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Digestibility of conventional, alternative and novel protein sources to be used as sustainable components of animal feed

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Abstract

The increasing global demand for more sustainable and efficient animal feed calls for the investigation of alternative protein sources to reduce the reliance on traditional ones. As poultry is the primary source of meat, there is an urgent need to prioritise protein quality in poultry feed. Currently, multiple *in vitro* techniques are used to assess protein quality and digestibility, but a consensus has yet to be established for chicken feed.

This study aimed to employ the INFOGEST static *in vitro* digestion model, a globally standardised protocol designed to mimic gastrointestinal digestion for assessing nutrient bioaccessibility, to evaluate the protein quality and digestibility of traditional, alternative and novel protein sources, and to compare the digestibility values derived from laboratory experiments with those obtained from an *in vivo* chicken trial.

Protein digestibility was assessed in a range of novel feed ingredients. These protein sources included commercial soybean and rapeseed products, yeast samples, microbial proteins, and casein as a reference. Results showed a strong positive correlation between the values obtained and those from the company supplier regarding crude protein content and amino acid content ($p < 0.001$). *Clostridium sp.* showed superior digestibility compared to sunflower meal ($p < 0.05$) and a higher DIAAS (0.70 and 0.49, respectively). Interestingly, *Clostridium sp* had a high lysine digestible indispensable amino acid ratio (DIAAR) of 1.87.

The digestible indispensable amino acid scores (DIAAS) for yeast protein isolates were exceptionally high, varying from 0.77 for dried pot ale yeast to 1.75 for brewer's

yeast 2, with lysine and sulphur-containing amino acids being the limiting amino acids, respectively.

The non-absorbable fraction weights of rapeseed meal, rapeseed protein concentrate, and sunflower protein concentrate after digestion were significantly higher ($p < 0.05$) than that of casein. Casein and sunflower meal lysine digestibility were significantly higher ($p < 0.05$) than that of other protein isolates, except for AlphaSoy Gold. Lysine digestibility of rapeseed meal was significantly lower than for AlphaSoy Gold, sunflower protein concentrate, sunflower meal and casein.

Lysine was the limiting amino acid for sunflower-based and rapeseed-based protein isolates, with DIAAS varying from 0.81 to 1.14. Pea protein concentrate and soya-based protein sources had a DIAAS between 0.52 and 0.90, with sulphur-containing amino as limiting amino acids.

Total amino acid digestibility of soybean meal (SBM), AlphaSoy and sunflower expeller at inclusion levels of 30%, 45% and 60% ranged from 60.80% to 100.00%. For most amino acids, SBM digestibility was significantly lower than AlphaSoy and sunflower expeller ($p < 0.05$), irrespective of the inclusion level. DIAAS values ranged from 0.32 – 0.58 for SBM, 0.59 – 0.80 for AlphaSoy and 0.67 – 0.76 for sunflower expeller. Sulphur-containing amino acids were the limiting factor for all, except for sunflower expeller, where lysine was limiting.

Protein digestibility was assessed in a range of bacteria using the same *in vitro* method. Bacteria showed variability in amino acid content, digestibility, and DIAAS values using the *in vitro* digestion method. *Thermus thermophilus* and *Psychrobacter frigidicola* showed relatively high total amino acid digestibility (84.0% and 88.4%,

respectively), significantly higher than *Cupriavidus necator* with polyhydroxybutyrate (PHB) (54.3%, $p < 0.05$). Sulphur-containing and aromatic amino acids were the most common limiting factors across bacteria, with DIAAS values ranging from 0.34 for *Thermus thermophilus* to 0.72 for *Methylococcus capsulatus*.

The correlation between PHB content and total amino acid digestibility in *Cupriavidus necator* showed a strong negative correlation ($r = -0.8211$) but the correlation was not statistically significant ($p = 0.1790$).

A chicken trial (Ross 308) was conducted using formulated feeds composed of SBM, AlphaSoy or sunflower expeller, with inclusion levels of 30%, 45%, or 60% for each protein source. *In vivo* digestibility was determined as apparent ileal digestibility (AID) using ileal digesta collected from the chicken trial, while *in vitro* digestibility was assessed using the INFOGEST static *in vitro* digestion model. *In vitro* digestibility values slightly exceeded *in vivo* values, with minimal differences. However, *in vitro* methods consistently overestimated the digestibility of lysine, leucine, valine, isoleucine, and phenylalanine, while underestimating methionine, threonine, and histidine.

In conclusion, this study illustrates the effectiveness of the INFOGEST model in distinguishing protein quality among various protein sources and emphasises the importance of adopting standardised evaluation methods to advance the creation of sustainable feed solutions.

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Lastly, to my partner Gabriel. I still cannot believe we met during this trying time of my life, but I am eternally grateful to have you by my side. Your unwavering support and presence have made the last year fundamentally better than the start of my PhD.

As a non-native English speaker, AI-based tools (Grammarly and ChatGPT) helped me improve the grammar and clarity of the text in this thesis. At the time of submission, the work presented in this thesis has not been previously published. Several manuscripts are in preparation for submission to peer-reviewed journals.

Covid-impact log

The work carried out in this thesis was slightly impacted by the COVID-19 pandemic. I began my thesis in February 2021 while in France and was only able to travel to England in May 2021, although travel from France to the UK was still subject to significant restrictions due to the COVID-19 pandemic. At that time, the North Laboratory in the School of Biosciences had recently been refurbished, and some analytical equipment was installed only several months later.

Lab training did not begin until late August 2021, as the laboratory was still operating with limited access. Training opportunities were further constrained because priority was given to PhD students nearing the end of their projects, as they had experienced prolonged periods without lab access.

The supply of general laboratory consumables and specific reagents also faced delays. Although this period out of the lab allowed me to focus on the literature review, the interruptions in training delayed the start of work on real samples until April 2022. Additionally, power cuts and floods occasionally disrupted the lab building, further complicating the completion of lab work.

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List of abbreviations

AAA	Aromatic Amino Acids
ANF	Anti-Nutritional Factor
ANOVA	Analysis of Variance
CP	Crude Protein
DH	Degree of Hydrolysis
DIAAR	Digestible Indispensable Amino Acid Ratio
DIAAS	Digestible Indispensable Amino Acid Score
FCR	Feed Conversion Ratio
IAA	Indispensable Amino Acid
LAA	Limiting Amino Acid
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
NPCF	Nitrogen-to-Protein Conversion Factor
NPN	Non-Protein Nitrogen
OPA	*o*-PhthaldiAldehyde
PAP	Processed Animal Proteins
PCDAAS	Protein Digestibility-Corrected Amino Acid Score
PHB	PolyHydroxyButyrate
SAA	Sulphur-containing Amino Acids
SBM	Soybean Meal
SD	Standard Deviation
SEM	Standard Error of the Mean
SOP	Standard Operating Procedure
SRM	Standard Reference Material
TIM	TNO Intestinal Model

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1. Introduction

1.1. The growing demand for poultry

Human population growth, urbanisation, and rising incomes continue to drive the global demand for livestock products. Over the past decade, this dietary shift toward greater consumption of animal products has persisted, with global per capita meat intake rising from 34.0 kg retail weight equivalent (r.w.e.) in the 2013–2015 base period to 35.3 kg r.w.e. by 2025, with a 1.3 kg increase primarily driven by poultry (*OECD-FAO Agricultural Outlook 2021-2030*, 2021).

Developing countries accounted for the bulk of this growth, with per capita consumption climbing from 26.6 kg r.w.e. to 28.0 kg r.w.e., explained by rapid population expansion, urbanisation, and income gains in regions like Asia, Latin America, the Middle East, and Sub-Saharan Africa. In these contexts, increased meat consumption remains beneficial, providing high-quality protein and contributing to the reduction of child stunting by improving nutrient availability during complementary feeding (Adesogan et al., 2020).

Another shift has been noticed, a change from red to white meat. Chickens are more cost-effective to raise due to their superior feed-to-meat conversion efficiency compared to pigs and cattle (FAO, 2011). By 2034, poultry meat is projected to provide 45% of the protein consumed from all meat sources (*OECD-FAO Agricultural Outlook 2025-2034*, 2025), as shown in Figure 1.1. Due to inflation, combined with global wage stagnation, people opt for less expensive meats. (*The State of Food Security and Nutrition in the World 2025*, 2025).

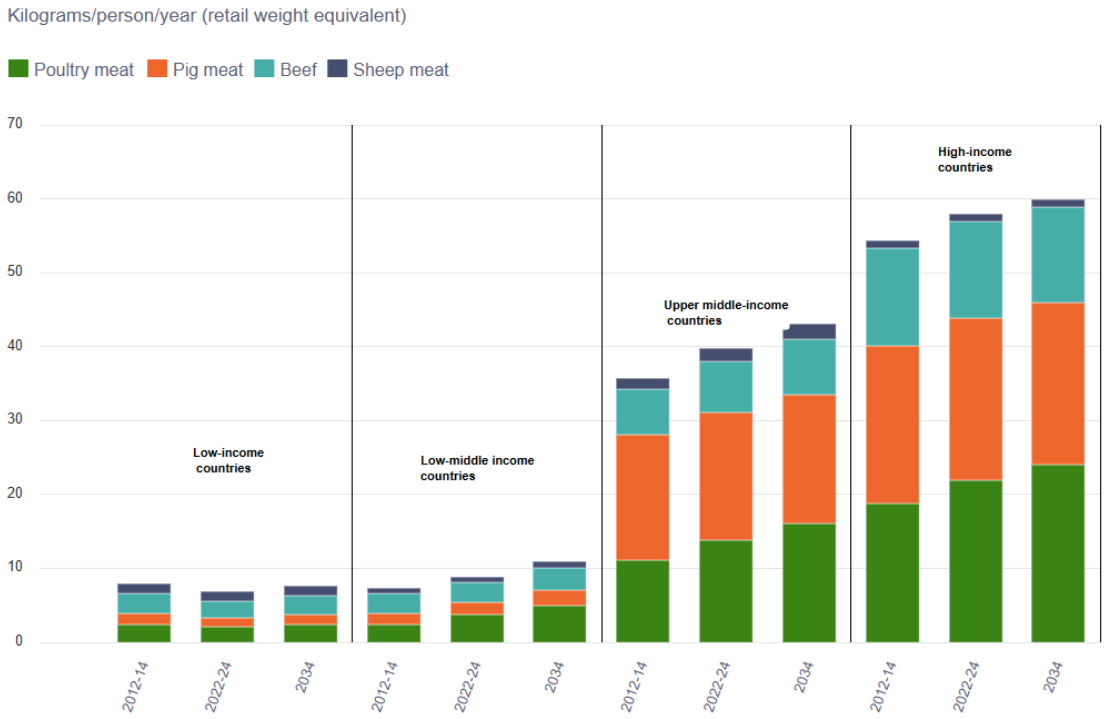


Figure 1.1: Per capita meat consumption by income group and meat type. Adapted from *OECD-FAO Agricultural Outlook 2025-2034*, 2025. The trend for poultry meat shows a clear increase across all income groups from 2012-14 to 2034.

Poultry is generally perceived as healthier than pork and beef. This is largely due to its lower content of saturated fats. Saturated fat is associated with increased levels of LDL cholesterol, which can contribute to heart disease (Siri-Tarino et al., 2010). White meat such as chicken breast contains about 27–29% of saturated fat relative to its total fat content, whereas cuts of pork and beef typically contain 40–50% saturated fat (Connolly & Campbell, 2023). Lower saturated fat intake is associated with a reduced risk of cardiovascular disease, which contributes to the perception of poultry as the healthier option. Poultry meat also tends to have fewer calories than comparable cuts of pork or beef. For instance, skinless chicken breast has about 165 kcal per 100 g, whereas lean beef can be between 270 and 280 kcal per 100 g (Petersen & Hirsch, 2023).

Poultry meat is associated with a much lower carbon footprint than red meat, yet it still imposes significant environmental burdens. For example, a review article from

2025 notes that in one life-cycle assessment, 1 kg of fried chicken equated to 5.86 kg CO_2-eq of global-warming impact, while an average kg of beef (excluding methane) was 36 kg CO_2-eq, which is roughly four times higher (Oleinikova et al., 2025). When methane emissions are included, beef's footprint can reach 100 kg CO_2-eq per kg. To meet global demand, world poultry meat production went from 9 million tonnes to 133 million tonnes between 1961 and 2020. The most recent data for 2024 estimated global poultry meat production at 149.7 million tonnes (FAO, 2024).

Genetic selection has enabled the production of Ross 308 broilers with faster growth rates younger slaughter ages and improved feed conversion ratios (FCR), meaning that chickens can convert feed into body mass more efficiently. Ross 308 broilers are bred to produce consistent results in growth rate, body weight and FCR. They reach the market weight of 2.5 kg in 35 to 42 days and often have an FCR below 2.0 in optimised systems, implying that less feed is required to produce 1 kg of body weight compared to other chicken breeds (Aviagen, 2022b; Brameld & Parr, 2016).

By comparison, the Cobb 500 broiler strain also shows very high performance: for example, in a field-study Cobb 500 birds achieved higher final body weights and slightly better FCR than Ross 308 under certain conditions (Pascalau et al., 2017). Among the two, Ross 308 may exhibit advantages under controlled environments (slightly better efficiency or robustness), while Cobb 500 may reach higher weights or faster early growth in some systems with a weight of 2.5 kg in 35 days (open housing under less-controlled conditions). According to a recent article comparing Cobb-500 and Ross 308, the Cobb strain achieved an FCR of 1.51 at 42 days whereas

the Ross 308 reached an FCR of 1.57 for Ross 308 in that study (Pandit et al., 2024). To improve efficiency whilst reducing costs and developing sustainability, the need for high-quality protein sources in chicken feed is fundamental.

Protein is crucial for improved egg quality and higher egg output for laying hens. A high-quality protein will also support the immune system and prevent stress by enhancing chicken resilience towards environmental and disease challenges (Beski et al., 2015; El-Shall et al., 2023; Teyssier et al., 2022). Muscle development and meat quality are improved with protein sources of high quality, as well as fertility and hatchability. A nutritious feed will also contribute to the sustainability of poultry farming by reducing the need for larger quantities of feed and minimising water usage, as chickens use the nutrients more effectively, particularly amino acids (Cappelaere et al., 2021).

1.2.Amino acids in poultry nutrition

Proteins are macromolecules that comprise one or more long chains of amino acid residues. The body uses amino acids to build new proteins, repair tissues, and support metabolic processes. Amino acids are organic compounds composed of both amino ($-NH_2$) and carboxyl groups (-COOH) and are crucial for various physiological functions in poultry. These include growth, muscle development, immune function, and overall health (Wu, 2009).

There are three categories to classify amino acids: indispensable, dispensable and conditionally indispensable. The classification of amino acids can vary depending on specific conditions, such as the growth rate of the broilers and the criteria used to determine amino acid adequacy.

Indispensable amino acids imply that they are derived from the diet, as the organism cannot synthesise the carbon skeleton of those amino acids or synthesise them in amounts adequate to meet the requirements. As with humans, indispensable amino acids for chickens include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Alagawany et al., 2020; Reeds, 2000).

The conditionally indispensable amino acids are amino acids which may become indispensable under conditions of rapid growth, stress, illness, where the rate of amino acid synthesis may not meet the requirements of the broiler. These amino acids cannot be synthesised in sufficient amounts by the body and must be obtained from the diet.

Arginine is considered an indispensable amino acid for chickens. This is due to the lack of a functional urea cycle in birds, which is necessary for sufficient arginine synthesis (Brugaletta et al., 2023). This amino acid plays a key role in nitric oxide synthesis, blood flow regulation and immune function in broilers (Fathima et al., 2023). Cysteine and tyrosine are considered conditionally indispensable because their synthesis depends on adequate levels of methionine and phenylalanine, respectively.

In contrast, glutamine and proline are generally listed as dispensable for young broiler chickens under normal conditions, not for young human children (Wu et al., 2011). These amino acids can be synthesized in sufficient amounts and typically do not need to be supplied in the diet under normal physiological conditions. Table 1.1 shows the optimal ratios of true digestible amino acids in diets for broiler chickens.

Table 1.1: Optimal ratios of true digestible amino acids in diets for broiler chickens

Amino acid	Category	Age of broiler chickens		
		0 – 21 days	21 – 42 days	42 – 56 days
		% of digestible lysine in diet[1]		
Lysine	Indispensable	100	100	100
Methionine	Indispensable	40	42	42
Threonine	Indispensable	67	70	70
Valine	Indispensable	77	80	80
Leucine	Indispensable	109	109	109
Isoleucine	Indispensable	67	69	69
Histidine	Indispensable	35	35	35
Phenylalanine	Indispensable	60	60	60
Tryptophan	Indispensable	16	17	17
Arginine	Indispensable	105	108	108
Tyrosine	Conditionally indispensable	45	45	45
Cystine	Conditionally indispensable	32	33	33
Proline	Conditionally indispensable	184	184	184
Glycine	Conditionally indispensable	176	176	176
Serine	Dispensable	69	69	69
Glutamate	Dispensable	178	178	178
Glutamine	Dispensable	128	128	128
Alanine	Dispensable	102	102	102
Arginine	Dispensable	105	108	108
Asparagine	Dispensable	56	56	56
Aspartate	Dispensable	66	66	66

Adapted from Wu & Li, 2022.

[1]Values are based on true ileal digestible amino acids. The amounts of digestible lysine in diet (as-fed basis; 90% dry matter) is 1.12% between 0 and 21 days, 0.89% between 21 and 42 days and 0.76% between 42 and 56 days.

The dietary requirements of amino acids in broiler chickens are commonly expressed as ratios relative to lysine, which is considered the reference amino acid due to its central role in muscle protein synthesis and growth. This “ideal protein” approach

assumes that all indispensable and conditionally indispensable amino acids are provided in optimal proportions to lysine, ensuring that none are supplied in excess or deficiency.

In practice, maintaining these ideal ratios can be challenging because some amino acids are present in lower concentrations. Such amino acids are referred to as limiting amino acids, as their deficiency restricts protein synthesis, growth, and overall performance even when other amino acids are adequate. In poultry diets, lysine and methionine are generally the first limiting amino acids, particularly in formulations based primarily on plant protein sources such as soybean meal (Fernandez et al., 1994a).

Both lysine and methionine are major limiting amino acids in broiler diets and jointly influence growth performance and carcass traits in male broilers (Han & Baker, 1994; Wilfred et al., 2024). An adequate balance of indispensable amino acids in the diet is crucial for optimising poultry production, as deficiencies or imbalances can significantly reduce FCR, muscle development, and overall performance (Konashi et al., 1999).

Another aspect is the importance of formulating feed based on digestible amino acids rather than total amino acids, as poultry do not fully digest all amino acids in feed ingredients. For broiler chickens between 0 and 3 weeks, digestible lysine is expected to be 1.2 to 1.3% of the diet and digestible methionine 0.55% of the diet (Aviagen, 2022). These values represent the foundation for achieving optimal growth and feed efficiency.

Achieving these digestible amino acid levels require careful ingredient selection in feed formulation. For example, soybean meal is known to be a good source of digestible lysine and often is synthetically supplemented with methionine to balance the animal diet (Macelline et al., 2022). The amino acid ratios are important to consider. Excessive supplementation of one amino acid can interfere with the absorption of others. The lysine to methionine ratio is 40 to 45 units of digestible methionine for every 100 units of digestible lysine in the case of a starter chicken (NRC, 1994). Maintaining the correct amino acid balance mainly relies on the quality and composition of the protein sources used in poultry feed.

1.3. Protein sources for poultry feed

1.3.1. Challenges in conventional protein sources

Processed animal proteins (PAPs) were commonly used protein source in animal feeds, made from animal by-products including meat meal and bone meal. However, they were banned in the European Union in 2001 as a response to the Bovine Spongiform Encephalopathy (BSE) crisis, also known as "mad cow disease." The ban prohibited the use of PAPs in feed for farmed animals due to concerns about the spread of prion diseases (Brown et al., 2001).

With improved scientific understanding and stricter controls, some restrictions have been relaxed recently, under specific conditions. For instance, in 2021, the European Commission reintroduced the use of certain PAPs (e.g., pigs and porcine PAPs) and authorised insect PAPs for feeding non-ruminant livestock, like poultry and fish, under tightly regulated circumstances (Inaudi et al., 2024).

Post-Brexit Transmissible Spongiform Encephalopathy (TSE) regulations on PAPs remain strict in Great Britain, maintaining a ban on porcine and poultry PAPs in non-ruminant feed (pigs and poultry). A 2025 consultation by DEFRA and Scottish Government proposed controlled reintroduction of porcine PAPs in poultry feed and poultry PAPs in pig feed, but as of 2025, these changes are not yet implemented.

At present, plant-based sources are the primary sources of protein in animal feed, with soybean meal being the most popular choice thanks to its high protein content and well-balanced amino acid profile. Soybeans are primarily crushed for oil, producing soybean meal as a co-product. This meal, rich in protein (44 to 49%), is then efficiently valorised by feeding poultry, pigs, and fish, delivering high-quality animal protein to humans while closing the nutrient loop and minimising resource waste (Thakur & Hurburgh, 2007).

