein quality of these foods before and after processing using *in vitro* digestion models. During heat treatments, it is reported that many changes can happen to protein depending on various factors. First, from stable native states, proteins could be reorganized at multiple levels, from losing the tertiary structure from 55^{o}C, losing the secondary structure from 70^{o}C, cleavage of disulfides bonds from 75^{o}C, to aggregation

and chemical reactions from 100ºC (Davis & Williams, 1998). Moreover, protein/AA digestibility could be affected indirectly by thermal treatment via interaction with other compounds like carbohydrates and ANFs (Drulyte & Orlien, 2019; El-Adawy, 2002). Thus, raw, boiled and roasted wheat, rice, chickpea and yellow pea were selected in this study in order to assess the potential of *in vitro* digestion models in determination of protein/AAs digestibility with various common samples as well as evaluate the effect of heat treatments on the *in vitro* digestibility and protein qualityIn additional, the *in vitro* protein digestibility from the INFOGEST was measured by three methods, including the o-phthaldialdehyde assay, the Kjeldahl method, and the total amino acid analysis, allowing comparison for the most suitable workflow of the INFOGEST digestion model. Finally, the *in vitro* protein quality expressed as the PER, the PDCAAS, and the DIAAS of all samples were compared with their permitted protein classification. This part aimed to assess not only the impact of the processing method on protein content claims but also the differences between the protein contents claims of samples when evaluated by different protein quality methods.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals

All tests were run with reverse osmosis water, except for the AA analysis using Milli-Q water. All chemicals were purchased from Sigma (Oakville, Ontario, Canada), Lipotech (Marseille, France), Santa Cruz Biotechnology (Dallas, Texas, USA), and Waters Inc. (Mississauga, Ontario, Canada).

5.3.2. Samples and processing methods

There were 14 samples and casein included in study 5. Gelatin isolate was analyzed and digested in as-is received forms from commercial suppliers in 2022. Hard whole kernel wheat,

Jasmine polished rice, whole deshelled chickpea, split yellow pea, and All-Bran® wheat bran cereal were purchased from local supermarkets (Winnipeg, MB, Canada). All-Bran® was ground into 0.7 mm size and then used as-is. The remaining samples, including wheat, rice, chickpeas, and yellow peas, were subjected to heat treatment into three categories: raw, boiled, and roasted. The roasting process was as follows: 200 g of samples as-is were spread over a large baking sheet, then baked at 395ºF for 30 minutes. Roasted samples were cooled to room temperature before grinding into 0.7 mm size. In terms of boiling treatment, rice and wheat were rinsed with water and then boiled with two parts of water. After reaching boiling point, the temperature was lowered to medium to simmer until fully cooked (20 minutes for rice; 15 minutes for wheat). The excess water in the pots was drained. Only chickpea was soaked overnight (~ 18 hours) before boiling. Chickpeas and yellow peas were rinsed and then cooked in a pot with 2 inches of water. After being brought to the boiling points, pots were simmered for 30 minutes (pulses became soft). Excess water was drained, and samples were cooled to room temperature. All boiled samples were freeze-dried and then ground to a size of 0.7 mm. All samples were stored at -20ºC for later digestion and analysis.

5.3.3. Experimental design

Figure 13 demonstrates the total experimental design. In total, 14 samples and casein were digested with the pH-drop and the INFOGEST models.

Like study 4, multiple IV-PD results were measured by the pH-drop model and the INFOGEST model with the micro-Kjeldahl method, the o-phthaldialdehyde method, and the total AA analysis, with acquired IV-PD data, the IV-PDCAAS were determined. The adjusted IV-PER values were calculated based on the IV-PDCAAS, followed by the Canadian regulatory. The AA analysis of the INFOGEST digesta allowed the measurement of IV-AAD and IV-DIAAS values.

Finally, the permitted protein content claims from each IV-PDCAAS, IV-PER, and IV-DIAAS were determined. Full details of crude protein analysis, two *in vitro* digestion models, the OPA, the Kjeldahl, and the AA analyses, were previously provided in sections 4.3.4 to 4.3.10.

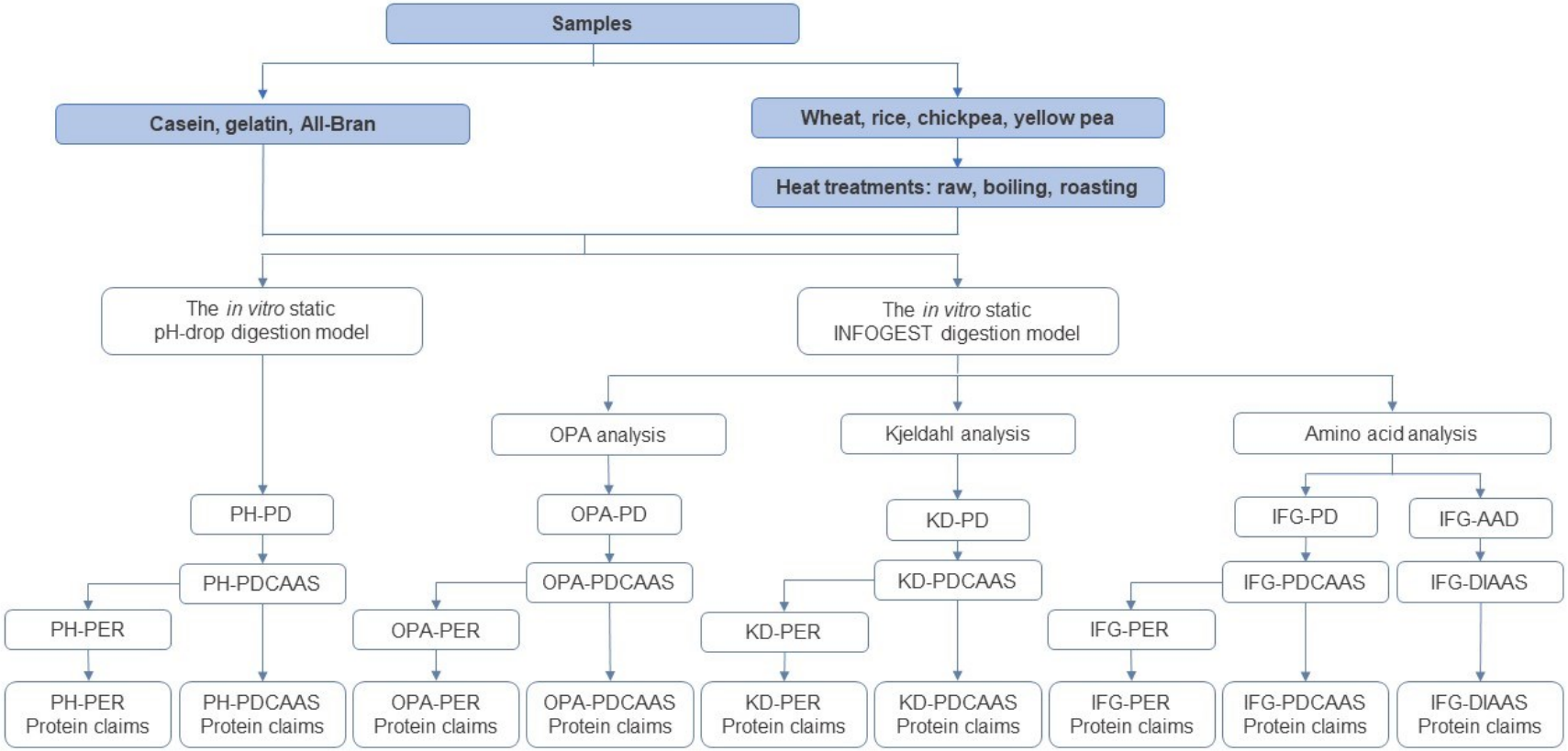


Figure 14. Study 5 experimental design

5.4. RESULTS AND DISCUSSION

5.4.1. Amino acid profile and scores in the PDCAAS method

The crude protein and AA content of all samples were presented as received in Table 15. Casein and gelatin had the highest protein contents among all samples at 84.9% and 86.3%, respectively. The All-bran sample had crude protein content similar to the raw wheat sample (16.2% vs. 16.6%), which is higher than the claimed value on the package (13.89%) (WK Kellogg Co, 2023). All unprocessed samples, including wheat (16.6%), rice (8.2%), chickpea (20.6%), and yellow pea (23.8%) samples had similar crude protein with reported information (15.4% vs. 7.04% vs 23.78% vs 21.91%) (Nosworthy, Franczyk, Medina, et al., 2017; Nosworthy, Neufeld, et al., 2017; U.S. Department of Agriculture, 2019b, 2023). Interestingly, processed cereal and pulses (boiled and roasted) samples showed slightly higher protein contents than raw samples, increasing from 0.6% to 3%. Moreover, boiled samples exhibited higher crude protein contents than roasted ones. While some studies reported decreases in protein contents after cooking due to the loss of water-soluble protein, the increases in protein contents in this study may be related to changes in the moisture contents of the samples during heating treatments and freeze-drying post-boiling process (Habiba, 2002; Nosworthy, Franczyk, Medina, et al., 2017).

From the AA profile, the AAS and limited AA were determined by the PDCAAS method of all samples presented in Table 16. Casein, all chickpea samples, raw yellow pea, and boiled yellow pea were limited in TRP. Meanwhile, the roasted yellow pea was reported to be limited in SAA. The limited AA was LYS for all wheat, rice, and All-bran samples. These results agreed with the previous reports on pulses, which are usually limited in TRP or SAA, while cereals usually lack LYS (Knight, 2000; McKevith, 2004; Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Sparvoli et al., 2015; Tiwari, Brijesh K.; Singh, 2012). Casein exhibited an excellent AAS at 1.14,

the highest among all samples. Gelatin had a significant deficiency in TRP and achieved an AAS of 0.003. The AAS of wheat samples was only higher than gelatin, ranging from 0.43 (raw wheat) to 0.44 (roasted wheat) and 0.49 (boiled wheat). Roasted treatment positively affected rice samples, increasing the AAS from 0.68 (raw rice) to 0.72 (roasted rice), while boiling treatment did not show changes in AAS. Untreated chickpeas had an AAS of 0.88, with roasting slightly increasing to 0.89, followed by boiling at 0.95. Yellow peas had an AAS at 0.82 in raw form, 0.83 in boiled form, and 0.87 in roasted form. Interestingly, the yellow pea was the only sample that showed changes in limited AA after processing, from TRP (raw) to SAA (roasted). For boiled and roasted yellow pea samples, in comparison to reported data for split yellow peas, these acquired AAS data were higher, but the limited AA information was similar (cooked split yellow pea: 0.78 – TRP; baked split yellow pea: 0.79 – SAA) (Nosworthy, Franczyk, Medina, et al., 2017).

These results indicate that processing does affect the crude protein and AA profile of all samples, leading to changes in AAS and limited AA determined by the PDCAAS method. Moreover, the effect differed depending on the sample and appeared small overall, with a maximum increase of 3% in crude protein and 0.07 in AAS between raw and boiled/roasted samples. The study included gelatin, which had a high crude protein (86.3%) yet was significantly deficient in TRP (AAS at 0.003), and All-Bran, which owns a low crude protein (16.2%) yet its AA scored at 0.72 with LYS limitation.

Table 15. Amino acid content g/100g as received of samples.

Sample	Crude protein (%)	HIS	SER	ARG	GLY	ASP	GLU	THR	ALA	PRO	CYS	LYS	TYR	MET	VAL	ILE	LEU	PHE	TRP
Casein	84.9	2.0	5.2	3.1	1.7	6.6	20.8	3.8	2.8	9.9	0.4	7.4	4.4	2.3	6.0	4.7	8.7	4.5	1.1
Wheat Raw	16.6	0.4	0.7	0.6	0.5	0.7	4.4	0.4	0.5	1.4	0.3	0.4	0.4	0.2	0.6	0.5	0.9	0.6	0.2
Wheat Boiled	18.3	0.4	0.8	0.7	0.7	0.9	5.8	0.5	0.6	1.9	0.3	0.5	0.5	0.2	0.7	0.6	1.2	0.8	0.2
Wheat Roasted	17.3	0.4	0.8	0.7	0.6	0.9	5.3	0.5	0.6	1.7	0.3	0.4	0.5	0.2	0.7	0.6	1.1	0.8	0.2
Rice Raw	8.2	0.2	0.4	0.6	0.3	0.8	1.5	0.3	0.5	0.4	0.1	0.3	0.4	0.1	0.5	0.3	0.7	0.4	0.1
Rice Boiled	9.5	0.2	0.5	0.7	0.4	0.9	1.8	0.3	0.5	0.4	0.1	0.4	0.4	0.2	0.6	0.4	0.8	0.5	0.1
Rice Roasted	9.3	0.2	0.5	0.8	0.4	1.0	1.8	0.3	0.6	0.4	0.1	0.4	0.5	0.2	0.6	0.4	0.9	0.5	0.1
Chickpea Raw	20.6	0.5	1.0	1.7	0.8	2.3	3.4	0.7	0.8	0.8	0.3	1.4	0.5	0.3	0.9	0.9	1.5	1.1	0.2
Chickpea Boiled	23.6	0.6	1.2	1.9	0.9	2.6	3.7	0.8	1.0	1.0	0.3	1.6	0.6	0.3	1.0	1.0	1.8	1.3	0.2
Chickpea Roasted	21.3	0.5	1.0	1.8	0.8	2.5	3.6	0.7	0.9	0.9	0.2	1.4	0.6	0.3	0.9	0.9	1.6	1.2	0.2
Yellowpea Raw	23.8	0.6	1.1	1.9	0.9	2.6	4.0	0.8	1.0	0.9	0.3	1.8	0.7	0.2	1.1	1.0	1.7	1.0	0.2
Yellowpea Boiled	25.8	0.6	1.2	2.0	1.0	2.9	4.3	0.9	1.1	1.0	0.3	2.0	0.8	0.3	1.2	1.1	1.9	1.2	0.2
Yellowpea Roasted	24.4	0.6	1.1	1.9	1.0	2.7	4.0	0.8	1.0	1.0	0.3	1.8	0.7	0.2	1.1	1.0	1.7	1.1	2.3
Gelatin	86.3	0.4	3.0	7.1	22.1	5.7	11.3	1.7	9.0	12.7	0.2	3.5	0.3	1.0	2.3	1.3	3.0	1.7	0.0
All Bran	16.2	0.4	0.7	1.0	0.8	1.2	3.0	0.5	0.8	0.9	0.2	0.7	0.4	0.2	0.7	0.5	1.0	0.6	0.2

ASP = Asparagine; THR = Threonine; SER = Serine; GLU = Glutamine; PRO = Proline; GLY = Glycine; ALA = Alanine; CYS = Cysteine; VAL = Valine; MET =Methionine; ILE = Isoleucine; LEU = Leucine; TYR = Tyrosine; PHE = Phenylalanine; HIS = Histidine; LYS = Lysine; ARG = Arginine; TRP = Tryptophan.

Table 16. Amino acid score of all samples

Sample	THR	VAL	SAA	ILE	LEU	AAA	HIS	LYS	TRP	AAS	Limiting AA
Casein	1.32	2.01	1.27	1.98	1.56	1.67	1.26	1.50	1.14	1.14	TRP
Wheat Raw	0.69	0.99	1.29	1.04	0.85	0.95	1.29	0.43	1.13	0.43	LYS
Wheat Boiled	0.80	1.16	1.25	1.22	1.00	1.12	1.22	0.49	1.03	0.49	LYS
Wheat Roasted	0.78	1.15	1.24	1.20	1.00	1.13	1.16	0.44	1.03	0.44	LYS
Rice Raw	0.99	1.66	1.29	1.51	1.28	1.54	1.12	0.68	1.18	0.68	LYS
Rice Boiled	1.00	1.70	1.34	1.50	1.30	1.50	1.18	0.68	1.20	0.68	LYS
Rice Roasted	1.07	1.82	1.34	1.61	1.40	1.63	1.15	0.72	1.14	0.72	LYS
Chickpea Raw	1.01	1.21	1.09	1.48	1.08	1.22	1.39	1.20	0.88	0.88	TRP
Chickpea Boiled	1.02	1.26	1.06	1.55	1.14	1.26	1.29	1.17	0.95	0.95	TRP
Chickpea Roasted	1.00	1.25	1.07	1.52	1.12	1.29	1.34	1.17	0.89	0.89	TRP
Yellowpea Raw	1.01	1.26	0.84	1.45	1.05	1.15	1.29	1.28	0.82	0.82	TRP
Yellowpea Boiled	1.05	1.33	0.89	1.51	1.11	1.22	1.32	1.32	0.83	0.83	TRP
Yellowpea Roasted	1.01	1.28	0.87	1.46	1.07	1.16	1.27	1.26	8.41	0.87	SAA
Gelatin	0.59	0.76	0.56	0.55	0.53	0.38	0.26	0.70	0.003	0.003	TRP
All Bran	0.97	1.31	1.00	1.13	0.94	0.98	1.25	0.72	1.16	0.72	LYS

THR = Threonine; VAL = Valine; SAA = Methionine and Cysteine; ILE = Isoleucine; LEU = Leucine; AAA = Phenylalanine and Tyrosine; HIS = Histidine; LYS = Lysine; TRP = Tryptophan; AAS = amino acid score.

5.4.2. The *in vitro* protein digestibility

The protein digestibility measured by four *in vitro* methods for all samples and collected true protein digestibility data from literature was shown in Table 17. Raw and heat-treated wheat samples demonstrated high IV-PD (82-100%). The IV-PD increased from 0.5-7.1 for boiling treatment and 0.7-12.5 for roasting treatment compared to raw wheat. Among four *in vitro* methods, the PH-PD displayed the slightest change after being processed with heat, while the Kjeldahl analysis of the INFOGEST digesta showed the biggest increases; however, no significant difference was observed. Additionally, the IV-PD measured by four different methods were statistically similar to each other as well as to the reported true protein digestibility for raw wheat (91.9%) and boiled wheat (92.6%) (Nosworthy et al., 2023). While there is no difference in IV-PD measured in boiled and roasted rice samples, the PH-PD of raw rice is significantly higher than OPA-PD, KD-PA, and IFG-PD values. Only the KD-PD and IGF-PD of boiled rice exhibited a statistical increase compared to raw rice. A study using the pH-drop model also reported that various boiling methods improved the IV-PD, which could be explained by protein denaturation and the destroying of presenting anti-nutritional factors (Sagum & Arcot, 2000). All four IV-PD of boiled rice were higher than the reported data for cooked rice (86.6%) (Rutherfurd et al., 2015). In terms of pulse samples, significant differences in values of IV-PD were shown for roasted chickpea and raw yellow pea samples. Improvements in PD were displayed in IFG-PD of chickpea, OPA-PD, and KD-PD of yellow pea samples. Previously, *in vivo* studies showed lower TPD of both chickpeas (87.23% – boiled, 84.63% – roasted) and yellow peas (89.04% - boiled, 86.84% - roasted) (Franczyk, 2018; Nosworthy, Franczyk, Medina, et al., 2017). Another study in 2002 reported a significant improvement in IV-PD of chickpeas after cooking treatment from 83.61% to 88.52%, with a suggestion that it is a result of protein denaturation and reduction of trypsin

inhibitor, tannins and phytic acids (El-Adawy, 2002). In terms of protein isolate samples, both casein and gelatin displayed high IV-PD, at 93.9-98.6% and 91.7-98.4%, respectively, and in agreement with the expected high TPD value of 99.3% for casein (Nosworthy et al., 2023). The All-bran samples exhibited significantly different IV-PD measured by four *in vitro* methods, where PH-PD (91.4%) and IFG-PD (100%) were higher than OPA-PD (79.2%) and KD-PD (71.9%), as well as report value from *in vivo* study (73%) (Sousa et al., 2023). It is also displayed that the PH-PD values were reported in a smaller range (89.2 - 93.6%) than IV-PD values from INFOGEST models, which were 65.2 - 96.9% for OPA method, 66.4 – 98.6% for Kjeldahl method and 73.4 - 100% for AA analysis method.

