Improving the digestibility of alternative proteins using high moisture extrusion for plant-based fish analogues

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KLTM02 – Degree Project in Food Engineering, Nutrition and Food Chemistry

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Abstract

The consumption of plant-based, food alternatives is increasing globally. Yet, the digestion of plant proteins is hindered by various antinutritional and external factors. Therefore, delving into the extent of plant protein digestibility in the human body becomes essential, alongside efforts to enhance this process. The main aim of this present work was to determine the digestibility of plant-based fish analogues based on a mixture of yellow pea (80%), and microalgae (1.85%) protein. Four samples were produced using the high moisture extrusion technique assigned to A, B, C, and D, with the extrusion temperature profiles decreasing sequentially for each sample by 5°C each zone. Protein digestibility of the obtained products was studied using an *in-vitro* digestion according to the guidelines of INFOGEST protocol. Real tuna underwent *in-vitro* digestion as a control for animal-derived protein source.

The protein content remaining after digestion was measured using the Bradford and Dumas method. The results obtained from both methods were compared and a similar trend was observed. The plant-based fish analogues and real tuna showed a decrease in protein content after the digestion by 76-77% and 84%, respectively, compared to the initial protein concentration. Statistical analysis proved that the temperature profile in high moisture extrusion has a significant effect on the protein content after digestion. Sample C had the second lowest temperature profile and highest digestion (zone 1 to 8 were 40°C, 60°C, 80°C, 115°C, 130°C, 140°C, 125°C, 110°C, respectively, and the cooling die at 35°C). The non-linear trend observed between extrusion temperature and digestibility rises the opportunity for optimisation of HME temperature profiles around C.

Additionally, after the base digestion, a plant-derived enzyme, bromelain in dose 1 mg/mL, was added to the oral phase. This was in attempt to improve the protein digestibility. Statistical analysis showed that there was no significant difference when bromelain was added, compared to those without. However, the observed trend did show a tendency for protein concentration to decrease. Hence, a higher bromelain concentration of up to 10 mg/mL is recommended for future experiments to obtain statistical significance. A texture analysis was conducted, indicating that sample C, with the highest digestibility, had the lowest toughness and firmness.

Keywords: high moisture extrusion, plant-based fish analogues, protein digestibility, *in-vitro* digestion, bromelain

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Popular Science Summary

Adding pineapple juice to a plant-based diet – what is the extent of plant protein digestibility, and how can it be improved?

The worldwide consumption of plant-based alternatives is increasing for various reasons. Main reasons for consumers have been linked to environmental concern, animal welfare and land usage. In this degree project, there was collaboration with Hooked Foods, a start-up company producing plant-based fish analogues. Compared to conventional fishing, the production of plant-based fish analogues may help to reduce by-catch, habitat loss, and biodiversity decline. However, wide observation has shown that plant-based proteins are not completely digested or absorbed in the body. Common factors hindering digestion are examples such as anti-nutritional factors found in plants, interfering with nutrient absorption and, hence, reducing their nutritional value. Therefore, the investigation of plant protein digestibility is highly relevant, not only to this degree project, but also for the health benefits that will come for plant-based consumers. Especially in finding ways to improve digestibility.

The plant-based fish analogues produced by Hooked Foods, were obtained using a high moisture extrusion machine that converts proteins into fibrous structures. Specifically for this degree project, the ingredients used were microalgae protein, yellow pea protein, pea fibre, and white fish flavouring. To obtain four different samples for the digestion experiments, the latter zones of the extrusion temperatures profiles were decreased sequentially. To investigate the digestibility of the samples, the human digestive system was mimicked in the laboratory. Digestive enzymes were added for the oral, gastric, and intestinal phase, while mixing was achieved by magnetic stirrers within each glass bottle. After performing the base digestion on the samples, a plant-derived enzyme, bromelain (found in pineapple) was added to try to improve the protein breakdown. To measure the extent of digestibility, protein analysis methods were carried out to measure the protein content in the final digestates.

The results from protein analysis revealed that the four samples were significantly different to each other, meaning that the temperature profile in high moisture extrusion has an impact on protein digestibility. However, there were no significant differences between the samples containing bromelain to the samples without. However, there was an observed tendency from the averages of the replicates, that the protein digestibility does manage to increase with the addition of bromelain and higher doses for this digestive enzyme need to be tested.

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List of acronyms

ANF	Antinutritional factors
BRM	Bromelain
BSA	Bovine Serum Albumin
GIT	Gastrointestinal tract
HME	High Moisture Extrusion
KW	Kruskal-Wallis
LC-MS	Liquid Chromatography Mass Spectroscopy
LD	Laser Diffraction
ns	not significant
OD	Optical Density
PBFA	Plant-based fish analogues
PEF	Pulsed Electric Field
PF	Protein Fibre
PM	Protein Masker
PPI	Pea Protein Isolate
SD	Standard deviation
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOP	Standard Operating Procedure
SPI	Soybean Protein Isolate
SSF	Simulated Salivary Fluid

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1. Introduction

The trend towards consuming plant-based foods is on the rise for various reasons. A start-up company in Stockholm, Hooked Foods, is at the forefront of this shift, offering plant-based fish analogues (PBFA) as an alternative to traditional ocean-caught fish. The company have done this knowing that the conventional method of fishing contributes to by-catch, habitat loss, and a decline in biodiversity [1]. Therefore, the offering of plant-based seafood rises the already increasing, human consumption of plant-based proteins. This degree project places emphasis on evaluating, as well as enhancing, the digestibility of these plant-based proteins within our human body.

Plant-based proteins have been a popular choice for many individuals with a motivated reason of being more environmentally conscious. The greenhouse gas emissions and land usage to produce a widely consumed, plant-based protein, for example, pea, are much lower than what is required for beef, 0.4 kg CO_2 eq. and 3.4 m^2 versus 50 kg CO_2 eq. and 164 m^2, respectively [2]. Hence, the consumption of alternative proteins contributes to alleviating the strain on our already environmentally challenged planet.

However, consuming plant-based proteins has caused some concern with regards to a human's ability to break down and utilise the protein. The presence of certain antinutritional factors (ANF), exemplified by phytates, tannins, trypsin inhibitors, and lectin [3], have shown to make them less bioavailable and hinder their digestibility. Not only this, but the lack of all essential amino acids in plant-based proteins renders them at a lower protein quality than all animal proteins which contain all essential amino acids [4]. Given that protein is an indispensable foundation for human life, there is great necessity to find ways of making them more digestible. The significance of this will not only extend to the ordinary person, but especially to the elderly population whose reduced stomach acid, diminished enzymes, and changes in gut motility, impede on their ability to efficiently digest protein [4]. The necessity also extends to the apt way of matching and combining the appropriate plant-based proteins, to ensure the fulfilled requirement of all essential amino acids needed for crucial bodily functions.

Excitingly, there have been certain enzymes within the category of 'non-animal derived', that have recently shown promise for their ability to enhance protein break down and hence, the digestibility of plant-based proteins when they are consumed together in a meal. With special mention, the enzyme bromelain (BRM), found in pineapple fruit and stem, has been proven to

not only enhance peptide cleavage in protein, but additionally has a positive impact on human health from its anti-inflammatory effects [5].

This research project aims to showcase the ability to empower plant-based proteins using additional, non-animal derived enzymes, while meeting the demand for sustainable food choices, and addressing any concerns about accessibility and nutritional benefits. The additional aim is for these optimized proteins to be utilised in Hooked Foods' plant-based seafood, aligning with their commitment to sustainable and eco-friendly food alternatives.

The overall conclusion will mark a crucial step in our movement towards a more inclusive and eco-friendly future of plant-based food that are readily absorbed by the human body.

1.1 Overall objectives

To investigate and increase the digestibility of alternative proteins using plant-based fish analogues, the following objectives were set.

- Research and decide on an ingredient recipe, using the combination of yellow pea, microalgae protein, fibres, etc.
- To perform and optimise HME by selecting an independent variable among the parameters of temperature, shear force, shear stress, retention time, and hence, obtain different samples. Set up a post-process method, including shredding and flavouring.
- Introduce extrusion samples into a mimic of the human digestive system (mouth, stomach, small intestine) by maintaining the temperature, pH, wiggling/mixing, and introducing body enzymes. Results are compared and evaluated to an animal-based product (tuna).
- Investigate whether the addition of plant-derived enzymes, for instance, in supplements or a juice, enhances the protein digestibility of the plant-based fish analogue samples.

2. Literature review

As the main focus of this degree project is to improve the digestibility of plant-based proteins within the human body. This literature review begins with a detailed look at the holistic functionality and importance of proteins for humans. It then narrows down to explore the two specific, plant-based proteins chosen for the digestibility challenge. Next, is an explanation of the process, and post-process methods of feeding the mixture of these plant-based proteins through a high moisture extruder. Following are the different mechanisms of how humans digest, why it is hard to digest, and how to improve the digestion of these plant-protein extrudates from the high moisture extrusion. The use of non-animal derived enzymes to aid with additional protein breakdown, are explored. A main focus was on the enzyme bromelain, with an explanation of its properties and mechanisms of protein breakdown.

2.1. Protein

2.1.1. Functionality and importance

Proteins play an indispensable role as a macronutrient for human nutrition. Their functionality is multifaceted, extending beyond only physiological processes [4]. Proteins are the building blocks of tissues and organs, acting as enzymes to accelerate metabolic processes and convert food into energy. They facilitate molecule transport, oxygenation, and immune response. As hormones, they regulate growth, development, and metabolism, while also transmitting signals. Furthermore, proteins enable muscle movement and regulate fluid balance and pH.

2.2. Plant-based protein

The majority of the protein in our bodies is derived through the consumption of food. These proteins are broken down into amino acids (AA) to be utilized in the body. However, the body can also synthesize certain amino acids on its own. Since the biochemical structure of proteins relies on chains of AAs, the types of AAs that our bodies cannot produce on their own are termed “essential amino acids". These must be obtained directly through the diet.
´Protein quality´ is a term given to assess the essential amino acid profile of a protein, and how well the body can digest and absorb it [4]. The consumption of proteins is not efficacious unless the body can use the protein effectively.

As previously mentioned, there is a notable increase in the human consumption of plant-based proteins. However, it is commonly known that most plant-based proteins are not ‘complete’ proteins. In other words, some do not contain all essential amino acids like animal proteins.

Plant-based proteins also have a weaker digestion and utilisation profile [4], which is a topic further discussed under Heading 2.4.2. Given the substantial shift to plant-based food, maximising the utilisation of these plant-based proteins becomes crucial for the fundamental importance of proteins mentioned above. Therefore, the careful selection of which exact proteins to use in plant-based foods recipes, forms the foundation of addressing this challenge.

To assess the digestibility challenge of this thesis, a mixture of two plant-based proteins were chosen, pea and microalgae protein. The literature below explores both protein foci, including their importance and why they were chosen.

2.2.1. Pea protein

Pea protein has grown to be the more popular choice for the main protein found in plant-based food alternatives. This is mainly due to its nutritional profile, functional properties, versatility as an ingredient, and sustainability considerations.

2.2.1.1. Nutritional profile

Pea (*Lathyrus* of *Fabaceae* family) stands out nutritionally with its high protein content ranging from 20-25% within the dry matter of pea seeds [7]. It is complemented by the macronutrients: carbohydrates in the form of starch (24-49%), dietary fibre (60-65%), and fats (1.5-2%). The minor constituents are vitamins, minerals, phytic acid, saponins, polyphenols, and oxalates. Peas are especially rich in vitamin B, which plays a vital role in supporting energy metabolism. The minerals present such as potassium, phosphorus, magnesium, and calcium [7], contribute to bone health, muscle function, and nerve transmission, highlighting their importance in maintaining bodily well-being. Additionally, polyphenols offer antioxidant properties which reduce oxidative stress and inflammation.