Soybean production, particularly in Brazil and Argentina, is a major driver of land use change (LUC)—the conversion of forests, savannas, and grasslands into cropland—which releases large amounts of stored carbon and accounts for up to 65–85% of the crop's total greenhouse gas emissions over a 20-year assessment period (H. Kim, 2022). In contrast, U.S. soybean production has near-zero direct LUC impact (less than 5%), as nearly all expansion since 2000 has occurred on existing cropland or previously cleared land, making it one of the most sustainably produced soy sources globally. Table 1.2 presents the crude protein content and indispensable amino acid composition of soybean meal from the three major global origins (United States, Brazil, and Argentina), expressed as % of crude protein (CP) on a dry matter (DM) basis from Thakur & Hurburgh, 2007.

Table 1.2: Crude protein and indispensable amino acid profiles of soybean meal from Us, Brazil and Argentina

Nutrient	U.S. soybean meal	Brazil soybean meal	Argentina soybean meal
Crude protein (% dry matter)	47.5 - 48.5	48.0 - 49.5	44.0 - 46.0
Lysine	6.2 – 6.4	5.9 – 6.1	6.0 – 6.2
Methionine	1.4	1.3	1.3
Cystine	1.5	1.4	1.4
Methionine + Cystine	2.8	2.7	2.7
Threonine	4.0	3.9	3.9
Tryptophan	1.4	1.3	1.3
Isoleucine	4.7	4.6	4.6
Valine	5.0	4.8	4.9
Standardised Ileal Digestibility (SID) - Lysine (Broilers, %)	93.1	89.4	88.2

Values are on a dry matter basis. Adapted from Thakur & Hurburgh, 2007.

Crude protein (CP) and amino acid profiles of soybean meal vary significantly by origin, with U.S. soybean meal offering the highest lysine (6.20 to 6.40% of CP) and digestibility (93.1%), followed by Argentine (6.0 to 6.2% of CP; 88.2%) and Brazilian (5.9 to 6.1% of CP; 89.4%) sources, requiring region-specific feed formulation to optimise nutrient use (Thakur & Hurburgh, 2007).

Soybean is heavily imported into Europe, the United States and South America (Song et al., 2021). The widespread use of pesticides and fertilizers in soybean farming contributes to water pollution and greenhouse gas emissions. Soybean meal price has also increased with the various inflations and crises affecting the world.

Other plant-based alternatives, like rapeseed and sunflower meals, also demand large amounts of land and water to produce (Govoni et al., 2024). Alongside these environmental concerns, using conventional protein sources in animal feed comes with additional challenges. As the competition for arable land between food and feed production grows, it puts increasing pressure on global food security.

Moreover, the nutritional quality of plant-based proteins can vary, often falling short of providing the indispensable amino acids needed for optimal animal growth and health. Many plant-based protein sources also contain anti-nutritional factors that can reduce nutrient availability and digestibility (Hertzler et al., 2020). To address these challenges, alternative protein sources that combine sustainability, high nutritional value, and minimal environmental impact should be further investigated for their future application in poultry feed.

Before exploring individual alternative protein sources, it is useful to define the key properties that a sustainable and nutritionally valuable protein source should possess. Table 1.3 summarises these desired characteristics, encompassing nutritional quality, environmental sustainability, food safety, economic viability, and resource efficiency. These criteria provide a framework for evaluating potential alternative protein candidates, helping to identify those that could meet the growing demand for poultry feed while minimising environmental impact and ensuring animal health.

Table 1.3: Desired properties of sustainable alternative protein sources for poultry feed

Category	Property	Purpose
Nutritional Quality	High protein content	Supports growth and production
	Balanced indispensable amino acids	Optimises feed efficiency and health
	High digestibility	Maximises nutrient absorption
Environmental Sustainability	Low land footprint	Reduces deforestation and land-use change
	Low water usage	Minimises freshwater demand
	Low greenhouse gas emissions	Reduces climate impact
Food Safety	Free from pathogens/prions	Ensures safe poultry products
	Low anti-nutritional factors	Improves nutrient availability
Economic & Supply	Cost-effective and scalable	Enables wide adoption
Circularity	Valorises waste/by-products	Enhances resource efficiency

1.3.1. Alternative protein sources

Sustainability in animal feed refers to the ability of protein sources to support poultry production while minimising environmental impact, conserving resources, and ensuring economic and food security for the long term. As conventional protein sources face environmental and nutritional limitations, identifying alternative proteins that are both nutritious and sustainable becomes essential.

Insects, algae, fungi-based, bacterial and underutilised crops show promise as efficient and sustainable protein sources. Table 1.4 lists these protein candidates with their benefits and challenges.

Table 1.4: Alternative protein sources as candidates for poultry feed

Protein Source	Type	Sustainability/Environmental Benefits	Nutritional Highlights	Challenges/Notes
Black Soldier Fly (BFL) larvae	Insect	Can be cultivated on approved organic substrates, circular economy, low land and water use	High protein, rich in indispensable amino acids, good digestibility	Regulatory approval, scale-up production, consumer acceptance
Mealworms	Insect	Efficient conversion of approved organic substrates, low environmental footprint	High protein, good amino acid profile	Regulatory approval, production scale, cost
Bacteria (*Methylococcus capsulatus, Cupriavidus necator, Paracoccus pantotrophus*)	Bacteria	Can be grown on methane or hydrogen, minimal land use	High protein, potential for tailored amino acid profiles	Scale-up, feed safety regulation, cost
Spirulina	Algae	Minimal land and freshwater usage, carbon storage potential	Rich in protein, vitamins, and minerals	Processing costs, digestibility
Chlorella	Algae	Growth in water, low land footprint, fast biomass production	High protein, indispensable amino acids, antioxidants	Harvesting and processing, cost
Mycoprotein (*Fusarium venenatum*)	Fungi	Produced via fermentation, low land and water use	High protein, good amino acid profile, low fat	Cost, regulatory approval, scale-up
Faba beans, peas, lupins	Underutilised crops	Nitrogen fixation, lower land and fertilizer input than soy	Indispensable amino acids, improved by processing	Anti-nutritional factors, variable digestibility, seasonal availability

Insects, such as black soldier fly (BFL) larvae and mealworms, can be cultivated on carefully controlled organic substrates, such as certain food processing by-products, in compliance with EU and UK feed hygiene regulations. This approach supports a circular economy and reduces the environmental footprint of protein production (Elahi et al., 2022; K. Hawkey et al., 2021; K. J. Hawkey et al., 2021). In the European Union, insect-derived proteins (from BFL larvae or mealworms) are now authorised for use in non-ruminant livestock (including poultry) feed under specific regulation, offering a circular-economy opportunity. In the UK, the use of processed insect-based proteins in feeds for poultry (and pigs) is not yet permitted.

Bacteria, such as *Methylococcus capsulatus* and *Cupriavidus necator*, can be cultivated using methane or hydrogen, representing a promising lead for sustainable protein production. However, challenges remain in terms of production scale-up and regulatory approval for use in animal feed (Kerckhof et al., 2021). *Cupriavidus necator* is also known for its capacity to accumulate intracellular polyhydroxybutyrate (PHB), a bioplastic that serves as an energy and carbon storage compound. While PHB accumulation is advantageous for industrial applications, its presence in microbial biomass can influence protein quality and digestibility when used as a feed ingredient (Ismail et al., 2024; Kunasundari et al., 2013).

Paracoccus pantotrophus is a versatile, non-pathogenic, Gram-negative bacterium known for its metabolic flexibility and ability to utilise a wide range of carbon and nitrogen sources (Pal et al., 2024a). It has been studied for biotechnological applications, including wastewater treatment, bioremediation, and microbial protein production (Huang et al., 2022). Its ability to grow on diverse substrates, including

agricultural residues, makes it a promising candidate for sustainable microbial protein generation, potentially offering high-quality protein with favourable amino acid composition (Pal et al., 2024b; Pyter et al., 2022).

One important nutritional consideration when evaluating insect and bacterial proteins is the proportion of non-protein nitrogen (NPN) they contain. NPN is the nitrogen that is measured as part of crude protein but does not contribute directly to amino acids usable for growth. For instance, a study found that the meal from BSL larvae contained approximately 3.0 % to 6.8 % NPN in the form of chitin and other nitrogenous compounds, meaning that the standard nitrogen-to-protein conversion would overestimate true protein content (Chobanova et al., 2023).

This over-estimation may partially explain why broilers fed a BSF larvae meal diet achieved lower weight gain and feed efficiency compared to a soybean meal control, even in isonitrogenic diets.

For bacteria, while total nitrogen may appear high, a portion of this may be NPN (nucleic acids, ammonia or cellular debris) and not all of it is converted into utilisable amino acids by poultry. Therefore, when assessing insect- or bacteria-based protein sources for poultry feed, it is important to adjust formulations for NPN content, ensure accurate amino acid profiling, and consider digestibility corrections. Without accounting for this, diets may appear equivalent on a crude protein basis but fall short in growth performance or nutrient utilisation.

Algae, such as Spirulina and Chlorella, are rich in proteins and have the potential to be cultivated with minimal land and water usage (Mosibo et al., 2024).

Fungi-based proteins, including mycoprotein from species like *Fusarium venenatum*, have gained attention for their high protein content and ability to be produced through fermentation processes (Tong et al., 2024).

Underutilised plant-based proteins are also being explored. Legumes such as faba beans, peas, and lupins can be grown in the UK and represent a locally produced, sustainable protein source for poultry feed due to their nitrogen-fixing ability and relatively high protein content (Costa et al., 2021a). However, their adoption in poultry diets remains limited compared to imported soybean meal because they contain anti-nutritional factors that reduce digestibility, have lower levels of certain essential amino acids (particularly methionine), and require additional processing to optimise nutritional value. Furthermore, variable yields and the need for processing infrastructure make large-scale incorporation into feed more challenging.

Despite these limitations, legumes remain a promising component of multi-source feed formulations aimed at improving amino acid balance while reducing reliance on imported proteins. A feed complemented with plant-based proteins from multiple sources might increase the amino acid content for specific amino acids in low concentrations, creating a more balanced chicken diet.

These novel protein sources combined with conventional plant-based proteins must undergo thorough evaluation for their nutritional quality, digestibility, and environmental impact. Understanding the interplay between protein digestibility, feed formulation, and animal health is crucial in meeting the growing global demand for poultry products while minimising environmental harm.

1.4. Digestion models

1.4.1. Chicken digestive system

Different animal species have unique digestive systems, and understanding these differences is essential for optimising protein feed formulations. Cows, as ruminants, have a specialised stomach with four compartments (rumen, reticulum, omasum, and abomasum), which allows them to efficiently ferment fibrous plant materials. Rumen bacteria break down cellulose and hemicellulose into volatile fatty acids and microbial protein, providing energy and amino acids for the animal.

Chickens are monogastric (non-ruminant) animals with a two-part stomach: the proventriculus for enzymatic digestion and the gizzard for mechanical grinding. Unlike ruminants, they lack microbial fermentation and rely mainly on enzymatic digestion, which limits fibre breakdown. As a result, their diets often include highly digestible protein sources and enzyme supplements to improve nutrient availability (Figure 1.2).

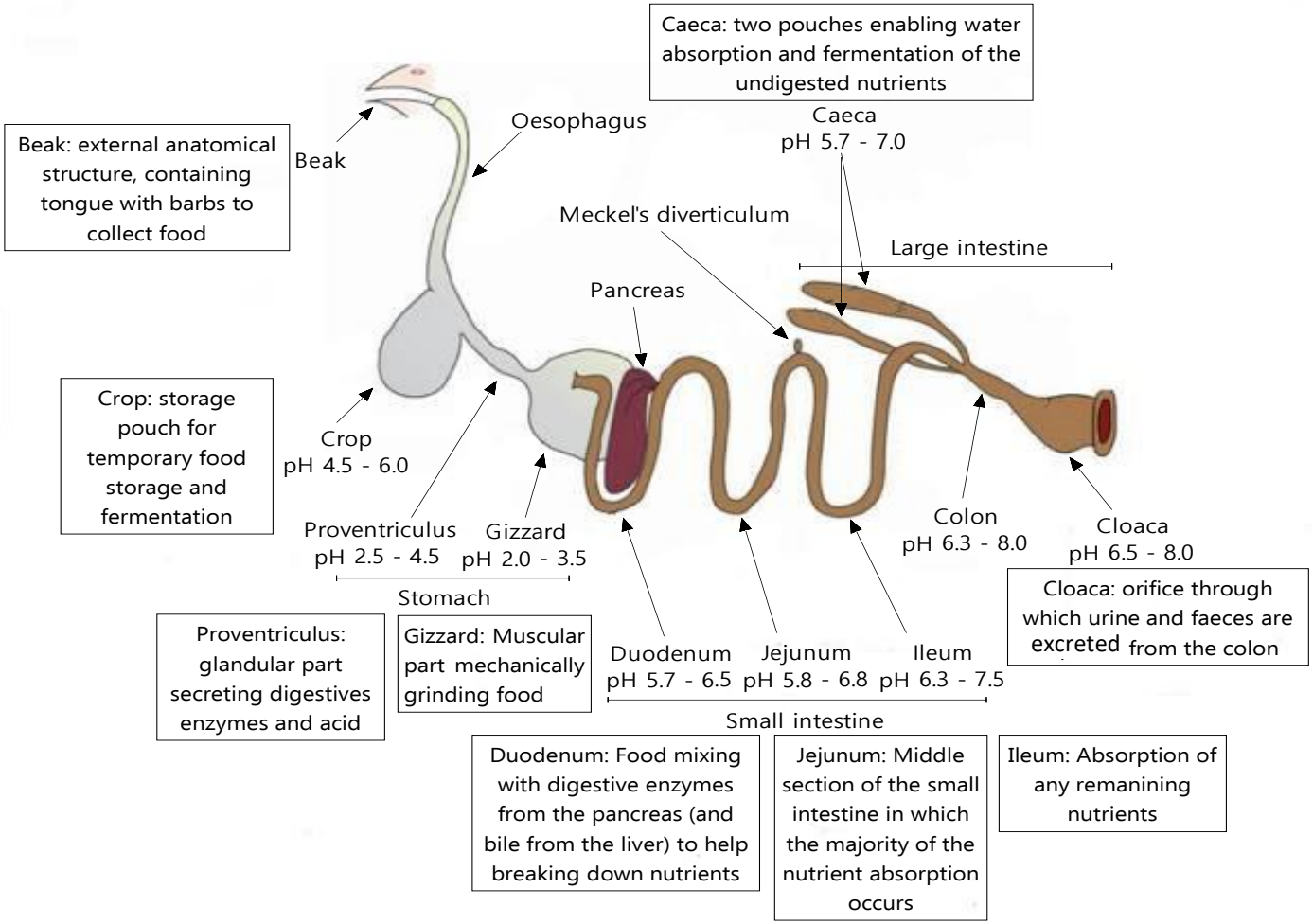


Figure 1.2: Anatomical structure and pH values of the chicken digestive system. Modified from Bindari & Gerber, 2022. The information included in the figure were extracted from Chang & Chen, 2000; Gauthier, 2002; Herwig et al., 2020; Liu et al., 2016; Mabelebele et al., 2014, 2017; Maoba et al., 2021; Riley & Austic, 1984; Ritzi et al., 2014.

Poultry are omnivores with a digestive system adapted to process both animal- and plant-based ingredients. While they can efficiently utilise many plant proteins, high-fibre or anti-nutritional components can limit nutrient availability. This is why poultry diets are often supplemented with enzymes such as phytase and xylanase, which help break down phytates and non-starch polysaccharides respectively, improving digestion and overall nutrient absorption. (Brameld & Parr, 2016; Costa et al., 2021b; Pirgozliev et al., 2022).

Phytase breaks down phytate, releasing minerals and phosphorus, making it digestible for poultry. Xylanase breaks down xylans (a type of fibre), improving digestibility of cereal grains.

Several differences exist between the chicken and the human digestive system. The chickens have a beak instead of teeth and a tongue with barbs to help collect food. Chickens are equipped with a crop. A crop is a storage pouch for temporary food storage and minor fermentation and is absent in the human digestive system.

Compared to humans, which have a single stomach, chickens have a two-part stomach consisting of the proventriculus and the gizzard. The proventriculus is the glandular portion that secretes digestive enzymes and acids to chemically break down food, while the gizzard is muscular and grinds feed mechanically. This combination allows chickens to process both animal- and plant-based ingredients, though they still lack the microbial fermentation capacity of ruminants like cattle, which enables more efficient breakdown of fibrous plant materials.

The caecum is a unique pouch at the beginning of the large intestine in the human digestive system, whereas the chicken possesses two ceca (van der Wielen et al., 2000), two pouches that facilitate fermentation of undigested nutrients.

Unlike mammals, chickens do not have a bladder, which contributes to their ability to conserve water and retain more of the nitrogen from the protein in their diet. This is important because nitrogen is essential for growth, but it must be excreted in a safe form since it can be toxic in large amounts within the body (Fígares et al., 1996; Vetere et al., 2025).

In birds, nitrogen is converted into uric acid in the kidneys and excreted via the cloaca. Uric acid is much less water-soluble than urea, the nitrogen waste excreted by mammals. As a result, chickens can excrete nitrogen with minimal water loss. This adaptation allows them to conserve water while still efficiently eliminating nitrogen from their bodies. Thus, chickens primarily excrete nitrogen waste as uric acid, not urea. This difference in nitrogen excretion is one reason why chickens retain more nitrogen from their food, supporting their growth.

Moreover, chickens have a shorter digestive tract and faster digesta transit time compared to humans. The average whole tract transit time in poultry is less than 3.5 hours, which is much faster than in humans (24 to 72 hours) (Pan & Yu, 2014). The chicken digestive system generally operates at around 40 to 42 °C (Aluwong et al., 2017; Troxell et al., 2015). The temperature in the chicken digestive system rises during the first four days after hatching (Lamoreux & Hutt, 1939). The pH ranges in each part of the chicken digestive system reported in Figure 1.2 reflect general trends observed across multiple studies and can vary based on factors such as diet, age, and

individual bird variation (Chang & Chen, 2000; Gauthier, 2002; Herwig et al., 2020; Y. Liu et al., 2016; Mabelebele et al., 2014; Maoba et al., 2021; Riley & Austic, 1984; Ritzi et al., 2014).

Understanding the chicken digestive system is essential for optimising feed formulations and improving protein digestion.

1.4.2. *In vivo* digestion models

To better understand how protein sources are digested in chickens, *in vivo* trials play a crucial role in assessing the efficiency of protein breakdown and absorption, much like in human digestive studies. This exploration necessitates a thorough understanding of protein digestibility, a crucial factor in determining the nutritional value of feed ingredients.

Digestibility refers to the extent to which a nutrient is absorbed in the gastrointestinal tract after ingestion. In the context of proteins, digestibility is a measure of how efficiently the animal can break down and absorb the amino acids from the protein source. A high crude protein content and high digestibility are desirable as they indicate that a larger proportion of the ingested protein is available for the animal's physiological processes (Toghyani et al., 2020). Crude protein is still widely used because it provides a quick, standardized estimate of total nitrogen in feed, allowing continuity with historical data and regulatory frameworks. However, it does not account for amino acid composition or digestibility, so precision feeding increasingly relies on digestible amino acids and protein quality measures for more accurate and efficient nutrition.

To assess protein digestibility more accurately, *in vivo* trials remain the gold standard. These trials involve feeding animals a test diet and measuring the difference between ingested and excreted nutrients by collecting faecal and ileal samples. For chickens, numerous studies have examined the digestibility of different protein sources, ranging from conventional plant-based options like soybean meal to novel alternatives like insect and algae proteins (Kaewtapee et al., 2023; Mosibo et al., 2024; Ravindran & Bryden, 1999; Sklan & Hurwitz, 1980; X. Wang & Parsons, 1998).

An *in vivo* study (Lemme et al., 2004) examined the digestibility of soybean meal, rapeseed meal, and sunflower meal in Ross 308 male broilers and found that alternative protein sources had lower pre-cecal digestibility compared to soybean meal (90%). Sunflower seed meal showed 84% digestibility and rapeseed meal showed 76% digestibility. The study also suggested that rapeseed meal proteins were more sensitive to heat damage compared to soybean and sunflower proteins.

Another *in vivo* study (Abdollahi et al., 2022) showed that proper heat treatment eliminated anti-nutritional factors (ANFs) in soybeans and improved amino acid digestibility. ANFs are compounds naturally present in plant-based feed ingredients that can reduce nutrient availability and limit protein utilisation (Samtiya et al., 2020). Phytates bind minerals such as calcium, zinc, and iron, and can also form complexes with proteins, reducing digestibility (Gilani et al., 2012). Tannins are polyphenolic compounds that can bind to proteins and digestive enzymes, inhibiting protein hydrolysis and decreasing amino acid absorption. Protease inhibitors, such as trypsin inhibitors, directly interfere with digestive enzymes, reducing protein breakdown in the gastrointestinal tract (Kuenz et al., 2022).

Various processing techniques, including heat treatment, enzymatic treatment, and fermentation, have been shown to reduce these ANFs and improve protein digestibility (Samtiya et al., 2020). Heat treatment, in particular, can denature protease inhibitors and reduce tannin activity, enhancing nutrient availability (Ojo, 2018). However, excessive heating may lead to Maillard reactions, forming complexes that reduce the availability of essential amino acids, such as lysine, thereby potentially compromising protein quality (Almeida et al., 2013a).