The changes in IV-PD after processing can be explained by the structural protein modification during heat treatments, and leading to solubility alteration (Noorbakhsh et al., 2010; Schmitt et al., 2010; Tornberg, 2005). It is reported that heat can denature proteins by losing their tertiary structure, unfolding processes, uncoiling, changing the protein α-helix and β-sheet ratio, and creating new links to react with sugars, leading to protein digestibility changes after heat processing (Davis & Williams, 1998; P. Yu, 2007; T. Yu et al., 2017). While protein digestibility enhancement could occur due to the loss of high structure and unfolding process, decreasing protein digestibility can be explained by protein aggregation or reducing accessibility sites for proteases (Carbonaro et al., 1997). It is agreed that all proteins are not affected similarly by heat; the changes depend on factors such as temperature, duration of heat exposure, native structure, and interaction with other compounds (Davis & Williams, 1998; Drulyte & Orlien, 2019; Zia-ur-Rehman; & Salariya, 2005). Moreover, many studies have reported that different processing methods will affect the anti-nutritional contents of pulses, thus altering the digestibility of foods (Shi et al., 2018; Zhou et al., 2024).

The relationship of the produced IV-PD data with available TPD results was displayed in Figure 15A-15D. It is noticeable that the coefficient of determination for all *in vitro* methods was small (0.013 - 0.474) and significantly smaller than the R^2 of IV-PD and TPD in study 4. One of the possible reasons was that the TPD in study 5 was measured with the same samples for the *in vitro* models, in addition to the different thermally treatment methods that were applied. Among four *in vitro* methods, the protein digestibility equation for the Kjeldahl method $R^2 = 0.474$ and OPA method $R^2= 0.277$ had the strongest relationship with the TPD than the AA analysis method $R^2 = 0.013$ and the pH-drop analysis $R^2 = 0.034$.

The agreements between the four *in vitro* methods were further analyzed with the Bland-Altman analysis (Figure 16A-16D) and Pearson correlation coefficient analysis matrix (Figure 17). All *in vitro* methods displayed from 2-3 points out of the 95% agreement with the TPD values. The OPA-PD and KD-PD exhibited better agreement with other methods, while the PH-PD and IFG-PD values had lower r values.

In conclusion, four *in vitro* methods determined a wide range of IV-PD from 65.2%-100%. No statistical differences were observed between IV-PD measured by pH-drop and INFOGEST model, except for raw rice, roasted chickpea, raw yellow pea, and All-Bran samples. The coefficient of variation of the pH-drop method showed better repeatability results than analysis by the INFOGEST methods.

Table 17. Summary of digestibility for all proteins, including literature data, the pH-drop and INFOGEST *in vitro* protein digestibility measured with the OPA method, Kjeldahl method, and AA analysis.

Sample	Literature TPD	PH-PD	STD	CV	OPA-PD	STD	CV	KD-PD	STD	CV	IFG-PD	STD	CV
Casein	99.3[1]	93.3[a]	0.2	0.3	96.9[a]	2.5	2.6	98.6[a]	2.1	2.1	98.4[a]	2.8	2.8
Wheat Raw	91.9[1]	89.7[a-x]	0.2	0.2	82.0[a-x]	5.1	10.8	83.9[a-x]	3.7	4.4	92.1[a-x]	13.8	15.0
Wheat Boiled	92.6[1]	90.2[a-x]	0.2	0.2	86.1[a-x]	2.0	2.4	88.3[a-x]	1.6	1.8	99.2[a-x]	1.3	1.4
Wheat Roasted	n/a	90.4[a-x]	0.0	0.1	93.4[a-x]	2.9	3.1	96.4[a-x]	3.1	3.2	100.0[a-x]	0.0	0.0
Rice Raw	n/a	90.4[a-x]	0.1	0.1	72.7[b-x]	2.9	3.9	73.7[b-x]	5.3	7.2	73.4[b-x]	3.8	5.2
Rice Boiled	86.6[2]	90.4[a-x]	0.1	0.1	86.7[a-x]	0.8	0.9	92.9[a-y]	6.5	7.0	94.6[a-y]	7.5	7.9
Rice Roasted	n/a	90.5[a-x]	0.1	0.1	84.6[a-x]	2.4	2.9	86.1[a-xy]	3.6	4.1	84.0[a-xy]	9.4	11.1
Chickpea Raw	n/a	89.3[a-x]	0.2	0.2	81.1[a-x]	5.2	6.4	83.5[a-x]	3.2	3.9	80.0[a-x]	7.0	8.7
Chickpea Boiled	87.2[3]	91.2[a-x]	0.1	0.1	86.9[a-x]	6.3	7.3	83.9[a-x]	6.0	7.2	83.3[a-xy]	3.0	3.6
Chickpea Roasted	84.6[3]	90.1[a-x]	0.1	0.1	85.4[ab-x]	5.7	6.7	87.5[abc-x]	3.8	4.3	100.0[ac-z]	0.0	0.0
Yellowpea Raw	n/a	89.2[a-x]	0.2	0.2	65.2[b-x]	8.3	12.7	66.4[bc-x]	0.9	1.3	84.4[a-x]	1.8	2.1
Yellowpea Boiled	89.0[4]	90.2[a-x]	0.2	0.2	82.4[a-y]	3.1	3.8	87.8[a-y]	0.7	0.8	94.0[a-x]	4.8	5.1
Yellowpea Roasted	86.8[4]	91.2[a-x]	0.1	0.1	89.5[a-y]	8.3	9.2	89.7[a-y]	2.7	3.0	86.1[a-x]	1.5	1.7
Gelatin	n/a	93.6[a]	0.2	0.2	91.7[a]	1.0	1.1	98.4[a]	1.6	1.6	92.7[a]	1.8	1.9
All Bran	73.0[5]	91.4[a]	0.1	0.1	79.2[ab]	9.1	11.5	71.9[bc]	2.3	3.2	100.0[a]	0.0	0.0

TPD = True protein digestibility; PH-PD = pH-drop in vitro protein digestibility; OPA-PD = OPA in vitro protein digestibility; KD-PD = Kjeldahl in vitro protein digestibility; IFG-PD = total amino acid in vitro protein digestibility; STD = Standard deviation; CV% = Coefficient of variation;

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

[a,b,c] *Means followed by different letters indicate a significant difference with the same sample measured by different methods ($p < 0.05$);*

[x,y,z] *Means followed by different letters indicate a significant difference with the raw and boiled/roasted wheat/rice/chickpea/yellow pea samples measured by the same method ($p < 0.05$);*

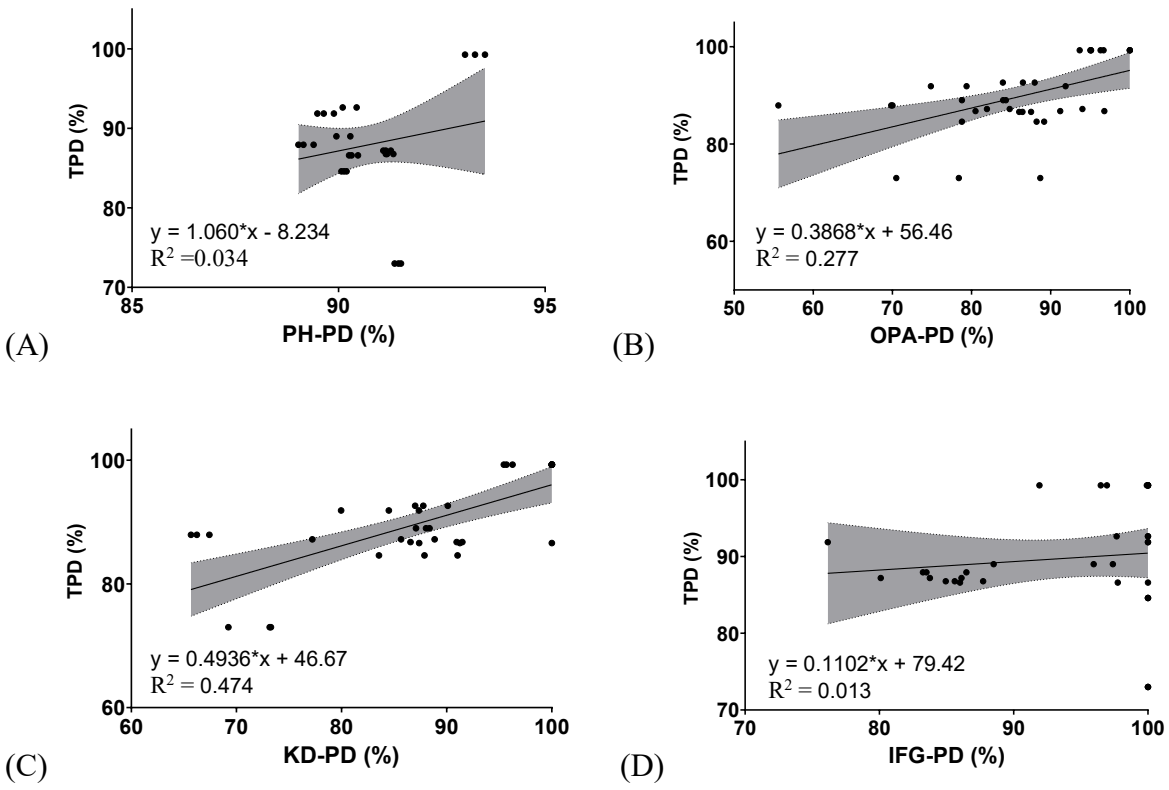


Figure 15. Relationship between literature *in vivo* true protein digestibility (TPD %) data and *in vitro* protein digestibility by (A) pH-drop digestion model (PH-PD %), (B) OPA method (OPA-PD %), (C) Kjeldahl method (KD-PD %), (D) total amino acid analysis (IFG-PD %) from INFOGEST digestion model, and including the regression line (black line), equation and coefficient of determination (R^2) in addition to the confidence (dotted line –gray area).

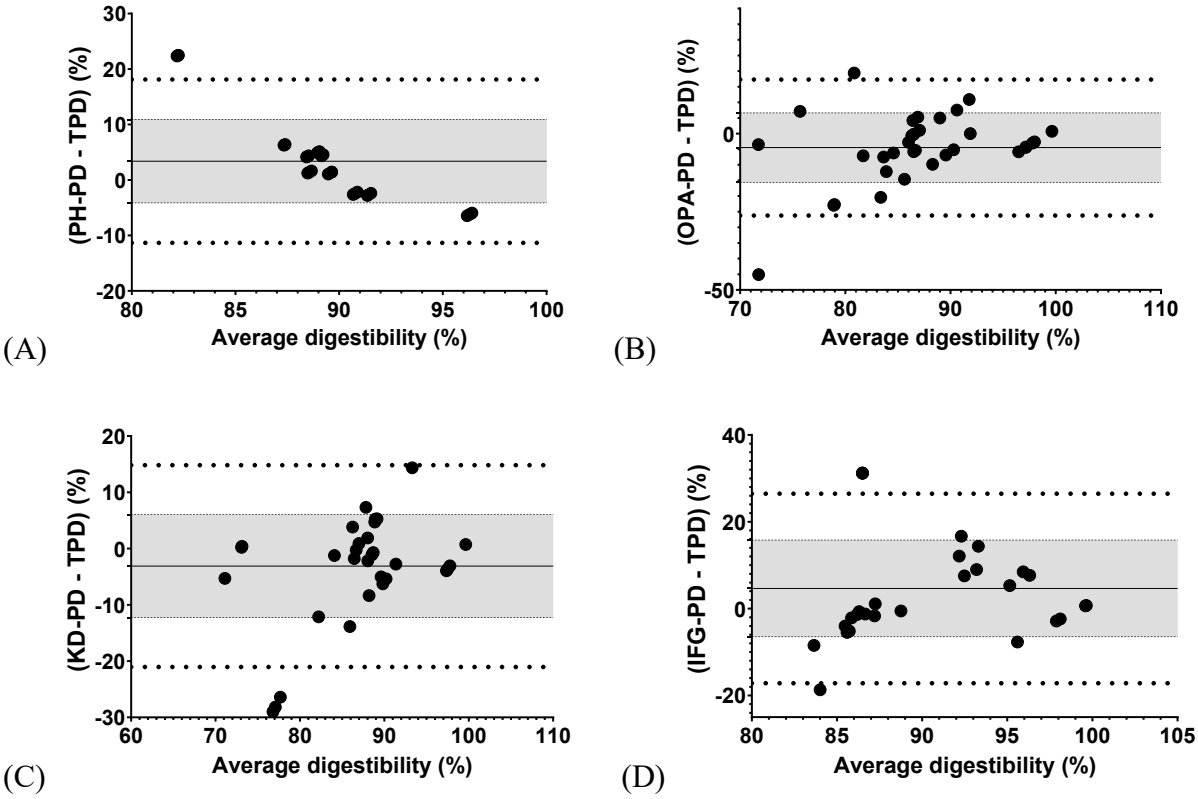


Figure 16. Bland-Altman for digestibility measures between the average and the percentage difference of the literature true protein digestibility and (A) pH-drop digestion model (PH-PD %), (B) OPA method (OPA-PD %), (C) Kjeldahl method (KD-PD %), (D) total amino acid analysis (IFG-PD %) from INFOGEST digestion model, with upper and lower limits of 95% agreement (dotted line), confidence line (dashed line), confidence area (gray area) in addition to bias (solid line).

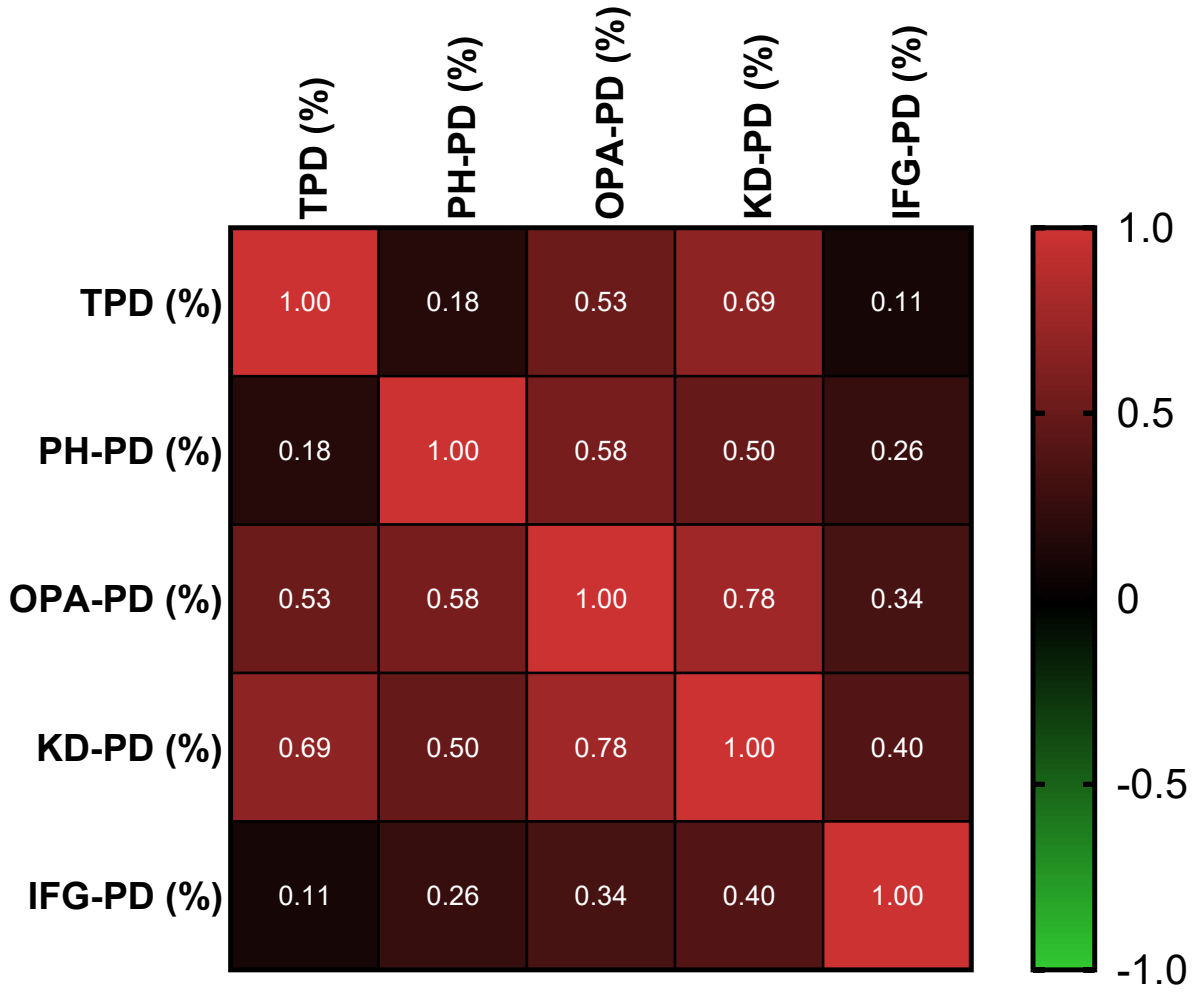


Figure 17. Correlation matrix between literature true protein digestibility (TPD %) and *in vitro* protein digestibility by pH-drop digestion model (PH-PD %), OPA method (OPA-PD %), Kjeldahl method (KD-PD %), and total amino acid analysis (IFG-PD %) from INFOGEST digestion model

5.4.3. The *in vitro* Protein Digestibility Corrected Amino Acid Score

As displayed in Table 18, the limited AA was similar to all samples between the *in vitro* test and reported data in the literature except for casein. The first limiting AA for cereal samples was LYS, while TRP was limiting in pulse samples. Yellow pea samples were limited in TRP in raw form and then changed to SAA after being roasted. The AAS measured with the PDCAAS method in the *in vitro* trial was close to the literature data. Similar trends to the IV-PD were observed in assessing IV-PDCAAS, as shown in Table 18. The IV-PDCAAS ranged from a low of 0.3 (gelatin) to a high of 112.1 (untruncated casein). Boiling increased the IV-PDCAAS to the highest for wheat (5.7-9.1%) and chickpea (6.3-11.2%), while rice and yellow pea benefited the most from roasting, an increase of 4.1-12.3% and 5.1-23.9% respectively. The significant improvement of IV-PDCAAS by thermal treatment was only observed in the INFOGEST digestion product analysis. Almost all samples had similar IV-PDCAAS measured by four *in vitro* methods, except for roasted chickpea, raw yellow pea and All-bran samples. IV-PDCAAS raw wheat samples, OPA-PDCAAS of boiled rice, OPA-PDCAAS of boiled yellow pea, KD-PDCAAS of All-bran, as well as PH-PDCAAS, OPA-PDCAAS, and KD-PDCAAS values of roasted chickpea were lower than previously reported (Franczyk, 2018; Nosworthy et al., 2023; Nosworthy, Franczyk, Medina, et al., 2017; Rutherfurd et al., 2015; Sousa et al., 2023).

The acquired IV-PDCAAS results displayed strong agreement with the reported *in vivo* PDCAAS data with all samples, exhibited by linear regression test (Figure 18), the Bland-Altman analysis (Figure 19A-19D) and Pearson correlation coefficient analysis matrix (Figure 20). All four *in vitro* methods reported high R^2 values relative to PDCAAS, from 0.9191 with the AA analysis method to 0.9640 with the Kjeldahl method. Between two *in vitro* models, the pH drop exhibited a stronger agreement with the PDCAAS. Both the AA analysis and the Kjeldahl method

only owned one point out of the 95% agreement range, while the OPA method exhibited three points out of the limitation. All *in vitro* methods displayed strong agreements with each other, with high r values higher than 0.96.

Table 18. Summary of amino acid score, limiting AA, and PDCAAS from literature and IV-PDCAAS for all proteins measured from four *in vitro* methodologies from the pH-drop digestion model, and INFOGEST digestion model with the OPA method, Kjeldahl method, and AA analysis.