2.2.1.2. Functional properties

Beyond its nutritional powerhouse, pea protein shows notable functional properties [7] that contribute to an optimal texture and mouthfeel for plant-based products. Starting off, pea protein has a strong water-binding capacity, which is crucial for water retention, gelling, and protein swelling. These factors aid in the structural integrity and maintenance of an optimal moisture content in the final food product.

Pea protein has an amphiphilic nature, which is characterized by the presence of both polar and non-polar groups. This, as well as its good oil-binding capacity, positions it as a highly

effective emulsifier. Emulsification properties become vital in many plant-based products to maintain stability, texture, mouthfeel, and to evenly disperse the flavours and ingredients. The emulsifying and water-holding capacities of pea protein are intricately linked to its solubility, which is, therefore, influenced by the proportion and distribution of these polar and non-polar groups within its structure. Solubility is also affected by intrinsic factors such as amino acid composition, isoelectric point, molecular flexibility, charge, as well as extrinsic factors: pH, temperature, and ionic strength [8]. Understanding these factors is crucial for food scientists and manufacturers to know how to optimize protein solubility, ensuring the execution of desirable sensory and functional properties in the final product.

2.2.1.3. Versatility as an ingredient

The relatively neutral [7] flavour of pea protein makes it a versatile ingredient in plant-based formulations. It can take on flavours and seasoning blends without dominating the overall taste. This neutral flavour is especially in yellow pea protein compared to that of green pea. Additionally, the colour effect of pea protein is minimal, which makes it an adaptable ingredient for a wide range of plant-based foods.

2.2.1.4. Pea vs. soy protein

Sustainability considerations

In 2019, the plant-based culinary industry relied heavily on soy protein as the main alternative protein for their food products [2]. However, recently, there has been a major shift and commercial investment in pea protein, not to mention its consumer acceptability. Large companies such as McDonald's and Beyond Meat have contributed to its growing market share [2]. Pea protein is especially beneficial in terms of environmental considerations. The greenhouse gas emissions, land use, and water requirement for pea protein is much lower than that of soy. Additionally, peas help with nitrogen fixation in the soil, reducing the need for synthetic fertilizers [2]. Therefore, both the industry momentum and environmental benefits for pea protein, make it a strategic and relevant focus for this degree project.

Allergens, GMO, Functionality

Unlike soy protein, pea protein is allergen friendly [2]. This is attractive for individuals with dietary restrictions and for companies who desire cleaner labelled products. Pea is also non-GMO, which is generally deemed to be a positive factor by the general public. Lastly, according to P. Shantakumar, et al., (2022) [7], pea protein is a better emulsifier and foaming agent than soy at a neutral pH, therefore, offering enhanced stability and versatility in food formulations. These properties can be improved by applying enzymatic treatments.

Health benefits

Pea protein offers many more advantageous health properties, including antioxidant, antihypertensive, anti-inflammatory, antidiabetic, and cholesterol reduction benefits [7]. The high protein content delays gastric emptying rates which exhibit appetite suppressing effects, moderates' glucose absorption and triggers the release of appetite-regulating hormones.

2.2.1.5. Yellow pea protein

Within the realm of pea protein types, yellow pea protein can be sourced locally in Sweden. Sourcing nearby is beneficial as it supports the local economy, reduces environmental impact, and allows for adaptability and flexibility. This ensures faster response times, a greater ability to meet specific requirements, and is also cost effective.

For this degree project, yellow pea protein has been chosen for the reasons which can be summarised into 1) growing traction of pea protein within the industry, 2) functionality, 3) positive ecological advantages, 4) hypoallergenic, GMO free 5) neutral taste and colour, 6) health benefits, 7) sourced locally, and 8) consumer acceptance.

2.2.1.6. Extraction of yellow pea isolate and effects on digestibility

Yellow pea isolate will be used as the main ingredient for the raw material feed to the extruder. Protein isolates are processed to remove most of the non-protein components, as such, the yellow pea isolate contains insignificant amounts of carbohydrates, fats, and fibres. The output of the extruder will be the start of the raw material sample used for enhancing digestibility. Isolates have a higher purity than the concentrates. This is advantageous because more refined ingredients give added value and improved technological functionality to the final product [9]. Using less refined ingredients, such as flours or concentrates, may be more environmentally friendly, but it may lead to challenges in the properties of the final product [9]. Additionally, as the main aim is to measure digestibility, using the isolate form of the protein will avoid the complexities of the other components (cellular structures and complexes with fibres, cell walls, lipids) which not only affects the nutritional and sensory properties, but also their technological functionality, like solubility, gelation, and hence, the final digestibility [4].

2.2.2. Microalgae protein

Due to the nature of HME, as well as to maintain pressure drop, a combination of two protein types was needed. Microalgae protein was chosen as the 'smaller proportion' protein to be added to yellow pea isolate, for reasons explained below.

Microalgae originate from seaweeds and are utilised in numerous products across the food industry. The use of microalgae in industry has proven to be beneficial for human health and the environment. This is especially due to their low requirement of land for cultivation, which yields a lower CO_2 footprint compared to that of animal-derived proteins [1]. Nutritionally, in general, microalgae are a rich source of nutrients including omega-3, omega-6 fatty acids, dietary fibre, several vitamins (e.g., A, C, D, E, K, and B vitamins) and some minerals (e.g., calcium, iron, magnesium, and potassium). This depends on the species, geographic location, growth season etc [40]. They are high purity compounds with low amounts of chemical contamination as well as serve as an antibacterial component and a useful food colorant [10]. In this degree project, *Chlorella* spp., belonging to the green microalgae (Chlorophyta), was used.

It has been shown that combination of varying concentrations of defatted *Nannochloropsis oceanica* biomass with pea protein isolate (PPI) in a plant-based fishcake resulted in different protein digestibilities [12]. The results showed an increase and decrease in digestibility when 10% and versus 30% of microalgae biomass was used, respectively. Due to its globular structure, the size of microalgae is larger than PPI. Hence, it weakens the bonds and decreases the clustering of pea proteins. This results in a more accessible surface area for digestive enzymes to break down more proteins [12]. For this reason, a smaller percentage, i.e., <10%, of microalgae was used for the PBFA in this degree project.

2.3. High moisture extrusion

Extrusion is used to convert proteins into fibrous structures. The technique is commonly used by the food industry to replicate conventional meat products. There are two types of extrusion-based methods that are well-known. Namely, high, and low moisture extrusion. The latter creates dry, texturized, vegetable proteins that are used for bread/sponge-like products, for instance, patties and burgers [13]. In this degree project, HME is rather utilized to obtain delicate, fibrous products containing a high moisture content >50 wt.% [13,18]. This is desired for the final products produced by Hooked Foods.

Generally, for plant-based meat analogues, the input materials consist of one plant protein combined with another. Or in many cases, with a polysaccharide that originates from the plant cell wall [14]. The protein material is usually in the form of a protein concentrate or isolate. The addition of water (50-60%) during the extrusion acts as a plasticizer and contributes to the final juiciness of the food product [14].

As seen in Figure 1 the proteins and other ingredients (e.g., pea fibre and protein masker) are first combined in their dry-state and then fed into the hopper of the extruder. Water is fed into the barrel and is mixed with proteins. Both protein powders melt together while passing through the screw extruder which is set at various temperatures. After cooking, the mixture is then forced through a cooling die to align and texturize the proteins. The cooling die also decreases the product expansion as well as promotes possible fibre formation [14,15] resulting in the final product.

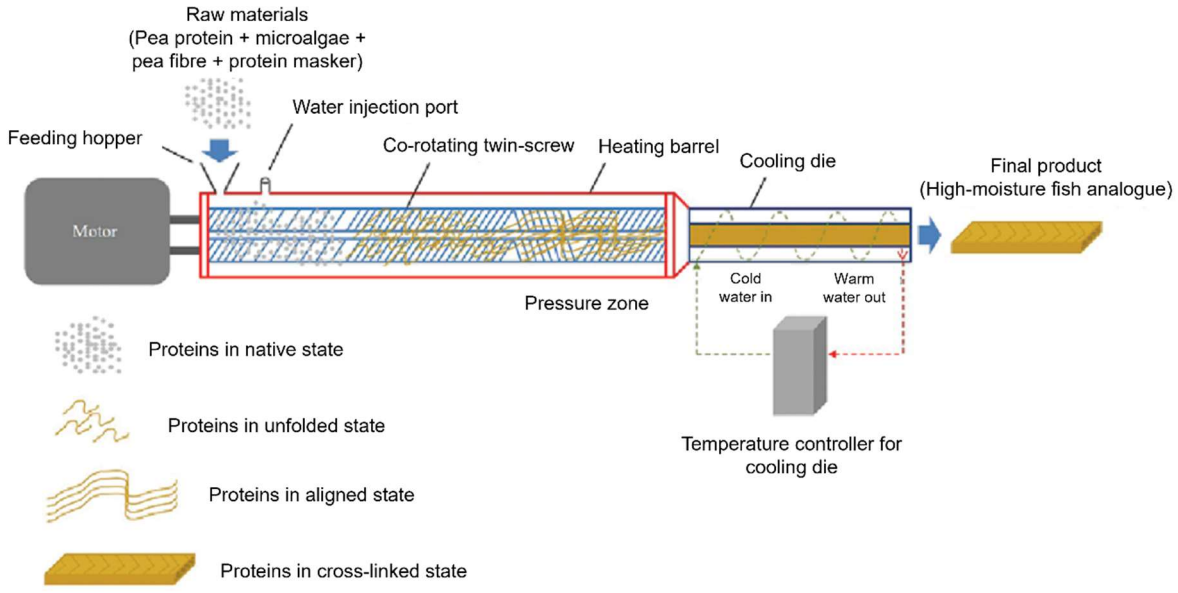

Figure 1: *Illustration of a HME based on and modified from T. Maung, et al., (2020) [16].*

Various process factors can be adjusted to create a product with the desired characteristics and texture. These include barrel temperature, screw speed, feed moisture, and feed rate [17]. The native state of plant-based proteins is often spherical. Thus, mimicking a fibrous structure and texture is achieved by unfolding and denaturing proteins at a sufficient temperature (see key in Figure 1 of unfolding proteins). Once the proteins unfold, the screw mechanism allows them to form a network and then aggregate together [14,18]. The aligning and cross-linking of the proteins can be attested to electrostatic, and hydrophobic forces, hydrogen, and disulphide bonds, van der Waals, and enzymatic reactions [18].

The twin-screw configuration, screw speed, and feed moisture were chosen based on what Hooked Foods uses to produce their plant-based tuna. These are all variables that can be optimised. However, the chosen configurations matched Hooked Foods' successful methods for achieving optimal tuna-based textures, as it was beyond this degree project's scope to explore alternatives.

2.3.1. The effect of temperature on digestibility

As previously mentioned, plant-based proteins are more difficult to digest and utilise. Hence, to improve their functionality, extrusion is often used. However, the point of interest is how the temperature, together within extrusion techniques, play a role in protein denaturation and reassembly upon extrusion processing, to produce a protein structure that may, or not, be more digestible.