It has also been shown that fibre-rich ingredients could impact digestion rates and protein digestibility in chickens (Manyeula et al., 2019). These findings highlight the need for precision in formulating chicken diets and carefully considering the nutritional profile, digestibility, and processing of feed ingredients.

While *in vivo* trials remain the gold standard for assessing protein digestibility in chickens, their use raises important ethical considerations. Researchers must adhere to the principles of the 3Rs, Replacement, Reduction, and Refinement, by considering alternatives such as *in vitro* digestion models, minimising the number of animals used through careful experimental design, and reducing stress or discomfort via appropriate husbandry, veterinary care, and non-invasive sampling methods (‘*The Principles of Humane Experimental Technique’,* 1959). Such experiments are also subject to strict regulatory approval, with institutional Animal Ethics Committees in the EU and UK requiring justification for the scientific need to use animals and ensuring that welfare standards are met.

Ethical justification for these trials is typically based on the importance of obtaining reliable data for feed formulation, which can optimise nutrient utilisation, support

animal health, and reduce environmental impact. By balancing scientific objectives with animal welfare, *in vivo* trials provide crucial insights into protein digestibility while maintaining ethical responsibility.

1.4.3. *In vitro* digestion models

In vitro digestion models have emerged as useful tools for studying the nutritional quality of feed ingredients, including protein digestibility, in a controlled laboratory-based environment. These models simulate gastrointestinal processes, offering a practical and cost-effective alternative to *in vivo* methods, particularly when preliminary evaluations or ethical considerations limit animal testing. Despite their limitations in fully replicating the complexities of the animal digestive system, ongoing advancements in *in vitro* methodologies have significantly enhanced their relevance and accuracy in animal nutrition research.

The development of *in vitro* digestion models has been intertwined with the understanding of gastrointestinal physiology and biochemistry. Early models focussed primarily on simulating simple enzymatic hydrolysis processes, often neglecting the dynamic interactions between feed ingredients and the digestive environment (Gehrt, 1972; Johnston & Coon, 1979).

Over time, *in vitro* models have evolved to incorporate more sophisticated features, such as sequential digestion phases, pH changes, and the inclusion of bile salts, which better mimic the digestive processes in non-ruminant animals like chickens (Palić et al., 2012; Zaefarian et al., 2021).

The static *in vitro* model from the COST INFOGEST network is a widely adopted standardised protocol and a milestone for *in vitro* digestion research (Minekus et al.,

2014). Originally developed for human nutrition studies, this method has been adapted for animal studies, providing a consistent framework for assessing protein digestibility.

The flow diagram of the INFOGEST 2.0 digestion method, by Brodkorb et al (2019) is shown in Figure 1.3.

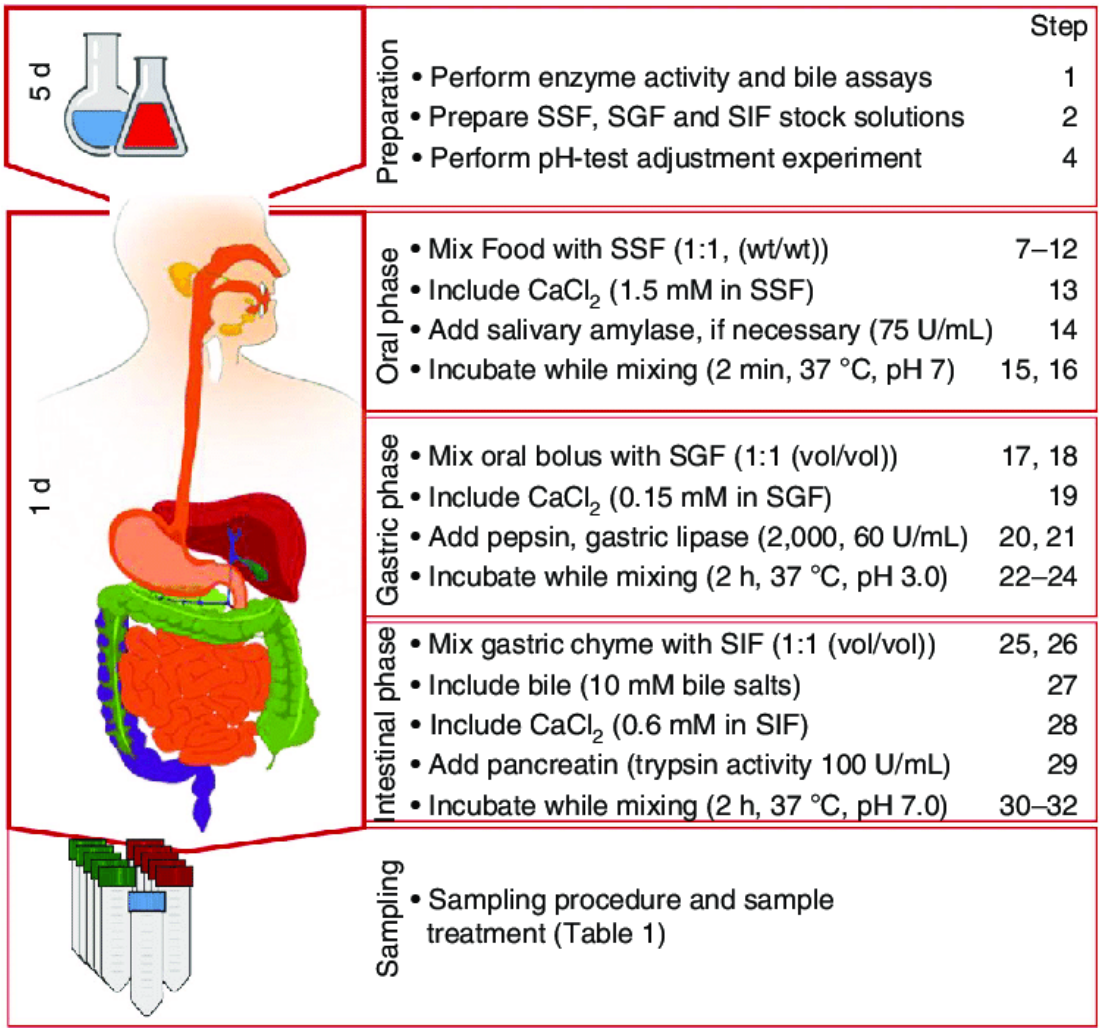


Figure 1.3: Flow diagram of the INFOGEST 2.0 digestion method (from Brodkorb et al., 2019). The expected time frame (left) and stages and corresponding step numbers in the procedure (right) are given. SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid.

The INFOGEST digestion method incorporates oral, gastric, and intestinal phases of digestion, using specific enzymes, buffers, and bile salts to simulate the sequential breakdown of macromolecules.

In vitro models offer several advantages, including cost and time efficiency, controlled environment and ethical considerations, and align with the principles of the 3Rs in animal research.

However, *in vitro* models can sometimes fail to replicate the complexity of the animal's digestive system, including factors like gut motility, microbial interactions, and nutrient absorption mechanisms (Egger et al., 2017). The variations in animal studies between adapted protocols using the INFOGEST protocol can lead to inconsistent results across studies, depending on parameters such as enzyme concentrations or digestion times (Mulet-Cabero et al., 2020).

Most *in vitro* models do not replicate the microbial fermentation processes occurring in the ceca of chickens, potentially underestimating the digestibility of certain feed ingredients. To address these limitations, advanced *in vitro* models have been developed such as dynamic digestion systems named TIM-1 and TIM-2 (TNO Intestinal Models). These systems replicate the peristaltic movements, vary the pH and gradually release digestive enzymes in the gastrointestinal phase (Singh, 2024).

Microbial fermentation simulations and high-throughput systems are also being explored to capture the contributions of gut microbiota to nutrient digestion and to simultaneously evaluate numerous feed ingredients (Asare et al., 2021; Card et al., 2017).

Other approaches involving combined i*n vitro* and *in silico* methods or computational models offer promising results for predicting the digestibility and nutritional value of feed ingredients (Gerrits et al., 2021). Despite being much more realistic to biological digestive systems, these advanced systems require specialised equipment and expertise. Establishing universally accepted guidelines for *in vitro* digestion studies with rigorous validation through *in vivo* trials will improve comparability and reproducibility across research groups and ensure the accuracy and applicability of these models in real-world poultry production.

1.5.Summary

Due to the increasing global demand for poultry meat driven by population growth, urbanisation, rising affluence, inflation and the necessity to reduce the land and environmental impact of agricultural production systems, there is an urgent need to improve poultry feed efficiency, particularly protein efficiency. One promising lead is the use of alternative protein sources with a high nutritional value and digestibility. Currently, methods used to evaluate the digestibility and amino acid content in animal feed rely on *in vivo* trials, *in vitro* digestion models adapted for animal studies or a combination of them.

However, variations in laboratory protocols highlight the need for a standardised *in vitro* digestion method for animal feed. Table 1.5 summarises the key variations in *in vitro* protein digestibility protocols commonly reported in poultry feed studies. Differences in enzyme type and concentration, pH conditions, incubation time, temperature, sample pre-treatment, and measurement methods can all significantly influence digestibility outcomes.

Table 1.5: Key variations in *in vitro* protein digestibility protocols for poultry feed studies

Parameter	Common variations in protocols	Potential impact on digestibility results	References
Enzyme source	Pepsin, trypsin, pancreatin, microbial enzymes	Different enzyme activities can over- or underestimate protein hydrolysis	Ravindran & Bryden, 1999; Sklan & Hurwitz, 1980
Enzyme concentration	Low, medium, high (U/mL or mg/mL)	Higher concentration may increase hydrolysis artificially, low may underestimate digestibility	Kaewtapee et al., 2023
pH conditions	Fixed pH, pH shift to mimic stomach and intestine	Digestibility can vary with pH; some proteins denature or aggregate	X. Wang & Parsons, 1998
Incubation time	1–24 hours, single or sequential digestion	Short times may not allow complete hydrolysis, long times may overestimate digestibility	Manyeula et al., 2019
Temperature	37–42 °C, constant or varying	Enzyme activity is temperature-dependent, chicken body temperature is 40 to 42 °C	Pan & Yu, 2014
Sample pre-treatment	Grinding, cooking, extrusion, autoclaving	Processing can affect protein structure and enzyme accessibility	Abdollahi et al., 2022
Measurement method	Nitrogen release, amino acid quantification, OPA assay	Different endpoints yield varying digestibility values	Toghyani et al., 2020

Highlighting these protocol variations underscores the need for a standardised method, such as the INFOGEST model, to reliably evaluate and compare the protein quality of both conventional and alternative feed ingredients.

What is unique about this work is the use of a standardised INFOGEST *in vitro* digestion model to evaluate a diverse set of protein sources, including traditional plant-based feeds, alternative protein sources and novel proteins with varying compositions, and the direct comparison of these laboratory digestibility results with *in vivo* data from a broiler chicken trial, providing a comprehensive assessment of protein quality and nutritional value for practical poultry feed applications.

This thesis aims to use the INFOGEST model to determine the protein quality of a range of traditional and alternative protein sources in chicken feed, compare the digestibility values from the *in vitro* model with the digestibility data from a chicken trial and evaluate the nutritional value and digestibility of bacterial protein sources using the INFOGEST model.

1.6. Work conducted in this thesis

The exploration of sustainable and high-quality protein sources for chicken feed is an ongoing challenge, which requires a deep understanding of protein digestibility and innovative approaches in both measurement techniques and the development of novel protein sources. With the use of alternative protein sources gaining attention, it is important to assess the protein quality of a wide range of protein sources in chicken feed.

One of the challenges is to find well-established techniques that would be suitable to determine protein digestibility in animal feed. This would allow the determination of

protein quality from a vast range of various sources. Using the INFOGEST digestion method, the three experimental chapters presented in this thesis will investigate (1) the protein quality of a range of protein feed ingredients, (2) the protein quality of a range of bacteria and (3) the comparison between *in vitro* digestibility data and *in vivo* digestibility values from a chicken trial.

Thesis aims

- To determine the protein quality of traditional protein sources for animal feed with the INFOGEST in vitro digestion method
- To determine the protein quality of bacterial protein sources with the INFOGEST in vitro digestion method
- To determine the protein quality of formulated feeds containing two conventional and one alternative protein sources and to compare *in vitro* digestibility data from the INFOGEST in vitro digestion method to *in vivo* digestibility values from a chicken trial

Thesis hypothesis

Using the INFOGEST *in vitro* digestion method, traditional, alternative, and bacterial protein sources will differ in protein digestibility and amino acid availability, and *in vitro* digestibility values will positively correlate with digestibility measured in a chicken trial.

2. Determining *in vitro* protein digestibility of a range of protein feed ingredients

2.1. Introduction

The indispensable amino acid composition and digestibility of a feed ingredient determines its protein quality. Among the twenty amino acids necessary for poultry health and growth, nine cannot be synthesised by chickens, or not in sufficient quantity to meet chicken requirements and must be obtained through the diet (Bhagavan, 2002). Arginine is sometimes considered as another indispensable amino acid (Fathima et al., 2023). These indispensable amino acids are thus targeted in feed formulation.

Methionine, lysine and threonine concentrations tend to be lacking in animal feed ingredients, especially plant-based ones (Fernandez et al., 1994b; Ojano-Dirain & Waldroup, 2001). These indispensable amino acids must be provided in the diet for metabolic purposes and proper growth. As the broiler sector is expected to contribute to the growing demand for animal meat, the quality of their protein is fundamental. These sources of protein need to offer an adequate amino acid profile and to be sustainable to respond to climate change and social obstacles.

The main sources of protein currently used in poultry diets involve a combination of plant-based and animal-based protein sources, with soybean meal being the most common protein source. Novel feed sources, such as insects, algae, fungi and bacteria, are being considered for feed formulation and for undergoing digestibility assays.

Bacteria are increasingly being explored as alternative ingredients for animal feed due to their high nutritional value and amino acid profiles. Among these, *Clostridium* species have emerged as a promising source of lysine, one of the main limiting amino acids in feed. While not all *Clostridium* species are safe, non-toxigenic strains can be used to produce lysine-rich protein ingredients, providing a potential route for improving feed formulations.

Due to the cost and time-consumption of *in vivo* digestibility assays, *in vitro* assays are prioritised to screen a large range of samples as a first step (Bryan & Classen, 2020a). Beyond practical considerations, the ethical implications of animal experimentation and adherence to the 3Rs principle further justify the preference for *in vitro* methods. This approach allows the selection of the most suitable candidates within novel sources of protein and to target them for feed formulation.

Utilisability is a key measure of protein quality, reflecting how efficiently protein is absorbed, accounting for both digestibility and amino acid composition. Various methods assess protein quality, with the Protein Digestibility Corrected Amino Acid Score (PDCAAS) being widely used. This score was adopted by the FAO/WHO in 1991 as the preferred method for assessing protein value (FAO, 1991).

The PDCAAS is calculated by dividing the amount of the limiting amino acid in 1 g of test protein by the amount of that amino acid in 1 g of reference protein and then multiplying the result by the true faecal digestibility percentage. However, this score has several limitations mainly due to the physiological differences between the small and large intestines.

The microbial fermentation occurring in the colon can alter the nitrogen content of undigested proteins, potentially overestimating digestibility (Hendriks et al., 2012). True faecal nitrogen digestibility does not account for indispensable amino acids that are not absorbed in the ileum and are lost in the colon.

In contrast, ileal digestibility is measured at the end of the small intestine and provides a more accurate assessment of protein and amino acid absorption. This resulted in the Digestible Indispensable Amino Acid Score (DIAAS) gradually replacing the PCDAAS (Mathai et al., 2017; Moughan & Lim, 2024). The DIAAS is based on the nutrient requirement of a 6-month to 3-year-old human child with human milk and enables ranking and complementation of protein sources.

Several *in vitro* methods are being used to estimate protein digestibility, but no consensus method has been achieved yet for chicken digestion. As *in vitro* digestive chicken models tend to vary, the model used here was the human INFOGEST consensus method (Minekus et al., 2014) using revisions by Brodkorb et al., 2019, Sousa et al., 2023 and Muleya et al., 2023. This allowed comparison with existing data and better robustness, as well as a first estimation of protein quality.

This study described in this chapter evaluates the human INFOGEST digestion methodology for comparative ranking between various protein isolates.

Aims

- To evaluate the protein quality of protein isolates for animal feed using the INFOGEST method
- To assess digestibility reproducibility and consistency of the INFOGEST in vitro digestion method with casein as a reference protein

Hypothesis

The methodology allows for comparative ranking between various protein isolates for animal feed. The human INFOGEST digestion model accurately reflects amino acid content and protein digestibility and enables comparative evaluation of feed ingredients against a reference protein.

2.2.Materials and methods

All chemicals and reagents were purchased from Merck (Dorset, UK) unless stated otherwise. A range of novel feed ingredients was provided by AB Agri (UK) in November 2022 and March 2023. These protein sources included commercial soybean and rapeseed products, yeast samples, microbial proteins, and casein as a reference.

2.2.1. Materials

The protein sources are listed in Table 2.1. AlphaSoy A3, AlphaSoy Gold A2 and Calysta were commercially available at the date they were obtained. All yeast samples were *Saccharomyces cerevisae* strains. All samples were minimally processed and obtained in powder form.

Casein was used as a protein reference for digestibility as it is known to be highly digestible. It should be noted that it was not a certified reference material. The samples labelled with “bench” were from small batches produced at a laboratory level at AB Agri. The “pilot” samples were from larger batches, produced at a semi-industrial scale.

Table 2.1: List of samples and description

Sample code	Description	Feed material	Batch number[1]
LIV-02-SFM-B (Bench)	Sunflower meal, co-product of oil extraction	Yes	1
LIV-03-SF-PRO-B (Bench)	Sunflower protein concentrate, purified product from sunflower meal	Yes	1
LIV-01-SPC-LT	Microbial protein (*Clostridium sp.*) by-product from ethanol production	No	1
LIV-10-IDY-LC	Dry bioethanol yeast from sugarcane-based bio-ethanol production	Yes	2
LIV-09-HY 40	Hydrolysed bioethanol yeast from sugarcane-based bio-ethanol production	Yes	2
LIV-15-PA-RD-Y	Dried pot ale yeast, by-product of whiskey production	Yes	2
LIV-11-BY-RD	Brewer's yeast 1, by-product of the brewing process, roller-dried	Yes	2
LIV-12-BY-SD	Brewer's yeast 2, by-product of the brewing process, spray dried	Yes	2
SBM	Soybean meal (SBM), co-product of soya oil extraction	Yes	3
A3	AlphaSoy A3, enzymatic thermo-mechanical treatment of soybean meal	Yes	3
A2	AlphaSoy Gold A2, co-processing method combining soybean meal with selected yeast fractions and the use of enzymatic thermo-mechanical treatment	Yes	3
LIV-04-RM-B (Bench)	Rapeseed meal, co-product of oil extraction	Yes	3
LIV-05-RM-PRO-B (Bench)	Rapeseed protein concentrate, purified product from rapeseed meal	Yes	3
LIV-03-SF-PRO-PT (Pilot)	Sunflower protein concentrate, purified product from sunflower meal	Yes	3
LIV-02-SFM-PT (Pilot)	Sunflower meal, co-product of oil extraction	Yes	3
LIV-08-PEA-PRO	Pea protein concentrate made from yellow peas	Yes	3
Casein	Pure, high-quality protein from bovine milk (no 10545691, Thermo Scientific, MA, US)	No	2, 3

[1]The samples were processed in 3 batches: batch 1 (3 samples digested across 3 days), batch 2 (5 samples digested across a separate set of 4 days) and batch 3 (8 samples digested across 4 days)

2.2.2. Crude protein content

Prior to this analysis, 1 ± 0.1 g of each sample was weighed in a 1.5 mL microcentrifuge tube. The tubes were placed in a freeze-dryer (Christ, Germany) and

left for 48 hours (< 0.4 mbar, -55°C) until they reached a constant weight. The crude protein content was determined using an elemental analyser (EA 1112, Thermo Scientific, MA, USA), following the Dumas method. Total nitrogen content (%) was measured for each sample. Based on the assumption that protein contains 16% nitrogen by weight (w/w), a nitrogen-to-protein conversion factor of 6.25 was applied to calculate the crude protein content (%).

The elemental analyser operates with a 64-combustion reactor, which combusts samples at 1000°C. The resulting products, including CO_2, H_2O, N_2 and NO_x, are carried through a series of reagents by helium gas. Copper is used to reduce NO_x to N_2, and the gas stream is passed through traps for H_2O and CO_2 before entering a gas chromatography (GC) column. Finally, N_2 is detected using a Thermal Conductivity Detector. Aspartic acid 10.52% (w/w, D5055, Elemental Microanalysis, Devon) was used as the bypass, standard, and quality control material.

Both the standards and the samples were prepared by weighing 50 mg into tin capsules (Elemental Microanalysis, Devon), which were then sealed and compressed into discs using a sealing device and cylindrical pressing tool. The prepared capsules were stored in a desiccator until analysis. All samples were prepared on a dry-weight basis. At the beginning of each sample batch, a 50 mg bypass sample of aspartic acid was analysed first.