*AAS = Amino acid score; Limited AA = Limited amino acid; PDCAAS = Protein Digestible Corrected**;** PH-PDCAAS = PDCAAS value measured with the pH-drop model; OPA-PDCAAS = PDCAAS*

Sample	**Literature *in vivo* data**			**Measured with *in vitro* test**													
	AAS	**LAA**	**PDCAAS**	**AAS**	**LAA**	**PH - PDCAAS**	**STD**	**CV (%)**	**OPA - PDCAAS**	**STD**	**CV (%)**	**KD - PDCAAS**	**STD**	**CV (%)**	**IFG - PDCAAS**	**STD**	**CV (%)**
Casein	0.98[1]	SAA[1]	100.0[1-a]	1.14	TRP	100.0[a]	0.0	0.0	100.0[a]	0.0	0.0	100.0[a]	0.0	0.0	100.0[a]	0.0	0.0
Casein *untruncated*	0.98[1]	SAA[1]	104.9[1-a]	1.14	TRP	106.3[a]	0.6	0.5	110.3[a]	2.9	2.6	112.4[a]	2.4	2.1	112.1[a]	3.2	2.8
Wheat Raw	0.44[1]	LYS[1]	40.0[1-a]	0.43	LYS	38.9[a-x]	0.1	0.2	35.6[a-x]	3.8	10.8	36.4[a-x]	1.6	4.4	39.9[a-x]	6.0	15.0
Wheat Boiled	0.42[1]	LYS[1]	38.8[1-a]	0.49	LYS	44.6[a-x]	0.1	0.2	42.6[a-y]	1.0	2.4	43.6[a-y]	0.8	1.8	49.0[a-y]	0.7	1.4
Wheat Roasted	n/a	n/a	n/a	0.44	LYS	39.6[a-x]	0.0	0.1	41.0[a-x]	1.3	3.1	42.3[a-x]	1.4	3.2	43.9[a-x]	0.0	0.0
Rice Raw	n/a	n/a	n/a	0.68	LYS	61.4[a-x]	0.1	0.1	49.4[b-x]	1.9	3.9	50.1[b-x]	3.6	7.2	49.9[b-x]	2.6	5.2
Rice Boiled	0.56[2]	LYS[2]	61.6[2-a]	0.68	LYS	61.8[a-x]	0.1	0.1	59.3[a-y]	0.5	0.9	63.6[a-y]	4.4	7.0	64.7[a-y]	5.1	7.9
Rice Roasted	n/a	n/a	n/a	0.72	LYS	65.5[a-x]	0.1	0.1	61.3[a-y]	1.8	2.9	62.4[a-y]	2.6	4.1	60.8[a-y]	6.8	11.1

value measured with the OPA method; KD-PDCAAS = PDCAAS value measured with the Kjeldahl method; IFG-PDCAAS = PDCAAS value measured with the total amino acid analysis; STD = Standard deviation; CV% = Coefficient of variation; TRP = Tryptophan; LYS = Lysine; SAA = Methionine and Cysteine.

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

[a,b,c] *Means followed by different letters indicate a significant difference with the same sample measured by different methods ($p < 0.05$);*

[x,y,z] *Means followed by different letters indicate a significant difference with the raw and boiled/roasted wheat/rice/chickpea/yellow pea samples measured by the same method ($p < 0.05$);*

Table 18. Summary of amino acid score, limiting AA, and PDCAAS from literature and IV-PDCAAS for all proteins measured from four *in vitro* methodologies from the pH-drop digestion model, and INFOGEST digestion model with the OPA method, Kjeldahl method, and AA analysis (continue)

Sample	Literature *in vivo* data			Measured with *in vitro* test													
	AAS	LAA	PDCAAS	AAS	LAA	PH - PDCAAS	STD	CV (%)	OPA - PDCAAS	STD	CV (%)	KD - PDCAAS	STD	CV (%)	IFG - PDCAAS	STD	CV (%)
Chickpea Raw	n/a	n/a	n/a	0.88	TRP	78.7^{a-x}	0.2	0.2	71.4^{a-x}	4.6	6.4	73.5^{a-x}	2.8	3.9	70.5^{a-x}	6.2	8.7
Chickpea Boiled	0.86[3]	TRP[3]	75.2^{3-a}	0.95	TRP	86.7^{a-x}	0.1	0.1	82.6^{a-xy}	6.0	7.3	79.8^{a-x}	5.7	7.2	79.2^{a-xy}	2.9	3.6
Chickpea Roasted	0.95[3]	TRP	80.0^{3-a}	0.89	TRP	79.9^{a-x}	0.1	0.1	75.7^{ab-xy}	5.1	6.7	77.6^{abc-x}	3.3	4.3	88.7^{a-y}	0.0	0.0
Yellowpea Raw	n/a	n/a	n/a	0.82	TRP	73.5^{a-x}	0.2	0.2	53.7^{b-x}	6.8	12.7	54.7^{bc-x}	0.7	1.3	69.5^{a-x}	1.5	2.1
Yellowpea Boiled	0.78[4]	TRP[4]	69.2^{4-a}	0.83	TRP	75.0^{a-x}	0.2	0.2	68.5^{a-y}	2.6	3.8	73.1^{a-y}	0.6	0.8	78.2^{a-xy}	4.0	5.1
Yellowpea Roasted	0.79[4]	SAA[4]	68.9^{4-a}	0.87	SAA	79.1^{a-x}	0.1	0.1	77.6^{a-y}	7.2	9.2	77.7^{a-y}	2.3	3.0	74.6^{a-xy}	1.3	1.7
Gelatin	n/a	n/a	n/a	0.00	TRP	0.3^{a}	0.0	0.2	0.3^{a}	0.0	1.1	0.3^{a}	0.0	1.6	0.3^{a}	0.0	1.9
All Bran	0.48[5]	LYS[5]	52.5^{5-a}	0.72	LYS	65.7^{ab}	0.1	0.1	56.9^{abc}	6.6	11.5	51.6^{ac}	1.7	3.2	71.8^{b}	0.0	0.0

*AAS = Amino acid score; Limited AA = Limiting amino acid; PDCAAS = Protein Digestible Corrected**;** PH-PDCAAS = PDCAAS value measured with the pH-drop model; OPA-PDCAAS = PDCAAS value measured with the OPA method; KD-PDCAAS = PDCAAS value measured with the Kjeldahl method; IFG-PDCAAS = PDCAAS value measured with the total amino acid analysis; STD = Standard deviation; CV% = Coefficient of variation; TRP = Tryptophan; LYS = Lysine; SAA = Methionine and Cysteine.*

[1] (Nosworthy et al., 2023); *[2]* (Rutherfurd et al., 2015)*; [3]* (Franczyk, 2018)*; [4]* (Nosworthy, Franczyk, Medina, et al., 2017)*; [5]* (Sousa et al., 2023)*;*

[a,b,c] Means followed by different letters indicate a significant difference with the same sample measured by different methods ($p < 0.05$);

[x,y,z] Means followed by different letters indicate a significant difference with the raw and boiled/roasted wheat/rice/chickpea/yellow pea samples measured by the same method ($p < 0.05$);

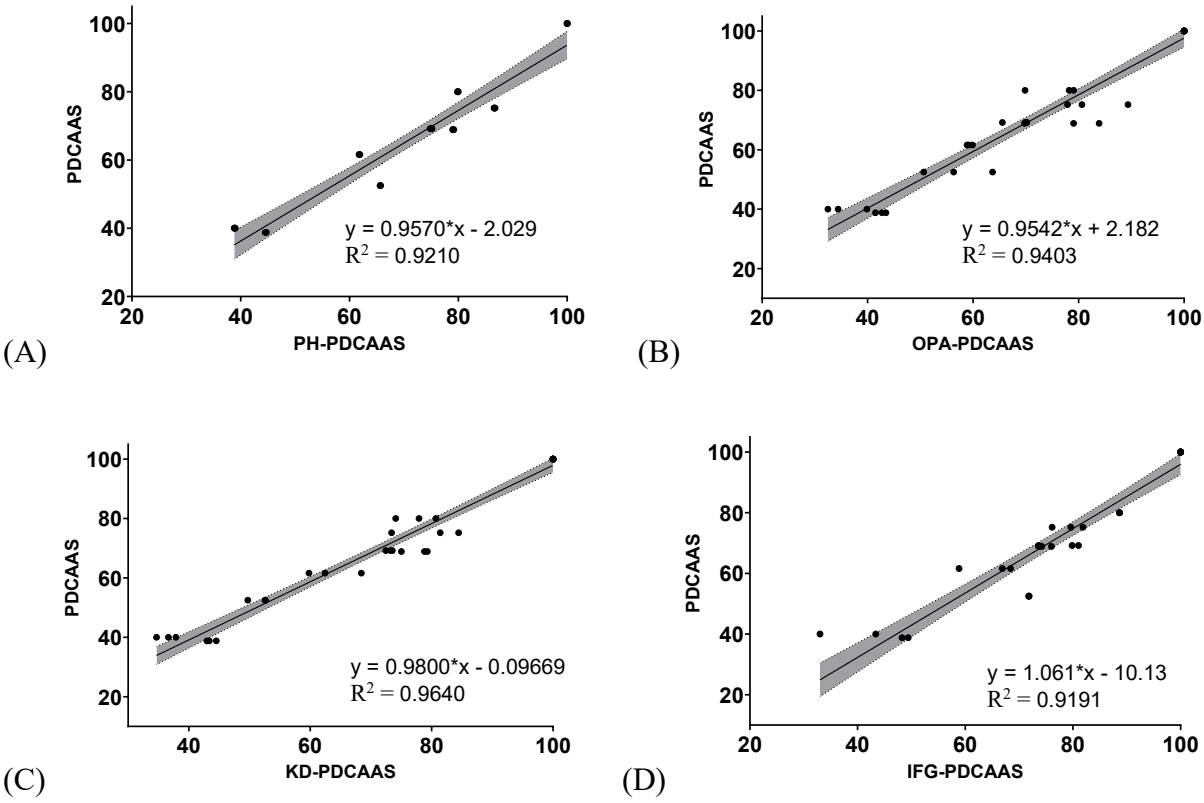


Figure 18. Relationship between literature *in vivo* PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model, and including the regression line (black line), equation and coefficient of determination (R^2) in addition to the confidence (dotted line –gray area).

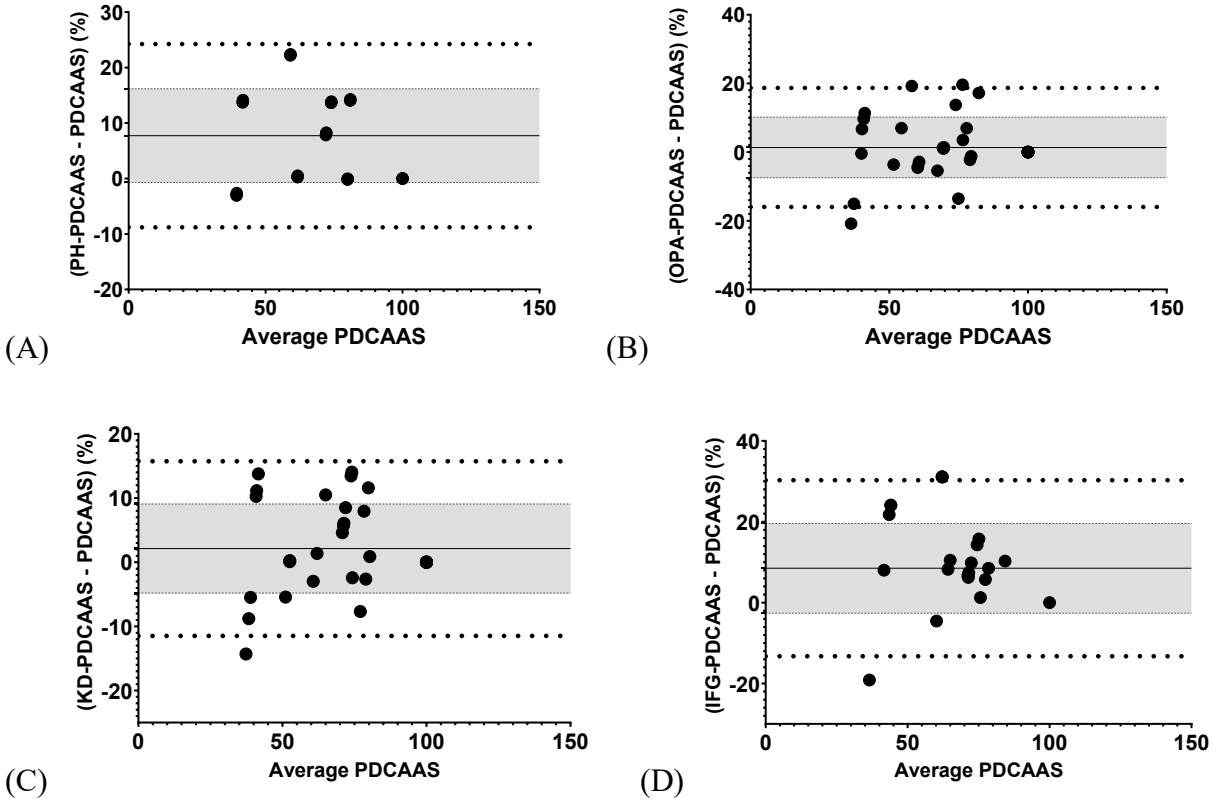


Figure 19. Bland-Altman for digestibility measures between the average and the percentage difference of literature *in vivo* PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model, with upper and lower limits of 95% agreement (dotted line), confidence line (dashed line), confidence area (gray area) in addition to bias (solid line).

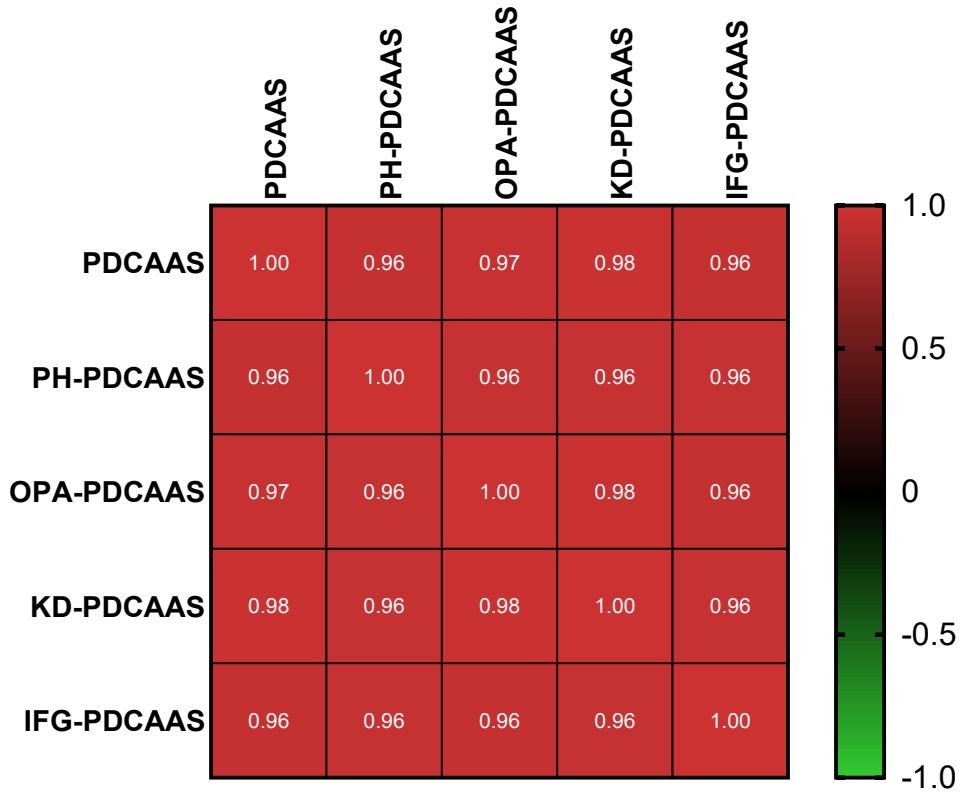


Figure 20. Correlation matrix between literature PDCAAS data and *in vitro* results measured by (A) pH-drop digestion model (PH-PDCAAS), (B) OPA method (OPA-PDCAAS), (C) Kjeldahl method (KD-PDCAAS), (D) total amino acid analysis (IFG-PDCAAS) from INFOGEST digestion model.

5.4.4. The *in vitro* amino acid digestibility and the Digestible Indispensable Amino Acid Score

The digestibility of individual IAAs determined with AA analysis of the INFOGEST digesta was summarized in Table 19. All IAAs of casein displayed high digestibility, with the lowest 93.1% of ILE. On the contrary, gelatin exhibited a relatively low digestibility, as TRP had the smallest digestibility percentage at 0.1%. The All-bran sample had a wide range of digestibility among all IAAs, with the highest of 62.1% (LEU) and the lowest of 11.4% (TRP). TRP was the IAA with the lowest digestibility in all cereal and pulses samples, while LEU or AAA reported the highest AAD. Post-thermal treatment, not all IAAs displayed an increase in digestibility, most commonly in HIS, TRP, SAA, and AAA.

Table 20 displays the summary of DIAAR, IV-DIAAS, limited AA, and the classification of protein content claims for all samples according to the IV-DIAAS method. Casein was the only sample categorized as an excellent source of protein, with IV-DIAAS at 118 and the limitation in SAA. The limitation in TRP of gelatin was exhibited strongly with IV-DIAAS only at 0.1. Meanwhile, the all-bran wheat-based cereal sample showed a LYS deficiency with IV-DIAAS at 22.3. Other cereal samples, including wheat and rice, also displayed limitations in LYS. After cooking, wheat samples showed an improvement of IV-DIAAS from 43.1 (raw) to 47.8 (boiling) or 43.6 (roasting). In the case of rice, the IV-DIAAS of raw rice (61.7) was increased by boiling (62.3) but decreased by roasting (48.4). A study about grains with rats reported similar limiting AA (LYS) but lower DIAAS values for cooked polished rice (37) and cooked whole wheat (20) (Han et al., 2019).

Similarly, chickpea samples experienced the sample pattern with a rise by boiling (+10.5%) and a drop by roasting (-9.9%) in IV-DIAAS. Only raw and boiled chickpeas were classified as

high-quality protein, while casein was the only excellent source, and all other samples were not qualified for any protein content claims. A change of limiting AA was also reported in chickpea samples from VAL in raw to LYS in boiled and SAA in roasted form. Meanwhile, Han et al., reported that steamed chickpeas had a DIAAS value of 67 with a limitation in LYS on growing pigs (Han et al., 2020). Yellow peas also showed limited TRP in raw form and then changed to SAA after boiling and LYS after roasting. The IV-DIAAS of yellow peas was improved from 67.3 (raw) to 71.6 (boiled) and 68.5 (roasted). A study about raw yellow yea on growing pigs reported the same limiting AA (TRP) but with a much lower DIAAS value at 27.27 (Cargo-Froom et al., 2023).