A study from R. del Rio, et al., (2022) [19] investigated whether changing temperature and heating time influenced the protein digestibility. Generally, protein denaturation enhances the digestibility to a certain degree, whereas more extensive heat treatment can cause protein aggregation which could impair digestibility. In this study, soybean protein isolate (SPI) is used, and the protein digestibility increased when heated. However, the optimal temperature and heating time was concluded to be dependent on the nature and type of protein.

The process of HME involves optimizing both temperature and screw speed to achieve the most digestible protein structure post-extrusion. Temperature plays a crucial role in protein denaturation, causing the proteins to unfold and hydrophobic bonds to break [20]. This initial heat treatment sets the stage for subsequent protein aggregation. As the material passes through the extruder, the screw mechanisms exert shear force, facilitating the aggregation of denatured proteins. By carefully controlling both temperature and screw speed, an optimal

environment can be created for protein aggregation and structure formation, ultimately leading to extrudates with enhanced digestibility [20].

2.4. Digestive system

2.4.1. Protein digestion and absorption mechanisms

Taken from E. Goodman, (2010) [21], when a human starts to ingest food, the initial phase of digestion commences in the mouth. However, this represents a mere mechanical and physical breakdown of the food, with no significant impact on the chemical structure of the proteins. The actual chemical digestion begins in the stomach, where the protease enzyme, pepsin gets secreted in the presence of food in the form of pepsinogen. At the same time, gastric acid, i.e., hydrochloric acid (HCl), is released from the parietal cells to alter the conformation of pepsinogen, so that it can cleave itself to become its active form, pepsin. Gastric acid also denatures protein, partially unfolding them, so that the pepsin has better access to the peptide bonds. Pepsin then begins the hydrolysis of these peptide bonds to form polypeptides.

From the stomach, the polypeptides enter the small intestine. The first part of the small intestine, the duodenum, is the starting point where both protein digestion and absorption occur. Protein digestion starts with the help of pancreatic enzymes called trypsinogen, chymotrypsinogen, and carboxypeptidase, which are all precursors of their active forms. These proenzymes, along with pancreatic bicarbonate, make up pancreatic juice. The bicarbonate raises the pH to an ideal activity level for the pancreatic proteases. The enzyme precursors are activated by special enzymes on the surfaces of the intestinal cells, called enterokinase. Enterokinase converts trypsinogen to trypsin (i.e., its active form), which then hydrolyses more peptide bonds of dietary protein.

Further breakdown of these small peptides is needed for the body to be able to absorb them. This happened with help of brush-border membrane enzymes, called endopeptidases. Once AAs are in their free form, they are absorbed by enterocytes into the blood stream, where they will be carried to the liver. From there they will be synthesised to new proteins or stored in other tissues [21].

2.4.2. Factors hindering plant protein digestibility

There are many reasons that explain plant-based proteins limited digestibility and utilisation. Referring again to the term ‘protein quality’, plant-based proteins often lack one or more essential amino acids, making them incomplete proteins, and hence, a poor-quality protein

[22]. Not only this, but the utilisation of the proteins within our bodies, is lacking because of the presence of certain ANFs making them less bioavailable and digestible.

Examples of ANFs, and how they hinder protein digestibility are as follows: 1) trypsin inhibitors interfere with the activity of trypsin and slow down the breakdown of proteins to polypeptides, 2) tannins form complexes with proteins and make them less accessible to digestive enzymes, 3) phytic acid binds to minerals like calcium, iron and zinc, forming insoluble complexes and making the minerals less bioavailable, they also reduce protein solubility, alter protein structure, and bind to enzymes that then hinders the breakdown of proteins into AAs 4) uricogenic nucleobases which are involved in the formation of uric acid that at high levels, can potentially impair digestive enzyme activity [22].

Despite the widespread occurrence of these ANF's, various mechanisms have been shown to mitigate, or minimise, their presence and impact on digestibility. Processes such as baking, and boiling are such examples [23]. However, the chosen method may lead to changes in protein content, structure, and other components in the matrix. Some processing of foods can form ANF's, such as Maillard reaction products, Protein-bound D-amino acids, and lysinoalanine [23]. Nevertheless, these effects have been less researched on foods with a widespread protein source. However, effects of processes done directly on whole protein sources (e.g., peas, lentils, etc.), protein isolates, or protein concentrates, are more commonly known. Hence, soaking and extrusion have been shown to be effective at decreasing ANF's and improve the nutritional quality [23] of whole protein sources.

2.5. Protein digestibility tests

Unlike the *in-vivo* approach, which is to evaluate digestion within living organisms (e.g., test animals or humans), the *in-vitro* method, replicates the human digestion outside of the body, such as in a laboratory.

The most straightforward and effective approach to assess the digestibility of dietary amino acids, is by measuring their reduction in quantity between the mouth and the rectum [24]. The reason the large intestine is not included is due to the extensive microbiota present which majorly influence the amino acid composition of the faecal material. For instance, there may be a net synthesis or net loss of AAs in the large intestine, or the AA either gets degraded to, or absorbed from ammonia. Therefore, it cannot be assumed that the loss of AAs within the large intestine is the same thing as absorption [24].

A wide range of gastrointestinal models have been performed, ranging from single static systems to multi-compartmental and dynamic systems [25]. Previous *in-vitro* digestibility tests have been achieved with interesting variation between models depending on the study purpose. *In-vitro* models need to account for the chemical composition of the digestive solutions in each phase, the type and enzyme concentrations, the salts and buffers used, the biological polymers, the surface-active components, and so on [25].

An *in-vitro* study was performed involving a two-stage, gastric and intestinal digestion with a pepsin-HCl mixture, followed by a neutralisation, and then a digestion in pancreatin or trypsin [26]. The results showed a good correlation to *in-vivo* tests done on rat faecal nitrogen digestibility, and the methods were highly reproducible. In general, the *in-vitro* approach has been found to be somewhat easy to perform, reproducible, and low in cost [26]. Standardised models are available worldwide nowadays, such as the International Network of Excellence on the Fate of Food in the Gastrointestinal Tract (INFOGEST) protocol [27]. Studies such as Z. Zhang, et al., (2023) [28] have used this model and have given guidance and success factors following this protocol.

The INFOGEST protocol was followed to perform this degree project.

2.6. Use of non-animal enzymes for enhanced digestion

There are certain enzymes that have been shown to help to improve human protein digestion. In this degree project, the investigation of plant-derived proteases, such as bromelain, was used to determine if supplementation of enzyme of plant origin would improve the digestion of a PBFA.

2.6.1. Properties of bromelain

Bromelain originates from pineapple fruit or stem. It catalyses proteolytic processes by helping break down proteins into AAs. Bromelain also contains substances that are beneficial for human health. Studies have shown that it has anti-inflammatory effects and can be an effective treatment for several diseases [29].

The ideal pH values for bromelain range between 3 and 8, with an average temperature between 37 and 70°C [30]. This makes it appropriate for the human digestive system as the pH in the stomach and small intestine are around 2 and 7, respectively. Therefore, the enzyme will be active within these ranges during digestion.

2.6.2. Absorption and bioavailability

Bromelain can be absorbed to a certain extent without losing its proteolytic power or cause adverse effects in the human body [29]. A study has shown that the consumption of not more than 12 g/day bromelain does not have any major impact [31]. Bromelain has proven to be well-absorbed after the oral phase. According to several *in-vitro* studies, it has been shown that approximately 30% of bromelain's bioavailability was stable in synthetic stomach juice [19]. Main absorption occurs in the gastrointestinal tract (GIT) phase and about 40% of the higher molecular weight substances are absorbed in the intestine [30].

On the contrary, bromelain can be relatively sensitive. Especially to a highly acidic environment, other existing substances, and temperatures in the gastric phase, resulting in a potential reduction of its protease activity. These changes lead to a decrease in benefits in the above-mentioned applications [32].

2.6.3. Mechanism of bromelain cleavage

Figure 2 shows that bromelain can cleave peptide bonds lysine, alanine, and threonine by hydrolysis [33]. This study also proved that bromelain softens meat fibres, managed to change the microstructure of the tenderized meat when incorporated and had a high degree of protein hydrolysis when utilized in an *in-vitro* digestion [33].

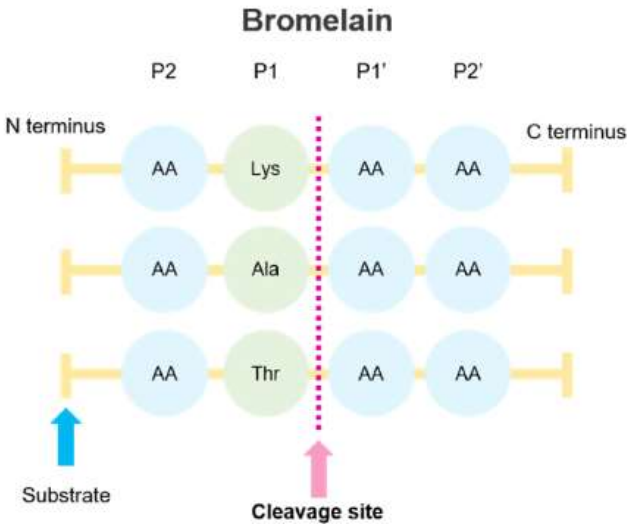

Figure 2: *The cleaving mechanism of bromelain [33].*

3. Materials and methods

This section outlines the experimental procedures conducted during high moisture extrusion, the post-process methods following extrusion, the *in-vitro* simulated digestion of the extrusion extrudate, and finally, the protein analysis post-digestion.

3.1. High moisture extrusion

3.1.1. Food prototype material

The food composition to the high moisture extruder is shown in Table 1. In agreement with the literature review, the two, main protein types in the feed consisted of yellow pea protein isolate, and microalgae protein isolate. Additionally, pea fibre and a protein masker were added in ratios that matched similarly to the Hooked Foods recipe for plant-based tuna. The fibres were added for textural properties, and the protein masker was used for adjusting the aroma consequences. All the powders were weighed using a lab scale (LKB sartorius) and mixed. According to the literature review, the microalgae protein was kept to a minimum.

Table 1: *Material composition to the extruder.*

Ingredient	Amount (%)	Supplier
Yellow pea protein isolate	80	Cosucra
Pea fibre	17	Cosucra
Microalgae protein isolate	1.85	Aliga
Protein masker	1.15	Lucta

3.1.2. Methodology of the extruder

3.1.2.1. Powder feeder and twin-screw

Before running the experiments, the powder feeder was calibrated to adjust to the new texture and density of the material (Table 1). Once the motor speed reached 90% capacity, the maximum output from the feeder read 20.95 kg/h. This resulted in a feeding speed of 800 g/h. The twin-screws were set to a motor speed of 500 rpm.

3.1.2.2. Water

Water was injected into the system at a rate of 945 mL/h. In the beginning, the powder to water ratio was set to 800:945.

3.1.2.3. Temperature

The initial temperatures were set on the high moisture extruder (Thermofisher Scientific, Process 16) from zone 1 to zone 8 as: 40°C, 60°C, 80°C, 115°C, 135°C, 145°C, 140°C, 120°C, respectively. These temperatures were chosen based on the protocol that Hooked Foods uses to produce their plant-based 'tuna' analogue. The cooling system was set to 45°C, and the extruder was left to pre-heat for 10 minutes.

Four extrusion runs (A, B, C, and D) were conducted in total, with the only variable being the temperature. Following each run, the temperatures of zones 5 to 8 and the cooling die temperature were systematically reduced, as outlined in Table 2. After each manual adjustment of temperature, a stabilization period of 5 minutes was given for the entire system before samples were collected.