This was followed by two empty tin capsules, serving as negative controls and recorded as blanks. Subsequently, two aspartic acid samples were analysed as nitrogen standards, followed by two as quality control checks. The remaining samples were loaded into the auto-sampler, and a chromatogram was generated for each,

highlighting the nitrogen peak. To maintain quality control, two aspartic acid samples were analysed after every 10 samples, and the results were verified. All measurements were conducted in duplicate for each sample. Triplicates were not deemed necessary, as preliminary analyses showed very low variability between duplicates, indicating high precision and reproducibility of the Dumas method.

AB Agri provided the crude protein values for their samples. However, the detailed analytical procedure was not disclosed.

2.2.3. *In vitro* digestion method

The *In vitro* digestion method was performed on each protein isolate, based on the INFOGEST static *in vitro* simulation of gastrointestinal food digestion from Brodkorb et al. (2019) and following an optimised analytical workflow recently described by Sousa et al. (2022) and Muleya et al. (2023), with some minor adjustments. For LIV-02-SFM-B, LIV-03-SF-PRO-B and LIV-01-SPC-LT, the *in vitro* digestions were completed on three different days with three replicates per day (nine biological replicates). This is referred to as batch one.

For the samples LIV-10-IDY-LC, LIV-09-HY 40, LIV-15-PA-RD-Y, LIV-11-BY-RD and LIV-12-BY-SD, four biological replicates were generated through four *in vitro* digestions on different days. This is referred to as batch two.

For SBM, A3, A2, LIV-04-RM-B, LIV-05-RM-PRO-B, LIV-03-SF PRO-PT, LIV-02-SFM-PT, LIV-08-PEA-PRO, four biological replicates were generated through four *in vitro* digestions on different days. This is referred to as batch three. Figure 2.1 illustrates the *in vitro* digestion model, showing the sequential oral, gastric, and intestinal phases used to simulate human digestion.

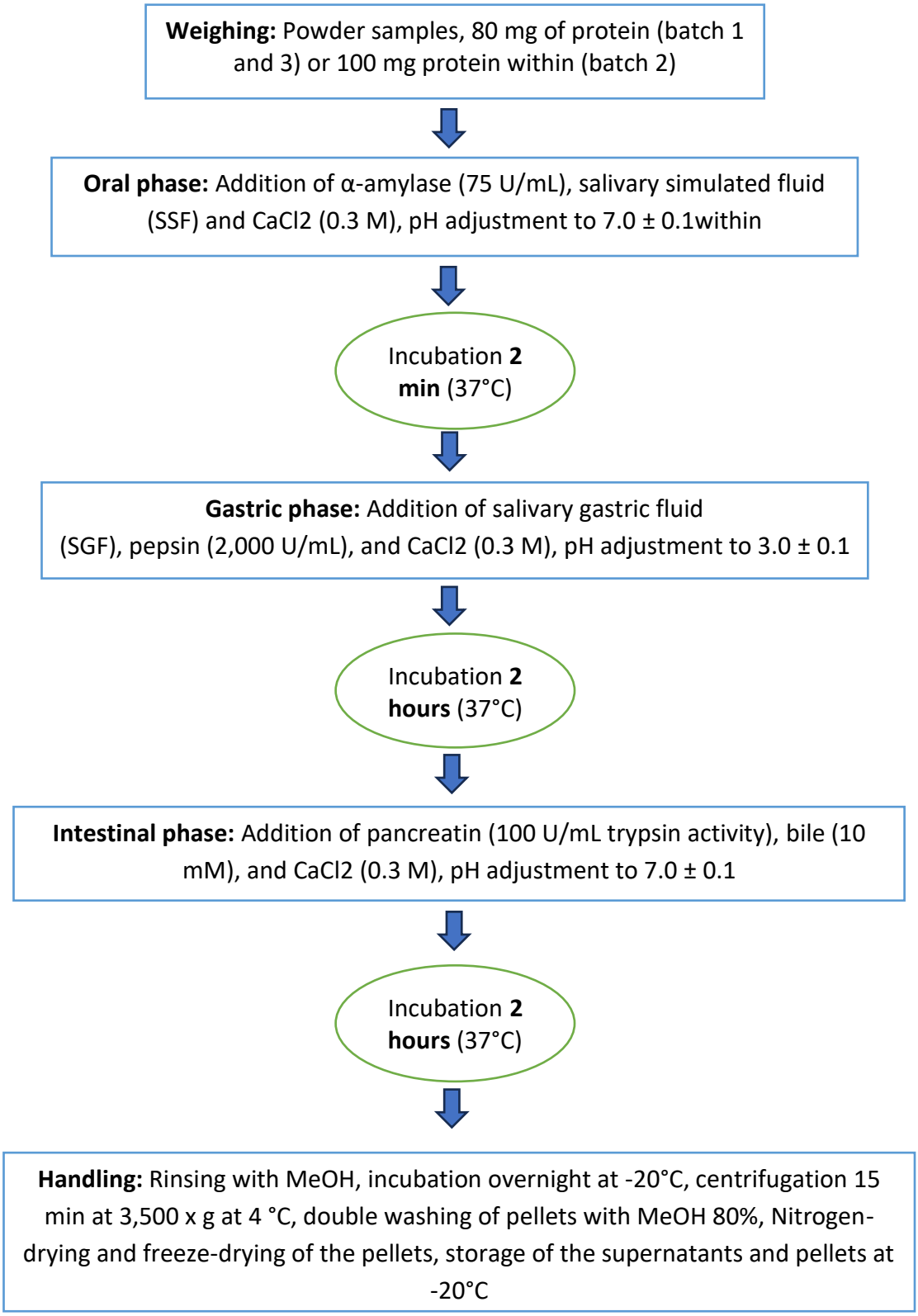


Figure 2.1: Flow diagram of the INFOGEST 2.0 *in vitro* digestion method (adapted from Brodkorb et al., 2019).

A blank digestion was also included in each digestion to take into account the endogenous free amino acids in the reagents. Casein was digested in batches two and three to monitor the success of the digestion.

Each protein isolate was weighed with 10% protein or 100 mg of protein per digestion sample for batches one and three, and 8% protein or 80 mg of protein per digestion sample for batch two. Both protein concentrations (8% and 10%) fall within the plateau region of the digestibility curve.

Higher protein concentrations may lead to reduced enzyme accessibility to protein substrates and possible protein aggregation during digestion (Berrazaga et al., 2020; Duque-Estrada et al., 2023; Joye, 2019). Good recovery of protein digestibility and high accuracy was found when using casein with a total protein of 8% (unpublished data, Dr Molly Muleya, 2022), becoming the standard protein weight in this study.

The sample weight was adjusted to 1 g by adding MilliQ water (18.2 MΩ cm). During the oral phase, 1 mL of simulated salivary fluid (pH 7.0) was mixed with the 1 g sample containing protein, containing 1.5 mM $CaCl_2$ (final concentration) and α-amylase from *Bacillus sp*. (Sigma-Aldrich, cat. no. A1031) (75 U/mL in the final mixture). The mixture was incubated in a shaking water bath at 37 °C for 2 minutes.

Next, the gastric phase involved adding 2 mL of simulated gastric fluid, containing 0.15 mM $CaCl_2$ (final concentration) and pepsin from porcine gastric mucosa (Sigma-Aldrich, cat. no. P7012) (2000 U/mL in the final mixture). The pH was adjusted to pH 3 ± 0.10 using 2 M HCl. The titration was completed using a pH electrode using a pH electrode (pH Sensor InLab® Expert Pro, Mettler Toledo, cat. no. 51343101). The

mixture was incubated at 37 °C in an end-over-end rotator for 2 hours. The pepsin activity was halted by raising the pH to 7 ± 0.10 with 2 M NaOH.

The intestinal digestion phase began with the addition of 4 mL of simulated intestinal fluid. This fluid included pancreatin from the porcine pancreas (Sigma-Aldrich, cat. no. P754) (100 U/mL trypsin activity in the final mixture), bovine bile (Sigma-Aldrich, cat. no. B3883) (10 mM in the final mixture), and 0.6 mM $CaCl_2$ (final concentration). The mixture was incubated again for 2 hours at 37 °C in an end-over-end rotator.

Upon completing the intestinal phase, the samples were placed on ice. Subsequently, 32 mL of ice-cold absolute methanol was added to achieve a final concentration of 80% (v/v) methanol, followed by incubation at −20 °C overnight. To separate the absorbable fraction (free amino acids and peptides) from the unabsorbable fraction (undigested proteins and large peptides), the samples were centrifuged at 3,500×g for 15 minutes at 4 °C. The supernatants (absorbable fraction (free amino acids and peptides)) were carefully transferred to fresh tubes and stored at −20 °C for further analysis.

The remaining pellet (unabsorbable fraction (undigested proteins and large peptides)) was washed twice with 5 mL of 80% (v/v) methanol, followed by centrifugation (3,500×g, 10 minutes, 4 °C) after each wash. The wash solutions were discarded after each centrifugation. The pellets were dried using nitrogen and freeze-dried to a constant weight, before being stored at −20 °C until further analysis. A mass balance approach was used throughout to track the weights of the supernatants and pellets, using equations 2.1 and 2.2 for calculations.

Equation 2.1: $$Weight\ of\ supernatant\ (g) = W2 - W3$$

Equation 2.2: $$Weight\ of\ pellet\ (g) = W3 - W1$$

W1 is the weight of the empty tube before *in vitro* digestion (g), W2 is the weight of the digesta with methanol (g) and W3 is the weight of the dried pellet after freeze drying (g).

2.2.4. OPA method

For batches one and three, the degree of hydrolysis (DH) was estimated on the absorbable fraction (supernatants from the digestion) using the o-phthaldialdehyde (OPA) assay, which gives the proportion of cleaved peptide bonds in a protein hydrolysate. The method relies on the determination of the free primary amine concentration (Adler-Nissen, 1979; Nielsen et al., 2001). Table 2.2 shows the solutions needed to prepare the OPA reagent.

Table 2.2: List of solutions prepared for OPA reagent

Solution	Concentration	Volume for OPA reagent (mL)
Di-sodiumtetraborate-decahydrate solution (Borate-solution)	0.1 mol/L	12.5
Sodium dodecylsulfate solution (SDS)	10% (w/w)	2.5
O-phthaldialdehyde (OPA) solution	40 g/L in Ethanol	0.5
Mercapto-ethansulfonic acid solution (Na-MES-solution)	200 g/L	0.5
Triton X-100 solution	100 g/L	1.25
MilliQ water	18.2 MΩ cm	25

Each solution was prepared in water unless specified otherwise. L-glutamic acid stock solution (100 mmol/L) was used as the primary amine to prepare the standard curve, diluted in perchloric acid (0.5 mol/L).

The serial dilution was performed as reported in Table 2.3.

Table 2.3: Standard curve for OPA assay

No of Standard	Concentration of standard (mmol/L)	Volume and standard No for serial dilution		Volume of perchloric acid 0.5 mol/L (μL)
		Standard No	Volume (μL)	
1	8	Stock	80	920
2	4	1	500	500
3	2	2	500	500
4	1	3	500	500
5	0.5	4	500	500
6	0.25	5	500	500
7	0	Blank	0	1000

232 μL of the freshly prepared OPA working solution was mixed with 8 μL of the sample, blank (perchloric acid 0.5 mol/L) or standards in a microplate (96-well, Sarstedt, Germany), mixed and incubated for 10 minutes at 30 °C in the dark. The absorption was measured at a wavelength of 335 nm on a spectrophotometer (LKB Ultrospec III, Pharmacia, Sweden) at room temperature, which was zeroed on the calibration blank setting.

Based on the linear equation from the standard curve, the L-glutamic acid concentration (hence the primary amine concentration) was calculated for each sample, correcting for the blank values from the standard curve and accounting for the dilution factor and volume of the absorbable fraction.

After correcting for values from the blanks for each digestion, the primary amine concentration per g of protein (NH2 mM/g protein) was calculated using the

proportion of protein and absorbable fraction volume. This is also known as h, or the number of hydrolysed peptide bonds after digestion.

To determine the degree of hydrolysis (DH), h_{tot} was also calculated. H_{tot} represents the total number of peptide bonds present before digestion. This is achieved by multiplying the amino acid content sum (in g/mol) in the sample before digestion by 1000 to obtain the amino acid content sum in mM/g protein. The h_{tot} value of 8 mM/g protein is an acceptable range for casein in hydrolysis studies.

The degree of hydrolysis was then determined following this equation:

Equation 2.3: $$DH\% = \frac{\mathrm{h}}{\mathrm{h}_{tot}} \times 100$$

2.2.5. Amino acid analysis

To evaluate protein quality, the amino acid content of the protein isolates and their corresponding digestibility were measured. This required an acid hydrolysis procedure to break proteins down into free amino acids, which was applied to both the protein isolates and the *in vitro* digesta fractions (absorbable and non-absorbable fractions).

To ensure the accuracy of the hydrolysis and amino acid analysis, a standard reference material (soy flour, SRM 3234, National Institute of Standards and Technology, Maryland, USA) was analysed alongside the samples. The method was based on published methods (Desmons et al., 2020; Muleya et al., 2023).

1. Amino acid oxidation

For the protein isolates and non-absorbable fractions, 50 mg materials were weighed into hydrolysis glass tubes. In the case of the absorbable fractions, 500 µL was

measured into 2 mL micro-centrifuge tubes and dried for 4 hours using a vacuum concentrator (Savant Thermo Scientific SpeedVac SPD140DDA).

Performic acid (10% hydrogen peroxide (v/v) in formic acid) was prepared and incubated at 27 °C for 1 hour. Once cooled down, the solution was added to the samples (2.5 mL for the protein isolates or non-absorbable fractions, and 2 mL for the absorbable fractions). The tubes were left open and incubated at 4 °C for 16–18 hours.

After incubation, 0.42 g of sodium metabisulfite ($Na_2S_2O_5$) was added to neutralise excess performic acid. This oxidation step stabilises sulphur-containing amino acids by converting methionine into methionine sulfone and cysteine into cysteic acid. Cystine and cysteine were both determined as cysteic acid in hydrolysates of oxidised samples but calculated as cystine by using the molecular weight of 240.3. Methionine was determined as methionine sulfone in the oxidised sample but calculated as methionine by using molecular weight of 149.21.

2. Amino acid hydrolysis

A 6 M HCl solution containing 1% phenol (w/v) (to prevent amino acid oxidation) was added to each sample (0.5 mL), followed by 12 M HCl (2.5 mL for the protein isolates and non-absorbable fractions, and 2 mL for the absorbable fractions) to achieve a final concentration of 6 M HCl.

The tubes were tightly capped and hydrolysed in an oven at 110 °C for 24 hours. After cooling, the hydrolysates were transferred to new tubes with 20 mM ammonium formate (pH 2.75) used as a rinse solution. The pH was adjusted to 2.75 using 4 M

ammonium formate, and the volume was made up to 50 mL with 20 mM ammonium formate.

The samples were centrifuged at 3,000×g for 10 minutes at room temperature, and the resulting supernatants were filtered through a 0.22 µm syringe filter. The filtered supernatants were stored at −20 °C until amino acid analysis.

The acid hydrolysis process deamidates asparagine into aspartic acid and glutamine into glutamic acid, meaning that the measured levels of aspartic and glutamic acids include contributions from their respective amide forms. Additionally, tryptophan is destroyed during acid hydrolysis and was not determined. This applies to all samples in this thesis.

3. LC-MSMS analysis

A 200 µL aliquot of each filtered supernatant was transferred into HPLC vials, followed by the addition of 200 µL of an internal standard solution containing isotopically labelled (13C, 15N) cell-free target amino acids. To generate an amino acid standard curve, the Supelco amino acid standard mix was supplemented with l-cysteic acid and methionine sulfone at similar concentrations.

Target amino acids in both the samples and standards were analysed using a Thermo Scientific™ Acclaim™ Trinity P1 mixed-mode column (150 mm × 2.1 mm, 3 µM) at 30 °C on a Thermo-Fisher Vanquish (uHPLC) system, coupled to an Altis Triple Quadrupole Mass Spectrometer (MS/MS) equipped with a heated electrospray ionization source.

Detection of amino acids was performed in positive electrospray ionization mode using multiple reaction monitoring. Chromatographic separation was achieved using

a programmed gradient elution method (outlined in Table 2.4), with an injection volume of 1 µL for each sample or standard.

Table 2.4: PLC gradient elution programme followed

Time (min)	Flow rate (ml/min)	Mobile phase A (%)[1]	Mobile Phase B (%)[2]
0	0.3	100	0
5	0.3	100	0
7	0.3	0	100
14	0.3	0	100
14.5	0.35	100	0
16.5	0.35	100	0
17	0.3	100	0
18	0.3	100	0

[1]Mobile phase A: 20 mM ammonium formate in water (pH 2.75)

[2]Mobile phase B: 100 mM ammonium formate in water and acetonitrile (80: 20, v/v)

The source conditions were as follows: spray voltage 3,500 V, spray current 63.4 µA, ion transfer tube temperature 325 °C, vaporiser temperature 370 °C, sheath gas 5.58 L/min, auxiliary gas 7.97 L/min, ion transfer tube DC 15 V, RF Lens Amplitude 47 V. Nitrogen gas was produced using a nitrogen generator (Genius NM32LA, Peak Scientific Instruments Ltd). For each amino acid transition of interest, the collision energies were optimised, and one transition was used as the quantifier.

Quantification for all targeted amino acids was achieved using the concentration vs peak area ratio (the integrated peak area of the analyte relative to that of the internal standard). Data acquisition was performed using Thermo Xcalibur™ mass spectrometry data system and data was processed using Thermo Tracefinder™ 4.1 application for batches one and three, and Chromeleon™ 7 for batch 2.

Due to the loss of asparagine, glutamine and tryptophan in the acid hydrolysis process, 17 amino acids could be analysed, including both indispensable amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, and valine and dispensable amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, proline and serine.

Recovery of amino acids was calculated using the soy SRM recovery (%) relative to its batch. All the data was normalised by the expected recovery of the SRM and gross amino acid compositions of the substrates were expressed as g/100g of dry weight.

Amino acid composition data from AB Agri were made available for comparison, but the underlying analytical methods were not provided.

2.2.6. Calculation of amino acid digestibility and digestible indispensable amino acid score (DIAAS)

To calculate the amount of individual amino acid digestibility, the amount of individual amino acids (mg) in the digesta supernatants and pellets obtained from LC-MS/MS was calculated after accounting for dilution factors and the weights obtained in the mass balance (equations 2.1 and 2.2). The following equation was used to calculate amino acid digestibility:

Equation 2.4: $$Amino\ acid\ digestibility\ \% = \frac{Ps - Bs}{(Ps - Bs) + \max(0; Pp - Bp)} \times 100$$

where Ps is the protein isolate supernatant (mg), Bs is the blank supernatant (mg), Pp is the protein isolate pellet (mg) and Bp is the blank pellet (mg). The blank refers to the blank used to account for endogenous amino acids. If Bp > Pp, the value was truncated to 0. The total amino acid digestibility was also calculated by considering

the sum of all measured amino acids in both the digesta supernatants and pellets and applying equation 2.4 to calculate the digestibility. DIAAS was calculated in two steps according to equations 2.5 and 2.6:

Equation 2.5:

$$In\ vitro\ DIAA\ (mg/g\ protein)\ =\ IAA\ (mg/g\ protein)\ \times\ in\ vitro\ digestibility\ coefficient\ of\ IAA$$

where DIAA is the digestible indispensable amino acid, IAA is the mg of indispensable amino acid per g of protein. IAA is calculated based on the gross IAA and protein compositions of the substrates and the *in vitro* digestibility coefficient determined in equation 2.4.

Equation 2.6: $$In\ vitro\ DIAAR = \frac{In\ vitro\ DIAA\ (mg/gprotein)}{IAA\ of\ reference\ protein\ (mg/gprotein)}$$

where DIAAR is the digestible indispensable amino acid ratio and the reference protein refers to the scoring pattern of a 6-month to 3-year-old child with human milk, recommended by the Food and Agriculture Organisation of the United Nations (FAO, 2011). The indispensable amino acid values from the scoring pattern are shown in Table 2.5. Other scoring patterns for adults and infants less than 6 months old can also be used.

Table 2.5: Indispensable amino acid (IAA) composition of the reference protein (mg/g protein) in a 6-month to 3-year-old child with human milk

Indispensable Amino Acid (IAA)	Content (mg/g protein)
Histidine	20
Isoleucine	32
Leucine	66
Lysine	57
Sulphur-containing amino acids (SAA)	27
Aromatic amino acids (AAA)	31
Valine	43

Modified from FAO, 2011.

DIAAS is obtained by considering the minimum DIAAR, which is a value that can range from 0 to above 1, and specifying the amino acid with the lowest DIAAR indicating the limiting amino acid of the substrate. A low-quality protein has a DIAAS below 0.50, a moderate-quality protein has a DIAAS between 0.50 and 0.74, a good-protein quality has a DIAAS between 0.75 and 0.99. A DIAAS above 1.00 shows that the protein is of high quality.

2.2.7. Data analysis

Prior to statistical analysis, data were checked for normality and homogeneity of variance using GraphPad Prism, and the experimental design ensured independence of measurements. Data met the assumptions required for parametric testing.

All graphs were produced using Graph Pad Prism 10.3.1. Genstat (24th Edition) was used for statistical analyses. T-test, one-way, unbalanced design and two-way ANOVA were applied followed by, when appropriate, a Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$.