Table 19. Summary of amino acid digestibility [value ± standard deviation (CV%)] from INFOGEST digesta

Sample	Amino acid digestibility (%)								
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA
Casein	100 ± 0 (0)	96 ± 5.3 (5.6)	98.1 ± 2.5 (2.6)	97.9 ± 3 (3)	93.1 ± 5.8 (6.2)	97.1 ± 4 (4.1)	96.9 ± 4.8 (5)	100 ± 0 (0)	98.8 ± 1.8 (1.8)
Wheat Raw	21.5 ± 0.7 (3)	23.9 ± 4.9 (20.5)	24.6 ± 5.2 (21)	35.2 ± 7.1 (20.2)	31 ± 3.7 (12)	59 ± 7.7 (13)	11.9 ± 0.6 (4.8)	28.3 ± 0.8 (2.8)	55.7 ± 20.7 (37.2)
Wheat Boiled	19.7 ± 1.3 (6.3)	25.9 ± 0.3 (1)	27.3 ± 0.3 (0.9)	38 ± 1.5 (3.8)	31.2 ± 1.5 (4.6)	62.1 ± 2.2 (3.6)	8.3 ± 1.5 (17.2)	27.2 ± 1.4 (5)	67.6 ± 0 (0)
Wheat Roasted	22 ± 0 (0)	26 ± 0 (0)	24.9 ± 0 (0)	39.4 ± 0 (0)	32.9 ± 0 (0)	64.2 ± 0 (0)	9.5 ± 1.7 (17.1)	28.5 ± 1.8 (6.3)	69.3 ± 0 (0)
Rice Raw	21.5 ± 0.7 (3)	23.9 ± 4.9 (20.5)	24.6 ± 5.2 (21)	35.2 ± 7.1 (20.2)	31 ± 3.7 (12)	59 ± 7.7 (13)	11.9 ± 0.6 (4.8)	28.3 ± 0.8 (2.8)	55.7 ± 20.7 (37.2)
Rice Boiled	22.5 ± 0 (0)	30.4 ± 0.4 (1.1)	35.6 ± 0 (0)	50.3 ± 4.3 (8.5)	37 ± 1.2 (3.1)	62.6 ± 10 (16)	13.2 ± 0 (0)	29.8 ± 1 (3.1)	53.4 ± 8.7 (16.4)
Rice Roasted	21.5 ± 0.6 (2.6)	26 ± 0.8 (3)	27.6 ± 2.2 (7.7)	50 ± 3.4 (6.8)	32.9 ± 2.5 (7.5)	71.9 ± 1.9 (2.6)	10.2 ± 3.1 (30.3)	33.5 ± 0 (0)	77.7 ± 4.8 (6.1)
Chickpea Raw	25.3 ± 2 (7.6)	27.9 ± 2.7 (9.6)	47.2 ± 5.4 (11.5)	33.1 ± 3.9 (11.7)	30.5 ± 4.2 (13.8)	60.3 ± 3.9 (6.4)	6.9 ± 2.5 (35.4)	23 ± 0.5 (2.2)	59.3 ± 9 (15.2)
Chickpea Boiled	24.3 ± 0.7 (2.6)	27.8 ± 0.9 (3)	50.1 ± 3.9 (7.7)	38.8 ± 2.6 (6.8)	35.4 ± 2.7 (7.5)	65.7 ± 1.7 (2.6)	7.5 ± 2.3 (30.3)	26.5 ± 0 (0)	67.1 ± 4.1 (6.1)
Chickpea Roasted	20.3 ± 2.5 (12.2)	34.2 ± 0 (0)	66.5 ± 2.5 (3.8)	43.8 ± 0 (0)	42.7 ± 0 (0)	73.7 ± 0.1 (0.1)	8.7 ± 1.6 (17.6)	18.2 ± 4.4 (24.2)	81.5 ± 0 (0)

HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine

Table 19. Summary of amino acid digestibility [value ± standard deviation (CV%)] from INFOGEST digesta (continue)

Sample	Amino acid digestibility (%)								
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA
Yellowpea Raw	24.5 ± 0 (0)	28.2 ± 1.5 (5.3)	58.7 ± 1.5 (2.5)	38.5 ± 0.7 (1.7)	33.8 ± 1.3 (3.8)	60.7 ± 1.9 (3.1)	5.8 ± 1 (17.5)	21.1 ± 0 (0)	58.7 ± 5.6 (9.6)
Yellowpea Boiled	22.1 ± 1.4 (6)	33.9 ± 2.4 (6.9)	69.1 ± 3.2 (4.6)	43.9 ± 2.7 (6.2)	40.6 ± 3.2 (7.7)	70.2 ± 3.8 (5.3)	7.6 ± 1.4 (18.4)	19.4 ± 2.4 (12.1)	73.4 ± 3.6 (4.9)
Yellowpea Roasted	24.2 ± 0 (0)	30 ± 0.7 (2.2)	39 ± 3.2 (8)	37.7 ± 2.3 (6)	31.2 ± 1.9 (5.9)	64.5 ± 1.3 (1.9)	6.1 ± 0.4 (5.7)	21 ± 0.5 (2.3)	66.2 ± 2.3 (3.4)
Gelatin	3.9 ± 0.6 (14.3)	16.3 ± 0.5 (3)	38.7 ± 1.2 (3.1)	23.8 ± 1.1 (4.7)	13.1 ± 0.6 (4.4)	31.4 ± 1 (3.1)	0.1 ± 0.1 (157.8)	11.5 ± 0.8 (6.6)	14.2 ± 5.4 (38.1)
All Bran	23.5 ± 0.5 (1.8)	33.1 ± 0 (0)	19 ± 5.5 (28.7)	46 ± 0 (0)	31.8 ± 0 (0)	62.1 ± 0.1 (0.1)	11.4 ± 1.3 (11.3)	25.1 ± 0 (0)	61.8 ± 0 (0)

HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine

Table 20. Summary of DIAAR (value ± standard deviation), limiting AA, DIAAS, and protein classification from INFOGEST digesta.

Sample	DIAAR									DIAAS	LAA	Classification
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA			
Casein	119.6 ± 0	138.6 ± 7.7	149.6 ± 3.9	160.5 ± 4.8	161.4 ± 10	166.6 ± 6.8	142.8 ± 7.1	118 ± 0	199.4 ± 3.5	118	SAA	Excellent quality
Wheat Raw	107.3 ± 3.2	76.9 ± 15.8	43.1 ± 9.1	81.7 ± 16.5	96.7 ± 11.6	98.2 ± 12.8	138.9 ± 6.7	104.7 ± 2.9	107 ± 39.8	43.1	LYS	No claim
Wheat Boiled	98.2 ± 6.2	83.4 ± 0.8	47.8 ± 0.5	88.2 ± 3.3	97.3 ± 4.4	103.4 ± 3.7	97.5 ± 16.8	100.6 ± 5	129.8 ± 0	47.8	LYS	No claim
Wheat Roasted	109.8 ± 0	83.8 ± 0	43.6 ± 0	91.5 ± 0	102.6 ± 0	106.9 ± 0	111 ± 19	105.2 ± 6.6	133.2 ± 0	43.6	LYS	No claim
Rice Raw	64.1 ± 2.2	76.7 ± 10.6	61.7 ± 3.6	99.3 ± 6.6	78.9 ± 22.7	68.5 ± 46	91.6 ± 6.3	95.8 ± 12	129.1 ± 23.5	61.7	LYS	No claim
Rice Boiled	112.4 ± 0	97.9 ± 1	62.3 ± 0	117 ± 9.9	115.6 ± 3.5	104.2 ± 16.6	154.9 ± 0	110.1 ± 3.4	102.5 ± 16.7	62.3	LYS	No claim
Rice Roasted	107.5 ± 2.8	83.6 ± 2.5	48.4 ± 3.7	116.2 ± 7.9	102.7 ± 7.8	119.7 ± 3.1	119 ± 36.1	123.8 ± 0	149.2 ± 9.1	48.4	LYS	No claim
Chickpea Raw	126.3 ± 9.6	89.7 ± 8.6	82.7 ± 9.5	77 ± 9.1	95.3 ± 13.1	100.4 ± 6.4	81.1 ± 28.7	84.9 ± 1.9	113.9 ± 17.3	77	VAL	High quality
Chickpea Boiled	121.2 ± 3.1	89.4 ± 2.7	87.8 ± 6.8	90.3 ± 6.1	110.6 ± 8.3	109.5 ± 2.8	87.5 ± 26.5	98.1 ± 0	129 ± 7.9	87.5	LYS	High quality
Chickpea Roasted	101.2 ± 12.3	110.1 ± 0	116.7 ± 4.4	101.7 ± 0	133.4 ± 0	122.7 ± 0.2	68.1 ± 60.3	67.1 ± 16.2	156.6 ± 0	67.1	SAA	No claim

LAA = limited AA; HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine; DIAAS = Digestible Indispensible Amino Acid Score; Limited AA = Limiting amino acid;

Table 20. Summary of DIAAR (value ± standard deviation), limited AA, DIAAS, and protein classification from INFOGEST digesta (continue)

Sample	DIAAR									DIAAS	LAA	Classification
	HIS	THR	LYS	VAL	ILE	LEU	TRP	SAA	AAA			
Yellowpea Raw	122.2 ± 0	90.9 ± 4.8	102.8 ± 2.6	89.6 ± 1.6	105.5 ± 4	101.1 ± 3.2	67.3 ± 11.8	77.9 ± 0	112.8 ± 10.8	67.3	TRP	No claim
Yellowpea Boiled	110.1 ± 6.5	109.3 ± 7.6	121.2 ± 5.5	101.9 ± 6.3	126.7 ± 9.7	116.9 ± 6.2	89.3 ± 16.4	71.6 ± 8.6	141.1 ± 6.9	71.6	SAA	No claim
Yellowpea Roasted	120.6 ± 0	96.7 ± 2.2	68.5 ± 5.5	87.5 ± 5.3	97.4 ± 5.7	107.4 ± 2.1	71.4 ± 4.1	77.7 ± 1.8	127.2 ± 4.4	68.5	LYS	No claim
Gelatin	19.4 ± 2.8	52.4 ± 1.6	67.8 ± 2.1	55.4 ± 2.6	40.8 ± 1.8	52.3 ± 1.7	0.1 ± 0.2	42.4 ± 2.8	27.3 ± 10.4	0.1	TRP	No claim
All Bran	117.3 ± 2.1	106.8 ± 0	22.3 ± 20.4	107 ± 0	99.1 ± 0	103.4 ± 0.1	133.7 ± 15.1	92.6 ± 0	118.7 ± 0	22.3	LYS	No claim

LAA = limited AA; HIS = Histidine; THR = Threonine; LYS = Lysine; VAL = Valine; ILE = Isoleucine; LEU = Leucine; TRP = Tryptophan; SAA = Methionine and Cysteine; AAA = Phenylalanine and Tyrosine; DIAAS = Digestible Indispensible Amino Acid Score; Limited AA = Limiting amino acid

5.4.5. Comparison between protein quality and protein claim indications

With the acquired IV-PDCAAS values from the pH-drop and the INFOGEST digestion models, the protein content claims of each sample were determined based on the Reference Amounts Customarily Consumed (RACC) information and the USA regulations in Table 21 (USA Food and Drug Administration, 2018). In the case of casein, due to the lack of RACC published information, the reference amount of "Dried texturized soy protein and soy protein isolate" of 20 grams was utilized as the replacement for calculation (Government of Canada, 2022). Moreover, for processed samples of rice, wheat, yellow pea, and chickpea, the same RACC amount of the same ingredients in raw forms were used. Overall, samples were categorized into all available three claim groups: "Excellent source of protein", "Good source of protein", and "No claim". Casein was classified as an "Excellent source of protein" in all four *in vitro* methods. This result was similar to previously published data for this protein (Nosworthy et al., 2023). Casein was a popular ingredient with a high percentage of crude protein, high quality, and stability, and it has been a popular control in many studies. This result further proved that casein can be applied as a control for studies of *in vitro* methodologies to assess protein digestibility and quality. There is no reported data for *in vivo* PDCAAS and protein content claim for gelatin. However, all three *in vitro* methodologies utilized in this study concluded that gelatin was not qualified for protein content claims despite owning a high protein content. The lack of TRP played an essential role in determining the IV-PDCAAS; hence, the protein content claims are based on *in vitro* methodologies. All-bran was ranked as a "Good source of protein" from both 4 *in vitro* methodologies and the published *in vivo* data (Sousa et al., 2023). All-bran samples only owned 16.2% of crude protein, while gelatin owned 86.3% (Table 15); however, the balanced AA profile of All-bran significantly contributed to the better PDCAAS and protein content claims. the raw

wheat did not qualify for protein content claims in all *in vitro* methodologies, which was in agreement with the *in vivo* conclusion of protein content claims (Nosworthy et al., 2023). Both the roasted and boiled wheat were ranked without protein content claims with *in vitro* methodologies, showing the effect of processing could not improve protein content claims in the case of wheat. Compared to published data, all *in vitro* methods ranked boiled wheat with similar claims by *in vivo* result ("No claim") (Nosworthy et al., 2023). Rice samples were in a similar situation, whereas all the raw and processed samples were classified as "No claims," similarly by all *in vitro* methodologies. The protein content claims were similar to the reported claims from an *in vivo* study in 2015 (Rutherfurd et al., 2015). In terms of pulses, chickpea and yellow pea samples were generally ranked equally or higher than cereal samples. In more detail, all three types of chickpea were ranked as a "Good source of protein" by all four *in vitro* methods. Boiling treatment has proven to be an effective processing method, increasing the corrected protein content (g) values yet cannot change the claim to higher grade. The acquired results for boiled and roasted chickpeas agreed with a published thesis 2018 with *in vivo* studies on rats (Franczyk, 2018). Raw yellow pea was the only sample that showed disagreement regarding protein content claims between *in vivo* methods. While the OPA and the Kjeldahl analysis of INFOGEST digestion products concluded that raw yellow pea was not qualified for any protein content claims, the AA analysis of INFOGEST digesta and the pH-drop model reported raw yellow pea as a "Good source of protein". Unfortunately, there is no published *in vivo* data for protein content claims of raw yellow pea for comparison purpose due to challenges in feeding yellow pea for animals. This showed the advantage of *in vitro* methodologies, in which can avoid the situation when the animals refuse to consume or should not consume the testing materials. After processing with heat, roasted and boiled yellow pea were classified as a "Good source of protein" with higher values of "Corrected

protein content (g)", and similar to published *in vivo* data (Nosworthy, Franczyk, Medina, et al., 2017).

Following the regulation of the Canadian government, the adjusted PER values were calculated for the acquired IV-PDCAAS values and utilized to determine the Canadian protein content claims for all samples (Table 22). The RACC information was used if a reasonable daily intake was unavailable (Schedule K) (Government of Canada, 2016, 2022; Marinangeli & House, 2017). Similar to Table 23, 20 g as the reference amount of "Dried texturized soy protein and soy protein isolate" was applied for casein due to the lack of available information for daily use. In general, 4 *in vitro* methodologies concluded the same protein content claims for all samples. Compared to published data from *in vivo* studies, all samples have similar protein content (Franczyk, 2018; Nosworthy et al., 2023; Nosworthy, Neufeld, et al., 2017; Rutherfurd et al., 2015; Sousa et al., 2023). Both boiling and roasting increased the protein ratings of all samples yet cannot improve the protein content claims compared to raw samples.

The radar chart (Figure 21) illustrated the impact of applying different protein quality evaluation assays to determine the protein content claims for all samples. Both the acquired *in vitro* data from this study and available *in vivo* published data were used in the chart, with some missing points for *in vivo* lines. For most samples, the concluded protein content claims by *in vitro* and *in vivo* PDCAAS were the same with a few exceptions, as aforementioned. Comparing the three assays, it is observed that the PDCAAS methods covered the outer ring (Excellent/High protein claim) more than the other two evaluation assays. While the DIAAS and the PER methods mainly categorized samples with "No claim" or "High protein" area, casein was the only sample ranked as an "Excellent source of protein".

Overall, in this study, all four *in vitro* methods evaluated most of the samples with the same protein content claims in three popular protein quality evaluation assays: the PDCAAS, the PER, and the DIAAS. All samples were categorized into all three available groups in each assay, from "No claim" to "High/Good source of protein", and "Excellent source of protein". These conclusions agreed with published protein content claims measured with *in vivo* studies, except for the PDCAAS result of raw yellow pea. The *in vitro* data from the OPA and Kjeldahl methods classify raw yellow pea with higher protein content claims than the *in vitro* data from the other methods. Between raw and heat-treated samples, both the boiling and roasting treatments increased the "Corrected protein content (g)" and "Protein rating" of raw samples but not enough to improve the protein content claims determined by the DIAAS, the PDCAAS, and the PER method. Finally, it is observed that the PDCAAS assay can give samples with higher protein content claims than the DIAAS and the PER methods.

Table 21. Summary of reference amounts customarily consumed (RACC), PDCAAS, corrected protein content, and protein content claim statements on samples by the USA regulations

Sample	RACC	PDCAAS	Corrected protein content (g)	Claim	PH-PDCAAS	Corrected protein content (g)	Claim	OPA-PDCAAS	Corrected protein content (g)	Claim	KD-PDCAAS	Corrected protein content (g)	Claim	IFG-PDCAAS	Corrected protein content (g)	Claim
Casein	20[a]	100.0[1]	17.0	Excellent source	100	17.0	Excellent source	100	17.0	Excellent source	100	17.0	Excellent source	100	17.0	Excellent source
Wheat Raw	45	40.0[1]	3.0	No claim	38.9	2.9	No claim	35.6	2.7	No claim	36.4	2.7	No claim	43.4	3.2	No claim
Wheat Boiled	45[b]	38.8[1]	3.2	No claim	44.5	3.7	No claim	42.6	3.5	No claim	43.6	3.6	No claim	49.4	4.1	No claim
Wheat Roasted	45[b]	n/a	n/a	n/a	39.6	3.1	No claim	41.0	3.2	No claim	42.3	3.3	No claim	43.9	3.4	No claim
Rice Raw	45	n/a	n/a	n/a	62.0	2.3	No claim	49.4	1.8	No claim	50.1	1.9	No claim	49.9	1.8	No claim
Rice Boiled	45[b]	61.6[2]	2.6	No claim	61.8	2.6	No claim	59.3	2.5	No claim	63.6	2.7	No claim	64.7	2.8	No claim
Rice Roasted	45[b]	n/a	n/a	n/a	65.5	2.7	No claim	61.3	2.6	No claim	62.4	2.6	No claim	60.8	2.6	No claim

[a] *Use Reference amount of "Dried texturized soy protein and soy protein isolate";* [b] *Using RACC amount with raw material;*

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

Table 21. Summary of reference amounts customarily consumed (RACC), PDCAAS, corrected protein content, and protein content claim statements on samples by the USA regulations (continued)

Sample	RACC	PDCAAS	Corrected protein content (g)	Claim	PH-PDCAAS	Corrected protein content (g)	Claim	OPA-PDCAAS	Corrected protein content (g)	Claim	KD-PDCAAS	Corrected protein content (g)	Claim	IFG-PDCAAS	Corrected protein content (g)	Claim
Chickpea Raw	35	n/a	n/a	n/a	78.6	5.7	Good source	71.4	5.2	Good source	73.5	5.3	Good source	70.5	5.1	Good source
Chickpea Boiled	35[b]	75.2[3]	6.2	Good source	86.7	7.2	Good source	82.6	6.8	Good source	79.8	6.6	Good source	79.8	6.6	Good source
Chickpea Roasted	35[b]	80.0[3]	6.0	Good source	79.9	6.0	Good source	75.7	5.6	Good source	77.6	5.8	Good source	88.7	6.6	Good source
Yellowpea Raw	35	n/a	n/a	n/a	73.5	6.1	Good source	57.6	4.8	No claim	54.7	4.6	No claim	69.5	5.8	Good source
Yellowpea Boiled	35[b]	69.2[4]	6.2	Good source	75.1	6.8	Good source	68.5	6.2	Good source	73.1	6.6	Good source	73.1	6.6	Good source
Yellowpea Roasted	35[b]	68.9[4]	5.9	Good source	79.1	6.8	Good source	77.6	6.6	Good source	77.7	6.6	Good source	74.6	6.4	Good source
Gelatin	20[a]	n/a	n/a	n/a	0.3	0.1	No claim	0.3	0.1	No claim	0.3	0.1	No claim	0.3	0.1	No claim
All Bran	60	52.5[5]	5.1	Good source	65.7	6.4	Good source	56.9	5.5	Good source	52.6	5.1	Good source	71.8	7.0	Good source

[a] *Use Reference amount of "Dried texturized soy protein and soy protein isolate";* [b] *Using RACC amount with raw material;*

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

Table 22. Protein content claim statements on sampled based on the PER method and Canadian regulations