Table 2: *Set temperatures of each zone and cooling die in the extruder.*

	Temperature (°C)								
Product	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**	**Zone 6**	**Zone 7**	**Zone 8**	**Cooling die**
A	40	60	80	115	135	145	140	120	45
B	40	60	80	115	130	140	135	115	40
C	40	60	80	115	130	140	125	110	35
D	40	60	80	115	130	140	120	100	30

3.2. Post-process methods

3.2.1. Texture analysis

The texture of the extrusion products (A, B, C, and D) was analysed using a texturize analyser (Stable Micro Systems). For each extrudate, a piece measuring approximately 25x23 mm was cut out, and the force was measured over time using a vertical blade cutting in both the cross-sectional and longitudinal directions. This process determined the anisotropic properties of the extrudates, and a graph of all the data was generated by Exponent Connect Lite Software.

Texture analysis was then divided into two categories of interest, namely toughness and firmness. The data collected for firmness (unit: N) and toughness (unit: Ns) was plotted against time. Toughness was calculated as the integration of the positive area under the curve, while firmness was the highest positive point on the curve. Samples A, B, C, and D were measured in duplicate for both cross-sectional and longitudinal directions. Table 16 with raw data can be found in Appendix G, with a mean and SD calculated over the duplicates.

3.2.2. Shredding and flavouring

After texture analysis, all samples (A, B, C, and D) were sent through a shredding machine (Russel Hobbs), until their shape and size resembled more like tuna, i.e., rod-shaped/flakes. White fish powder flavouring (1.85 wt.%) was added thereafter.

3.3. *In-vitro* digestion

The methodology used for the *in-vitro* digestion was based on the INFOGEST protocol [27], which included the three phases: oral, gastric, and small intestine.

3.3.1. Sample preparation

3.3.1.1. Digestive Fluids

The three digestive fluids that were used for the oral, gastric, and small intestine phases were simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), respectively. These were prepared first according to the information of stock solutions in Table 3 and were stored in the refrigerator thereafter.

Table 3: *Electrolyte stock solutions volumes needed for 400 mL of diluted digestion fluids.*

Chemicals			**Amounts added in digestive fluids (mL)**		
Formula	**Concentration (g/L)**	**Molarity (M)**	**SSF (pH7)**	**SGF (pH3)**	**SIF (pH7)**
KCl	37.3	0.5	15.1	6.9	6.8
KH_2PO_4	68	0.5	3.7	0.9	0.8
$NaHCO_3$	84	1	6.8	12.5	42.5
NaCl	117	2	-	11.8	9.6
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	48	0.5	0.06	0.5	-
HCl	37	5	0.135	1.95	1.05
*$CaCl_2(H_2O)_2$	44.1	0.3	0.025	0.005	0.04

*To avoid precipitation, $CaCl_2(H_2O)_2$ was excluded when preparing the digestive fluids, and only added during its occurrence on the protocol for each digestive phase.

3.3.1.2. Enzyme preparation

According to INFOGEST protocol, salivary α-amylase was necessary for the oral phase, pepsin and gastric lipase for the gastric phase, and trypsin in pancreatin, along with bile salts, for the intestinal phase. However, due to lack of availability, gastric lipase was omitted from the gastric phase. Nevertheless, this posed no significance as the food product does not

contain any lipids. The enzymatic activities and volumes needed for each phase, are displayed in the first three columns of Table 4.

Enzymes were kept in solid form in the fridge or freezer. Individual stock solutions were made for each enzyme at their correct concentration. The types of enzymes used in the INFOGEST protocol [27] have different specific enzymatic activities (U/mg) to those used in this experiment. Hence, to keep the same final enzymatic activity (U/mL) occurring in the digestion, (as well as the volume of enzyme to be added), new concentrations were calculated based on Equation 1:

$$Concentration\ \left(\frac{mg}{mL}\right) = \frac{Enzyme\ Activity\ \left(\frac{U}{mL}\right) \times Total\ Volume\ (mL)}{Specific\ activity\ \left(\frac{U}{mg}\right) \times Volume\ added\ (mL)} \qquad \textit{Equation 1}$$

Table 4: *Enzymatic activities and volumes according to INFOGEST protocol [27].*

Type of enzyme	Enzyme activity or bile concentration	Enzyme / bile to be added	Calculated concentration (mg/mL)*	Digestion phase	Final volume in digestion phase
Salivary α-amylase	75 U/mL	0.75 mL	0.87	Oral	10 mL
Pepsin	2000 U/mL	0.667 mL	5	Gastric	20 mL
Trypsin in pancreatin	100 U/mL	5.0 mL	4	Intestinal	40 mL
Bile salts	10 mM	3.0 mL	5.235 g/100 mL	Intestinal	

*See Appendix A for individual enzyme calculations.

3.3.1.3. Food samples from extrusion

Prior to digestion, 5 g of each sample post extrusion named, A, B, C, and D, respectively, were weighed and crushed into a crumbly structure using a pestle and mortar. Each sample was then transferred into its labelled, 50 mL glass bottle, where their entire digestion would occur.

Real tuna preserved in salt water, was purchased from ICA supermarket. This was included as a reference of animal-derived protein for digestion. As much water was removed as possible to get the sample at minimum moisture. Then, the digestion was carried out following the same protocol on the real tuna as was with the PBFA.

Table 5 shows an overview of the types of solutions and volumes needed for each digestion phase. Explanations of the methodology associated with each phase, are underneath.

Table 5: *Volumes added to each sample glass bottle in each phase.*

	Oral	**Gastric**	**Intestinal**
Solution	**Volume (mL)**	**Volume (mL)**	**Volume (mL)**
Water	0.225	0.448	3.16
SSF	4	-	-
SGF	-	8	-
SIF	-	-	8
Salivary α-amylase	0.75	-	-
Pepsin	-	0.667	-
Trypsin in pancreatin	-	-	5
Bile salts	-	-	3
$CaCl_2(H_2O)_2$	0.025	0.005	0.04

3.3.2. Oral phase

To begin, the 5 g of each food sample A, B, C, D, were mixed with SSF in a 1:1 (wt./wt.) ratio. According to INFOGEST, the created bolus should be a tomato-paste or mustard-like consistency. Therefore, additional water was needed. According to the first trial-run performed, it was noted that the addition of water to yield a final volume of 20 mL in the glass bottle, was sufficient for each run thereafter.

Next, $CaCl_2(H_2O)_2$ was added according to the amount specified in Table 5. Salivary alpha-amylase was added to degrade (if any) carbohydrates in the food sample. The bolus mixtures were then placed in a water bath shaker (Thermomix 1419) set at 37°C for 2 minutes, where the digestion began. During the initial set-up of the oral phase, SGF stock solution was added to the water bath to pre-heat to 37°C.

3.3.3. Gastric phase

SGF was added in a of 1:1 (vol/vol) to each sample from the oral phase. The pH was adjusted to be in the range of 2-3, using 5 M HCl, bearing in mind that pH stabilization for solid foods can take up to 5 minutes. The pH was measured using a pH-meter (Standard pH Meter, MeterLab PHM210). Afterwards, $CaCl_2(H_2O)_2$ was added, as well as the porcine pepsin, according to Table 5. A timer of 2 hours was started as soon as pepsin was added to the samples. The pH was checked and re-adjusted to around 2.5 and the specified amount of

water was added. The samples were placed back in the water bath shaker set at 37°C for the remainder of the 2 hours. During the gastric phase, SIF was pre-heated to 37°C.

3.3.4. Intestinal phase

SIF was added in a of 1:1 (vol/vol) to each sample from the gastric phase. The pH of the gastric chyme was adjusted to 7 by adding 5 M NaOH. Subsequently, the bile salt solution was added, and the digestion mixture placed in a water bath shaker for 30 minutes at 37°C for the bile to solubilise. After this, $CaCl_2(H_2O)_2$, trypsin, and water were added, respectively. A timer of 2 hours was started as soon as trypsin was added to the samples. The pH was again checked and re-adjusted to 7. The samples were then incubated at 37°C for 2 hours.

3.3.5. Ending the digestion process

The digestion was ended by halting the enzymatic activity. This was done by placing all samples in an ice bath and increasing the pH up to 9 using 5 M NaOH. All samples were centrifuged, with the supernatant separated, labelled, and stored in a freezer at -18°C, for subsequent analysis.

3.4. Supplementation of bromelain

In total, the digestion process was repeated 4 times for each prototype produced, A, B, C, and D on 4 different occasions, yielding samples A1, A2, A3, A4, B1, …, etc. (see Appendix B, Table 10). This was classified as the ‘base digestion’. Following this, only samples C and D were chosen in the performance of digestion again with the addition of bromelain to investigate if the digestion of protein can be further improved (see Appendix B, Table 11). Furthermore, it was decided to introduce bromelain to the sample of real tuna as well. However, this protein content was obtained using the Dumas method.

Realistically, consumers will ingest bromelain together with their food/meal. Therefore, the enzymatic activity of bromelain starts from the oral phase. Hence, it was added there accordingly. The final concentration of bromelain in the starting oral mixture should be 1 mg/mL. Too high of a concentration has proven to increase the mucosal permeability [34], and so, 1 mg/mL was chosen as a starting point. See Appendix A for calculations and volumes of bromelain added. The digestion for C and D with bromelain was repeated 4 times on 4 different days, yielding C5, C6, C7, C8, D5, … etc. The digestion of tuna with bromelain was performed on one day in duplicate, i.e., Tu1, Tu2, Tu+BRM1, Tu+BRM2.

3.5. Protein analysis

3.5.1. Bradford method

To analyse the protein content of the digestates after digestion, the Bradford's assay method was used. To begin, Coomassie Blue G-250 Plus™ Protein Assay Reagent (ThermoScientific) was taken out the refrigerator and kept at room temperature (RT) for some minutes. Then, sampling from the digestates, A, B, C, and D (i.e., from each glass bottle) was done by pipetting 1.5 ml into Eppendorf tubes and labelled accordingly. The rest of the digestate was transferred into a cyclic tube with a red screw cap and saved in the freezer for subsequent analysis.

The 1.5 ml Eppendorf tubes were then vortexed (CAT VM3) for 20 seconds and then centrifuged for 5 minutes at 3500 rpm. Samples were diluted in a ratio of 85:15, water to sample. The samples were then centrifuged and 10 µL of the supernatant was pipetted (Eppendorf Research) into a microplate. This pipetting process specifically targeted the soluble protein fraction to minimize errors and ensure that all solids were avoided. As a standard, known concentrations of bovine serum albumin (BSA) was used. All raw data can be found in Appendix B, Table 10.

300 µL of Coomassie blue was then added onto each. The microplate was placed on a microplate shaker for 30 seconds and left at RT for 10 minutes before read in spectrophotometer (SPECTROstar Nano, BMG LABTECH). The optical density was measured at 595 nm.

A 4-parameter fit was used to obtain the standard curve of protein concentration against optical density, using samples of known concentration of purified protein. In this case, BSA (ThermoScientific) standard was used. Then, using extrapolation, the protein concentrations of the unknown samples were then obtained. The BSA standard series are displayed in Table 9 in Appendix B.