2.3. Results

The results are presented in three parts: crude protein content, amino acid composition, and amino acid digestibility with DIAAS values. Three batches of protein sources were studied: batch one with sunflower and bacterial proteins, batch two with yeast-derived samples, and batch three with plant protein isolates. Casein was tested and used a reference protein. Amino acid content was compared between experimentally determined data (Dumas method) and supplier values. Key outcomes, including *in vitro* digestibility, DIAAR/DIAAS, and degree of hydrolysis, are summarised in the corresponding figures and tables.

2.3.1. Crude protein content

In general, there was good agreement between the crude protein contents measured using the Dumas method on a dry matter basis, of which there were two technical replicates, and those provided by the company (Figure 2.2).

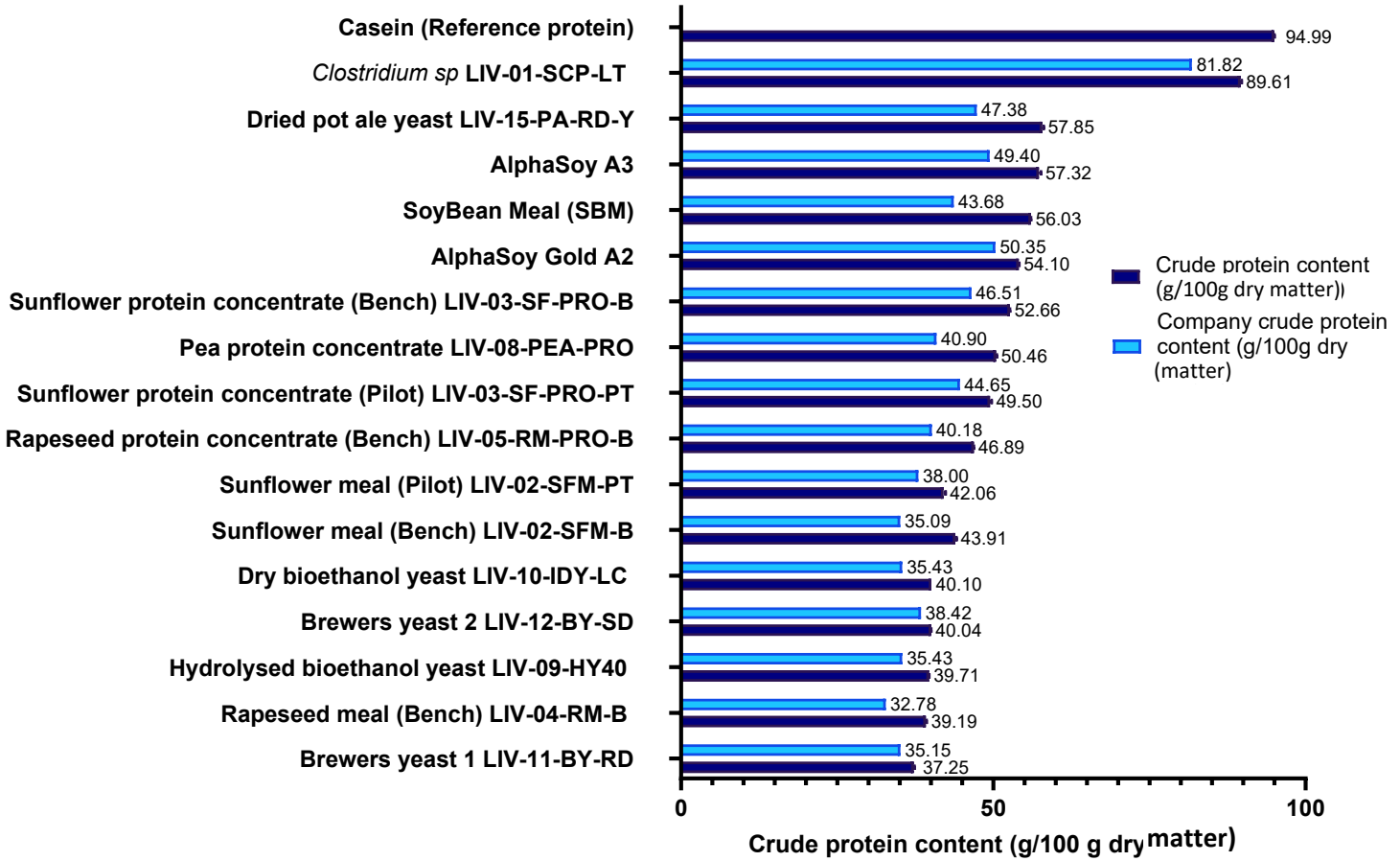


Figure 2.2: Crude protein content (g/100g dry matter) of samples using the Dumas method (N-analyser). Data is shown as mean with SEM, n = 2 technical replicates per protein isolate. The samples were analysed as they were when received, after a freeze-drying step.

The crude protein contents determined in the lab were higher than those provided by AB Agri and varied between 37.25 and 89.61 g/100g dry weight. Casein had a high crude protein content of 94.99 g/100g dry weight. A strong positive correlation was observed between the two sets of values (Figure 2.3), $R^2 = 0.9478$ ($p < 0.001$).

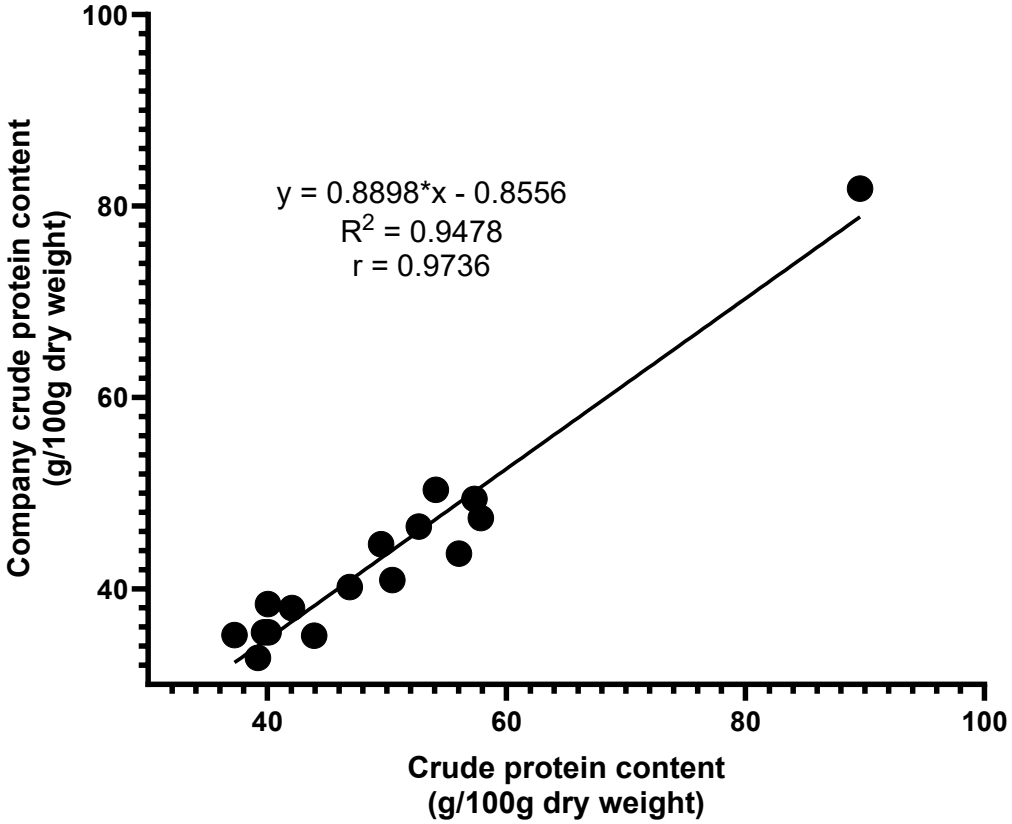


Figure 2.3: Crude protein content correlation between experimental determined data (Dumas method) or obtained data from the company. Each point represents a sample.

2.3.2. Amino acid content of protein isolates

Table 2.6 shows the amino acid content of protein isolates in batch one.

Table 2.6: Amino acid content (g/100g of protein) of protein isolates in batch one

	Amino acid content (g/100g of protein)[1]					
Amino acid	***Clostridium sp* LIV-01-SPC-LT**	**Company values**	**Sunflower meal (bench) LIV-02-SFM-B**	**Company values**	**Sunflower protein concentrate (Bench) LIV-03-SF-PRO-B**	**Company values**
Indispensable amino acids						
Lysine	10.16 ± 0.15	9.50	3.20 ± 0.36	3.82	3.92 ± 0.03	3.78
Methionine	2.28 ± 0.08	2.85	1.60 ± 0.14	2.45	2.06 ± 0.06	2.45
Threonine	3.20 ± 0.08	4.94	2.96 ± 0.20	3.93	3.15 ± 0.04	3.81
Valine	5.78 ± 0.03	6.42	4.37 ± 0.52	5.13	5.18 ± 0.09	4.90
Leucine	7.00 ± 0.20	7.93	5.00 ± 0.58	6.73	5.91 ± 0.30	6.51
Isoleucine	5.68 ± 0.24	6.84	3.22 ± 0.45	4.30	4.09 ± 0.05	4.24
Histidine	1.45 ± 0.00	1.65	2.08 ± 0.24	2.59	2.55 ± 0.02	2.62
Phenylalanine	3.89 ± 0.06	4.05	3.82 ± 0.42	4.70	4.75 ± 0.04	4.77
Dispensable amino acids						
Tyrosine	3.72 ± 0.15	2.81	2.13 ± 0.35	2.25	2.78 ± 0.11	2.36
Cystine	1.01 ± 0.05	1.33	1.09 ± 0.16	1.71	1.40 ± 0.05	1.46
Serine	3.79 ± 0.16	3.78	3.61 ± 0.48	4.59	4.51 ± 0.11	4.41
Glycine	3.37 ± 0.29	4.83	4.36 ± 0.43	6.16	4.58 ± 0.25	5.96
Glutamic acid	12.29 ± 0.08	11.70	18.72 ± 2.06	20.29	23.21 ± 0.03	20.96
Proline	3.30 ± 0.02	3.67	3.83 ± 0.45	4.50	4.76 ± 0.02	4.79
Alanine	6.58 ± 0.22	6.61	4.08 ± 0.41	4.59	5.04 ± 0.35	4.47
Arginine	3.84 ± 0.02	3.89	6.86 ± 0.81	8.49	8.87 ± 0.09	8.66
Aspartic acid	10.36 ± 0.15	11.32	7.75 ± 0.94	9.43	9.23 ± 0.21	9.37

[1] Data is shown as mean with SEM, n = 3 technical replicates per protein isolate

LIV-01-SPC-LT showed a very high lysine content, three times higher than LIV-02-SFM-B and LIV-03-SF-PRO-B. The amino acid content of protein isolates in batch two and batch three are shown in Table 2.7 and Table 2.8 respectively, alongside their company values when given.

Table 2.7: Amino acid content of protein isolates in batch two

	Amino acid content (g/100g of protein)[1]									
Amino acid	**Dry bioethanol yeast LIV-10-IDY-LC**	**Company values**	**Hydrolysed bioethanol yeast LIV-09-HY-40**	**Company values**	**Dried pot ale yeast LIV-15-PA-RD-Y**	**Company values**	**Brewer's yeast 1 LIV-11-BY-RD**	**Company values**	**Brewer's yeast 2 LIV-12-BY-SD Company values**	**Company values**
Dispensable amino acids										
Lysine	6.97 ± 0.26	7.85	5.93 ± 0.18	7.85	4.80 ± 0.36	5.98	6.34 ± 0.16	6.32	5.66 ± 0.84	6.04
Methionine	1.74 ± 0.21	1.73	1.57 ± 0.15	1.73	1.38 ± 0.12	1.54	1.60 ± 0.06	1.45	1.99 ± 0.41	1.41
Threonine	4.74 ± 0.36	5.49	3.87 ± 0.18	5.49	3.42 ± 0.30	4.26	4.15 ± 0.13	4.44	3.72 ± 0.52	4.27
Valine	5.70 ± 0.29	6.10	4.93 ± 0.10	6.10	3.58 ± 0.47	4.83	5.02 ± 0.08	5.15	4.71 ± 0.92	5.05
Leucine	7.95 ± 2.04	7.29	8.10 ± 0.10	7.29	5.14 ± 1.61	6.57	7.27 ± 1.44	6.34	5.98 ± 1.78	6.12
Isoleucine	7.37 ± 1.07	5.23	6.44 ± 1.37	5.23	4.20 ± 0.69	4.24	4.76 ± 0.25	4.24	4.66 ± 0.54	4.11
Histidine	2.96 ± 0.86	2.23	1.65 ± 0.29	2.23	2.00 ± 0.60	1.99	2.09 ± 0.03	2.16	2.14 ± 0.11	2.03
Phenylalanine	4.79 ± 0.64	4.35	3.38 ± 0.65	4.35	3.30 ± 0.26	3.94	4.47 ± 0.11	3.81	3.86 ± 0.42	3.64
Dispensable amino acids										
Tyrosine	5.17 ± 1.82	3.15	2.79 ± 0.18	3.15	4.00 ± 1.45	2.39	3.56 ± 0.35	2.59	4.40 ± 0.68	2.42
Cystine	0.59 ± 0.46	0.70	0.28 ± 0.16	0.70	0.98 ± 0.35	1.10	0.48 ± 0.29	0.80	0.71 ± 0.30	0.96
Serine	7.94 ± 0.94	5.75	6.79 ± 1.11	5.75	6.04 ± 0.97	4.44	5.55 ± 0.22	4.69	5.88 ± 0.52	4.50
Glycine	6.67 ± 2.35	4.52	3.55 ± 0.36	4.52	3.04 ± 0.15	3.85	4.24 ± 0.15	4.27	4.93 ± 0.60	4.19
Glutamic acid	9.86 ± 4.94	11.06	13.44 ± 0.84	11.06	9.66 ± 1.30	10.18	14.23 ± 0.44	11.72	11.62 ± 3.26	12.18
Proline	5.83 ± 1.29	3.59	3.56 ± 0.28	3.59	3.95 ± 0.38	3.47	4.84 ± 0.22	4.67	4.32 ± 0.26	4.32
Alanine	7.99 ± 0.19	6.69	5.01 ± 0.26	6.69	5.51 ± 1.21	5.15	7.50 ± 0.64	6.17	5.42 ± 0.55	6.01
Arginine	5.95 ± 1.54	4.17	5.91 ± 1.87	4.17	6.12 ± 2.81	4.1	6.67 ± 1.39	4.13	5.08 ± 0.26	4.71
Aspartic acid	11.29 ± 1.12	10.52	10.27 ± 1.28	10.52	8.25 ± 1.23	8.26	11.85 ± 0.97	8.82	9.44 ± 2.22	8.49

[1] Data is shown as mean with SEM, n = 3 technical replicates per protein isolate

Table 2.8: Amino acid content (g/100g protein) of protein isolates in batch three

	Amino acid content (g/100g of protein)[1]												
Amino acid	**Soybean meal (SBM)**	**AlphaSoy A3**	**Company values**	**AlphaSoy Gold A2**	**Company values**	**Rapeseed meal (bench) LIV-04-RM-B**	**Company values**	**Rapeseed protein concentrate (Bench) LIV-05-RM-PRO-B**	**Company values**	**Sunflower protein concentrate (Pilot) LIV-03-SF-PRO-PT**	**Sunflower meal (Pilot) LIV-02-SFM-PT**	**Pea protein concentrate LIV-08-PEA-PRO**	**Company values**
						Indispensable amino acids							
Lysine	5.31 ± 0.08	5.90 ± 0.20	6.32	5.91 ± 0.30	6.32	5.09 ± 0.13	5.43	5.20 ± 0.19	5.55	3.68 ± 0.20	3.62 ± 0.07	8.28 ± 0.42	8.44
Methionine	1.10 ± 0.02	1.12 ± 0.05	1.39	1.10 ± 0.09	1.39	1.62 ± 0.08	2.14	1.72 ± 0.08	2.19	1.97 ± 0.09	1.94 ± 0.07	0.98 ± 0.06	1.22
Threonine	2.96 ± 0.01	2.92 ± 0.12	4.15	2.95 ± 0.15	4.15	3.67 ± 0.07	4.67	3.39 ± 0.13	4.51	3.12 ± 0.03	3.29 ± 0.05	3.28 ± 0.05	4.16
Valine	4.29 ± 0.13	4.63 ± 0.29	5.24	4.34 ± 0.22	5.24	4.91 ± 0.18	5.43	4.89 ± 0.09	2.66	4.90 ± 0.27	4.88 ± 0.10	4.91 ± 0.21	4.96
Leucine	6.22 ± 0.28	7.10 ± 0.42	8.34	6.57 ± 0.68	8.34	5.97 ± 0.24	7.32	6.07 ± 0.33	7.49	5.88 ± 0.07	5.30 ± 0.28	6.57 ± 0.56	8.22
Isoleucine	3.78 ± 0.22	4.31 ± 0.30	5.00	3.87 ± 0.27	5.00	3.24 ± 0.16	4.21	3.18 ± 0.15	4.18	3.90 ± 0.26	3.60 ± 0.05	4.15 ± 0.19	4.65
Histidine	2.22 ± 0.08	2.34 ± 0.12	2.78	2.26 ± 0.19	2.78	2.38 ± 0.14	2.81	2.59 ± 0.11	2.91	2.43 ± 0.11	2.39 ± 0.06	2.57 ± 0.04	2.91
Phenylalanine	4.58 ± 0.06	4.96 ± 0.29	5.48	4.70 ± 0.24	5.48	3.69 ± 0.10	4.09	3.84 ± 0.13	4.21	4.54 ± 0.19	4.35 ± 0.11	5.22 ± 0.26	5.31
						Dispensable amino acids							
Tyrosine	3.06 ± 0.15	3.48 ± 0.31	4.00	3.07 ± 0.27	4.00	2.69 ± 0.21	2.53	2.75 ± 0.13	1.29	2.53 ± 0.23	2.61 ± 0.02	3.62 ± 0.18	2.27
Cystine	1.02 ± 0.01	1.13 ± 0.08	1.43	0.96 ± 0.06	1.43	1.85 ± 0.08	2.53	1.81 ± 0.09	2.44	1.30 ± 0.08	1.15 ± 0.03	1.03 ± 0.05	1.47
Serine	4.88 ± 0.15	4.76 ± 0.18	5.06	4.85 ± 0.54	5.06	4.20 ± 0.14	4.61	4.21 ± 0.30	4.46	4.29 ± 0.13	4.37 ± 0.13	5.19 ± 0.28	5.43
Glycine	3.46 ± 0.06	3.26 ± 0.31	4.23	3.91 ± 0.69	4.23	4.08 ± 0.17	5.40	5.14 ± 0.48	5.45	4.46 ± 0.41	4.58 ± 0.69	3.89 ± 0.18	4.77
Glutamic acid	19.20 ± 0.05	19.93 ± 0.92	18.74	18.94 ± 0.96	18.74	17.46 ± 0.83	17.78	18.61 ± 0.50	18.84	22.27 ± 1.00	21.46 ± 0.61	19.57 ± 0.73	18.58
Proline	4.97 ± 0.04	5.23 ± 0.28	5.09	4.96 ± 0.25	5.09	6.25 ± 0.30	6.68	6.15 ± 0.20	7.04	4.51 ± 0.22	4.38 ± 0.10	4.63 ± 0.17	4.79
Alanine	3.97 ± 0.19	4.28 ± 0.14	4.36	4.26 ± 0.12	4.36	4.52 ± 0.11	4.67	4.58 ± 0.18	4.78	4.49 ± 0.02	4.66 ± 0.07	4.87 ± 0.16	4.74
Arginine	6.45 ± 0.10	6.96 ± 0.42	7.55	6.76 ± 0.39	7.55	5.32 ± 0.23	5.95	5.70 ± 0.15	6.35	8.40 ± 0.46	7.82 ± 0.19	9.01 ± 0.29	9.88
Aspartic acid	9.95 ± 0.45	10.73 ± 0.57	11.69	10.29 ± 0.72	11.69	7.06 ± 0.17	7.69	6.92 ± 0.22	7.57	8.91 ± 0.21	8.84 ± 0.07	10.91 ± 0.77	13.13

[1] Data is shown as mean with SEM, n = 3 technical replicates per protein isolate

The yeast samples had a lysine content between 4.80 and 6.97 g/100g protein (between 5.98 and 7.85 g/100 protein for AB Agri values). The amino acid content values for SBM, LIV-03-SF-PRO-PT and LIV-02-SFM-PT in batch three were omitted by the company supplier.

Overall, there was a positive correlation between the two sets of values ($p < 0.001$), with an R^2 going from 0.8128 to 0.9904, as reported in Table 2.9.