Food	Reasonable daily intake or reference amount	Adjusted PER	Protein rating	Claim	Adjusted PH-PER	Protein rating	Claim	Adjusted OPA-PER	Protein rating	Claim	Adjusted KD-PER	Protein rating	Claim	Adjusted TAA-PER	Protein rating	Claim
Casein	20[a]	2.5[1]	42.4	Excellent source	2.5	42.4	Excellent source	2.5	42.4	Excellent source	2.5	42.4	Excellent source	2.5	42.4	Excellent source
Wheat Raw	45.0	1.0[1]	7.5	No claim	1.0	7.3	No claim	0.9	6.6	No claim	0.9	6.8	No claim	1.1	8.1	No claim
Wheat Boiled	45.0[b]	1.0[1]	8.0	No claim	1.1	9.2	No claim	1.1	8.8	No claim	1.1	9.0	No claim	1.2	10.2	No claim
Wheat Roasted	45.0[b]	n/a	n/a	n/a	1.0	7.7	No claim	1.0	8.0	No claim	1.1	8.2	No claim	1.1	8.5	No claim
Rice Raw	45.0	n/a	n/a	n/a	1.6	5.7	No claim	1.2	4.5	No claim	1.3	4.6	No claim	1.2	4.6	No claim
Rice Boiled	45.0[b]	1.5[2]	6.6	No claim	1.5	6.6	No claim	1.5	6.3	No claim	1.6	6.8	No claim	1.6	6.9	No claim
Rice Roasted	45.0[b]	n/a	n/a	n/a	1.6	6.9	No claim	1.5	6.4	No claim	1.6	6.5	No claim	1.5	6.4	No claim

[a] *Use Reference amount of "Dried texturized soy protein and soy protein isolate";* [b] *Using RACC amount with raw material;*[c] *Use g/250ml information from* (Nosworthy, Neufeld, et al., 2017) *for chickpea and yellow pea boiled to change Reasonable daily intake or reference amount from ml to g;*

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

Table 22. Protein content claim statements on sampled based on the PER method and Canadian regulations (continue)

Food	Reasonable daily intake or reference amount	Adjusted PER	Protein rating	Claim	Adjusted PH-PER	Protein rating	Claim	Adjusted OPA-PER	Protein rating	Claim	Adjusted KD-PER	Protein rating	Claim	Adjusted TAA-PER	Protein rating	Claim
Chickpea Raw	35.0	n/a	n/a	n/a	2.0	14.2	No claim	1.8	12.9	No claim	1.8	13.3	No claim	1.8	12.7	No claim
Chickpea Boiled	35.0[b]	1.9[3]	15.6	No claim	2.2	17.9	No claim	2.1	17.1	No claim	2.0	16.5	No claim	2.0	16.5	No claim
Chickpea Roasted	35.0[b]	2.0[3]	14.9	No claim	2.0	14.9	No claim	1.9	14.1	No claim	1.9	14.5	No claim	2.2	16.5	No claim
Yellowpea Raw	35.0	n/a	n/a	n/a	1.8	15.3	No claim	1.4	12.0	No claim	1.4	11.4	No claim	1.7	14.5	No claim
Yellowpea Boiled	35.0[b]	1.7[4]	15.6	No claim	1.9	16.9	No claim	1.7	15.4	No claim	1.8	16.5	No claim	1.8	16.5	No claim
Yellowpea Roasted	35.0[b]	1.7[4]	14.7	No claim	2.0	16.9	No claim	1.9	16.6	No claim	1.9	16.6	No claim	1.9	16.0	No claim
Gelatin	20.0[a]	n/a	n/a	n/a	0.01	0.1	No claim	0.01	0.1	No claim	0.01	0.1	No claim	0.01	0.1	No claim
All Bran	28.0	1.3[5]	5.9	No claim	1.6	7.4	No claim	1.4	6.4	No claim	1.3	6.0	No claim	1.8	8.1	No claim

[a] *Use Reference amount of "Dried texturized soy protein and soy protein isolate";* [b] *Using RACC amount with raw material;*

[1] (Nosworthy et al., 2023); [2] (Rutherfurd et al., 2015)*;* [3] (Franczyk, 2018)*;* [4] (Nosworthy, Franczyk, Medina, et al., 2017)*;* [5] (Sousa et al., 2023)*;*

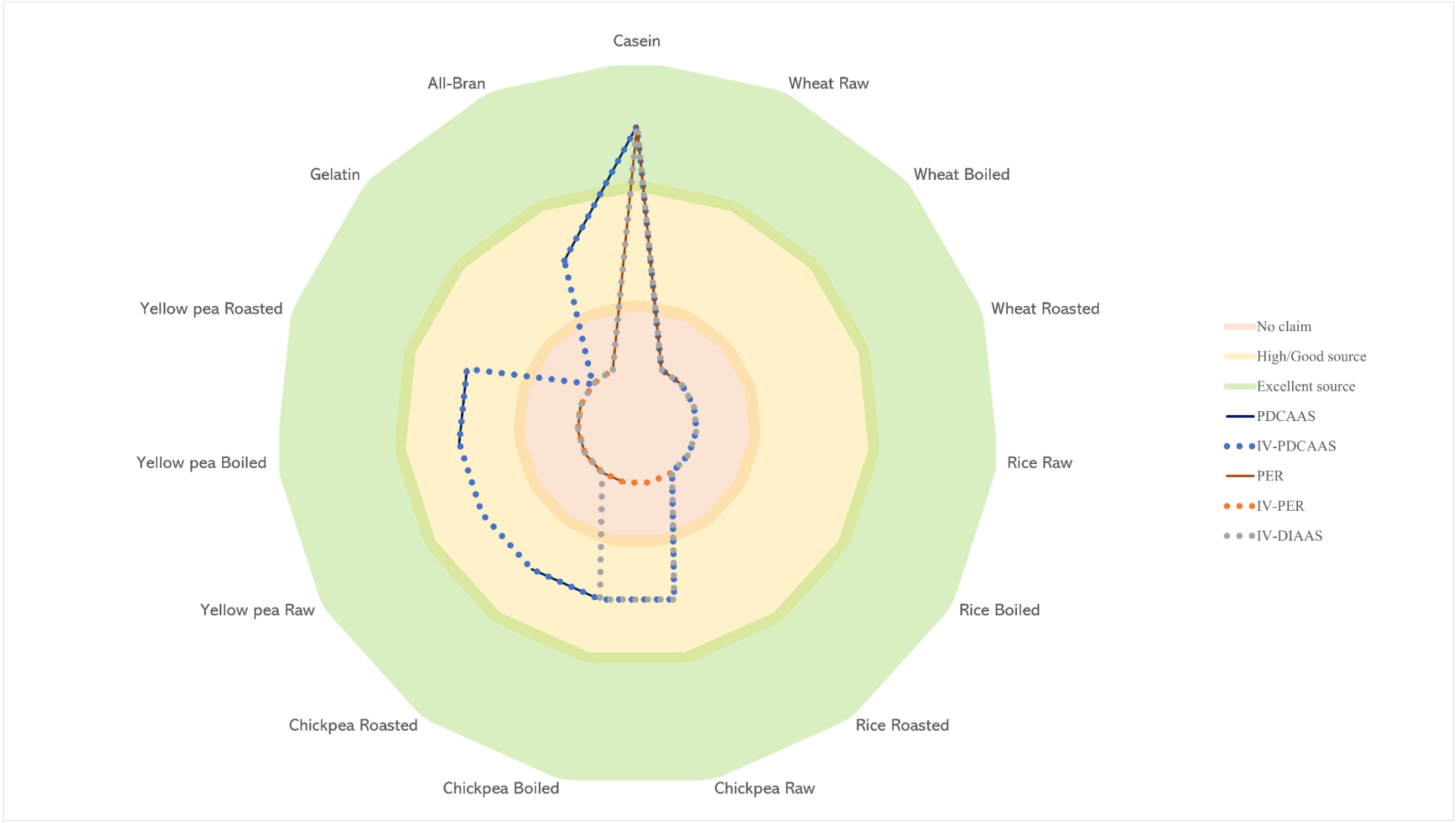


Figure 21. Summary chart of protein content claims of all samples from IV-PDCAAS, IV-PER, and IV-DIAAS

5.5. CONCLUSION

This study aimed to not only investigate the potential of two *in vitro* static digestion models, the pH-drop and the INFOGEST methods, in determining *in vitro* digestibility and protein quality but also assess the impact of thermal treatment on IV-PD, IV-AAD, and IV-PQ.

The acquired results proved that all four *in vitro* methods displayed a wide range of IV-PD from 65.2% to 100%. The coefficient of variation of the pH-drop method showed better repeatability results than analysis by the INFOGEST methods. Assessing by the PDCAAS method, the first limiting AA for cereal samples was LYS, while TRP was limiting in pulse samples. The IV-PDCAAS ranged from a low of 0.3 (gelatin) to a high of 112.1 (untruncated casein). Comparing four *in vitro* data, almost all samples had similar IV-PDCAAS except roasted chickpea, raw yellow pea and All-bran samples. Even though there are changes after heat treatment, the improvement of IV-PDCAAS by boiling and roasting was only observed in the analysis of the INFOGEST digestion products. There are differences between available *in vivo* PDCAAS from the literature and acquired IV-PDCAAS values. Analyzing the relationship by the linear regression, the Bland-Altman analysis, and Pearson correlation coefficient analysis matrix test proved that all IV-PDCAAS had a strong correlation with *in vivo* data, with high R^2 value from 0.92 (AA analysis method) to 0.96 (Kjeldahl method) (Franczyk, 2018; Nosworthy et al., 2023; Nosworthy, Franczyk, Medina, et al., 2017; Rutherfurd et al., 2015; Sousa et al., 2023)

Regarding IV-DIAAS, casein had the highest IV-DIAAS at 118 with SAA limitation, while gelatin owned the lowest IV-DIAAS at 0.1 with TRP limitation. Raw and boiled chickpeas were classified as high-quality protein, while casein was the only excellent source, and all other samples were not qualified for any protein content claims. Similar to the PDCAAS method, All-bran and cereal samples were limiting in LYS. At the same time, pulse samples experimented with a change

of limiting AA after heat processing. Boiling treatment increased the IV-DIAAS of cereal and pulse samples, while roasting only improved the IV-DIAAS for wheat and yellow pea.

In terms of protein content claims, overall, all four *in vitro* methods used in this study yielded the same claims for most of the samples by all three popular protein quality evaluation assays: the PDCAAS, the PER, and the DIAAS. The *in vitro* conclusions agree with *in vivo* reported data, except where *in vivo* conclusions ranked boiled wheat and boiled chickpea higher than *in vivo* protein content claims. Additionally, the boiling treatment improved the protein content claims more effectively than roasting. Finally, it is observed that the PDCAAS assay can give samples with higher protein content claims than the DIAAS and the PER methods.

These results indicate that both *in vitro* static pH-drop and INFOGEST digestion model can be applied to determine the *in vitro* protein digestibility, amino acid digestibility, and protein quality with various samples that showed a wide range of crude protein content, AA profile, digestibility, and quality. Moreover, it was observed that the *in vitro* tools could measure the effect of heat treatments on *in vitro* digestibility and protein quality. Finally, the *in vitro* permitted protein classification protein of all samples by the PER, the PDCAAS, and the DIAAS methods showed that the DIAAS method usually ranked samples with lower claims than the PDCAAS method.

CHAPTER 6: GENERAL CONCLUSION

Firstly, regarding hypothesis 1, there were strong correlations between IV-PD and IV-PDCAAS measures from the *in vitro* digestion models and *in vivo* data. The results from the analysis of the INFOGEST digestion products exhibited a closer relationship, emphasizing its efficacy in predicting *in vivo* outcomes for protein digestibility. Secondly, hypothesis 2 was supported by the study results, demonstrating the versatility of the *in vitro* static INFOGEST digesta analysis. This model provides the ability to assess not only IV-PD but also IV-AAD; hence, it can be applied to determine both IV-PDCAAS and IV-DIAAS. Next, the data in Chapter 5 proved that heat treatment such as boiling and roasting significantly affected protein content, digestibility and PQ of cereal and legume samples. Moreover, the findings in Chapter 5 aligned with Hypothesis 3, showing that the influences of thermal treatment can be observed by *in vitro* assessments of the INFOGEST analysis. The final hypothesis was proved in Chapters 4 and 5, where the outcome showed the significant impact of PQ assessment methods permitted protein content claims. The study highlighted the need for careful consideration when choosing assessment methods due to their subsequent influence on PQ determinations and permitted claims. Overall, the study results substantiated all the initial hypotheses, proving the significant potential of *in vitro* methodologies in protein quality assessments.

Apart from the initial hypotheses, the results showed additional outcomes beyond *in vivo* and *in vitro* method comparisons. At first, it is observed that casein can be an effective control for *in vitro* digestion studies, displaying consistent IV-PD, IV-AAD, and IV-PQ across all testing methods. Next, the study included samples with a wide range of crude protein contents, AA profiles, protein/AA digestibility, protein quality, and protein content claims. Further expanding the diversity of samples in future research not only continues to explore the potential of *in vitro* methodologies but also allows for the examination of samples that have challenges for animal research. Additionally, the study showed

differences in limiting amino acids between IV-PDCAAS and IV-DIAAS methods for some samples, possibly arising from differences in AA reference patterns between the two assays.

Chapter 4 in this study was constrained by the exclusive usage of in-housed *in vivo* data for statistical comparisons, and non-disclosure of sample types. While these limitations created difficulties for readers in verifying and utilizing the data, it is important to note that the primary objective of Chapter 4 was to assess the potential of *in vitro* methodologies rather than focusing on the specific characteristics of the samples themselves. Hence, samples were selected from previous in-house *in vivo* trials. This approach allowed for the evaluation of the analytical methods by establishing the relationship between the *in vitro* dataset and the *in vivo* dataset of the exact same samples.

During post-analysis, we noted that the bile concentration used for the INFOGEST digestion process in this study was lower than required in the original protocol (Brodkorb et al., 2019). Bile primarily serves as a detergent to facilitate digestion and absorption of dietary lipids by breaking down large fat globules into smaller droplets and forming micelles with the emulsified lipids (Danielsson, 1963; Iqbal & Hussain, 2009; Keller, 2013). While bile does not directly participate in protein digestion, its reduced utilization could potentially pose limitations , as suggested by an *in vitro* study on beta-lactoglobulin, bovine serum albumin, and myoglobin (Gass et al., 2007). While most of the samples in this study have low-fat content, which may mitigate some effects, the decreased presence of bile might indirectly affect protein digestion. Therefore, a reduced bile supply could potentially hinder the optimal absorption of these protein-derived nutrients. However, our data reveal a consistent bile concentration between the blank and real samples throughout the digestion process, suggesting minimal impact on protein digestibility.

The aim of developing an *in vitro* methodology for assessing digestibility and protein quality is to find an alternative method that is superior to traditional *in vivo* approaches in terms of cost, time, complexity, and ethics while maintaining equal accuracy and precision. This study investigated two

in vitro static digestion models with four methodologies for determining IV-PD and IV-AAD in assessing IV-PQ. The pH-drop and INFOGEST emerged as promising tools for evaluating IV-PDCAAS, showcasing strong potential with results exhibiting high repeatability and minimal variation. Despite a weaker correlation between IV-PD results and in-house *in vivo* data of the same samples in Chapter 4 or *in vivo* reference data from the literature in Chapter 5, all *in vitro* tests demonstrated robust agreement with PDCAAS values. Notably, the INFOGEST model, mainly through OPA and AA analysis, exhibited a stronger relationship with *in vivo* data than the pH-drop model. However, the latter method demonstrated better repeatability, which can be attributed to its more straightforward protocol. As a recently established model lacking an official protocol for measuring digestibility, the INFOGEST model offers ample opportunities for future improvements in accuracy. These improvements can focus on refining post-digestion treatments for samples and potentially modifying analysis methods to suit their digestion products. Additionally, individual AA analysis of the INFOGEST digesta offers a significant additional value in determining IV-AAD. This comprehensive value is crucial to FAO's recommendation to replace the PDCAAS method with the DIAAS method. Through AA analysis, researchers can measure both *in vitro* protein quality values simultaneously, while the pH-drop and the other two analyses with the INFOGEST were limited to the IV-PDCAAS only. Moreover, the AA analysis possibly allows the research of AA digestibility of biologically available amino acids, offering a more comprehensive overview of protein quality.

In conclusion, the pH-drop and the INFOGEST digestion models present potential tools for determining IV-PD and IV-PDCAAS. While the data indicated comparable performance, the choice of which *in vitro* models depends on various factors. The pH-drop model is suitable for basic PD studies with time or resource limitations. In contrast, the INFOGEST model, particularly

through AA analysis, offers detailed analysis crucial for comprehensive assessments. Strategic model selection should align with study objectives, regulatory considerations, and the evolvement of industry standards.

CHAPTER 7: FUTURE DIRECTIONS

From the initial purpose of investigating the potential of the pH-drop and the INFOGEST models in assessing *in vitro* protein quality, this study proved that both *in vitro* static models can be valuable screening tools for researchers and industries interested in protein quality assessment. In the study, there were a total of 22 samples digested by both the pH-drop and the INFOGEST samples. Even though all samples belonged to a wide range of digestibility and protein quality, the total amount was small and thus can not fully represent the diversity of food in general. Moreover, some samples lacked *in vivo* digestibility and protein quality data, especially the DIAAS values. Therefore, in order to consolidate the applications of the i*n vitro* digestion model in assessing protein quality more broadly, the following directions can be considered to develop this topic further:

- Expanding on sample diversity with different plant/animal based samples, protein isolation/concentration, protein blends, processed sample (heat/non-heat), whole cooked meals, et cetera;
- Focusing on digesting samples with available *in vivo* data for protein digestibility, amino acid digestibility and protein quality, aiming to investigate the regression relationship between *in vivo* and *in vitro* results;
- Assessing the ability of the *in vitro* digestion model to determine protein digestibility, amino acid digestibility and protein quality of samples which are challenging for bioassay studies, such as nitrogen-free samples/diets or samples/diets which animals refuse to consume;
- Comparing the potential of pH-drop and INFOGEST herein with a more sophisticated *in vitro* digestion model TIM-1 in assessing *in vitro* protein quality

- Assessing the potential of the INFOGEST digestion model with additional compartments of absorbance pathways or gastrointestinal tracts such as brush-border membrane peptidase, intestinal lumen cells, or large intestine digestion compartment with fermentation activity;
- Investigating the ability of the pH-drop and the INFOGEST models in measuring the amino acid digestibility of reactive amino acids, especially with heat-treated samples, hence further correcting the protein quality of samples;
- Application of the release of anti-nutritional factors with the *in vitro* digestion models at various digestion stages and assessing the relationship between the amount of anti-nutritional factors and the *in vitro* protein quality;
- Investigating the ability of *in vitro* digestion models to determine the impact of various factors on *in vitro* digestibility and protein quality, such as particle size;
- Continually studying the impact of using different protein quality evaluation assays, focusing on the PDCAAS, the PER, and the DIAAS methods, in determining the protein content claims with *in vitro* methodologies;

REFERENCES

Alonso, R., Aguirre, A., & Marzo, F. (2000). Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry*, *68*(2), 159–165. https://doi.org/10.1016/S0308-8146(99)00169-7

Aluko, R. E., & Monu, E. (2003). Functional and Bioactive Properties of Quinoa Seed Protein Hydrolysates. *Journal of Food Science*, *68*(4), 1254–1258. https://doi.org/10.1111/j.1365-2621.2003.tb09635.x

AOAC. (1995). *Official Methods of Analysis. 16th Edition*. Association of Official Analytical Chemists.

Bailey, H. M., Mathai, J. K., Berg, E. P., & Stein, H. H. (2020). Pork Products Have Digestible Indispensable Amino Acid Scores (DIAAS) That Are Greater Than 100 When Determined in Pigs, but Processing Does Not Always Increase DIAAS. *The Journal of Nutrition*, *150*(3), 475–482. https://doi.org/10.1093/jn/nxz284

Bailey, H. M., & Stein, H. H. (2020). Raw and roasted pistachio nuts (Pistacia vera L.) are 'good' sources of protein based on their digestible indispensable amino acid score as determined in pigs. *Journal of the Science of Food and Agriculture*, *100*(10), 3878–3885. https://doi.org/10.1002/jsfa.10429

Baudoin, J. P., & Maquet, A. (1999). Improvement of protein and amino acid contents in seeds of food legumes. A case study in Phaseolus. *Biotechnology, Agronomy, Society and Environment*, *3*(4), 220–224.