3.5.2. Freeze-drying

The Dumas method was chosen as an additional method to confirm the protein content within each sample. The saved frozen samples: A, B, C, and D after digestion; and C, D, and tuna with bromelain after digestion, were thawed and centrifuged (ALC refrigerated centrifuge PK130R) for 5 minutes at 3500 rpm. 9mL of the supernatant was pipetted into aluminum discs and 1 g of starch (Merck) was added. The samples were frozen and then freeze-dried in a freeze-dryer (Heta Drywinner) for 88 hours and 40 minutes.

Additionally, all the initial samples (A-initial, B-initial, C-initial, D-initial, and Tuna-initial) were freeze-dried. This was done so that the percentage difference in protein content could also be deducted.

3.5.3. Dumas protein analyser

Powdered freeze-dried samples, 25 mg of each tested product were in duplicates. The nature of the Dumas method analyses the total nitrogen content, which can then be converted to a protein percentage using aspartic acid calibration standards. The calibration standards were permitted to have a SD of approximately ± 0.2.

After the results were obtained, the percentage of protein was calculated, considering the ratio of starch within the samples, see Appendix C. It was assumed that the same percentage of water left during freeze-drying for all samples. This was calculated to approximately 35%. These percentages were compared with the results obtained from Bradford, which gives protein concentration in µg/mL.

3.6. Statistical analysis

The software, GraphPadPrisma (Version 10), was used to perform the statistical analysis on all the results. Results are demonstrated on a wet basis. The scatter plots are presented as mean ± standard deviation (SD). Differences within and among the groups were compared and statistically tested. An unpaired t-test was performed to see the differences between two groups. Whereas multiple groups were compared using a one-way ANOVA. However, the ANOVA only adheres to groups that are normally distributed. If not normally distributed, a Kruskal-Wallis (KW) was performed. Finally, a post-hoc test, for further analysis into the type of differences, was done. Significant differences were evaluated when $p<0.05$ (confidence interval of 95%). Outliers were visually identified on the scatter plots; none were found and therefore no formal outlier test was performed.

4. Results and discussion

4.1. Base digestion

The base digestion included the four samples (A, B, C, and D) carried out on four different days, hence, A1-A4, B1-B4, etc. For reliability, each sample was pipetted in triplicates. Within the nature of the Bradford method, the protocol recommends micro-plating each sample in duplicates. This means that A1 yielded 6 different values. In total, A then had six times four values, making 24 values for each A, B, C, and D. All raw data can be found in Appendix B. Real tuna was selected as a control for animal-derived protein source, and this was also digested.

4.1.1. Average trend of base digestion

The first step was to assess whether the results from the four different samples adhered to a normal distribution. Thus, a normality test was performed. As indicated in Table 13, Appendix D, only variables C and D indicated normal distribution at a confidence interval of 95%.

One of the objectives was to determine whether there was a significant difference between the treatments A, B, C, and D. With thorough adherence to statistics, due to samples A and B exhibiting no normal distribution, a KW test was performed, with the only variable being protein concentration. The following median box plot was formed (Figure 3).

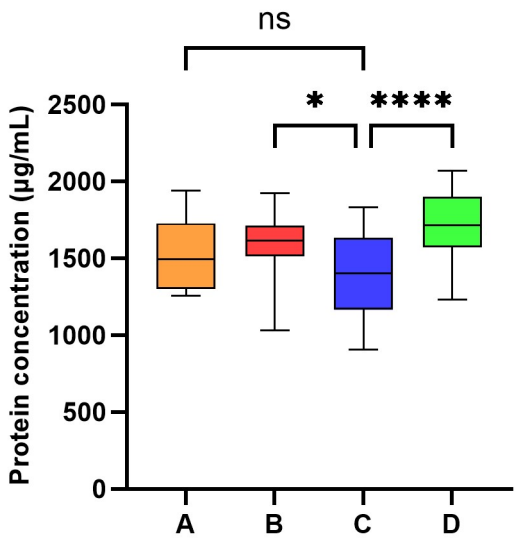

Figure 3: *Differences in protein concentration between samples A, B, C and D according to Bradford method. Results presented as mean ± SD. Significance between groups manifested by asterisk, where * p<0.05, **** p<0.0001.*

The results showed that all samples were significantly different at $p<0.05$. This allows the conclusion to be drawn that the temperature profile in HME has an influence on protein digestibility. The averages of all repeated samples showed that sample C had the lowest protein concentration, and hence, the most optimal temperature profile (zone 1 to 8 as 40°C, 60°C, 80°C, 115°C, 130°C, 140°C, 125°C, 110°C, respectively, and cooling die at 35°C. Interestingly, there is no linear trend between a decreasing HME temperature with an increasing digestibility, as C has a higher HME temperature than D. This shows an opportunity of optimization for an ideal HME temperature, which from these results, lies between C and D (Table 2 in section "Materials and methods").

As sample C was of interest, it was decided to test the difference between sample C in relation to the other samples. Therefore, a post hoc was conducted. This showed a significant difference between C and B, as well as C and D. However, sample A exhibited no significant difference to C. This can be seen from the displayed square brackets in Figure 3.

4.1.2. Digestion run variations

Next, it was decided to see if there were any differences between the results of the same sample performed on different digestion days, A1 - Day1 vs. A2 - Day2. A one-way ANOVA test was conducted for this purpose, with the only variable being protein concentration. As a representative example of the scatter plots obtained, see sample C in Figure 4, the rest of which can be found in Appendix E.

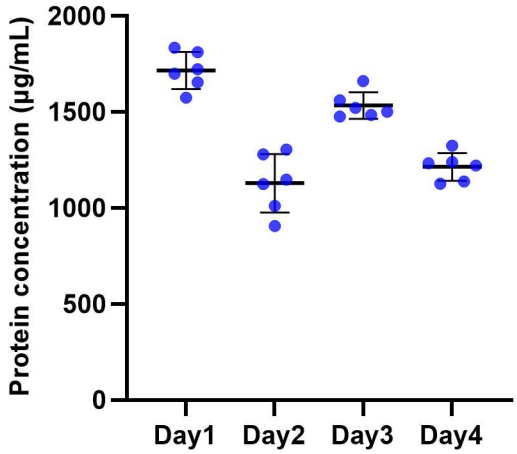

Figure 4: *The different digestion days for sample C showing a variation in protein concentration (µg/mL).*

Within the same digestion day

From the graph, it is evident that results from within the same day were grouped. The average of all respective SDs for A, B, C, and D was low at 8%, with the highest at 25%. However, the nature of the differences was attributed to the insolubility of pea protein, creating samples in suspension, non-homogeneous digestates and hence, dissimilar samples when pipetting. To minimise this as much as possible, all samples were centrifuged, and only the soluble protein was pipetted, to avoid errors arising from the solids.

Between the days (A1 vs A2 vs…)

Digestion was performed on different days to minimise the 'batch effect'. From the scatterplot, differences between the days were more varied. At $p<0.05$ all days were significantly different to each other. This was observed for all samples. Many reasons may attribute this. Firstly, no sample was standardised to the same particle size [28] leading to some days receiving larger clumps of solids, affecting the surface area of attack for enzymes. Secondly, samples A, B, C, D were stored in a sealed container in the fridge. This meant that when it came to fourth day (Day4) of digestion, (digestions were not done on consecutive days), samples A4-D4 were affected by storage life. According to a study that explored the influence of storage time (at 4 and 0°C) on mackerel protein breakdown [35], the results revealed that protein degradation was observed. Additionally, another study confirmed that the storage time decreased the protein content in several types of legumes [23]. Therefore, for future experiments, it is recommended that to decrease the effect of protein degradation in storage, all samples should minimally be stored in the freezer (-18°C).

4.2. Digestion with bromelain supplementation

Following the base digestion, samples C and D were chosen for further improvement of protein digestibility with the addition of bromelain.

4.2.1. Statistical analysis of digestion with bromelain

To reveal the effects of adding bromelain, it was decided to see whether Samples C and D, with and without the addition of bromelain, showed a significant difference. An unpaired t-test was therefore performed, assuming the same SD.

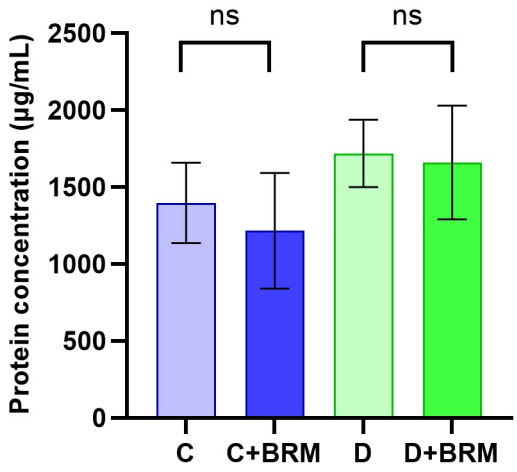

Figure 5: *In-vitro digestion of sample C and D, with and without the addition of 1 mg/mL bromelain (BRM).*

Figure 5 shows that C with bromelain compared to C without bromelain was not significantly different, at $p<0.05$. The same result was revealed for sample D, see Appendix F, Table 14 and 15. However, by calculating the percentage difference, based on their averages, it was observed that bromelain could potentially increase the protein digestibility of sample C by 13.0% ± 36% and sample D by 3.44% ± 51%.

Despite, there being no significant difference, bromelain is still recommended, as previously mentioned with its observed tendency to increase protein breakdown and from the reasons explained in section 2.6 “Literature review”. It has been found that 1 mg/mL had a positive effect on the protein digestibility in mice [34]. However, the results from this present project showed that 1 mg/mL was not sufficient to get a significant difference. According to O. Kostiuchenko, et al., (2022) [34], 10 mg/mL of bromelain was the maximum concentration tested, and this had a suboptimal effect on the permeability of the intestinal mucosa. The enzyme increased the permeability and additionally caused an increase in drug uptake as well, making the function of additional plant enzymes crucially dose dependent [34]. Therefore, it would be suggested that for future experiments with bromelain, to try the addition of different concentrations between 1 mg/mL and 10 mg/mL.

4.3. Dumas method

4.3.1. Protein content Dumas versus Bradford method

As previously discussed, when using the Bradford method, the obtained protein concentrations showed somewhat fluctuating results and high error bars between the

replicates. Therefore, for a confirmational analysis, the protein content of all samples was re-analysed using the Dumas method.

The results from both methods, Bradford and Dumas, are shown in Figure 6A and 6B, respectively. As the two methods measured the protein content in different units, i.e., protein concentration, µg/mL (Bradford), and percentage protein %, (Dumas), the average trend between the two methods was observed.