Table 2.9: Amino acid content correlation between experimental determined data (Dumas method) or obtained data from the company

Sample	r value	R^2 value
Dried pot ale yeast LIV-15-PA-RD-Y	0.9015	0.8128
Dry bioethanol yeast LIV-10-IDY-LC	0.9087	0.8256
Hydrolysed bioethanol yeast LIV-09-HY40	0.9295	0.8641
Brewer's yeast 2 LIV-12-BY-SD	0.9627	0.9267
Brewer's yeast 1 LIV-11-BY-RD	0.9727	0.9462
Microbial protein (*Clostridium sp.)* LIV-01-SPC-LT	0.9740	0.9487
Rapeseed protein concentrate (Bench) LIV-05-RM-PRO-B	0.9741	0.9490
Pea protein concentrate LIV-08-PEA-PRO	0.9820	0.9644
AlphaSoy Gold A2	0.9921	0.9843
AlphaSoy A3	0.9932	0.9865
Sunflower protein concentrate (Bench) LIV-03-SF-PRO-B	0.9937	0.9875
Rapeseed meal (Bench) LIV-04-RM-B	0.9940	0.9880
Sunflower meal (Bench) LIV-02-SFM-B	0.9952	0.9904

As an example, the correlation for the two protein isolates with the lowest correlation value (LIV-15-PA-RD-Y, LIV-10-IDY-LC) and the correlation for the two protein isolates with the highest correlation value (LIV-04-RM-B, LIV-02-SFM-B) were illustrated in Figure 2.4.

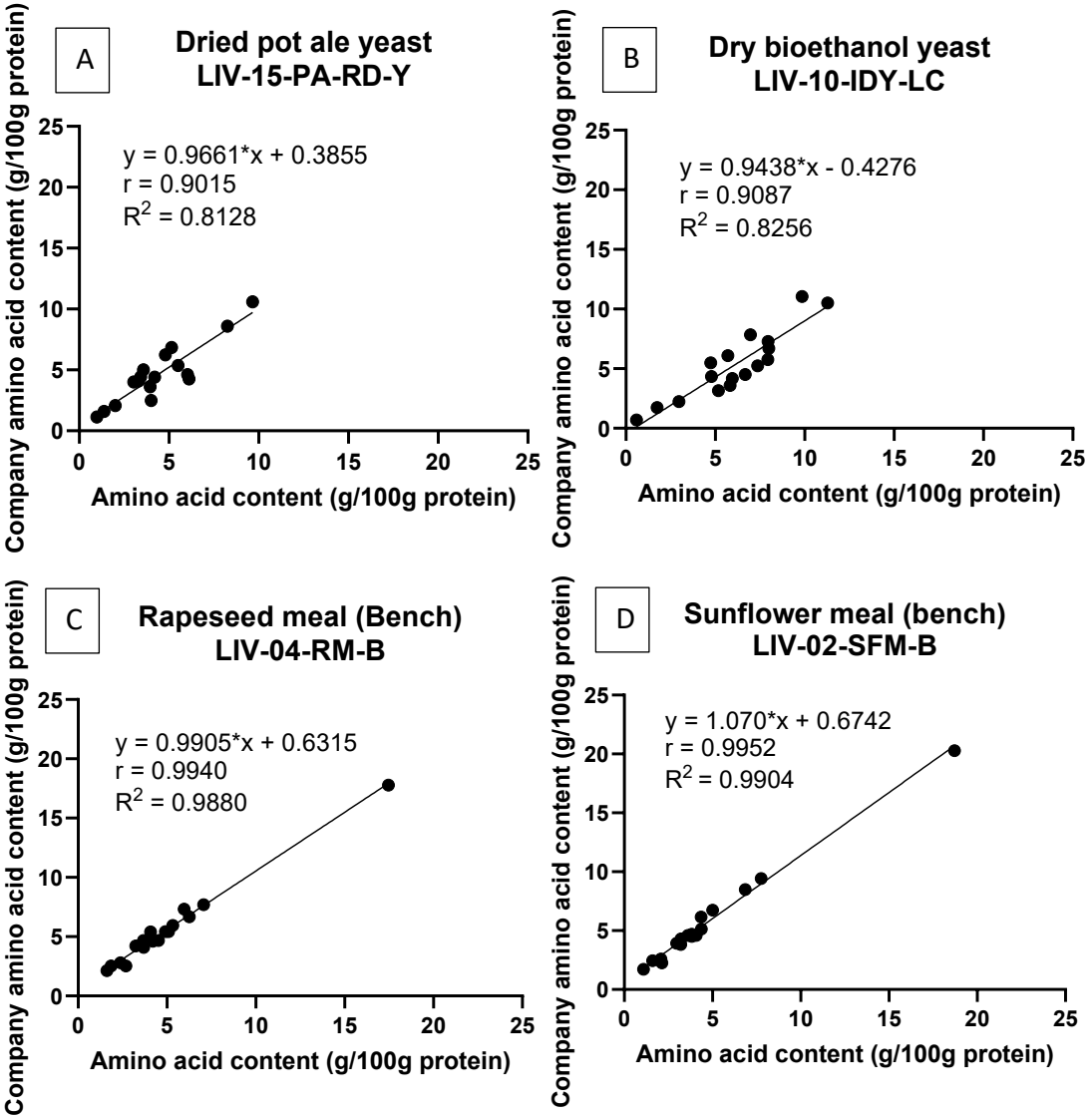


Figure 2.4: Amino acid content correlation between experimental determined data (Dumas method) or obtained data from the company for four protein isolates. Each point represents an amino acid. A. Dried pot ale yeast LIV-15-PA-RD-Y correlation. B. Dry bioethanol yeast LIV-10-IDY-LC. C. Rapeseed meal (Bench) LIV-04-RM-B correlation. D. Sunflower meal (Bench) LIV-02-SFM-B.

2.3.3. Amino acid digestibility and DIAAS of sunflower meal, sunflower protein concentrate and bacterial protein sources in batch one

Table 2.10 shows the comparison of LIV-02-SFM-B, LIV-03-SF-PRO-B, and LIV-01-SPC-LT digestibility for batch one.

Table 2.10: Various protein isolates amino acid *in vitro* digestibility (batch one)

	Digestibility (%)[1]			
Amino acid	**Sunflower meal (bench) LIV-02-SFM-B**	**Sunflower protein concentrate (Bench) LIV- 03-SF-PRO-B**	***Clostridium* sp LIV-01-SPC-LT**	**p-value[2]**
Indispensable amino acids				
Lysine	35.40 ± 3.32^{a}	55.19 ± 5.31^{b}	86.83 ± 1.73^{c}	<0.001*
Methionine	62.14 ± 4.29^{a}	73.94 ± 5.81^{a}	93.10 ± 3.48^{b}	0.002*
Threonine	69.30 ± 4.10^{a}	79.51 ± 5.70^{ab}	89.72 ± 2.70^{b}	0.032*
Valine	71.63 ± 3.63	79.17 ± 6.27	87.05 ± 4.44	0.165
Leucine	57.50 ± 4.74^{a}	74.34 ± 7.12^{ab}	83.12 ± 2.87^{b}	0.021*
Isoleucine	43.66 ± 5.71^{a}	63.72 ± 6.55^{ab}	81.33 ± 3.83^{b}	0.001*
Histidine	41.35 ± 2.72^{a}	58.93 ± 4.87^{ab}	79.27 ± 9.70^{b}	<0.001*
Phenylalanine	59.79 ± 4.74^{a}	76.20 ± 6.17^{ab}	81.55 ± 3.55^{b}	0.027*
Dispensable amino acids				
Tyrosine	41.35 ± 6.07^{a}	62.73 ± 8.55^{ab}	75.18 ± 6.57^{b}	0.019*
Cystine	28.24 ± 3.93^{a}	49.62 ± 7.35^{a}	78.37 ± 9.71^{b}	<0.001*
Serine	40.85 ± 2.69^{a}	58.68 ± 6.41^{b}	69.43 ± 3.48^{b}	0.003*
Glycine	49.01 ± 2.96^{a}	64.99 ± 4.08^{b}	81.55 ± 2.62^{c}	<0.001*
Glutamic acid	77.66 ± 2.88	86.18 ± 3.78	82.58 ± 2.67	0.191
Proline	60.11 ± 4.03^{a}	74.22 ± 5.79^{ab}	83.37 ± 3.78^{b}	0.015*
Alanine	60.31 ± 7.96	72.90 ± 8.22	72.47 ± 4.30	0.437
Arginine	59.81 ± 3.45	72.38 ± 5.79	73.28 ± 4.13	0.113
Aspartic acid	52.38 ± 4.24^{a}	69.78 ± 6.46^{ab}	81.67 ± 1.86^{b}	0.004*
Total amino acid	60.01 ± 4.08^{a}	74.76 ± 5.85^{ab}	82.53 ± 2.73^{b}	0.015*

[1] Data is shown as mean with SEM, n=9 technical replicates per protein isolate

[2] p-value for unbalanced design one-way ANOVA

* Significant difference. Different letters $^{a, b, c}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

For LIV-02-SFM-B, LIV-03-SF-PRO-B, and LIV-01-SPC-LT, the crude protein content was 43.91, 52.66 and 89.61 g/100g dry weight, respectively. There was a significant difference in digestibility across the samples for most of the amino acids ($p < 0.05$).

LIV-02-SFM-B had significantly lower threonine, leucine, isoleucine, histidine, phenylalanine, tyrosine, proline, aspartic acid, and total amino acid digestibility than LIV-01-SPC-LT ($p < 0.05$), but not LIV-03-SF-PRO-B.

The lysine and glycine digestibility of LIV-03-SF-PRO-B were significantly higher than those of LIV-02-SFM-B, but significantly lower than those of LIV-01-SPC-LT ($p < 0.05$). LIV-01-SPC-LT exhibited significantly higher methionine and cystine digestibility ($p < 0.05$) than the other protein isolates. Serine digestibility was significantly lower for LIV-02-SFM-B ($p < 0.05$) than for the other protein isolates.

The DIAAR and DIAAS values for batch one are reported in Table 2.11.

Table 2.11: DIAAR and DIAAS of feed ingredient yeast protein isolates (batch one)

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting AA (LAA)	Expected LAA
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43			
Sunflower meal (Bench) LIV-02-SFM-B	1.07	1.09	1.08	**0.49**[1]	1.19	1.51	1.64	1.80	**0.49**	**Lysine**	Lysine
Clostridium sp LIV-01-SCP-LT	**0.70**	1.75	1.07	1.87	1.30	1.39	1.12	1.42	**0.70**	**Histidine**	N/A
Sunflower protein concentrate (Bench) LIV-03-SF-PRO-B	1.85	2.01	1.64	**0.94**	2.03	2.54	1.99	2.35	**0.94**	**Lysine**	Lysine

[1] Values in bold represent the lowest DIAAR and the DIAAS

For the protein isolates tested in batch one, the DIAAS varied from 0.49 to 0.94 for LIV-02-SFM-B and LIV-03-SF-PRO-B, respectively, with lysine as a limiting amino acid. In contrast, the limiting amino acid for LIV-01-SCP-LT was histidine, with a DIAAS of 0.70. Interestingly, LIV-01-SCP-LT had a high lysine DIAAR of 1.87.

2.3.4. Casein as a reference protein

For casein, there was good agreement between the measured amino acid contents and those found in the literature (Table 2.12).

Table 2.12: Casein amino acid content in comparison with reported values for casein in literature

			Published casein AA content (mg/g protein)		
Amino acid	**Casein (based on amino acids recovered) (mg/g total amino acids recovered)[1]**	**Casein (based on crude protein content) (mg/g crude protein)[2]**	**Liu *et al.* (2019)**	**Eklund *et al.* (2010)**	**deMan *et al.* (1999)**
Indispensable amino acids					
Lysine	79.08 ± 0.74	87.03 ± 5.13	85	89	89
Methionine	22.85 ± 0.33	25.13 ± 1.35	29	32	18
Threonine	30.37 ± 0.52	33.36 ± 1.49	46	47	44
Valine	61.72 ± 0.90	67.93 ± 4.08	76	73	68
Leucine	82.09 ± 5.56	101.96 ± 11.28	102	103	92
Isoleucine	45.48 ± 1.30	50.21 ± 4.24	59	57	38
Histidine	27.01 ± 0.50	29.66 ± 1.27	31	33	56
Phenylalanine	47.04 ± 0.71	51.69 ± 2.43	55	57	53
Dispensable amino acids					
Tyrosine	51.54 ± 1.34	56.91 ± 4.77	59	N/A	57
Cystine	3.05 ± 0.09	3.35 ± 0.22	4	5	3
Serine	52.71 ± 1.26	57.86 ± 2.27	65	75	53
Glycine	23.17 ± 2.76	25.14 ± 1.35	19	22	19
Glutamic acid	229.15 ± 1.45	265.50 ± 13.70	227	266	245
Proline	109.77 ± 0.73	127.49 ± 6.76	105	120	135
Alanine	32.24 ± 1.22	35.51 ± 2.76	32	36	31
Arginine	34.21 ± 0.19	37.64 ± 2.11	37	41	33
Aspartic acid	68.53 ± 1.00	75.45 ± 4.83	75	85	76

[1,2] Data is shown as mean with SEM, n = 3 technical replicates per casein batch

The strong positive correlation between the measured amino acid contents and those found in the literature ($p < 0.001$) is reported in Figure 2.5.

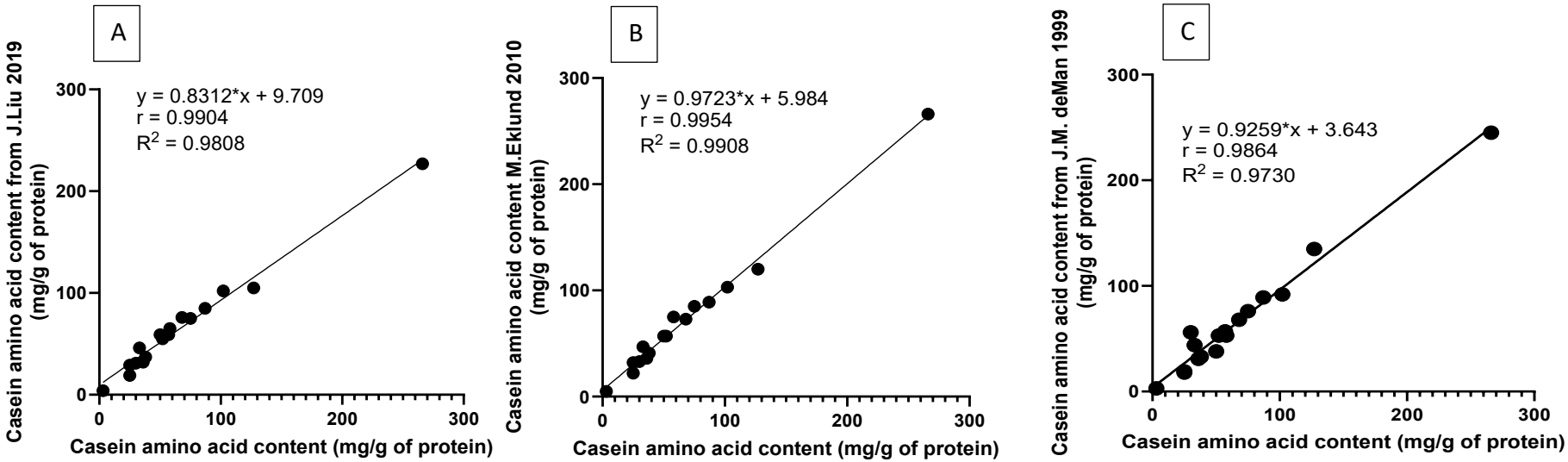


Figure 2.5: Casein amino acid content correlation between measured data and data found in literature. Each point represents an amino acid. A. Correlation to values reported by Liu *et al.,* (2019). B. Correlation to values reported by Eklund *at al*. (2010). C. Correlation to values reported by deMan *et al.* (1999).

The comparison of casein amino acid digestibility between batch two and batch three (Table 2.13) revealed no significant differences across all amino acids, except for proline (p = 0.038).

Table 2.13: Casein amino acid digestibility comparison between batch two and batch three

	Casein digestibility (%)[1]		
Amino acid	**Batch two**	**Batch three**	**p-value[2]**
	Indispensable amino acids		
Lysine	95.24 ± 1.24	92.88 ± 0.97	0.188
Methionine	96.51 ± 2.14	97.51 ± 1.19	0.696
Threonine	93.75 ± 2.35	94.08 ± 3.34	0.939
Valine	94.83 ± 1.36	91.69 ± 3.16	0.395
Leucine	95.47 ± 2.43	91.75 ± 1.12	0.216
Isoleucine	90.66 ± 3.22	92.85 ± 0.35	0.547
Histidine	96.16 ± 0.90	93.46 ± 0.96	0.088
Phenylalanine	96.78 ± 1.92	93.17 ± 0.42	0.152
	Dispensable amino acids		
Tyrosine	88.40 ± 2.71	91.37 ± 2.20	0.422
Cystine	83.18 ± 9.87	92.27 ± 7.57	0.524
Serine	N/A	83.80 ± 3.14	N/A
Glycine	97.21 ± 2.35	95.21 ± 2.44	0.595
Glutamic acid	94.08 ± 2.37	89.92 ± 4.20	0.421
Proline	97.30 ± 1.14	92.98 ± 1.15	0.038*
Alanine	91.96 ± 1.86	94.36 ± 1.90	0.401
Arginine	90.22 ± 3.66	90.13 ± 1.44	0.980
Aspartic acid	82.87 ± 6.34	91.49 ± 4.00	0.295
Total amino acid	94.03 ± 2.02	92.69 ± 0.31	0.919

[1] Data is shown as mean with SEM, n = 4 technical replicates per casein batch

[2] p value for t-test

* Significant difference

2.3.5. Amino acid digestibility and DIAAS of Saccharomyces cerevisiae protein isolates from different sources

Table 2.14 shows the comparison of protein isolates digestibility for the batch two, which focuses on *Saccharomyces cerevisiae*, a species of yeast.

Table 2.14: Feed ingredient yeast protein isolates amino acid *in vitro* digestibility (batch two)

	Digestibility (%)[1]						
Amino acid	**Dry bioethanol yeast LIV-10-IDY-LC**	**Hydrolysed bioethanol yeast LIV-09-HY-40**	**Dried pot ale yeast LIV-15-PA-RD-Y**	**Brewer's yeast 1 LIV-11-BY-RD**	**Brewer's yeast 2 LIV-12-BY-SD**	**Casein yeast batch**	**p-value[2]**
Indispensable amino acids							
Lysine	64.07 ± 6.08^{ab}	77.73 ± 1.77^{bc}	52.57 ± 10.00^{a}	69.33 ± 5.61^{ab}	73.17 ± 3.47^{abc}	95.25 ± 1.24^{c}	0.001*
Methionine	69.50 ± 6.44^{a}	81.83 ± 2.93^{ab}	75.70 ± 1.46^{ab}	80.40 ± 4.95^{ab}	70.63 ± 9.15^{a}	96.53 ± 2.14^{b}	0.014*
Threonine	52.00 ± 8.97^{a}	69.83 ± 6.08^{ab}	57.63 ± 4.34^{a}	65.05 ± 4.36^{ab}	61.40 ± 6.36^{ab}	93.75 ± 2.35^{c}	0.002*
Valine	58.68 ± 8.53^{a}	74.33 ± 10.28^{ab}	74.50 ± 1.10^{ab}	73.43 ± 5.49^{ab}	75.13 ± 3.63^{ab}	94.83 ± 1.36^{b}	0.016*
Leucine	70.75 ± 8.71^{ab}	85.98 ± 0.84^{ab}	84.50± 8.50^{ab}	69.05 ± 7.62^{a}	81.63 ± 1.15^{ab}	95.45 ± 2.43^{b}	0.032*
Isoleucine	55.55 ± 4.43^{a}	79.75 ± 3.87^{ab}	81.50 ± 8.24^{ab}	85.07 ± 5.22^{ab}	76.97 ± 12.76^{ab}	90.65 ± 3.22^{b}	0.015*
Histidine	63.33 ± 10.75^{a}	86.33 ± 4.59^{ab}	66.45 ± 2.43^{ab}	70.17 ± 8.83^{ab}	74.77 ± 3.20^{ab}	96.15 ± 0.90^{b}	0.014*
Phenylalanine	63.63 ± 10.93^{a}	81.73 ± 5.78^{ab}	74.80 ± 8.28^{ab}	71.15 ± 9.54^{ab}	68.75 ± 3.46^{ab}	96.80 ± 1.92^{b}	0.040*
Dispensable amino acids							
Tyrosine	50.60	80.00 ± 15.01	67.27 ± 17.64	82.05 ± 8.88	49.50 ± 3.29	88.38 ± 2.71	0.219
Cystine	70.00	71.12	70.80 ± 7.28	75.20	99.70	83.18 ± 9.87	0.728
Serine	68.97 ± 8.85	77.78 ± 4.43	72.23 ± 13.88	66.75 ± 10.64	67.35 ± 2.45	49.50 ± 2.26	0.402
Glycine	76.10 ± 8.44^{ab}	87.30 ± 3.63^{ab}	59.00 ± 10.37^{a}	79.53 ± 2.84^{ab}	77.60 ± 7.54^{ab}	97.20 ± 2.35^{b}	0.020*
Glutamic acid	63.58 ± 5.81^{a}	72.03 ± 3.72^{a}	68.03 ± 1.60^{a}	77.88 ± 3.66^{ab}	79.90 ± 0.66^{ab}	94.08 ± 2.37^{b}	<0.001*
Proline	70.13 ± 8.59^{ab}	84.07 ± 4.40^{ab}	60.03 ± 8.77^{a}	74.73 ± 4.26^{ab}	82.13 ± 3.19^{ab}	97.30 ± 1.14^{b}	0.005*
Alanine	73.95 ± 8.86^{ab}	83.98 ± 3.91^{ab}	63.47 ± 3.96^{a}	81.25 ± 4.89^{ab}	74.70 ± 2.07^{ab}	91.98 ± 1.86^{b}	0.028*
Arginine	61.07 ± 7.93	78.47 ± 4.89	82.53 ± 12.14	67.20 ± 5.35	79.20 ± 5.51	90.23 ± 3.66	0.083
Aspartic acid	51.50 ± 3.33^{a}	64.50 ± 7.23^{ab}	67.97 ± 6.20^{ab}	71.07 ± 3.35^{ab}	67.70 ± 4.30^{ab}	82.88 ± 6.34^{b}	0.022*
Total amino acid	55.70 ± 0.93^{a}	79.33 ± 2.80^{bc}	64.53 ± 7.39^{ab}	70.62 ± 4.17^{ab}	72.90 ± 2.75^{ab}	92.90 ± 2.02^{c}	<0.001*

[1]Data is shown as mean with SEM, n = 4 technical replicates per protein isolate

[2]p-value for unbalanced design one-way ANOVA (values shown as single measurements were excluded)

* Significant difference. Different letters $^{a, b, c}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

The crude protein content in batch two varied from 37.25 to 40.10 g/100g dry weight for all yeast samples, except for LIV-15-PA-RD-Y with a crude protein content of 57.85 g/100g dry weight. There was a significant difference in digestibility between the different protein sources ($p < 0.05$) and, as expected, casein gave the highest digestibility for all. Leucine showed the highest digestibility overall, reaching up to 95.45% in some samples. Tyrosine demonstrated the lowest digestibility among all amino acids measured.