Beijerinck, M. W. (1901). Uber oligonitrophile Mikroben. *Zentralbl. Bakterol. Parasitenkd. Infektionskr. Hyg. Abt. II.*, *7*, 561–582. https://cir.nii.ac.jp/crid/1573387449638826752.bib?lang=en

Bhagavan, N. V., & Ha, C.-E. (2015). Protein and Amino Acid Metabolism. In *Essentials of Medical Biochemistry* (pp. 227–268). Elsevier. https://doi.org/10.1016/B978-0-12-416687-5.00015-4

Bhutta, Z. A., & Sadiq, K. (2012). Protein Digestion and Bioavailability. *Encyclopedia of Human Nutrition*, *4–4*, 116–122. https://doi.org/10.1016/B978-0-12-375083-9.00240-3

Blanco, A., & Blanco, G. (2017). Digestion - Absorption. In *Medical Biochemistry* (pp. 251–273). Elsevier. https://doi.org/10.1016/B978-0-12-803550-4.00012-4

Boland, M. (2016). Human digestion - a processing perspective. *Journal of the Science of Food and Agriculture*, *96*(7), 2275–2283. https://doi.org/10.1002/jsfa.7601

Booij, L., Merens, W., Markus, C. R., & Van der Does, A. J. W. (2006). Diet rich in α-lactalbumin improves memory in unmedicated recovered depressed patients and matched controls. *Journal of Psychopharmacology*, *20*(4), 526–535. https://doi.org/10.1177/0269881105057466

Bouglé, D., & Bouhallab, S. (2017). Dietary bioactive peptides: Human studies. *Critical Reviews in Food Science and Nutrition*, *57*(2), 335–343. https://doi.org/10.1080/10408398.2013.873766

Breese Jones. (1931). *Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins*. US Department of Agriculture. https://babel.hathitrust.org/cgi/pt?id=uiug.30112002029335&seq=1

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*,

14(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Bryan, D. D. S. L., & Classen, H. L. (2020). In Vitro Methods of Assessing Protein Quality for Poultry. *Animals*, *10*(4), 551. https://doi.org/10.3390/ani10040551

Buamah, T. F., & Singsen, E. P. (1975). Studies on the protein efficiency ratio method for the evaluation of poultry feed supplements. Modifications associated with choice of dietary protein level for assay. *Journal of Nutrition*, *105*(6), 688–700. https://doi.org/10.1093/jn/105.6.688

Butts, C. A., James, K. A. C., Koolaard, J. P., Booth, C. L., Donaldson, H. E., Scott, M. F., & Moughan, P. J. (2002). The effect of digesta sampling time and dietary protein source on ileal nitrogen digestibility for the growing rat. *Journal of the Science of Food and Agriculture*, *82*(4), 343–350. https://doi.org/10.1002/jsfa.1044

Butts, C. A., Monro, J. A., & Moughan, P. J. (2012). In vitro determination of dietary protein and amino acid digestibility for humans. *The British Journal of Nutrition*, *108 Suppl*(S2), S282-7. https://doi.org/10.1017/S0007114512002310

Carbonaro, M., Cappelloni, M., Nicoli, S., Lucarini, M., & Carnovale, E. (1997). Solubility−Digestibility Relationship of Legume Proteins. *Journal of Agricultural and Food Chemistry*, *45*(9), 3387–3394. https://doi.org/10.1021/jf970070y

Cargo-Froom, C. L., Tansil, F., Columbus, D. A., Marinangeli, C. P. F., Kiarie, E. G., & Shoveller, A. K. (2023). Determination of standardized ileal digestibility of crude protein and amino acids and digestible indispensable amino acid score of faba beans, lentils, and yellow peas fed to growing pigs. *Canadian Journal of Animal Science*, *103*(3), 273–281. https://doi.org/10.1139/cjas-2023-0022

Carmody, R. N., & Wrangham, R. W. (2009). The energetic significance of cooking. *Journal of*

Human Evolution, *57*(4), 379–391. https://doi.org/10.1016/j.jhevol.2009.02.011

Carpenter, K. J. (1960). The estimation of the available lysine in animal-protein foods. *Biochemical Journal*, *77*(3), 604–610. https://doi.org/10.1042/bj0770604

Casas, G. A., Huang, C., & Stein, H. H. (2017). Nutritional value of soy protein concentrate ground to different particle sizes and fed to pigs1. *Journal of Animal Science*, *95*(2), 827–836. https://doi.org/10.2527/jas.2016.1083

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins. *Journal of Dairy Science*, *66*(6), 1219–1227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2

Codex Alimentarius - FAO. (1987). CODEX Standard for Follow-on Formula CODEX STAN 156-1987. In *FAO* (pp. 156–1987).

Cordonnier, C., Thévenot, J., Etienne-Mesmin, L., Denis, S., Alric, M., Livrelli, V., & Blanquet-Diot, S. (2015). Dynamic In Vitro Models of the Human Gastrointestinal Tract as Relevant Tools to Assess the Survival of Probiotic Strains and Their Interactions with Gut Microbiota. *Microorganisms*, *3*(4), 725–745. https://doi.org/10.3390/microorganisms3040725

Craddock, J. C., Genoni, A., Strutt, E. F., & Goldman, D. M. (2021). Limitations with the Digestible Indispensable Amino Acid Score (DIAAS) with Special Attention to Plant-Based Diets: a Review. *Current Nutrition Reports*, *10*(1), 93–98. https://doi.org/10.1007/s13668-020-00348-8

Damerau, K., Waha, K., & Herrero, M. (2019). The impact of nutrient-rich food choices on agricultural water-use efficiency. *Nature Sustainability*, *2*(3), 233–241. https://doi.org/10.1038/s41893-019-0242-1

Danielsson, H. (1963). Influence of Bile Acids on Digestion and Absorption of Lipids. *The American Journal of Clinical Nutrition*, *12*(3), 214–219. https://doi.org/10.1093/ajcn/12.3.214

Davis, P. J., & Williams, S. C. (1998). Protein modification by thermal processing. *Allergy*, *53*(s46), 102–105. https://doi.org/10.1111/j.1398-9995.1998.tb04975.x

Day, L., & Swanson, B. G. (2013). Functionality of protein-fortified extrudates. *Comprehensive Reviews in Food Science and Food Safety*, *12*(5), 546–564. https://doi.org/10.1111/1541-4337.12023

de Mejia, E. G., Bradford, T., & Hasler, C. (2003). The anticarcinogenic potential of soybean lectin and lunasin. *Nutrition Reviews*, *61*(7), 239–246. https://doi.org/10.1301/nr.2003.jul.239-246

de Mejia, E. G., & Dia, V. P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-κB pathway in the macrophage. *Peptides*, *30*(12), 2388–2398. https://doi.org/10.1016/j.peptides.2009.08.005

Donkoh, A., Moughan, P. J., & Smith, W. C. (1994). Comparison of the slaughter method and simple T-piece cannulation of the terminal ileum for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, *49*(1–2), 43–56. https://doi.org/10.1016/0377-8401(94)90080-9

Driscoll, D. M., & Copeland, P. R. (2003). Mechanism and regulation of selenoprotein synthesis. *Annual Review of Nutrition*, *23*(1), 17–40. https://doi.org/10.1146/annurev.nutr.23.011702.073318

Drulyte, D., & Orlien, V. (2019). The Effect of Processing on Digestion of Legume Proteins. *Foods*, *8*(6), 224. https://doi.org/10.3390/foods8060224

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., Denis, S., Ferrua,

M., Havenaar, R., Lelieveld, J., Mackie, A. R., Marzorati, M., Menard, O., Minekus, M., Miralles, B., Recio, I., & Van den Abbeele, P. (2019). Can dynamic in vitro digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition*, *59*(10), 1546–1562. https://doi.org/10.1080/10408398.2017.1421900

Dupont, Didier, & Mackie, A. R. (2015). Static and dynamic in vitro digestion models to study protein stability in the gastrointestinal tract. *Drug Discovery Today: Disease Models*, *17–18*(xx), 23–27. https://doi.org/10.1016/j.ddmod.2016.06.002

EFSA Panel on Dietetic Products Nutrition and Allergies. (2012). Scientific Opinion on Dietary Reference Values for protein. *EFSA Journal*, *10*(2), 2557. https://doi.org/10.2903/j.efsa.2012.2557

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., Dürr, D., Stoll, P., Vergères, G., & Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Research International (Ottawa, Ont.)*, *102*(4), 567–574. https://doi.org/10.1016/j.foodres.2017.09.047

El-Adawy, T. A. (2002). Nutritional composition and antinutritional factors of chickpeas (cicer arietinum L.) undergoing different cooking methods and germination. *Plant Foods for Human Nutrition*, *57*(1), 83–97. https://doi.org/10.1023/A:1013189620528

Elango, R. (2020). Methionine nutrition and metabolism: Insights from animal studies to inform human nutrition. *Journal of Nutrition*, *150*, 2518S-2523S. https://doi.org/10.1093/jn/nxaa155

FAO. (2013). Dietary protein quality evaluation in human nutrition. *FAO Food and Nutrition Paper*, *92*, 1–66. http://www.ncbi.nlm.nih.gov/pubmed/26369006

FAO. (2018). *Protein quality assessment in follow-up formula for young children and ready to use therapeutic foods*.

FAO. (2022). *The State of the World's Land and Water Resources for Food and Agriculture 2021 – Systems at breaking point*. FAO.

Fekadu Gemede, H. (2014). Antinutritional Factors in Plant Foods: Potential Health Benefits and Adverse Effects. *International Journal of Nutrition and Food Sciences*, *3*(4), 284. https://doi.org/10.11648/j.ijnfs.20140304.18

Feng, C., Sun, Z., Zhang, L., Feng, L., Zheng, J., Bai, W., Gu, C., Wang, Q., Xu, Z., & van der Werf, W. (2021). Maize/peanut intercropping increases land productivity: A meta-analysis. *Field Crops Research*, *270*(84), 108208. https://doi.org/10.1016/j.fcr.2021.108208

Fernández-García, E., Carvajal-Lérida, I., & Pérez-Gálvez, A. (2009). In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. *Nutrition Research*, *29*(11), 751–760. https://doi.org/10.1016/j.nutres.2009.09.016

Fiszman, S., & Tarrega, A. (2020). The Contribution of Food Oral Processing. In *The Contribution of Food Oral Processing*. MDPI. https://doi.org/10.3390/books978-3-03936-835-8

Franczyk, A. J. (2018). *Evaluation of in Vitro Methodology To Determine Protein Digestibility and Quality of Plant-Based Proteins* [University of Manitoba]. https://mspace.lib.umanitoba.ca/xmlui/handle/1993/33437

Freeman, H. J., Kim, Y. S., & Sleisenger, M. H. (1979). Protein digestion and absorption in man: Normal mechanisms and Protein-Energy malnutrition. *The American Journal of Medicine*, *67*(6), 1030–1036. https://doi.org/10.1016/0002-9343(79)90645-4

Friedman, M., & Brandon, D. L. (2001). Nutritional and Health Benefits of Soy Proteins. *Journal of Agricultural and Food Chemistry*, *49*(3), 1069–1086. https://doi.org/10.1021/jf0009246

Fuller, M. F., & Tomé, D. (2005). In vivo determination of amino acid bioavailability in humans and model animals. *Journal of AOAC International*, *88*(3), 923–934.

https://doi.org/10.1093/jaoac/88.3.923

Gass, J., Vora, H., Hofmann, A. F., Gray, G. M., & Khosla, C. (2007). Enhancement of dietary protein digestion by conjugated bile acids. *Gastroenterology*, *133*(1), 16–23. https://doi.org/10.1053/j.gastro.2007.04.008

Gilani, G. S., & Lee, N. (2003). Protein | Quality. In *Encyclopedia of Food Sciences and Nutrition* (pp. 4847–4854). Elsevier. https://doi.org/10.1016/B0-12-227055-X/00984-6

Gonzalez-Vega, J. C., & Stein, H. H. (2012). Amino acid digestibility in canola, cottonseed, and sunflower products fed to finishing pigs. *Journal of Animal Science*, *90*(12), 4391–4400. https://doi.org/10.2527/jas.2011-4631

González, A. D., Frostell, B., & Carlsson-Kanyama, A. (2011). Protein efficiency per unit energy and per unit greenhouse gas emissions: Potential contribution of diet choices to climate change mitigation. *Food Policy*, *36*(5), 562–570. https://doi.org/10.1016/j.foodpol.2011.07.003

Government of Canada. (2016). *Food and drug regulations: schedule K—reasonable daily intake for various foods*. https://laws-lois.justice.gc.ca/eng/regulations/c.r.c.,_c._870/page-121.html#h-580603

Government of Canada. (2022). *Food and drug regulations: schedule M—reference amounts.* https://www.canada.ca/en/health-canada/services/technical-documents-labelling-requirements/table-reference-amounts-food.html

Government of Canada - Canadian Food Inspection Agency. (2020). *Measuring the protein quality of foods*. https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/policies/measuring-protein-quality-foods.html

Government of Canada - Canadian Food Inspection Agency. (2021). *Elements within the Nutrition*

Facts table. https://inspection.canada.ca/food-label-requirements/labelling/industry/nutrition-labelling/elements-within-the-nutrition-facts-table/eng/1613599715710/1613599936553?chap=7#fn3-rf

Guillin, F. M., Gaudichon, C., Guérin-Deremaux, L., Lefranc-Millot, C., Airinei, G., Khodorova, N., Benamouzig, R., Pomport, P. H., Martin, J., & Calvez, J. (2022). Real ileal amino acid digestibility of pea protein compared to casein in healthy humans: a randomized trial. *The American Journal of Clinical Nutrition*, *115*(2), 353–363. https://doi.org/10.1093/AJCN/NQAB354

Habiba, R. A. (2002). Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods. *Food Chemistry*, *77*(2), 187–192. https://doi.org/10.1016/S0308-8146(01)00335-1

Hall, C., Hillen, C., & Garden Robinson, J. (2017). Composition, Nutritional Value, and Health Benefits of Pulses. *Cereal Chemistry*, *94*(1), 11–31. https://doi.org/10.1094/CCHEM-03-16-0069-FI

Han, F., Han, F., Wang, Y., Fan, L., Song, G., Chen, X., Jiang, P., Miao, H., & Han, Y. (2019). Digestible indispensable amino acid scores of nine cooked cereal grains. *British Journal of Nutrition*, *121*(1), 30–41. https://doi.org/10.1017/S0007114518003033

Han, F., Moughan, P. J., Li, J., & Pang, S. (2020). Digestible Indispensable Amino Acid Scores (DIAAS) of Six Cooked Chinese Pulses. *Nutrients*, *12*(12), 3831. https://doi.org/10.3390/nu12123831

Havenaar, R., De Jong, A., Koenen, M. E., Van Bilsen, J., Janssen, A. M., Labij, E., & Westerbeek, H. J. M. (2013). Digestibility of transglutaminase cross-linked caseinate versus native caseinate in an in vitro multicompartmental model simulating young child and adult

gastrointestinal conditions. *Journal of Agricultural and Food Chemistry*, *61*(31), 7636–7644. https://doi.org/10.1021/jf402824u

He, Y. (2012). Liquid-Based Microextraction Techniques for Environmental Analysis. *Comprehensive Sampling and Sample Preparation: Analytical Techniques for Scientists*, 835–862. https://doi.org/10.1016/B978-0-12-381373-2.00116-2

Health Canada. (1981). *Determination of Protein Rating FO-1* (pp. 1–6). https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/policies/measuring-protein-quality-foods.html

Henchion, M., Hayes, M., Mullen, A., Fenelon, M., & Tiwari, B. (2017). Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods*, *6*(7), 53. https://doi.org/10.3390/foods6070053

Herreman, L., Nommensen, P., Pennings, B., & Laus, M. C. (2020). Comprehensive overview of the quality of plant- And animal-sourced proteins based on the digestible indispensable amino acid score. *Food Science & Nutrition*, *8*(10), 5379–5391. https://doi.org/10.1002/fsn3.1809

Holmes, R., & Lobley, R. W. (1989). Intestinal brush border revisited. *Gut*, *30*(12), 1667–1678. https://doi.org/10.1136/gut.30.12.1667

House, J. D., Brodkorb, A., Messina, M., Braun, M., & Krul, E. S. (2023). Options for substantiating protein content claims for conventional foods. *Applied Physiology, Nutrition, and Metabolism*, *10*, 1–10. https://doi.org/10.1139/apnm-2023-0243

Hsu, H. W., Vavak, D. L., Satterlee, L. D., & Miller, G. A. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, *42*(5), 1269–1273. https://doi.org/10.1111/j.1365-2621.1977.tb14476.x

Hudson, J. L., Baum, J. I., Diaz, E. C., & Børsheim, E. (2021). Dietary Protein Requirements in

Children: Methods for Consideration. *Nutrients*, *13*(5), 1554. https://doi.org/10.3390/nu13051554

Humiski, L. M., & Aluko, R. E. (2007). Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *Journal of Food Science*, *72*(8). https://doi.org/10.1111/j.1750-3841.2007.00475.x

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry*, *125*(1), 1–12. https://doi.org/10.1016/j.foodchem.2010.08.036

Hurrell, R. F., & Carpenter, K. J. (1976). An approach to the rapid measurement of "reactive lysine" in foods by dye binding. *The Proceedings of the Nutrition Society*, *35*(1), 23A-24A. http://www.ncbi.nlm.nih.gov/pubmed/945896

Institute of Medicine. (2005). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. In *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)*. National Academies Press. https://doi.org/10.17226/10490

Iqbal, J., & Hussain, M. M. (2009). Intestinal lipid absorption. *American Journal of Physiology-Endocrinology and Metabolism*, *296*(6), E1183–E1194. https://doi.org/10.1152/ajpendo.90899.2008

ISO. (2016). *ISO 13904:2016 - Animal feeding stuffs — Determination of tryptophan content*. https://www.iso.org/standard/59955.html

Joint FAO/WHO/UNU Expert Consultation. (1985). *Energy and protein requirements : report of a Joint FAO/WHO/UNU Expert Consultation*. https://apps.who.int/iris/handle/10665/39527

Joint FAO/WHO. (1991). *Protein quality evaluation report of joint FAO/WHO expert consultation*

(Vol. 51). Rome, Italy: Food and Agriculture Organization of the United Nations.

Karwacka, M., Ciurzyńska, A., Lenart, A., & Janowicz, M. (2020). Sustainable Development in the Agri-Food Sector in Terms of the Carbon Footprint: A Review. *Sustainability*, *12*(16), 6463. https://doi.org/10.3390/su12166463

Keller, J. (2013). Gastrointestinal Digestion and Absorption. In *Encyclopedia of Biological Chemistry* (pp. 354–359). Elsevier. https://doi.org/10.1016/B978-0-12-378630-2.00106-7

Knight, R. (2000). *Linking Research and Marketing Opportunities for Pulses in the 21st Century: Proceedings of the Third International Food Legumes Research Conference* (R. Knight (ed.)). Springer Science & Business Media.