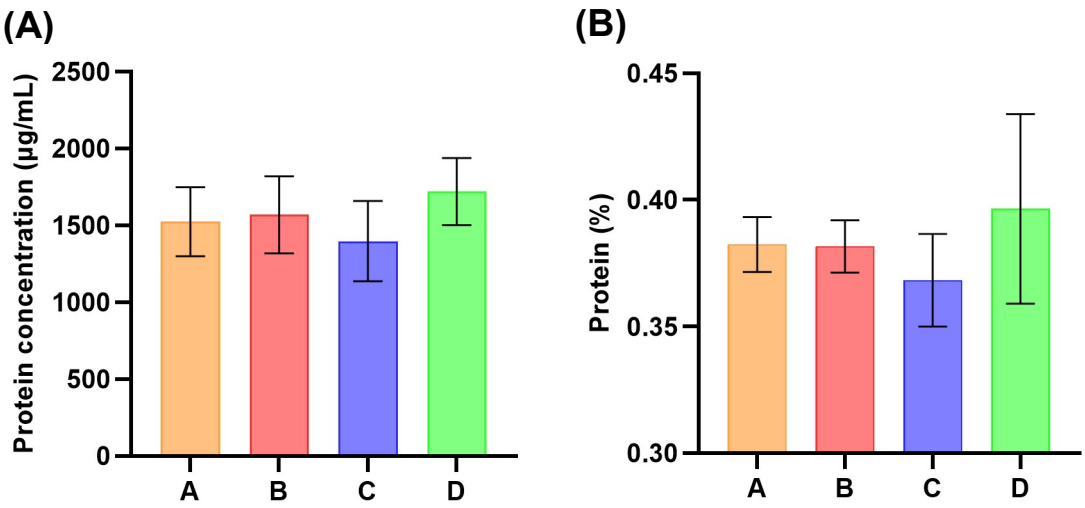

Figure 6: *Protein content post digestion obtained using the Bradford method (A) and Dumas method (B).*

The results of both methods revealed a similar trend. However, observations from the Dumas method showed that D had a very high standard deviation. Potential factors could include errors in sample weighing. Nevertheless, sample C, again, optimistically displayed the lowest protein concentration for both methods after *in-vitro* digestion. Therefore, sample C still proves to contain the best temperature profile during HME for protein digestibility.

4.4. Decrease in protein content during digestion

After A, B, C and D; real tuna underwent *in-vitro* digestion as well. The original and final protein content was analysed using Dumas. All results were measured in duplicates due to time restraints, and therefore, no statistical analysis was performed. Figure 7 shows the percentage differences calculated from the initial to the final protein content of all samples.

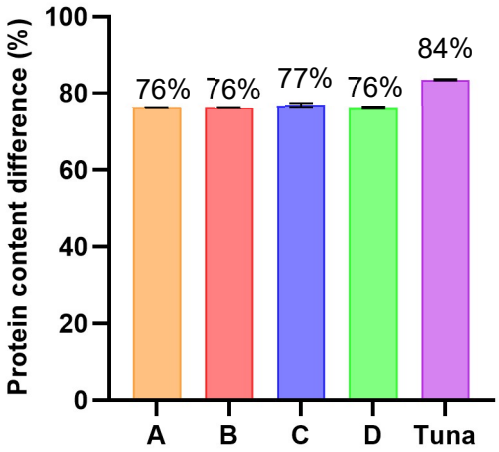

Figure 7: *The percentage decrease in protein content for each sample post digestion.*

The protein content of A, B, C, D, and real tuna managed to all decrease substantially, with tuna at the highest percentage. These findings confirm that animal-derived proteins are more easily digested in the human body, as also stated in several studies [22]. Notably, sample C decreased the most in protein content, compared to A, B, and D. Again, proving that it is the most easily digested of the samples, and hence, contains the most optimal temperature profile in HME.

4.4.1. Supplementation of bromelain

Dumas was performed on the samples including bromelain, however the exact nitrogen content of bromelain after digestion was not calculated. Nevertheless, Dumas was performed in any case (despite being slightly inaccurate) for general observation. It was seen, again, that bromelain produced samples with a lower protein content, see Figure 8 below. A discussion of how to progress with this topic in future experiments is under Heading 5 “Future improvements and recommendations” below.

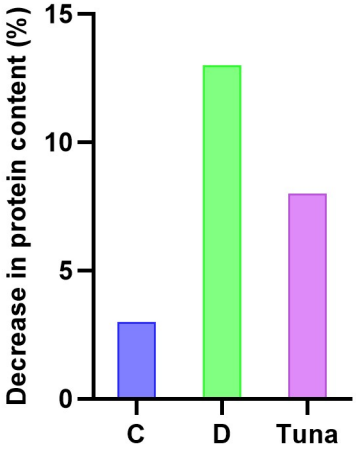

Figure 8: *Percentage decrease in protein content with the addition of bromelain.*

Regarding the real tuna, Figure 8 showed that bromelain had a positive effect on the animal-derived protein digestibility as well. This is an optimistic result, as it shows that bromelain can be consumed with a meal, to aid in, not only plant-based protein, but any protein digestion.

4.5. Texture relationships of base digestion

4.5.1. Toughness & Firmness

From the texture analysis, a relationship between texture and temperature for digestibility was addressed. As seen on Figure 9 and 10 the lowest toughness and firmness was attributed to sample C, which happens to be the sample with the highest protein breakdown. Again, providing a conclusion for the most optimal food product, i.e., a softer, more fluid product with a protein structure that is presumably more accessible for the enzymes to break down.

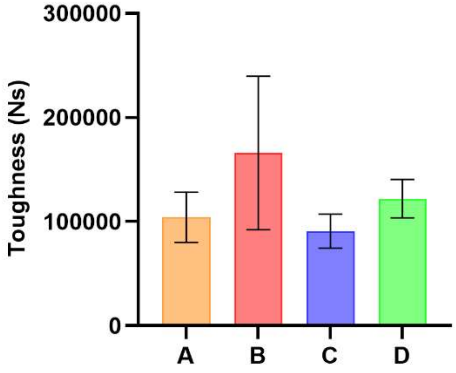

Figure 9: *Toughness comparison of sample A, B, C and D.*

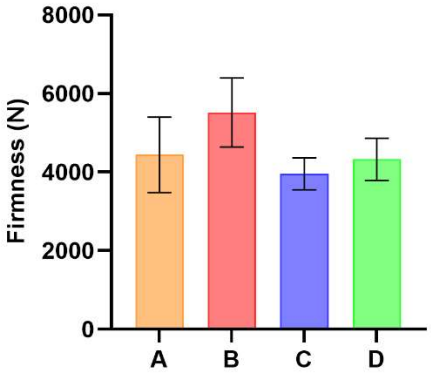

Figure 10: *Firmness comparison of sample A, B, C and D.*

4.6. Limitations and troubleshooting

During the experimental procedure, there were many unforeseen shortcomings that affected the outcome of the results. The following discussion categorises the occurrences regarding equipment used, enzymatic preparations, and sampling methods.

4.6.1. Equipment limitations

To represent the human digestive system most accurately, the equipment used should ideally mimic the actions performed in the mouth, stomach, and small intestine. That being, oscillating and reciprocating mechanisms to simulate the chewing action in the mouth [28], a rotating or agitating chamber to mimic the stomach, and a peristaltic pump to simulate peristalsis in the small intestine. More progressive technology that has tried to start adopting more of this

technique has been recently made available by SHIME® [38]. For contrast, an image of this technology is placed in comparison to what was used in this degree project, see Figure 11 and 12.

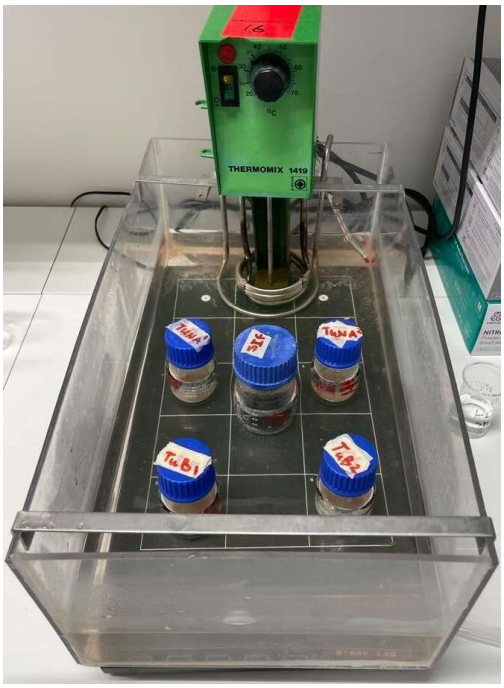

Figure 11: *Water bath experimental set up for this thesis experiment.*

Figure 12: *Experimental set up for SHIME® [38].*

What was available at the university was a water bath placed on a magnetic plate with six locations for the glass bottle samples to be placed. Inside each glass bottle was a small magnet that agitated and mixed the contents inside. Only having six locations available minimized the number of samples that could be digested at one time. Performing the samples on different days increased variability and errors.

More precise results would come from an upgrade of the equipment used. Especially from equipment that can more closely mimic what happens within the human body. However, utilizing the resources at hand, the results still showed that the enzymes and digestive fluids were able to break down the protein in the plant-based fish analogue, to an optimal extent.

4.6.2. Enzyme preparations

To make sure that all the enzymes were as close to their correct and calculated activity levels, each solution was prepared freshly each day before starting the digestion. Amylase was not used in samples that contained (i.e., C5-C8, D5-D7, TuB1, TuB2, Tu1, Tu2), whereas lipase was also not obtainable during this experiment, and hence, none of the samples contained it. Despite this, it was not deemed a significant issue as neither lipase nor amylase digest protein.

This has been mentioned in several other studies [25, 27, 28] as well. Nevertheless, for more accurate future experiments, is it recommended to use all enzymes that are present during the human digestion system.

4.6.3. Sampling and pea protein insolubility

Pea protein insolubility [7] was the biggest challenge. Its greatest effect was that the final digestate was largely in suspension and unhomogenized, and hence, gave major difficulties when sampling. Often, when trying to pipette out of the final sample, strands of protein fibres embedded the pipette, larger solids were collected. This led to the conclusion that the sampling method gave a relatively untrustworthy representation of final sample, and hence, fluctuating results when performing the Bradford method. This was seen in the statistical analysis.

A main take-away was that the initial sample material was not crushed to the same, or small enough particle size to begin with. With an inconsistent particle size, batches with smaller particles led to an easier target for enzymes during digestion, and hence, a greater protein breakdown. It is recommended that for future experiments, that particle size not be a variable. The samples should be all crushed/grinded to a standardized powder. This will eliminate difficulties of pipetting larger particles at the end and lessen the problem of pea protein insolubility.

A minor solution was to centrifuge the samples and pipette only the soluble fraction, i.e., the supernatant. However, centrifugation was only done for 5 minutes at 3500 rpm. It should be noted that centrifugation can occur for at least 30 minutes, and this is suggested for future.

5. Conclusion

The PBFA's were chosen to be based on a mixture of yellow pea protein (80%) and microalgae protein (1.85%). HME was used to produce samples A, B, C, and D, with the extrusion temperature profile chosen as the independent variable. Protein digestibility of the obtained products was succeeded using an *in-vitro* digestion according to INFOGEST protocol.

The temperature profile of HME was observed to have a significant effect on the texture, where sample with the lowest firmness and toughness was the most digestible. Furthermore, based on obtained results from the current project we can conclude that the second lowest temperature profile (i.e., sample C) was shown to have the highest digestibility, and hence, the most optimal temperature profile (zone 1 to 8 as 40°C, 60°C, 80°C, 115°C, 130°C, 140°C, 125°C, 110°C, respectively, and cooling die at 35°C) is recommended for raw material process in order to design a fish analogue with better protein digestibility. The protein content was detected using two different protein analysis methods, the Bradford, and the Dumas method, where both methods were found to be suitable to monitor changes in protein digestion of plant-based proteins.

Surprisingly, the digestion procedure performed on different days demonstrated the 'batch-effect', significantly affecting obtained results, therefore an optimization of standard operating procedure (SOP) needs to be applied to study digestion of pea-microalgae based materials. For instance, this effect was likely due to the nature of pea protein insolubility, and hence the suspended, unhomogenised digestate, yielding difficulties in the sampling method and fluctuating results measured specifically by Bradford method.

Since, real tuna was digested by a means of an animal-source control, it was observed that the tuna showed 84% of decreased protein content, while the PBFA managed decrease in protein content by 76-77%, depending on extrusion conditions, which is approximately 7-8% difference in digestion between PBFA and real tuna.