Total amino acid digestibility varied considerably between different yeast sources, with values ranging from approximately 63% to 94%. Except for LIV-09-HY-40, casein total amino acid digestibility was significantly higher than the other protein isolates ($p < 0.05$). LIV-10-IDY-LC had a significantly lower total amino acid digestibility than casein and LIV-09-HY-40 but was not significantly different from the other yeast isolates.

Lysine digestibility of LIV-15-PA-RD-Y was significantly lower than both LIV-09-HY-40 and casein ($p < 0.05$), but not from the other isolates. Casein had a significantly higher methionine digestibility than LIV-10-IDY-LC and LIV-12-BY-SD ($p < 0.05$), but either of these yeast isolates was different from the others. LIV-10-IDY-LC and LIV-15-PA-RD-Y had a significantly lower threonine digestibility than casein ($p < 0.05$) but were not different from the other isolates.

Table 2.15 shows the DIAAR and DIAAS of yeast protein isolates in batch two.

Table 2.15: DIAAR and DIAAS of feed ingredient yeast protein isolates (batch two)

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)	Expected LAA
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43			
Casein (Reference protein)	1.50	1.50	1.55	1.53	**1.05**[1]	2.03	**1.06**	1.58	**1.05/1.06**	**SAA/Threonine**	SAA
Dry bioethanol yeast LIV-10-IDY-LC	1.66	3.19	1.62	1.95	**1.24**	2.72	1.98	1.94	**1.24**	**SAA**	SAA, lysine
Hydrolysed bioethanol yeast LIV-09-HY40	1.80	4.04	2.66	2.04	**1.46**	2.42	2.19	2.15	**1.46**	**SAA**	SAA, lysine
Dried pot ale yeast LIV-15-PA-RD-Y	1.15	1.85	1.09	**0.77**	1.11	1.71	1.10	1.07	**0.77**	**Lysine**	SAA, lysine
Brewer's yeast 1 LIV-11-BY-RD	1.97	3.40	**1.66**	2.07	1.75	3.15	2.34	2.30	**1.66**	**Leucine**	SAA, lysine
Brewer's yeast 2 LIV-12-BY-SD	1.91	3.12	2.28	2.07	**1.75**	2.41	2.07	2.45	**1.75**	**SAA**	SAA, lysine

[1]Values in bold represent the lowest DIAAR and the DIAAS

Casein had sulphur-containing amino acids and threonine for limiting amino acids, with respective DIAAR of 1.05 and 1.06. The DIAAS values for the yeast protein isolates in batch two varied from 0.77 to 1.75. Sulphur-containing amino acids were the limiting amino acids for LIV-10-IDY-LC, LIV-09-HY40 and LIV-12-BY-SD (DIAAS of 1.24, 1.46 and 1.75 respectively). Lysine was the limiting amino acid for LIV-15-PA-RD-Y (DIAAS of 0.77). LIV-11-BY-RD had leucine for limiting amino acid, with a DIAAS of 1.66.

2.3.6. Pictures, amino acid digestibility, pellet weight and DIAAS of a range of protein isolates

For batch three, a range of plant-based protein isolates, which encompasses sunflower, rapeseed and soya samples, was analysed. The crude protein content of the protein isolates in batch three varied from 39.19 to 57.32 g/100g dry weight. The visual representation of the samples with pictures is shown in Figure 2.6.

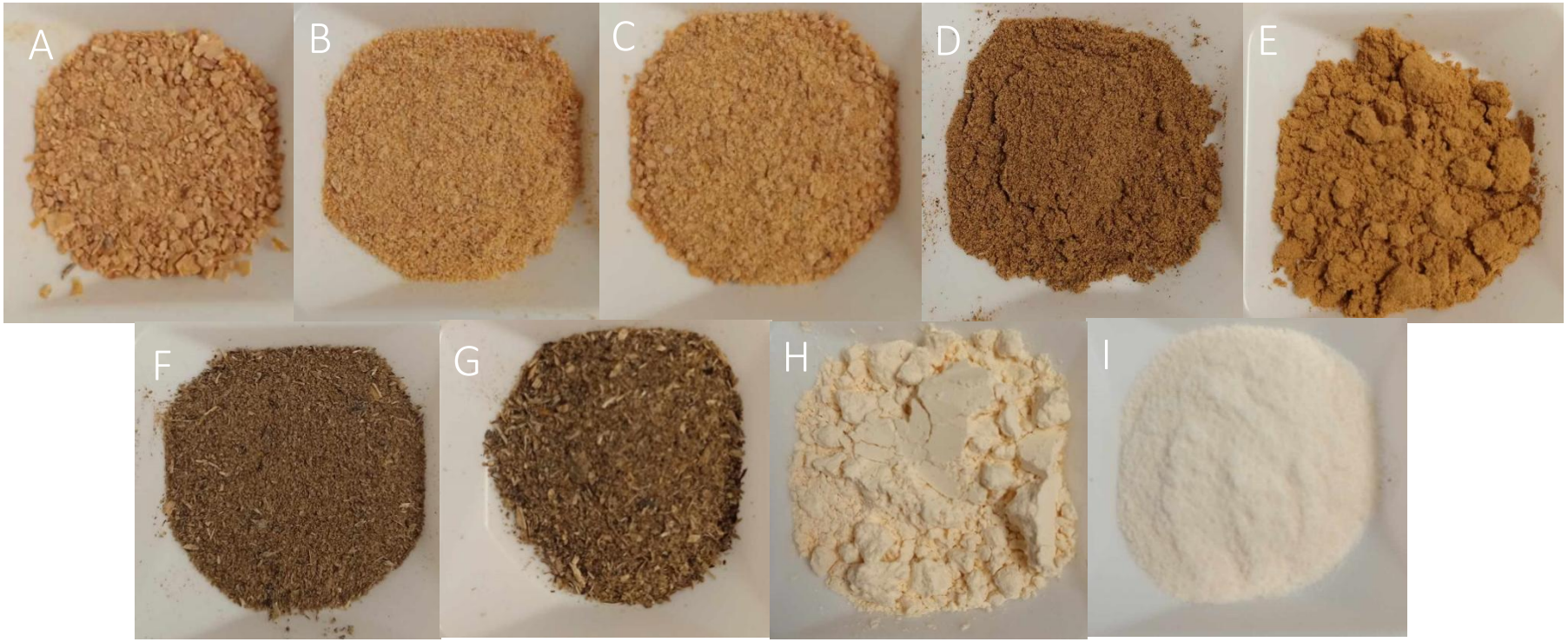


Figure 2.6: Nine protein isolates samples before digestion for batch three, showing varying colours and textures. A. Soybean meal. B. AlphaSoy A3. C. AlphaSoy Gold A2. D. Rapeseed meal (Bench) LIV-04-RM-B. E. Rapeseed protein concentrate (Bench) LIV-05-RM-PRO-B. F. Sunflower protein concentrate (Pilot) LIV-03-SF-PRO-PT. G. Sunflower meal (Pilot) LIV-02-SFM-PT. H. Pea protein concentrate LIV-08-PEA-PRO-B. I. Casein.

SBM (A) appeared as a light brown, coarse-textured sample. AlphaSoy A3 (B) was slightly darker with a uniform granulated texture, likely indicating further refinement. AlphaSoy Gold (C) was golden brown with fine granules, similar to AlphaSoy.

Rapeseed meal of bench scale (D) had a dark brown, denser structure, suggesting a potentially high fibre content. Rapeseed protein concentrate of bench scale (E) had a light orange-brown powder, much lighter than rapeseed meal of bench scale, and a fine texture.

Sunflower protein concentrate of pilot scale (F) was dark and coarse. The yellow fragments are likely residual fibre particles or plant material fragments. Sunflower meal of pilot scale (G) was dark brown, coarse and even more fibrous than the sunflower protein concentrate of pilot scale.

Pea protein concentrate (H) had a light beige, fluffy consistency, representing a high-purity protein isolate. Finally, casein (I), a white homogenous fine powder, served as the reference standard for high protein quality.

The pellet weight of protein isolates in batch three after *in vitro* digestion is shown in Figure 2.7.

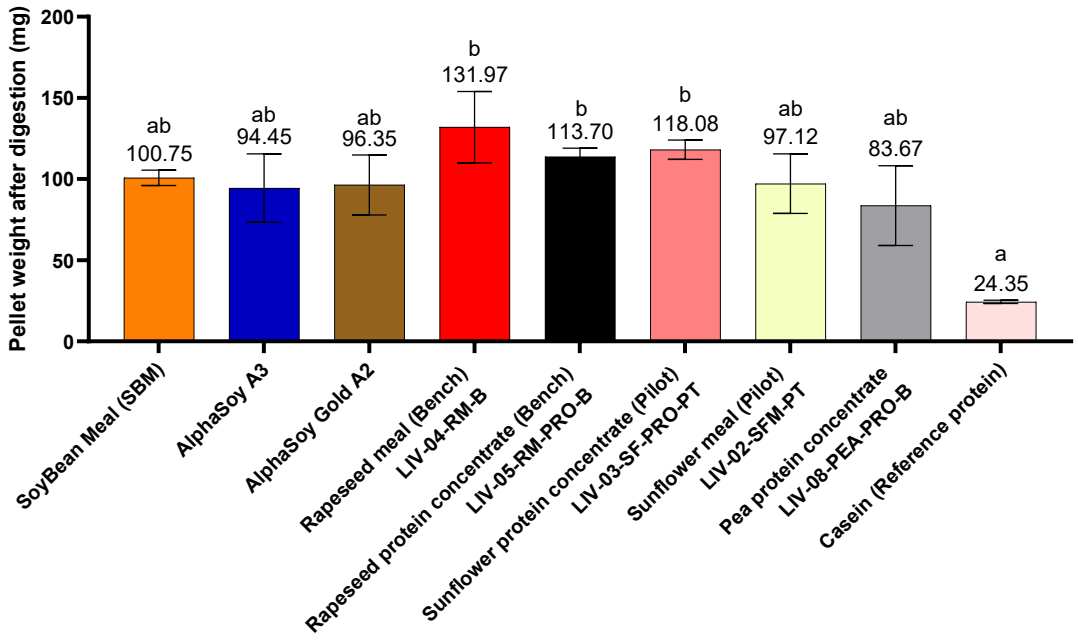


Figure 2.7: Pellet weight after digestion (mg) in batch three. Data is shown as mean with SEM, n = 4 technical replicates per protein isolate. There was a significant effect of protein ingredient source ($p = 0.006$), using a one-way ANOVA. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$). The results were adjusted by subtracting the blank.

The pellet weight represents the non-absorbable fraction left after digestion, composed of undigested protein and large peptides. The pellet weight values ranged from 24.35 mg for casein to 131.97 mg for LIV-04-RM-B. Casein pellet weight was significantly lower ($p < 0.05$) than the pellet weight of LIV-05-RM-PRO-B, LIV-03-SF-PRO-PT and LIV-04-RM-B.

Table 2.16 shows the comparison of protein isolates digestibility in batch three.

Table 2.16: Feed ingredient protein isolates amino acid *in vitro* digestibility (batch three)

	Digestibility (%)[1]									
Amino acid	**Soybean meal (SBM)**	**AlphaSoy A3**	**AlphaSoy Gold A2**	**Rapeseed meal (bench) LIV-04-RM-B**	**Rapeseed protein concentrate (Bench) LIV-05-RM-PRO-B**	**Sunflower protein concentrate (Pilot) LIV-03-SF-PRO-PT**	**Sunflower meal (Pilot) LIV-02-SFM-PT**	**Pea protein concentrate LIV-08-PEA-PRO**	**Casein**	**p-value[2]**
					Indispensable amino acids					
Lysine	55.08 ± 1.18 [ab]	56.84 ± 4.44 [ab]	68.89 ± 4.13 [bc]	38.80 ± 1.90 [a]	58.28 ± 5.53 [ab]	62.06 ± 4.73 [b]	84.57 ± 6.08 [cd]	62.90 ± 8.55 [ab]	92.88 ± 0.97 [d]	<0.001*
Methionine	72.32 ± 9.78	80.74 ± 6.53	83.40 ± 5.55	76.78 ± 5.57	76.55 ± 4.31	82.53 ± 3.75	94.87 ± 0.57	83.32 ± 7.75	97.52 ± 1.19	0.183
Threonine	84.62 ± 7.47	87.97± 9.98	92.97 ± 5.09	83.18 ± 3.45	86.35 ± 3.33	85.32 ± 6.58	90.34 ± 5.01	77.47 ± 17.29	94.08 ± 3.33	0.823
Valine	62.36 ± 10.11	70.39 ± 9.76	75.30 ± 10.04	69.04 ± 9.54	69.42 ± 7.86	77.57 ± 6.61	84.26 ± 6.45	60.69 ± 10.13	91.69 ± 3.15	0.223
Leucine	58.88 ± 2.48 [a]	71.69 ± 2.97 [abc]	79.77 ± 3.06 [abc]	63.57 ± 6.20 [ab]	70.59 ± 5.80 [abc]	74.21 ± 3.35 [abc]	83.48 ± 5.89 [bc]	60.62 ± 6.79 [a]	91.75 ± 1.13 [c]	<0.001*
Isoleucine	59.59 ± 2.31 [a]	70.34 ± 2.82 [ab]	77.95 ± 4.40 [ab]	50.47 ± 11.59 [a]	61.09 ± 4.97 [a]	71.56 ± 1.38 [ab]	79.84 ± 9.76 [ab]	59.53 ± 4.47 [a]	92.85 ± 0.36 [b]	<0.001*
Histidine	52.34 ± 6.46 [a]	63.79 ± 3.51 [abc]	63.33 ± 8.07 [abc]	56.21 ± 10.66 [ab]	64.74 ± 3.20 [abc]	68.89 ± 2.48 [abc]	83.75 ± 9.23 [bc]	56.53 ± 4.87 [ab]	93.46 ± 0.97 [c]	<0.001*
Phenylalanine	63.79 ± 3.99 [a]	75.05 ± 4.67 [ab]	80.56 ± 4.64 [ab]	65.30 ± 7.64 [a]	73.42 ± 4.92 [ab]	80.40 ± 2.74 [ab]	84.20 ± 7.82 [ab]	65.07 ± 7.40 [a]	93.17 ± 0.43 [ab]	0.006*
					Dispensable amino acids					
Tyrosine	70.40 ± 16.30	72.14 ± 27.9	83.00 ± 16.85	41.60 ± 3.19	73.70 ± 2.25	63.50 ± 2.54	74.60 ± 5.50	30.40	91.37 ± 2.19	0.189
Cystine	23.48 ± 6.30 [a]	26.69 ± 4.80 [a]	41.14 ± 4.35 [ab]	23.75 ± 6.66 [a]	41.20 ± 8.73 [ab]	40.18 ± 12.89 [a]	10.54 ± 0.65 [a]	67.00	92.27 ± 7.58 [b]	<0.001*
Serine	49.27 ± 4.34	63.65 ± 6.73	71.45 ± 6.83	49.45 ± 11.53	53.91 ± 8.68	66.10 ± 3.09	73.00 ± 11.32	54.64 ± 7.97	83.80 ± 3.14	0.038*
Glycine	39.87 ± 13.90 [ab]	51.51 ± 11.38 [ab]	65.51 ± 13.78 [ab]	44.75 ± 13.52 [ab]	52.74 ± 2.34 [ab]	60.97 ± 11.13 [ab]	58.03 ± 18.38 [ab]	31.22 ± 2.05 [a]	95.21 ± 2.45 [b]	0.041*
Glutamic acid	75.35 ± 4.21	82.86 ± 4.30	84.64 ± 3.78	80.11 ± 2.30	84.45 ± 2.74	86.06 ± 4.19	90.72 ± 3.25	72.89 ± 9.69	89.92 ± 4.18	0.083
Proline	64.05 ± 4.94 [ab]	67.29 ± 1.71 [ab]	76.51 ± 4.67 [abc]	69.23 ± 6.11 [ab]	75.67 ± 4.82 [abc]	76.53 ± 2.69 [abc]	84.34 ± 7.23 [bc]	60.48 ± 6.13 [a]	92.98 ± 1.16 [c]	0.001*
Alanine	80.80 ± 6.96	79.68 ± 11.79	87.34 ± 7.58	82.56 ± 5.13	80.59 ± 9.17	89.06 ± 4.46	92.13 ± 3.87	74.99 ± 13.70	94.36 ± 1.90	0.661
Arginine	52.23 ± 8.32 [a]	69.05 ± 3.64 [ab]	72.46 ± 3.17 [ab]	55.94 ± 9.79 [a]	68.22 ± 4.72 [ab]	76.91 ± 3.08 [ab]	79.48 ± 7.14 [ab]	65.55 ± 8.44 [ab]	90.13 ± 1.43 [b]	0.004*
Aspartic acid	59.41 ± 3.52 [a]	71.10 ± 6.01 [ab]	76.99 ± 3.93 [ab]	58.05 ± 8.92 [a]	64.97 ± 4.78 [ab]	73.48 ± 1.51 [ab]	79.45 ± 8.75 [ab]	65.64 ± 9.75 [ab]	91.49 ± 4.02 [b]	0.012*
Total amino acid	63.88 ± 2.43 [a]	73.01 ± 4.68 [ab]	78.60 ± 4.53 [ab]	66.61 ± 5.94 [a]	72.71 ± 4.66 [ab]	77.54 ± 3.13 [ab]	84.78 ± 5.80 [ab]	64.78 ± 8.82 [a]	92.69 ± 0.30 [b]	0.002*

[1]Data is shown as mean with SEM, n = 4 technical replicates per protein isolate

[2]p-value for unbalanced design one-way ANOVA (values shown as single measurements were excluded)

[*] Significant difference. Different letters [a, b, c, d] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$)

Samples with high pellet weights, LIV-05-RM-PRO-B, LIV-03-SF-PRO-PT and LIV-04-RM-B showed low lysine digestibility. Casein, with the lowest pellet weight, had significantly higher digestibility across most amino acids.

There were not as many significant different amino acid digestibilities across the different protein isolates as there were for yeast samples (Table 2.14). Cystine showed consistently lower digestibility across most protein sources, with values as low as 23.48% in soybean meal. Threonine demonstrated high digestibility across most protein sources with values above 77%.

Casein total amino acid digestibility was significantly higher ($p < 0.05$) than soybean meal, LIV-04-RM-B, and LIV-08-PEA-PRO total amino acid digestibility, with all the other isolates not being different from these three or casein. When considering the three individual amino acids which are usually limiting in plant-based protein sources, it was noticeable that there was a significant difference in the lysine digestibility ($p < 0.001$) but there was no significant difference between any of the isolates examined in batch three for methionine and threonine digestibility.

Lysine digestibility had the highest range of values across protein isolates, discounting casein, varying from 38.80% to 84.57%. Casein and LIV-02-SFM-PT lysine digestibility were significantly higher ($p < 0.05$) than that of all other protein isolates, except for AlphaSoy Gold A2. Lysine digestibility of LIV-04-RM-B was significantly lower than for A2, LIV-03-SF-PRO-PT, LIV-02-SFM-PT and casein.

The DIAAS values for batch three are reported in Table 2.17.