Kong, F., & Singh, R. P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. *Journal of Food Science*, *75*(9), E627-35. https://doi.org/10.1111/j.1750-3841.2010.01856.x

Krzycki, J. A. (2005). The direct genetic encoding of pyrrolysine. *Current Opinion in Microbiology*, *8*(6), 706–712. https://doi.org/10.1016/j.mib.2005.10.009

Kurpad, A. V. (2013). Protein: Quality and Sources. In *Encyclopedia of Human Nutrition* (Vols. 4–4, pp. 123–130). Elsevier. https://doi.org/10.1016/B978-0-12-375083-9.00241-5

Lee, W. T., Weisell, R., Albert, J., Tomé, D., Kurpad, A. V., & Uauy, R. (2016). Research Approaches and Methods for Evaluating the Protein Quality of Human Foods Proposed by an FAO Expert Working Group in 2014. *The Journal of Nutrition*, *146*(5), 929–932. https://doi.org/10.3945/jn.115.222109

Li, E., Zhu, T., Dong, W., & Huang, C. (2019). Effects of brown rice particle size on energy and nutrient digestibility in diets for young pigs and adult sows. *Animal Science Journal*, *90*(1), 108–116. https://doi.org/10.1111/asj.13127

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., Jones, P. J. H., & Aluko, R. E. (2011). Blood Pressure Lowering Effect of a Pea Protein Hydrolysate in Hypertensive Rats and Humans. *Journal of Agricultural and Food Chemistry*, *59*(18), 9854–9860. https://doi.org/10.1021/jf201911p

Macfarlane, G. T., & Macfarlane, S. (2011). Fermentation in the human large intestine: Its physiologic consequences and the potential contribution of prebiotics. *Journal of Clinical Gastroenterology*, *45*(SUPPL. 3), 120–127. https://doi.org/10.1097/MCG.0b013e31822fecfe

Mackie, A. (2020). Insights and gaps on protein digestion. *Current Opinion in Food Science*, *31*, 96–101. https://doi.org/10.1016/j.cofs.2020.03.006

Mackie, A., Mulet-Cabero, A.-I., & Torcello-Gómez, A. (2020). Simulating human digestion: developing our knowledge to create healthier and more sustainable foods. *Food & Function*, *11*(11), 9397–9431. https://doi.org/10.1039/d0fo01981j

Mæhre, H., Dalheim, L., Edvinsen, G., Elvevoll, E., & Jensen, I.-J. (2018). Protein Determination—Method Matters. *Foods*, *7*(1), 5. https://doi.org/10.3390/foods7010005

Mansilla, W. D., Marinangeli, C. P. F., Cargo-Froom, C., Franczyk, A., House, J. D., Elango, R., Columbus, D. A., Kiarie, E., Rogers, M., & Shoveller, A. K. (2020). Comparison of methodologies used to define the protein quality of human foods and support regulatory claims. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition et Metabolisme*, *45*(9), 917–926. https://doi.org/10.1139/apnm-2019-0757

Marinangeli, C. P. F., & House, J. D. (2017). Potential impact of the digestible indispensable amino acid score as a measure of protein quality on dietary regulations and health. *Nutrition Reviews*, *75*(8), 658–667. https://doi.org/10.1093/nutrit/nux025

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein - Beyond 6.25

and Jones' factors. *Critical Reviews in Food Science and Nutrition*, *48*(2), 177–184. https://doi.org/10.1080/10408390701279749

Marrelli, M., Conforti, F., Araniti, F., & Statti, G. A. (2016). Effects of Saponins on Lipid Metabolism: A Review of Potential Health Benefits in the Treatment of Obesity. *Molecules*, *21*(10), 1404. https://doi.org/10.3390/molecules21101404

Mason, M., & Palmer, R. (1973). The Influence of Bacterial Activity in the Alimentary Canal of Rats on Faecal Nitrogen Excretionv. *Acta Agriculturae Scandinavica*, *23*(3), 141–150. https://doi.org/10.1080/00015127309433211

Mathai, J. K., Liu, Y., & Stein, H. H. (2017). Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS). *British Journal of Nutrition*, *117*(4), 490–499. https://doi.org/10.1017/S0007114517000125

McKevith, B. (2004). Nutritional aspects of cereals. *Nutrition Bulletin*, *29*(2), 111–142. https://doi.org/10.1111/j.1467-3010.2004.00418.x

Ménard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., & Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. *Food Chemistry*, *240*(July 2017), 338–345. https://doi.org/10.1016/j.foodchem.2017.07.145

Mennah-Govela, Y. A., & Bornhorst, G. M. (2021). Food buffering capacity: quantification methods and its importance in digestion and health. *Food & Function*, *12*(2), 543–563. https://doi.org/10.1039/D0FO02415E

Mennah-Govela, Y. A., Singh, R. P., & Bornhorst, G. M. (2019). Buffering capacity of protein-based model food systems in the context of gastric digestion. *Food & Function*, *10*(9), 6074–

6087. https://doi.org/10.1039/C9FO01160A

Millward, D. J. (2012a). Identifying recommended dietary allowances for protein and amino acids: A critique of the 2007 WHO/FAO/UNU report. *British Journal of Nutrition*, *108*(SUPPL. 2). https://doi.org/10.1017/S0007114512002450

Millward, D. J. (2012b). Protein: Synthesis and Turnover. *Encyclopedia of Human Nutrition*, *4–4*(1995), 139–146. https://doi.org/10.1016/B978-0-12-375083-9.00243-9

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct.*, *5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G., & Huis in't Veld, J. H. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Applied Microbiology and Biotechnology*, *53*(1), 108–114. https://doi.org/10.1007/s002530051622

Minekus, Mans. (2015). The TNO Gastro-Intestinal Model (TIM). In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health* (pp. 37–46). Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_5

Minekus, Mans, Marteau, P., Havenaar, R., & Veld, J. H. J. H. in't. (1995). A Multicompartmental Dynamic Computer-controlled Model Simulating the Stomach and Small Intestine. *Alternatives to Laboratory Animals*, *23*(2), 197–209.

https://doi.org/10.1177/026119299502300205

Miner-Williams, W. M., Stevens, B. R., & Moughan, P. J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutrition Research Reviews*, *27*(2), 308–329. https://doi.org/10.1017/S0954422414000225

Moughan, P. J., Butts, C. A., van Wijk, H., Rowan, A. M., & Reynolds, G. W. (2005). An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. *The Journal of Nutrition*, *135*(3), 404–409. https://doi.org/10.1093/jn/135.3.404

Moughan, P. J., & Rutherfurd, S. M. (2008). Available lysine in foods: a brief historical overview. *Journal of AOAC International*, *91*(4), 901–906. https://doi.org/10.1093/jaoac/91.4.901

National Research Council. (1994). *National Research Council (US) Subcommittee on Poultry. Nutrient requirements of poultry: 9th revised edition,*. National Academies Press.

National Research Council. (1995). National Research Council (US) Subcommittee on Laboratory Animal Nutrition. Nutrient requirements of laboratory animals: 4th revised edition. In *The Quarterly Review of Biology* (Vol. 70, Issue 4). https://doi.org/10.1086/419223

Noorbakhsh, R., Mortazavi, S. A., Sankian, M., Shahidi, F., Maleki, S. J., Nasiraii, L. R., Falak, R., Sima, H. R., & Varasteh, A. (2010). Influence of Processing on the Allergenic Properties of Pistachio Nut Assessed in Vitro. *Journal of Agricultural and Food Chemistry*, *58*(18), 10231–10235. https://doi.org/10.1021/jf101364g

Nosworthy, M. G., Franczyk, A. J., Medina, G., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., & House, J. D. (2017). Effect of Processing on the in Vitro and in Vivo Protein Quality of Yellow and Green Split Peas (Pisum sativum). *Journal of Agricultural and Food Chemistry*, *65*(35), 7790–7796. https://doi.org/10.1021/acs.jafc.7b03597

Nosworthy, M. G., Franczyk, A., Zimoch-Korzycka, A., Appah, P., Utioh, A., Neufeld, J., &

House, J. D. (2017). Impact of Processing on the Protein Quality of Pinto Bean (Phaseolus vulgaris) and Buckwheat (Fagopyrum esculentum Moench) Flours and Blends, As Determined by in Vitro and in Vivo Methodologies. *Journal of Agricultural and Food Chemistry*, *65*(19), 3919–3925. https://doi.org/10.1021/acs.jafc.7b00697

Nosworthy, M. G., Hernandez-Alvarez, A. J., Franczyk, A. J., Medina, G., Neufeld, J., Arcand, Y., Ribéreau, S., Sánchez-Velázquez, O. A., & House, J. D. (2023). Effect of cooking on the in vitro and in vivo protein quality of soy, oat and wheat varieties. *Cereal Chemistry*, *100*(2), 460–472. https://doi.org/10.1002/cche.10623

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., & House, J. D. (2018). Effect of Processing on the In Vitro and In Vivo Protein Quality of Beans (Phaseolus vulgaris and Vicia Faba). *Nutrients*, *10*(6), 671. https://doi.org/10.3390/nu10060671

Nosworthy, M. G., Neufeld, J., Frohlich, P., Young, G., Malcolmson, L., & House, J. D. (2017). Determination of the protein quality of cooked Canadian pulses. *Food Science & Nutrition*, *5*(4), 896–903. https://doi.org/10.1002/fsn3.473

Nyachoti, C. M., Lange, C. F. M. de, McBride, B. W., & Schulze, H. (1997). Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: A review. *Canadian Journal of Animal Science*, *77*(1), 149–163. https://doi.org/10.4141/A96-044

Osborne, T. B., Mendel, L. B., & Ferry, E. L. (1919). A METHOD OF EXPRESSING NUMERICALLY THE GROWTH-PROMOTING VALUE OF PROTEINS. *Journal of Biological Chemistry*, *37*(2), 223–229. https://doi.org/10.1016/S0021-9258(18)86353-0

Ozorio, L., Mellinger-Silva, C., Cabral, L. M. C., Jardin, J., Boudry, G., & Dupont, D. (2020). The Influence of Peptidases in Intestinal Brush Border Membranes on the Absorption of

Oligopeptides from Whey Protein Hydrolysate: An Ex Vivo Study Using an Ussing Chamber. *Foods*, *9*(10), 1415. https://doi.org/10.3390/foods9101415

Paz-Yépez, C., Peinado, I., Heredia, A., & Andrés, A. (2019). Influence of particle size and intestinal conditions on in vitro lipid and protein digestibility of walnuts and peanuts. *Food Research International*, *119*(June 2018), 951–959. https://doi.org/10.1016/j.foodres.2018.11.014

Picariello, G., Miralles, B., Mamone, G., Sánchez-Rivera, L., Recio, I., Addeo, F., & Ferranti, P. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition & Food Research*, *59*(5), 948–956. https://doi.org/10.1002/mnfr.201400856

Poore, J., & Nemecek, T. (2018). Reducing food's environmental impacts through producers and consumers. *Science*, *360*(6392), 987–992. https://doi.org/10.1126/science.aaq0216

Postgate, J. R. (1998). *Nitrogen fixation*. Cambridge University Press.

Precedence Research. (2022). *Protein Ingredients Market - - Global Industry Analysis, Size, Share, Growth, Trends, Regional Outlook, and Forecast 2022 – 2030*. https://www.precedenceresearch.com/protein-ingredients-market

Rakić, S., Petrović, S., Kukić, J., Jadranin, M., Tešević, V., Povrenović, D., & Šiler-Marinković, S. (2007). Influence of thermal treatment on phenolic compounds and antioxidant properties of oak acorns from Serbia. *Food Chemistry*, *104*(2), 830–834. https://doi.org/10.1016/j.foodchem.2007.01.025

Rebello, C. J., Greenway, F. L., & Finley, J. W. (2014). A review of the nutritional value of legumes and their effects on obesity and its related co-morbidities. *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *15*(5), 392–407.

https://doi.org/10.1111/obr.12144

Reckling, M., Hecker, J.-M., Bergkvist, G., Watson, C. A., Zander, P., Schläfke, N., Stoddard, F. L., Eory, V., Topp, C. F. E., Maire, J., & Bachinger, J. (2016). A cropping system assessment framework—Evaluating effects of introducing legumes into crop rotations. *European Journal of Agronomy*, *76*, 186–197. https://doi.org/10.1016/j.eja.2015.11.005

Revilla, I. (2015). Impact of Thermal Processing on Faba Bean (Vicia faba) Composition. In *Processing and Impact on Active Components in Food* (pp. 337–343). Elsevier. https://doi.org/10.1016/B978-0-12-404699-3.00040-8

Rochefoucauld, F. de la. (n.d.). *No Title*.

Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K., & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *The British Journal of Nutrition*, *71*(1), 29–42. https://doi.org/10.1079/bjn19940108

Rutherfurd, S. M., Fanning, A. C., Miller, B. J., & Moughan, P. J. (2015). Protein Digestibility-Corrected Amino Acid Scores and Digestible Indispensable Amino Acid Scores Differentially Describe Protein Quality in Growing Male Rats. *The Journal of Nutrition*, *145*(2), 372–379. https://doi.org/10.3945/jn.114.195438

Sá, A. G. A., Hang, J., Jardine, L., Bett, K. E., & House, J. D. (2023). How different amino acid scoring patterns recommended by FAO / WHO can affect the nutritional quality and protein claims of lentils . *Sustainable Food Proteins*, *1*(2), 59–73. https://doi.org/10.1002/sfp2.1008

Sá, A. G. A., Wan, Z., Jha, A. B., Gali, K. K., Warkentin, T. D., & House, J. D. (2024). Influence of different amino acid scoring patterns on the protein quality of field peas. *Journal of Food Composition and Analysis*, *127*(November 2023), 105938.

https://doi.org/10.1016/j.jfca.2023.105938

Sagum, R., & Arcot, J. (2000). Effect of domestic processing methods on the starch, non-starch polysaccharides and in vitro starch and protein digestibility of three varieties of rice with varying levels of amylose. *Food Chemistry*, *70*(1), 107–111. https://doi.org/10.1016/S0308-8146(00)00041-8

Samtiya, M., Aluko, R. E., & Dhewa, T. (2020). Plant food anti-nutritional factors and their reduction strategies: an overview. *Food Production, Processing and Nutrition*, *2*(1), 6. https://doi.org/10.1186/s43014-020-0020-5

Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chemistry*, *239*, 486–494. https://doi.org/10.1016/j.foodchem.2017.06.134

Sarwar Gilani, G., Wu Xiao, C., Cockell, K. A., Gilani, G. S., Xiao, C. W., & Cockell, K. A. (2012). Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. *British Journal of Nutrition*, *108*(SUPPL. 2), S315-32. https://doi.org/10.1017/S0007114512002371

Schaafsma, G. (2012). Advantages and limitations of the protein digestibility-corrected amino acid score (PDCAAS) as a method for evaluating protein quality in human diets. *The British Journal of Nutrition*, *108 Suppl*(S2), S333-6. https://doi.org/10.1017/S0007114512002541

Schmitt, D. A., Nesbit, J. B., Hurlburt, B. K., Cheng, H., & Maleki, S. J. (2010). Processing Can Alter the Properties of Peanut Extract Preparations. *Journal of Agricultural and Food Chemistry*, *58*(2), 1138–1143. https://doi.org/10.1021/jf902694j

Searchinger, T., Hanson, C., Ranganathan, J., Lipinski, B., Waite, R., Winterbottom, R., Dinshaw,

A., Heimlich, R., Boval, M., Chemineau, P., Dumas, P., Guyomard, H., Kaushik, S., Makowski, D., Manceron, S., & Ben, T. (2014). Creating a sustainable food future. A menu of solutions to sustainably feed more than 9 billion people by 2050. World resources report 2013-14: interim findings. In *World Resources Institute* (Issue July). https://hal.archives-ouvertes.fr/hal-01129910/

Shi, L., Arntfield, S. D., & Nickerson, M. (2018). Changes in levels of phytic acid, lectins and oxalates during soaking and cooking of Canadian pulses. *Food Research International*, *107*(March), 660–668. https://doi.org/10.1016/j.foodres.2018.02.056

Siegert, W., Ganzer, C., Kluth, H., & Rodehutscord, M. (2018). Effect of particle size distribution of maize and soybean meal on the precaecal amino acid digestibility in broiler chickens. *British Poultry Science*, *59*(1), 68–75. https://doi.org/10.1080/00071668.2017.1380295

Singh, B., Singh, J. P., Singh, N., & Kaur, A. (2017). Saponins in pulses and their health promoting activities: A review. *Food Chemistry*, *233*, 540–549. https://doi.org/10.1016/j.foodchem.2017.04.161

Sousa, R., Portmann, R., Dubois, S., Recio, I., & Egger, L. (2020). Protein digestion of different protein sources using the INFOGEST static digestion model. *Food Research International (Ottawa, Ont.)*, *130*(8), 108996. https://doi.org/10.1016/j.foodres.2020.108996

Sousa, R., Recio, I., Heimo, D., Dubois, S., Moughan, P. J., Hodgkinson, S. M., Portmann, R., & Egger, L. (2023). In vitro digestibility of dietary proteins and in vitro DIAAS analytical workflow based on the INFOGEST static protocol and its validation with in vivo data. *Food Chemistry*, *404*(PB), 134720. https://doi.org/10.1016/j.foodchem.2022.134720

Sparvoli, F., Bollini, R., & Cominelli, E. (2015). Nutritional Value. In *Grain Legumes* (pp. 291–325). https://doi.org/10.1007/978-1-4939-2797-5_10

Staniak, M., Ksiak, J., & Bojarszczuk, J. (2014). Mixtures of Legumes with Cereals as a Source of Feed for Animals. In *Organic Agriculture Towards Sustainability* (pp. 125–145). InTech. https://doi.org/10.5772/58358

Statista. (2022). *Global animal and plant-based protein market size 2021-2030*. https://www.statista.com/statistics/1177892/global-plant-based-protein-market-value/

Stein, H. H., Fuller, M. F., Moughan, P. J., Sève, B., Mosenthin, R., Jansman, A. J. M., Fernández, J. A., & de Lange, C. F. M. (2007). Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. *Livestock Science*, *109*(1–3), 282–285. https://doi.org/10.1016/j.livsci.2007.01.019

Tinus, T., Damour, M., van Riel, V., & Sopade, P. A. (2012). Particle size-starch–protein digestibility relationships in cowpea (Vigna unguiculata). *Journal of Food Engineering*, *113*(2), 254–264. https://doi.org/10.1016/j.jfoodeng.2012.05.041

Tiwari, Brijesh K.; Singh, N. (2012). *Pulse chemistry and technology*. Cambridge: Royal Society of Chemistry.

Tornberg, E. (2005). Effects of heat on meat proteins – Implications on structure and quality of meat products. *Meat Science*, *70*(3), 493–508. https://doi.org/10.1016/j.meatsci.2004.11.021

U.S. Department of Agriculture, A. R. S. (2019a). *Fooddata Central - Nuts, pistachio nuts, raw*. https://fdc.nal.usda.gov/fdc-app.html#/food-details/170184/nutrients

U.S. Department of Agriculture, A. R. S. (2019b). *Fooddata Central - Wheat, hard red spring*. https://fdc.nal.usda.gov/fdc-app.html#/food-details/168889/nutrients

U.S. Department of Agriculture, A. R. S. (2023). *Fooddata Central - Rice, white, long grain, unenriched, raw*. https://fdc.nal.usda.gov/fdc-app.html#/food-details/2512381/nutrients

Udenigwe, C. C., & Aluko, R. E. (2010). Antioxidant and angiotensin converting enzyme-

inhibitory properties of a flaxseed protein-derived high fischer ratio peptide mixture. *Journal of Agricultural and Food Chemistry*, *58*(8), 4762–4768. https://doi.org/10.1021/jf100149w

Udenigwe, C. C., & Aluko, R. E. (2012). Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *Journal of Food Science*, *77*(1), R11–R24. https://doi.org/10.1111/j.1750-3841.2011.02455.x

United Nations. (2019). World Population Prospects 2019: Highlights. In P. D. Department of Economic and Social Affairs (Ed.), *United Nations*. http://www.ncbi.nlm.nih.gov/pubmed/12283219

USA Food and Drug Administration. (2018). *Reference Amounts Customarily Consumed: List of Products for Each Product Category: Guidance for Industry*. https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-101/subpart-A/section-101.12

USA Food and Drug Administration. (2021). *Electronic code of federal regulations fiber definitions*. https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-101/subpart-A/section-101.9

Vagadia, B. H., Vanga, S. K., & Raghavan, V. (2017). Inactivation methods of soybean trypsin inhibitor – A review. *Trends in Food Science & Technology*, *64*, 115–125. https://doi.org/10.1016/j.tifs.2017.02.003

Wen, S., Zhou, G., Li, L., Xu, X., Yu, X., Bai, Y., & Li, C. (2015). Effect of cooking on in vitro digestion of pork proteins: a peptidomic perspective. *Journal of Agricultural and Food Chemistry*, *63*(1), 250–261. https://doi.org/10.1021/jf505323g

WHO, & FAO. (2007). *Protein and amino acid requirements in human nutrition : report of a joint FAO/WHO/UNU expert consultation* (pp. xi, 265 p.). World Health Organization.