Furthermore, supplementation with bromelain enzyme in dose 1 mg/mL has not resulted in improved protein digestion, although show trends for the improvement as for PBFA as well as for real tuna therefore higher dose usage of bromelain is recommended in the further studies. In addition, high-end equipment that more accurately mimics the actions performed in the mouth, stomach, and small intestine is also recommended for further experiments that might increase accuracy of the experiments and minimise observed batch-effect.

6. Future improvements and recommendations

This section offers suggestions for future experiments, outlining improvements and recommendations.

6.1. Bromelain concentration

The concentration of bromelain used for the oral phase was set to 1 mg/mL. Results from this thesis revealed that there was an observed tendency for bromelain to further breakdown protein, however, statistically, there was no significant difference when bromelain was added. This means that protein digestibility was slightly improved but could be even more. It is suspected that this specific concentration did not suffice. Therefore, it is suggested to use multiple trials of higher concentrations to see which is the most optimal. However, it should be noted that the concentration should not exceed 10 mg/mL. Anything above this concentration has proven to damage the mucosa permeability, allowing bacteria to enter the lumen [34] and compromising human health.

In addition, other plant-derived enzymes (e.g., papain) [33] or microbial-derived enzymes (e.g., *Aspergillus* spp.) [37, 41] have demonstrated beneficial effects. These have been shown to enhance the protein digestibility of various protein sources, such as dairy, animal-derived, soy and pea proteins [37].

6.2. Other *in-vitro* digestion methods

The primary protocol utilised in this degree project was INFOGEST due to the availability of all necessary tools and equipment as illustrated in Figure 12. While SHIME® is another recommended method to use, it primarily focuses on the gut microbiota-host interactions [38], whereas INFOGEST places more emphasis on the gastrointestinal digestion [27]. Nevertheless, Lund University will soon obtain the SHIME® equipment, which should enhance method accuracy and results due to its advanced technology.

6.3. Particle size

It has been recommended that particle size reduction through processes such as crushing, or grinding be further investigated. Maintaining a standardised particle size is crucial for developing a reliable system, as it directly impacts the accessibility of enzymes responsible for protein degradation. Particle size and distribution can be measured using techniques like Laser Diffraction (LD) [28], or filtering methods such as sieving. Microfibre filters [39], for instance, can also be used to effectively remove larger particles from samples, allowing only particles of a specific diameter to pass through.

6.4. Antinutritional factors

Phytates are an example of ANF's that limit the extent of protein digestibility [36]. Hence, many methods have been put forward to limit the effect of these compounds. One way is to use high or low moisture extrusion, as mentioned in the article [36]. Other methods include fermentation and germination, heat and pressure treatments, particle size reduction and physical size separation, Pulsed Electric Field (PEF), and the use of specific enzymes. In the case of phytates, the enzyme phytase could be introduced to help with additional breakdown of the substance. However, as this thesis focused just on the extent of protein breakdown using HME temperatures and enzymes (e.g., bromelain), these additional methods could be implemented as a bonus to allow for protein breakdown to increase even more within the human body.

6.5. Protein analysers

Dumas was used to analyse the protein content in the samples as an additional, confirmational method to confirm the protein content/trend obtained from Bradford. Performing these measurements in triplicate would have been more appropriate if time permitted. However, there are further analytical methods, for example Liquid chromatography Mass Spectroscopy (LC-MS) [39], that could be used to further confirm and analyse the protein content post digestion.

6.6. Additional measurements and monitoring

It is recommended to perform a trial run on the pH adjudgment measurements prior to the entire experimental design. This would save time and limit the effect on enzyme activity as the amount of HCl and NaOH needed would be known prior to each stage.

In this degree project, only the soluble protein portion of the samples was measured. However, to give a comprehensive view of the whole digestion performance, it is recommended to account for the entire protein content in the digestates. This means that the insoluble protein should be measured too, using Dumas or another method of protein analysis.

Additionally, the control that is selected for using the digestion method needs to be a standardized control. For instance, sample A could be used as a control sample for further experiments.

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Appendices

A. Enzyme Calculations

Table 6: *Digestive enzyme types and purchase information.*

Type of enzyme	Purchase details
Salivary α-amylase	Alpha-amylase from human saliva, Sigma-Aldrich
Pepsin	Pepsin from porcine gastric mucosa, Merck
Gastric Lipase	Rabbit Gastric Extract, Lipolytech, RGE 15-100 MG, >15 U/mg
Trypsin in pancreatin	Pancreatin from porcine pancreas, Sigma-Aldrich
Bile salts	Bile salts, Sigma-Aldrich

Table 7: *Enzymatic activities and volumes according to the INFOGEST protocol.*

Type of enzyme	Enzyme activity or bile concentration	Volume of enzyme or bile to be added	Digestion phase	Final volume in digestion phase
Salivary α-amylase	75 U/mL	0.75 mL	Oral	10 mL
Pepsin	2000 U/mL	0.667 mL	Gastric	20 mL
Gastric Lipase	60 U/mL	0.48 mL	Gastric	
Trypsin in pancreatin	100 U/mL	5.0 mL	Intestinal	40 mL
Bile salts	10 mM	3.0 mL	Intestinal	

Enzymes were stored in solid form in the fridge or freezer. However, prior to mixing with the other digestion fluids, individual stock solutions were required for each enzyme, to get the correct concentration. The types of enzymes used in the INFOGEST protocol have different specific enzymatic activities (U/mg) to the ones used in this experiment. Hence, to maintain equal final enzymatic activity (U/mL) during the digestion process, (as well as consistent enzyme volumes), new concentrations were calculated using the following Equation 1:

$$Concentration\ \left(\frac{mg}{mL}\right) = \frac{Enzyme\ Activity\ \left(\frac{U}{mL}\right) \times Total\ Volume\ (mL)}{Specific\ activity\ \left(\frac{U}{mg}\right) \times Volume\ added\ (mL)} \qquad \textit{Equation 1}$$

Salivary Amylase

$$Concentration = \frac{75\ \left(\frac{U}{mL}\right) \times 10\ (mL)}{1149\ \left(\frac{U}{mg}\right) \times 0.75\ (mL)}$$

$$Concentration = 0.87 \frac{mg}{mL}$$

Pepsin

The specific activity mentioned on the bottle: 2 U/mg FIP [42]. However, the enzyme activity of products should be measured and reported in FCC units. Hence, to convert FIP to FCC, the following correlation was used:

$$1.67 \frac{U}{mg}\ FIP = 10\ 000 \frac{U}{mg} FCC$$

$$Specific\ activity\ FCC = 2 \frac{U}{mg} FIP \times \frac{10\ 000 \frac{U}{mg} FCC}{1.67 \frac{U}{mg} FIP}$$

$$Specific\ activity\ FCC = 11\ 976\ \frac{U}{mg}$$

$$Concentration = \frac{2000\ \left(\frac{U}{mL}\right) \times 20\ (mL)}{11\ 976\ \left(\frac{U}{mg}\right) \times 0.667\ (mL)}$$

$$Concentration = 5\ mg/mL$$

Gastric Lipase

$$Specific\ activity\ Rabbit\ Gastric\ Extracts\ 15 = 15\ \frac{U}{mg}$$

$$Concentration = \frac{60\ \left(\frac{U}{mL}\right) \times 20\ (mL)}{15\ \left(\frac{U}{mg}\right) \times 0.48\ (mL)}$$

$$Concentration = 166.67\ mg/mL$$

Trypsin in pancreatin

The specific activity mentioned on the bottle: 8 x USP specification. However, to convert to FCC, the following correlation was used.

$8 \times USP = 200 \frac{U}{mg} FCC$, where:

$USP = 25$

$Specific\ activity\ FCC = 200\ \frac{U}{mg}$

$$Concentration\ = \frac{100\ \left(\frac{U}{mL}\right) \times 40\ (mL)}{200\ \left(\frac{U}{mg}\right) \times 5\ (mL)}$$

$Concentration = 4\ mg/mL$

Bile salts

$Molecular\ weight\ =\ 392.58\ g/mol$

$1\ mol\ =\ 392.58\ g$

$If\ 20\ g\ of\ bile\ salts\ was\ taken, it\ would\ have\ mol = 50.9\ mmol$

$$Specific\ activity\ \left(\frac{mmol}{g}\right) = \frac{50.9\ mmol}{20g} = 2.547\ mmol/g$$

$$Concentration\ = \frac{10\ (mM) \times 40\ (mL)}{2.547\ (mmol/g) \times 3\ (mL)}$$

$Concentration = 5.234 \frac{g}{100\ mL}$

Bromelain

The final concentration of bromelain in the oral mixture should be 1 mg/ml. A stock solution of 10 mg/mL was made for bromelain. The final volume in the oral phase, according to the previous digestions, was 20 mL. Therefore, the amount of bromelain to add was calculated as follows:

$Concentration\ of\ bromelain\ needed = 1 \frac{mg}{mL}$

$Concentration\ of stock\ solution\ made = 10 \frac{mg}{mL}$

$Final\ volume\ in\ oral\ phase\ = 20\ mL$

$\left(\frac{10\ mg}{20\ mL}\right) \times Volume\ of\ bromelain\ to\ add\ (mL) = 1 \frac{mg}{mL}$

$Volume\ of\ bromelain\ to\ add\ (mL) = 2\ mL$

B. Bradford results

Table 8: *Calibration series for BSA standards.*

Series	Concentration of BSA standards (µg/mL)
Blank	0
1	50
2	125
3	250
4	500
5	750
6	1000
7	1250
8	1500
Stock	2000

Table 9: *Protein concentration of base samples A, B, C, and D using Bradford.*

Sample	Optical density at 595nm	Concentration (µg/ml)
A1-1	0.21045	1532.84
A1-1	0.22815	1639.173
A1-2	0.21925	1585.787
A1-2	0.22105	1596.593
A1-3	0.22235	1604.393
A1-3	0.21385	1553.313
B1-1	0.21655	1569.56
B1-1	0.21835	1580.373
B1-2	0.22695	1631.987
B1-2	0.23345	1670.913
B1-3	0.23305	1668.52
B1-3	0.21355	1551.513
C1-1	0.21725	1573.767
C1-1	0.23805	1698.433
C1-2	0.24205	1722.34
C1-2	0.23075	1654.747
C1-3	0.26075	1833.987
C1-3	0.25695	1811.313

D1-1	0.23725	1693.647
D1-1	0.22455	1617.593
D1-2	0.21615	1567.153
D1-2	0.21975	1588.787
D1-3	0.23775	1696.633
D1-3	0.25385	1792.807
A2-1	0.17375	1309.553
A2-1	0.19805	1457.92
A2-2	0.16505	1255.78
A2-2	0.17105	1292.907
A2-3	0.17605	1323.707
A2-3	0.18935	1405.067
B2-1	0.21695	1571.96
B2-1	0.22855	1641.573
B2-2	0.20505	1500.267
B2-2	0.23575	1684.673
B2-3	0.21345	1550.907
B2-3	0.22075	1594.793
C2-1	0.16885	1279.32
C2-1	0.17275	1303.393
C2-2	0.14405	1124.047
C2-2	0.14775	1147.487
C2-3	0.12655	1011.527
C2-3	0.11045	905.0267
D2-1	0.26825	1878.74
D2-1	0.27585	1924.107
D2-2	0.23845	1700.82
D2-2	0.26895	1882.92
D2-3	0.27515	1919.927
D2-3	0.27615	1925.893
A3-1	0.26755	1874.567
A3-1	0.27295	1906.793
A3-2	0.27895	1942.613
A3-2	0.24775	1756.393
A3-3	0.24995	1769.527

A3-3	0.25915	1824.44
B3-1	0.23585	1685.273
B3-1	0.25675	1810.12
B3-2	0.27625	1926.493
B3-2	0.26995	1888.887
B3-3	0.26905	1883.513
B3-3	0.24685	1751.013
C3-1	0.20505	1500.267
C3-1	0.23175	1660.74
C3-2	0.20225	1483.34
C3-2	0.21515	1561.14
C3-3	0.20105	1476.087
C3-3	0.20855	1521.387
D3-1	0.28295	1966.507
D3-1	0.26445	1856.067
D3-2	0.23905	1704.407
D3-2	0.27005	1889.487
D3-3	0.30005	2068.787
D3-3	0.27245	1903.807
A4-1	0.22395	1445.127
A4-1	0.20995	1364.873
A4-2	0.19415	1275.133
A4-2	0.19705	1291.54
A4-3	0.19415	1275.133
A4-3	0.19845	1299.473
B4-1	0.21355	1385.44
B4-1	0.27115	1721.413
B4-2	0.15505	1056.453
B4-2	0.15065	1032.107
B4-3	0.17195	1150.427
B4-3	0.17155	1148.193
C4-1	0.16975	1138.147
C4-1	0.20285	1324.44
C4-2	0.18785	1239.587
C4-2	0.16755	1125.887

C4-3	0.18655	1232.267
C4-3	0.18445	1220.46
D4-1	0.19145	1259.887
D4-1	0.18625	1230.58
D4-2	0.22575	1455.493
D4-2	0.24455	1564.573
D4-3	0.27145	1723.2
D4-3	0.22725	1464.147

Table 10: *Protein concentration of bromelain digestion C, and D using Bradford.*

Sample	Optical density at 595nm	Concentration (µg/ml)
C5-1	0.12115	870.0133
C5-1	0.13525	947.2667
C5-2	0.09665	736.5267
C5-2	0.10605	787.6533
C5-3	0.10525	783.3
C5-3	0.14785	1016.64
D5-1	0.19535	1281.92
D5-1	0.19745	1293.807
D5-2	0.16305	1100.84
D5-2	0.18585	1228.333
D5-3	0.18045	1197.993
D5-3	0.16195	1094.727
C6-1	0.11145	817.0667
C6-1	0.13095	923.6667
C6-2	0.14335	991.82
C6-2	0.10635	789.2867
C6-3	0.15595	1061.44
C6-3	0.14365	993.4733
D6-1	0.29005	1834.773
D6-1	0.25345	1616.713
D6-2	0.27535	1746.46
D6-2	0.35445	2234.413
D6-3	0.26055	1658.547
D6-3	0.28555	1807.633

C7-1	0.21685	1404.333
C7-1	0.26015	1656.187
C7-2	0.24695	1578.6
C7-2	0.26365	1676.88
C7-3	0.23975	1536.593
C7-3	0.26765	1700.6
D7-1	0.31445	1983.653
D7-1	0.31465	1984.887
D7-2	0.33695	2123.64
D7-2	0.31715	2000.313
D7-3	0.28595	1810.04
D7-3	0.29975	1893.607
C8-1	0.29295	1852.313
C8-1	0.29565	1868.68
C8-2	0.23635	1516.827
C8-2	0.21325	1383.727
C8-3	0.17655	1176.14
C8-3	0.16905	1134.247

C. Dumas results

Table 11: *Protein percentage obtained from Dumas for A, B, C, and D, bromelain C, D, and initials.*

Sample name	Weight (mg)	Protein factor	Nitrogen	Protein (%)
A1	25.1	6.25	1.02	9.58
A1dup	24.9	6.25	1.27	11.94
A2	24.9	6.25	3.44	21.50
A2dup	24.9	6.25	3.30	20.64
A3	24.9	6.25	3.16	19.75
A3dup	25.2	6.25	3.28	20.51
A4	25.2	6.25	3.20	20.02
A4dup	25	6.25	3.25	20.33
B1	25.1	6.25	3.21	20.04
B1dup	25	6.25	3.15	19.68

B2	25.1	6.25	3.32	20.72
B2dup	25.1	6.25	3.18	19.85
B3	24.9	6.25	3.37	21.04
B3dup	25.1	6.25	3.33	20.79
B4	25.1	6.25	3.12	19.51
B4dup	25.1	6.25	3.27	20.44
C1	25.2	6.25	3.24	20.24
C1dup	25.1	6.25	3.20	20.03
C2	25	6.25	3.20	19.97
C2dup	25.2	6.25	3.29	20.56
C3	25.2	6.25	3.07	19.16
C3dup	25.2	6.25	3.05	19.05
C4	25.2	6.25	2.92	18.27
C4dup	25	6.25	2.93	18.34
D1	24.8	6.25	3.90	24.40
D1dup	25	6.25	3.57	22.29
D2	25	6.25	3.31	20.70
D2dup	24.7	6.25	3.36	21.01
D3	25.1	6.25	3.07	19.21
D3dup	24.8	6.25	3.09	19.28
D4	25.2	6.25	2.69	16.84
D4dup	24.7	6.25	2.73	17.04
Ain	25	6.25	10.37	64.79
Aindup	24.8	6.25	10.43	65.17
Bin	24.8	6.25	10.44	65.22
Bindup	25	6.25	10.44	65.28
Cin	25	6.25	10.35	64.70
Cindup	24.8	6.25	10.11	63.19
Din	25.2	6.25	10.16	63.53
Dindup	24.8	6.25	10.18	63.65
Tunain	25	6.25	14.82	92.66
Tunaindup	24.9	6.25	14.70	91.85
C5	24.9	6.25	3.13	19.59
C5dup	24.8	6.25	3.09	19.34
C6	25.2	6.25	2.94	18.39

C6dup	25.1	6.25	2.97	18.56
D5	25.2	6.25	2.88	18.02
D5dup	25.2	6.25	2.81	17.55
D6	25.1	6.25	2.70	16.86
D6dup	24.9	6.25	2.61	16.31
Tuna1	24.8	6.25	3.23	20.18
Tuna1dup	25.3	6.25	3.27	20.43
Tuna2	24.7	6.25	3.11	19.43
Tuna2dup	24.7	6.25	3.26	20.36
TuB1	25.1	6.25	2.85	17.83
TuB1dup	25.2	6.25	2.86	17.86
TuB2	25	6.25	3.12	19.50
TuB2dup	25	6.25	2.81	17.57

D. Statistical analysis – normality test

Table 12: *Summary of normality test for samples A, B, C, and D.*

Test for normal distribution	A	B	C	D
Shapiro-Wilk test				
W	0.9042	0.8919	0.9623	0.9382
P value	0.0265	0.0145	0.4864	0.1484
Passed normality test (alpha=0.05)?	No	No	Yes	Yes
P value summary	*	*	ns	ns
Number of values	24	24	24	24

E. Statistical analysis – base digestion

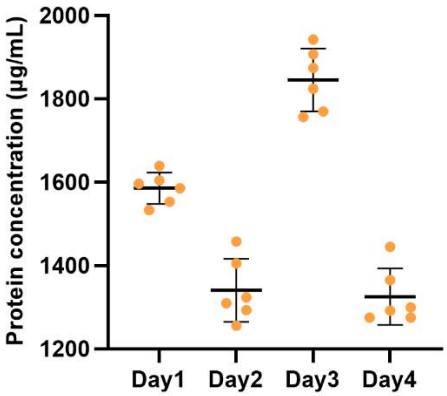


Figure 13: *Variation in protein concentration (µg/mL) of sample A plotted per day.*

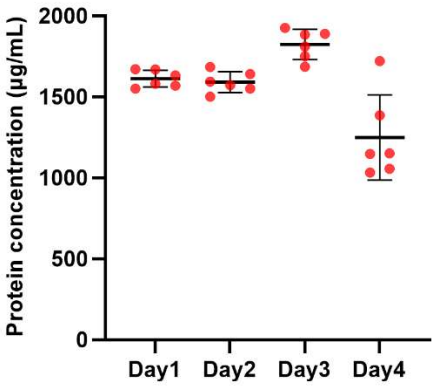


Figure 14: *Variation in protein concentration (µg/mL) of sample B plotted per day.*

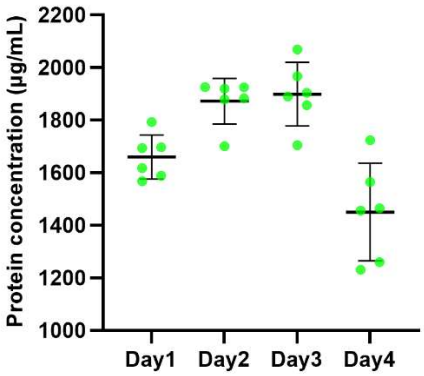


Figure 15: *Variation in protein concentration (µg/mL) of sample D plotted per day.*

F. Statistical analysis – supplementation of bromelain

Table 13: *Unpaired t-test C without and C with bromelain.*

P value	0.0589
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=1,937, df=46

Table 14: *Unpaired t-test D without and D with bromelain.*

P value	0.5206
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0,6481, df=40

G. Texture analysis – firmness and toughness.

Table 15: *Texture results toughness and firmness of A, B, C, and D.*

Sample	Toughness (Nm)	Toughness (Nm)	Firmness – mean force (g)	Firmness (g)
A along*	7 836 250	10 624 369 ± 2 453 087	4 979.44 ± 324.47	4 440.40 ± 961.42
A across**	12 018 429 ± 612 260.2		4 979.44 ± 162.23	
B along	15 478 962 ± 3 060 093	14 421 792 ± 3 447 432.8	5 241.95 ± 258.54	5 516.47 ± 878.44
B across	13 364 622 ± 4 671 164		5 791 ± 1 321.42	
C along	9 623 488 ± 1 423 082	9 257 244 ± 1 675 610	4 374.03 ± 545.29	4 027.07 ± 511.12
C across	8 890 999 ± 2 421 014		3 680.12 ± 69.91	
D along	13 455 371 ± 820 627.1	12 448 444 ± 1 874 420.3	4 008.17 ± 56.44	4 326.10 ± 534.76
D across	10 318 498 ± 164 177.7		4 644.04 ± 671.10	

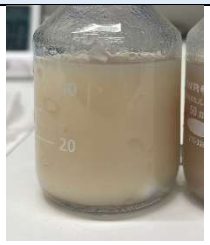
*along means the cut was made parallel to the direction of the fibres

**across means the cut was made perpendicular to the direction of the fibres

H. Appearance during digestion

The overall appearance including colour and particle size of the PBFA and real tuna were roughly monitored during the different phases, as shown in Table 6. In the oral phase, large chunks were visible after mastication was simulated. The plant-based fish analogue had a lower moisture content compared to the real tuna, in which more substances were involved, making it easier to grind. The colours of the PBFA and real tuna mixture were beige and brownish-pink, respectively. However, after digestion, the appearance had changed to a more brownish-beige colour. In addition, the particles notably got smaller due to the addition of enzymes in each phase.

Table 16: *Monitored appearance of plant-based fish analogue and real tuna throughout the gastrointestinal tract.*

	Oral phase	**Gastric phase**	**Intestinal phase**
Plant-based fish analogue			
Real Tuna