Wickham, M. J. S., Faulks, R. M., Mann, J., & Mandalari, G. (2012). The Design, Operation, and Application of a Dynamic Gastric Model. *Dissolution Technologies*, *19*(3), 15–22. https://doi.org/10.14227/DT190312P15

Wiggins, A. K. A., Anderson, G. H., & House, J. D. (2019). Research and regulatory gaps for the substantiation of protein content claims on foods. *Applied Physiology, Nutrition, and Metabolism*, *44*(1), 95–98. https://doi.org/10.1139/apnm-2018-0429

WK Kellogg Co. (2023). *No Title*. https://www.allbran.ca/en_CA/products/all-bran-original-cereal-product.html

Wolfe, R. R., Rutherfurd, S. M., Kim, I.-Y., & Moughan, P. J. (2016). Protein quality as determined by the Digestible Indispensable Amino Acid Score: evaluation of factors underlying the calculation. *Nutrition Reviews*, *74*(9), 584–599. https://doi.org/10.1093/nutrit/nuw022

Young, V. R., & Pellett, P. L. (1994). Plant proteins in relation to human protein and amino acid nutrition. *The American Journal of Clinical Nutrition*, *59*(5), 1203S-1212S. https://doi.org/10.1093/ajcn/59.5.1203S

Yu, P. (2007). Protein Molecular Structures, Protein SubFractions, and Protein Availability Affected by Heat Processing: A Review. *American Journal of Biochemistry and Biotechnology*, *3*(2), 66–86. https://doi.org/10.3844/ajbbsp.2007.66.86

Yu, T., Morton, J. D., Clerens, S., & Dyer, J. M. (2017). Cooking-Induced Protein Modifications in Meat. *Comprehensive Reviews in Food Science and Food Safety*, *16*(1), 141–159. https://doi.org/10.1111/1541-4337.12243

Zhang, Y. T., Yu, B., Lu, Y. H., Wang, J., Liang, J. B., Tufarelli, V., Laudadio, V., & Liao, X. D. (2017). Optimization of the Fermentation Conditions to Reduce Anti-Nutritive Factors in

Soybean Meal. *Journal of Food Processing and Preservation*, *41*(5), e13114. https://doi.org/10.1111/jfpp.13114

Zhou, J., Li, M., Bai, Q., de Souza, T. S. P., Barrow, C., Dunshea, F., & Suleria, H. A. R. (2024). Effects of Different Processing Methods on Pulses Phytochemicals: An Overview. *Food Reviews International*, *40*(4), 1138–1195. https://doi.org/10.1080/87559129.2023.2212041

Zia-ur-Rehman;, & Salariya, A. M. (2005). The effects of hydrothermal processing on antinutrients, protein and starch digestibility of food legumes. *International Journal of Food Science & Technology*, *40*(7), 695–700. https://doi.org/10.1111/j.1365-2621.2005.00978.x

APPENDICES

APPENDIX A – AMYLASE ACTIVITY ASSAY

Unit definition: One unit of amylase releases 1.0 mg of maltose equivalent from starch in 3 minutes at pH 6.9 and 20 °C.

Reagents and solution

- 0.2 % w/v maltose standard
- 2M NaOH
- The color solution: 96 mM 3,5-dinitrosalicylic acid and 5.3 M sodium potassium tartrate solution
- Buffer solution: 6.7 mM NaCl in 20 mM sodium phosphate buffer
- 1.0 % (wt/vol) soluble potato starch solution in the buffer solution
- The enzyme solution: dilute (mg) enzyme in water for an estimated 1 unit amylase activity/ml solution

Procedure to measure the standard curve

- Dilute the 0.2 % w/v maltose standard maltose with water as below

Maltose tube	1 (Blank)	2	3	4	5	6	7	8
H_2O (mL)	1.0	0.975	0.9	0.8	0.7	0.6	0.5	-
0.2 % w/v maltose solution (mL)	-	0.025	0.1	0.2	0.3	0.4	0.5	1.0
Total volume (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

- Add 0.5 mL of the colour solution to each maltose standard
- Boil the standards at 100 °C for 15 minutes
- Place the tubes on ice until they reach room temperature
- Add 4.5 mL of H_2O and mix well

- Read the absorbance at 540 nm and 20°C and plot the standard curve.

Procedure to measure enzyme solution

- Add 0.5 mL of the 1.0 % (wt/vol) substrate soluble potato starch solution into reaction tubes
- Mix and incubate at 20°C for 5 minutes to achieve temperature equilibration
- Add the first time enzyme solution according to the scheme : 0 - 0.25 - 0.3 - 0.35 mL
- Mix and incubate at 20°C for precisely 3 minutes
- Immediately stop the reaction by adding 0.5 mL of the colour solution
- Add enzyme the second time to make enzyme volume to 0.5 mL in total (0.5 - 0.25 - 0.2 - 0.15 mL)
- Boil all the tubes at 100°C for exactly 15 minutes
- Place the tubes on ice until they reach room temperature
- Add 4.5 mL of H_2O and mix the reaction
- Record the absorbance at 540 nm and 20°C.
- Total reaction for each tube

Amylase tube	1 (Blank)	2	3	4
Substrate – the potato starch solution (mL)	0.5	0.5	0.5	0.5
1st enzyme solution (mL)	-	0.25	0.3	0.35
Colour solution (mL)	0.5	0.5	0.5	0.5
2nd time of enzyme (mL)	0.5	0.25	0.2	0.15
H_2O (mL)	4.5	4.5	4.5	4.5

Calculations

- Standard Curve: $\Delta A540_{Standard} = \Delta A540_{Standard} - \Delta A540_{Standard\ Blank}$
- Establish a linear regression: $\Delta A540_{Standard} = a \times [\text{maltose (mg)}] - b$
- Enzyme activity:$\Delta A540_{Sample} = \Delta A5400_{Test} - \Delta A540_{Test\ Blank}$

$$\frac{Units}{mg\ powder} = \frac{[A_{540}Test - A_{540}Test\ Blank] - b}{(a \times X)}$$

a: the linear regression slope of ΔA540nm *vs* the quantity of maltose (mg).

b: the linear regression intercept of ΔA540nm *vs* the quantity of maltose (mg).

X: the quantity of amylase (mg) added **before stopping the reaction**

APPENDIX B – GASTRIC LIPASE ENZYME ACTIVITY ASSAY

Unit definition: One unit of lipase releases 1 μmol of butyric acid per minute at 37 °C at 6.0 for human gastric lipase and 5.5 for rabbit gastric lipase.

Reagents and solution

- Tributyrin purity ≥ 99%
- 0.1 M HCl
- 0.1 M NaOH
- The assay solution: 150 mM NaCl, 2 mM sodium taurodeoxycholate, and 1 μM bovine serum albumin in water
- The enzyme solution: 1mg/mL of lipase in water

Procedure

- Add 14.5 mL of the assay solution and 0.5 mL of tributyrin into a 20 mL beaker
- Add 8 mL of water to a 50 mL beaker
- Put the 20 mL reaction beaker inside the 50mL beaker
- Add the magnetic stirrer to the 20 mL beaker
- Place the 2-beaker on the hot place and measure the pH temperature of the reaction vessel
- Mixing until tributyrin disperses into the assay solution as a fine oil-in-water emulsion
- For the blank test: measure the pH and monitor the volume of 0.1M NaOH that needs to keep pH at 5.5 at 37°C for 10 minutes
- For the enzyme test:
    - Adding titrant solution (0.1 M NaOH) to monitor the pH and adjust it at pH 8
    - Add 50 – 75 - 100 μL of the enzyme solution

- Monitor the rate of 0.1M NaOH required to maintain the pH 5.5 at 37°C for 10 minutes

Calculations

$$\frac{Units}{mg\ powder} = \frac{R(NaOH) \times 1000}{v\ \times\ [E]} \times F$$

R(NaOH): the rate [µmol per minute] of NaOH delivery i.e., µmol free fatty acid titrated per minute

v: the volume [µL] of enzyme solution added in the vessel

[E]: the concentration [mg powder/mL] of the enzyme solution

F: the correction factor to take into account the partial ionization of fatty acids at the pH of the assay, i.e. at pH 5.5 F = 1.12

APPENDIX C – PEPSIN ACTIVITY ASSAY

Unit definition: One unit produces the amount of TCA–soluble products as ΔA280 of 0.001 per min at pH 2.0 and 37 °C

Reagents and solution

- 10 mM HCl
- 2% (wt/vol) hemoglobin in water
- 10 mM Tris + 150 mM NaCl buffer solution
- 5% (wt/vol) trichloroacetic acid in water
- The enzyme solution: 1mg/mL of pepsin or lipase in water

Note

The rabbit gastric lipase product also contained pepsin; hence, the pepsin activity unit of the lipase also needs to be measured.

Procedure

- Dilution of the pepsin and lipase solution to a suitable concentration
- Pipette 500 μL of 2% hemoglobin to 2 mL tubes.
- Place the tubes at 37^{o}C for 4 minutes to reach temperature equilibration
- Add 100 μL of each pepsin/lipase dilution to the tube, except the blanks
- Incubate for 10 minutes at 37 °C
- Add 1 mL of 5% (wt/vol) trichloroacetic acid to every tube to stop the reaction.
- For blank tubes, 100 μL of enzyme solution is added after the addition of trichloroacetic acid solution
- Centrifuge all tubes at 6,000g for 30 minutes to precipitate the hemoglobin
- Read the absorbance of the supernatant at 280 nm

Calculations

$$Units/mg = \frac{[(A280\ test - A280\ blank)]\ x\ 1000}{(\Delta t\ x\ X\ x\ 0.001)}$$

Δt: 10 minutes

X: concentration of pepsin powder in the final reaction mixture (quartz cuvette) [µg/ml]

1000: dilution factor to convert µg to mg

0.001 = A280 per unit of pepsin

Ensure the activity obtained is the same for each tested pepsin concentration as belonging to the linear evolution of pepsin concentration. If no linear part is found, it is necessary to repeat with more dilute enzyme solutions.

APPENDIX D – TRYPSIN ACTIVITY ASSAY

Unit definition: One unit of trypsin hydrolyses 1 μmol of TAME per minute at 25°C and pH 8.1.

Reagents and solution

- 1 mM HCl
- 46 mM Tris/HCl buffer with 11.5 mM $CaCl_2$
- 10 mM p-Toluene-sulfonyl-L-arginine methyl ester (TAME) in water
- The enzyme solution:
  - Prepared a 1mg/mL of pancreatin solution in water
  - Mix well
  - Apply ultrasound treatment (45 Hz,124 130 W) for 5 minutes
  - Centrifuge the pancreatin solution at 2000xG for 5 minutes
  - Transfer the pancreatin supernatant, then keep the solution on ice for the assay

Procedure

- Prepare a pancreatin dilution from the pancreatin supernatant solution with 1 mM HCl.
- Pipette 1.3 ml of the Tris/HCl buffer and 150 μL of the substrate TAME solution into cuvettes
- Incubate at 25°C for 4 minutes to achieve the temperature equilibration
- Add 50 μL of each concentration of pancreatin dilution except the blanks
- Mix pancreatin before pipetting it to the enzyme reaction vessel
- Mix reaction vessel by inversion/pipetting

- For blank tests, replace the enzyme amount (50 µL) with Tris/HCl buffer for the reaction
- Record in the continuum the absorbance increase at 247 nm during 10 min

Calculations

Plot the absorption versus the time in minutes and determine the slope ΔA247 from the initial linear part of the curve. If no linear part is found, the test should be repeatted with a lower or higher amount of enzyme

$$Units/mg = \frac{[(\Delta A247\ test - \Delta A247\ blank)]x\ 1000\ x\ 1.5}{(540\ x\ X)}$$

ΔA247: the slope of the initial linear portion of the curve [unit absorbance/minute]

540: molar extinction coefficient of TAME at 247 nm.

1.5: Volume (in millilitres) of reaction mix (Tris/HCl + TAME + enzyme)

X: quantity of trypsin [mg] in the final reaction inside the quartz cuvette

APPENDIX E – THE INFOGEST 2.0 DIGESTION STEPS

Preparation

- Thaw and pre-warm all three SDF at 37 °C
- Prepare all enzyme and bile solutions according to acquired enzyme activity results and bile concentration test
- Pre-warm incubator at 37 °C
- Thaw cookies samples
- Weight sample to equivalent 40 mg of crude protein,
- Dissolve the sample and cookies in 1 ml water for each digestion

Digestion

- Dilute digestion tube with 0.8 mL SSF and 5 µL of $CaCl_2$
- Add 0.1 mL of prepared amylase solution and 0.095 ml of water
- Incubate while mixing for 2 minutes at 37 °C
- Add 1.6 mL SGF and 1 µL of $CaCl_2$
- Adjust the pH of the digestion tube to 3.0, and record the added volume of NaOH/HCl
- Add water 0.299 ml – added volume of NaOH/HCl) to achieve a 1× concentration of SGF
- Add 0.1 mL of prepared pepsin solution and 0.1 mL of prepared gastric lipase solution
- Incubate for 2 hours at 37 °C while mixing
- Add 1.7 mL SIF and 8 µL of $CaCl_2$
- Adjust the pH of the digestion tube to 7.0, and record the added volume of NaOH/HCl
- Add water (0.792 ml – added volume of NaOH/HCl) to achieve a 1× concentration of SGF

- Add 1 mL of prepared pancreatin solution and 0.5 mL of prepared bile solution
- Incubate for 2 hours at 37 °C while mixing.

Post-digestion

- Boil the digesta to 100°C for 5 minutes to inactive all enzyme activities.
- Separate the digesta into digestible and undigestible fractions by MeOH precipitation.
    - Add MeOH to digesta to reach the final concentration of 80% (vol/vol).
    - Incubate digesta at -20°C for 1 hour, allowing the precipitation of large molecules.
    - Centrifuge all tubes at 2,000 g at 4°C for 15 minutes.
    - The supernatants were collected into new tubes without taking the interface.
    - Wash the pellets twice with MeOH (100%) and centrifuge 2,000 g at 4°C for 5 minutes.
    - Dry the pellets with the Thermo Savant DNA120 SpeedVac Concentrator and Savant™ UVS400 Universal SpeedVac™ Vacuum System (Thermo Fisher Scientific) in moderated heat for 8 hours until completely dry.
    - Record the added MeOH volume, the total collected supernatant, and the dried pellet weight
    - Store both supernatants and pellets at -20°C for other analysis.

APPENDIX F – THE OPA ASSAY

Phase 1

- Pre-warm oven to 110°C
- Weight dried pellets into hydrolysis tubes (2-4mg)
- Mix supernatant samples well and add 200 µL to a hydrolysis tube
- Tightly wrap 4 rounds of Teflon tape over the glass threads
- Add two drops of octanol
- Add 3.8mL of 6.3N HCl
- Purge nitrogen gas into the tube for about 7 seconds, then seal the tube immediately with the cap afterwards.
- Add a copper jacket over test sample tubes in a heat-resistant polypropylene rack.
- Place samples in the oven with a metal sheet overtop for 24 hours

Phase 2

- Carefully remove the tube from the oven and allow the sample to cool
- Remove Teflon tape
- Add 3.9mL of 25% NaOH to each sample tube
- Vortex then pours the hydrolysate into a 50mL beaker with a magnetic stir bar.
- Wash the tube inside three times, each time by adding 4mL of water, vortex to mix well, and then transfer the water to the same beaker.
- Stir sample well in 2 minutes at 300 RPM
- Filter the hydrolysate with a 0.22 µm filter to a new 2 mL cryogenic vial

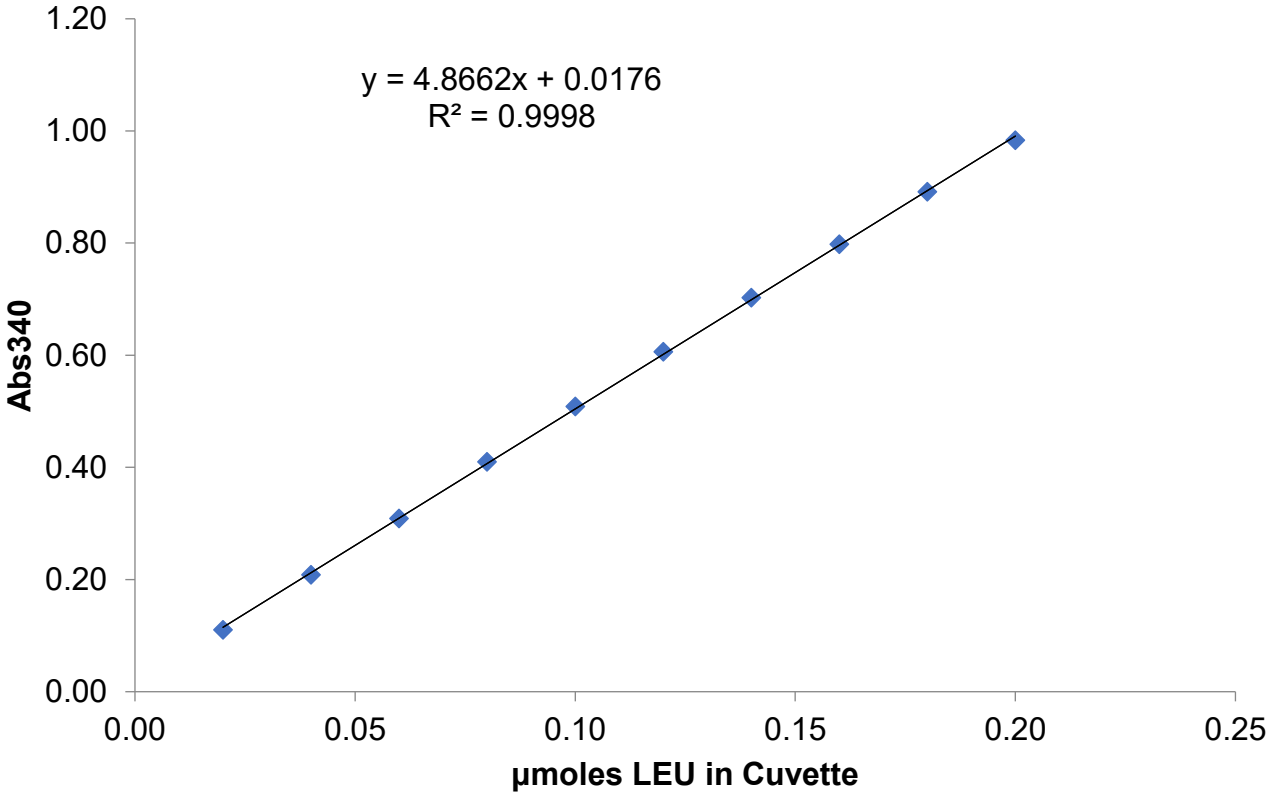
OPA solution preparation (for 50 ml solution)

- Add 25 mL of 0.1M sodium tetraborate, 2.5 mL of 20% SDS and 21.4 mL water to an amber flask and stir at 150-200 RPM at room temperature.
- Inside a fume hood, add 100µL of β-mercaptoethanol into the amber
- Dilute 60 mg o-phthaldialdehyde (OPA) in 1.5 mL methanol in a brown plastic vial
- Add 1mL of OPA solution to the amber flask
- Continually stir the solution for at least 1 hour prior to use

Measuring

- Add 200 µL of 0.1M sodium tetraborate buffer and 1mL of OPA solution into the cuvette
- Quickly mix the solution with a pipette 8-10 times
- Read the absorbance at 340 nm after 2 minutes as the "Blank" value
- Prepare 10 concentrations of L-Leucine standard from 0.1mM to 1mM in 0.1M sodium tetraborate buffer
- Add 200 µL of each L-Leucine standard from least to most and 1mL of OPA solution into the cuvette
- Pipette mix 8-10 times and read the absorbance at 340 nm after 2 minutes as the "Standard" value
- Repeat the process for measuring samples using 200µL of each filtered hydrolysate sample from Phase 2
- Repeat the measure order of sample and standard backward to complete the forward/reverse order

APPENDIX J – THE ABSORBANCE OF THE L-LEUCINE STANDARD CURVE



APPENDIX H - UPLC CHROMATOGRAM OF CASEIN SAMPLE WITH REGULAR HYDROLYSIS PROTOCOL