Table 2.17: DIAAR and DIAAS of protein isolate in batch three

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)	Expected LAA
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43			
Casein (Reference protein)	1.62	1.71	1.66	1.66	**1.20**[1]	2.26	**1.19**	1.70	**1.19/1.20**	**Threonine /SAA**	SAA
Soybean meal (SBM)	1.04	1.26	0.99	0.92	**0.69**	1.74	1.44	1.11	**0.69**	**SAA**	SAA
AlphaSoy A3	1.30	1.65	1.35	1.03	**0.78**	2.09	1.45	1.32	**0.78**	**SAA**	SAA
AlphaSoy Gold A2	1.32	1.74	1.62	1.32	**0.90**	2.25	1.63	1.40	**0.90**	**SAA**	SAA
Rapeseed meal (Bench) LIV-04-RM-B	1.71	1.30	1.47	**1.14**	1.59	1.94	2.51	2.01	**1.14**	**Lysine**	Lysine
Rapeseed protein concentrate (Bench) LIV-05-RM PRO-B	1.78	1.29	1.39	**1.13**	1.63	1.99	2.01	1.68	**1.13**	**Lysine**	Lysine
Sunflower protein concentrate (Pilot) LIV-03-SF-PRO-PT	1.69	1.76	1.33	**0.81**	1.61	2.04	1.74	1.78	**0.81**	**Lysine**	Lysine
Sunflower meal (Pilot) LIV-02-SFM-PT	2.38	2.14	1.60	**1.08**	1.61	2.56	2.28	2.41	**1.08**	**Lysine**	Lysine
Pea protein concentrate LIV-08-PEA-PRO-B	1.44	1.53	1.20	1.81	**0.52**	1.71	1.62	1.37	**0.52**	**SAA**	SAA

[1] Values in bold represent the lowest DIAAR and the DIAAS

As with batch 2, casein had sulphur-containing amino acids and threonine for limiting amino acids, with respective DIAAR of 1.19 and 1.20. For the plant-based protein isolates tested in batch 3, the DIAAS varied from 0.52 to 1.14.

Lysine was the limiting amino acid for LIV-03-SF-PRO-PT, LIV-02-SFM-PT, LIV-05-RM PRO-B and LIV-04-RM-B (DIAAS of 0.81, 1.08, 1.13 and 1.14 respectively). LIV-08-PEA-PRO-B, SBM, A3 and A2 had sulphur-containing amino acids for limiting amino acids, with respective DIAAS of 0.52, 0.69, 0.78 and 0.90.

2.3.7. Degree of hydrolysis of protein isolates in batches one and three

The degree of hydrolysis for batch one is shown in Figure 2.8.

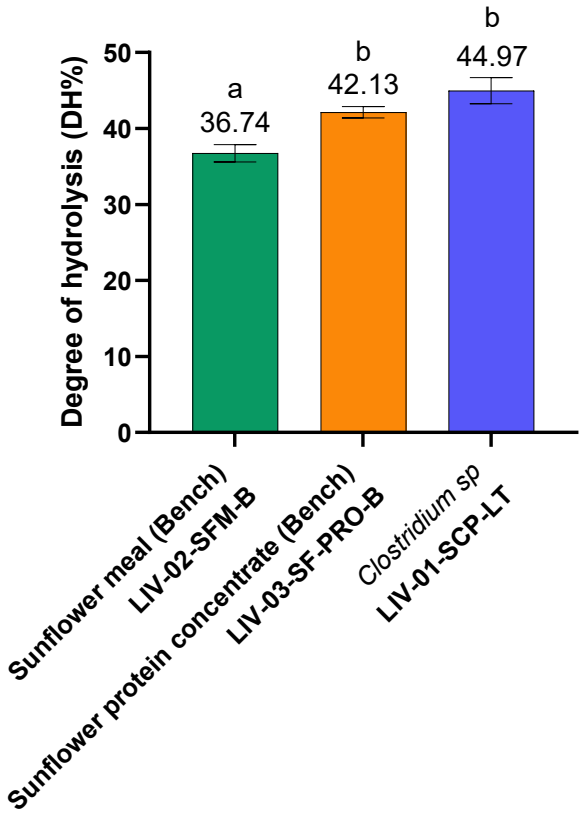


Figure 2.8: Degree of hydrolysis (%) in batch one, data is shown as mean with SEM, n=9 technical replicates per protein isolate. There was a significant effect of protein

ingredient source ($p < 0.001$), using a one-way ANOVA. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

The degree of hydrolysis values ranged narrowly between 36.74 and 44.92%. LIV-02-SFM-B had a significantly lower DH% than that of the other protein isolates ($p < 0.05$). Figure 2.9 shows the degree of hydrolysis for batch three.

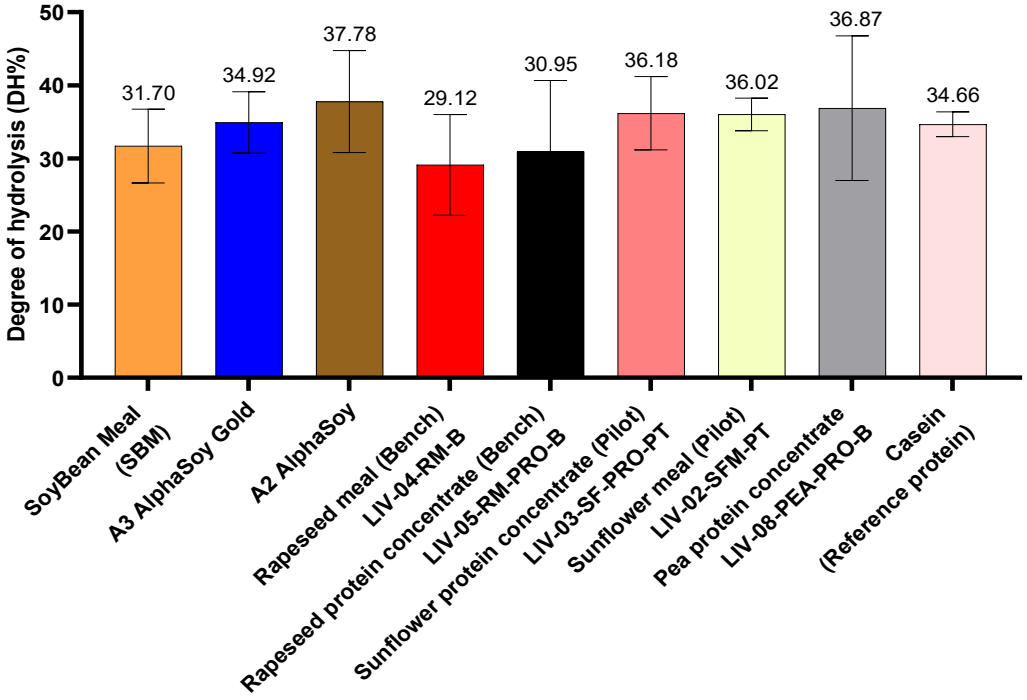


Figure 2.9: Degree of hydrolysis (%) in batch three, data is shown as mean with SEM, n = 4 technical replicates per protein isolate. The values of the degree of hydrolysis varied from 29.12% with LIV-04-RM-B to 37.78% with A2. No significant differences were observed in degree of hydrolysis among all protein isolates ($p = 0.877$).

2.4. Discussion

Hypothesis: the methods used can assess crude protein and amino acid content and values are consistent with existing data.

Seventeen protein isolates were analysed in this study, with the inclusion of casein. All samples were relatively high in protein and highly digestible. Overall, there was good agreement between obtained crude protein content and crude protein content from AB Agri.

Clostridium sp. (LIV-01-SPC-LT) was found to have a high protein content, which can partly be explained by the presence of a significant amount of non-protein nitrogen (NPN) compounds, such as nucleic acids. Nucleic acids store and transmit genetic information and are involved in various cellular functions. These compounds, although not actual proteins, contribute to the total nitrogen detected during protein analysis.

Nucleic acids like DNA and RNA are particularly rich in nitrogen and can inflate the measured protein levels in such samples. This highlights the need to consider the types of nitrogen present when interpreting protein content, as non-protein nitrogen can affect both the nutritional quality and functional characteristics of the isolate.

Using the Dumas method with a nitrogen-to-protein conversion factor (NPCF) of 6.25, the obtained crude protein content was systematically slightly higher compared to the values reported by AB Agri. Since both analyses employed the same method and conversion factor, the discrepancy likely comes from differences in sample preparation differences, calibration or sensitivity. Samples could have been handled differently, with

a slight mix before weighing out the samples for AB Agri. Differences observed may partially reflect unknown methodological differences, as AB Agri complete protocols were not accessible.

AB Agri may use a different reference material than Aspartic acid 10.52% or handle the same standard differently, resulting in lower nitrogen readings. The equipment used in the lab might have a higher sensitivity, capturing more nitrogen including traces than AB Agri's equipment. To confirm the cause of the discrepancy, calibration standards and standard operating procedures (SOPs) for the Dumas method should be cross-checked between labs.

The use of 6.25 of NPCF for all protein isolates is a simple standardised factor that allows a direct comparison between samples. However, animal proteins have nitrogen contents closer to the 6.25 assumption, which is not the case of plant proteins, often containing non-protein-nitrogen components (free amino acids, nucleic acids), which can alter the actual protein content (Balogh-Hartmann et al., 2024; Mæhre et al., 2018). More accurate NPCFs should be used for different protein isolates, to prevent over- or under-estimation of protein content.

For instance, Sriperm et al., (2011) determined that the NPCF of soybean meal should be 5.64. Maubois & Lorient, (2016) found that the NPCF of milk proteins should be around 6.37. The average NPCF was of 3.99 for mushrooms and 5.47 for wheat, according to Fujihara (2010), and 4.76 for *Tenebrio molitor* according to Boulos et al. (2020a). Overall,

this emphasises that the use of a universal conversion factor can introduce significant errors, underscoring the need for source-specific NPCFs in protein analysis.

For the amino acid content, there was good agreement between obtained value and amino acid content from AB Agri. The consistently high correlation coefficients ($r > 0.9000$) across all samples confirm that the analytical methods employed in both laboratories yield highly comparable results. The slight inter-lab variability for yeast samples is consistent with prior research on yeast proteins, where the variability between laboratories has been attributed to differences in cell wall disruption and protein accessibility during hydrolysis (Zupan et al., 2009).

Amino acids that are hydrophilic, charged, or prone to forming bonds with the cell wall matrix containing β-glucans and mannoproteins are more likely to exhibit incomplete recovery except if robust cell wall disruption methods are employed, such as sonication or enzymatic treatments (Bzducha-Wróbel et al., 2014). To confirm this, an additional pre-treatment could be used to enhance yeast cell wall disruption before analysing amino acid content.

The similarity in values seems to reflect consistency in analytical methods and calibration, as well as the reliability of amino acid quantification across labs. However, definitive conclusions about relative performance require access to the original methods or standardised cross-laboratory validation.

Hypothesis: casein can be used as a protein isolate reference across the methods and is consistent between batches.

The sum of amino acids recovered during analysis should be used carefully as an indicator of true protein content. Amino acids may oxidise or degrade during sample preparation, leading to discrepancies. Furthermore, some amino acids such as cystine are susceptible to degradation or incomplete release during hydrolysis. Tryptophan was also not measured; therefore, the total amino acid sum would be underestimated. Using crude protein as the basis aligns with standard practices in protein evaluation. Calculated from total nitrogen content, crude protein provides a more universally accepted reference for comparing protein quality across studies (Boulos et al., 2020b; Reilly et al., 2020; Sweeney & Rexroad, 1987).

Casein amino acid content, based on crude protein content, shows consistent results with results from previous studies (deMan, 1999; Eklund et al., 2010; J. Liu et al., 2019). The absence of serine digestibility data for batch three is attributed to software integration issues in TraceFinder following a software switch. Serine elutes early during LC-MS/MS analysis, which makes its peak more susceptible to poor resolution, baseline instability, and overlapping with unretained compounds.

The baseline drift during column equilibration and the narrow poorly resolved peak of serine exacerbate the inability of the software to accurately identify and quantify serine. Addressing these issues would require optimisation of chromatographic conditions, refinement of integration settings, and validation of software performance for early-eluting compounds.

The casein amino acid consistency across digestibility batches demonstrates that it is a reliable reference protein for digestion studies and that digestibility results are reproducible between batches.

Hypothesis: the digestion method allows for grading differentiation between protein isolates of the same species.

Hydrolysed bioethanol yeast (LIV-09-HY 40) was more digestible than dry bioethanol yeast (LIV-10-IDY-LC) in terms of total amino acid digestibility. This suggests greater accessibility of amino acids for enzymatic digestion. This can be explained by the hydrolysis process step, making the free amino acids more accessible. On the other hand, dry bioethanol yeast might have a resistant cell wall from ethanol fermentation and drying, making it more difficult to digest.

Lysine, methionine and threonine digestibility were the main focus for indispensable amino acids, as they are the limiting amino acids for most animal feeds. The dried pot ale yeast (LIV-15-PA-RD-Y) is a liquid by-product of distillation that is less refined. Its lysine digestibility was significantly lower than the one of hydrolysed bioethanol yeast and casein.

This protein isolate might contain unhydrolysed β-glucans and mannans on the cell wall. As the dried pot ale yeast has not undergone hydrolysis, the proteins in it may remain in larger, more complex structures, which can make it harder for the digestive enzymes to break down. This would directly affect the availability of lysine and other amino acids.

Brewer's yeast 1 (LIV-11-BY-RD) and 2 (LIV-12-BY-SD) undergo autolysis, enabling a partial cell wall breakdown, making them highly digestible (Gao et al., 2024; J. Wang et al., 2018). Despite these differences between yeast samples, no significant difference was observed for methionine nor threonine digestibility, except when compared with casein. However, the DIAAS gives the ability to rank the samples, with dried pot ale yeast having the lowest DIAAS (0.77), and Brewer's yeast 2 having the highest DIAAS (1.75).

The primary driver of the DIAAS in the yeast samples is digestibility, influenced by processing. The low digestibility of some amino acids and total amino acid in dried pot ale yeast likely contributes to the lower DIAAS for that sample. Brewer's yeast, with its partially broken-down cell wall, might allow the digestive system to absorb amino acids more efficiently, which is why it shows a high digestibility and a higher DIAAS, even though its amino acid composition is not drastically different from other yeast samples.

Hydrolysis and autolysis increase amino acid availability, improving the DIAAS. Alternatively, an unhydrolysed and less refined sample such as the dried pot ale yeast has a resistant cell wall disrupting digestion, leading to lower digestibility and a lower DIAAS. The digestion method enables progressive distinction between protein isolates from the same species.

Hypothesis: the digestion method allows for gradual differentiation across a wide range of protein isolates.

Batch one was composed of one microbial protein, one sunflower meal, and one sunflower protein concentrate. The microbial protein had a lysine content three times

higher than that of the sunflower proteins in batch one, and a significantly higher lysine digestibility, resulting in a high lysine DIAAR of 1.87. In contrast, it exhibited a low histidine content. This demonstrates that the key factor for the DIAAS in this batch is the amino acid content. *Clostridium sp.* emerges as a valuable lysine source, relevant for animal feed formulations, as lysine is one of the main limiting amino acids in animal feed. When using Clostridium-derived lysine for animal feed, only non-toxigenic strains should be used, and proper processing is required to ensure safety.

In the initial phase of the study (batch one), although variability was observed between the initial nine replicates, the trends and patterns in the data remained consistent, suggesting that the variability was not significant enough to affect the overall conclusions (SEM < 10%).

Given resource constraints (time and budget), it was determined that reducing the number of replicates to four would still provide reliable results. As the INFOGEST network is a standardised protocol for *in vitro* digestion model to minimise variability between laboratories and differences between experimental groups were sufficient enough to require fewer replicates (different protein type or different treatment/fermentation processes), four technical replicates were generated for each sample.

Batch three consisted of three soya-based samples, two sunflower-based samples, two rapeseed-based samples, and one pea-based sample. The lower total amino acid digestibility of rapeseed meal (LIV-04-RM-B) can be attributed to its complex plant-based

matrices, including anti-nutritional factors such as fibre, mainly composed of non-starch polysaccharides, which hinder enzymatic access to proteins (Rezvani et al., 2012). This is reflected in the pellet weight, which, while not statistically significant, shows a trend towards reduced digestibility due to the presence of undigested material.

Coarse particles with visible fragments, likely residual fibre, exhibited higher pellet weights, indicating a greater non-absorbable fraction post-digestion. In contrast, fine, homogeneous samples such as casein and pea protein showed lower pellet weights, reflecting better digestion and absorption. Particle size played a critical role in these outcomes, with smaller, uniform particles facilitating greater enzymatic accessibility.

While the impact of particle size on digestibility is acknowledged, unsieved samples provided a more representative model of the feed typically given to chickens. Commercial poultry feed naturally includes a mix of coarse and fine particles, reflecting the inherent variability in particle size. By avoiding sieving, the study ensured that digestion conditions aligned more closely with real-world feeding scenarios, gathering data with greater practical relevance. Sieving would have artificially standardised particle size distribution, potentially skewing results and reducing their applicability to commercial settings.

Despite being protein isolates, some sources contained elements that could inhibit or disrupt the digestion process. For example, s), a well-regarded plant protein source, likely exhibited moderate digestibility due to residual anti-nutritional compounds such as trypsin inhibitors. Trypsin inhibitors are a type of protease inhibitor specifically targeting

trypsin, which is a digestible enzyme essential for protein breakdown. Trypsin inhibitors are commonly found in legumes such as peas and soybeans, grains, seeds and nuts (Avilés-Gaxiola et al., 2018).

Fermentation and heat can mitigate the presence of anti-nutritional factors as many trypsin inhibitors are heat-sensitive (Salter & Lopez-Viso, 2021). However, heat processing can lead to the formation of Maillard reaction products, where reducing sugars react with amino acids like lysine, reducing their availability for digestion. The DIAAS of 0.69 for soybean meal, associated with sulphur-containing amino acids such as methionine, highlights its potential need for methionine supplementation in animal feed. However, the high DIAAR of soya-based ingredients, apart from their limiting amino acid, validate their high protein quality.

The enzymatic and thermo-mechanical treatments applied to Alpha Soy and AlphaSoy Gold significantly improved lysine and methionine digestibility, as these processes reduced anti-nutritional factors such as trypsin inhibitors and phytates, which are present in higher amounts in untreated soybean meal.

While sunflower protein concentrate typically has a high crude protein content, this does not always translate into higher digestibility or DIAAS. Harsh processing can degrade protein quality, as observed in sunflower protein concentrate and pea protein concentrate, Sunflower protein concentrate demonstrated lower digestibility and DIAAS compared to its less-processed counterparts.

The pellet weight results aligned with the digestibility values, where samples such as sunflower protein concentrate exhibited significantly higher pellet weights, indicating a greater fraction of undigested material. This residual material likely prevented enzymatic access to amino acids, as reflected in the lower lysine digestibility. In contrast, casein, with its smooth and uniform texture, had the lowest pellet weight and highest total amino acid digestibility, highlighting the impact of physical properties on protein quality and digestibility outcomes.

Overall, the determined limiting amino acid for each protein isolate matched expectations, further suggesting that the digestion method combined with LC-MS/MS analysis enables a gradual and accurate differentiation between various protein isolates.

Hypothesis: the degree of hydrolysis allows for gradual differentiation across a wide range of protein isolates.

The OPA assay is a time and money-saving assay, which could be a good tool to discriminate samples. OPA, in the presence of reduced sulfhydryl groups, reacts with the primary amino groups found in terminal amino acids and the ε-amino group of lysine to form fluorescent moieties. However, OPA does not react directly with proline and reacts poorly with cysteine (Lee & Drescher, 1979). The overall amino acid profile of each sample can therefore influence how detectable certain amino acids are, resulting in variation in the overall results.

Due to intra-variability between samples, no significant difference was observed for batch three. Even within the same protein isolate, there can be slight differences in

composition or processing outcomes such as batch-to-batch variation in protein concentration or structure. This can be explained by the matrix effect in which components from the samples can interfere with the method. Residual non-protein components like fibre, lipids, or carbohydrates, may affect the accessibility or reactivity of amino groups during the OPA reaction (Gałęzowska et al., 2021; Lai et al., 1991).

The significant difference in the degree of hydrolysis in batch one is mainly attributed to the biological difference in amino acid content and protein source, between the microbial protein and the sunflower proteins. This shows that amino acid analysis is paramount for protein isolates to pick up differences between protein isolates from the same source as the OPA method might be not sensitive enough.

The methodology allows for comparative ranking across all protein isolates

All protein isolates DIAAS highlight the moderate to high protein quality of most samples, with Brewer's yeast 1 (LIV-11-BY-RD) and 2 (LIV-12-BY-SD) being up to 70% higher than casein. It has been recently shown that yeast samples have a high protein quality (Ma et al., 2023).

The DIAAS allows for rapid identification of potential complementation between several protein isolates to improve protein quality. Furthermore, DIAAS is theoretically based on ileal digestibility, which is obtained by *in vivo* assays. Samples of interest would need to go through *in vivo* digestions to prove the original estimation from this system.

In vivo systems for determining digestibility and protein quality are the gold standard for measuring actual nutrient absorption, but they come with a variety of challenges and

limitations. The key aspects of those limitations are the carrier matrices, endogenous losses, ethics, animal variation, and cost (Santos-Sánchez et al., 2024).

Carrier matrices are commonly used to deliver protein isolates to animals; however, these matrices can interfere with digestion (Akkerdaas et al., 2022; Zahir et al., 2018). This could skew the results if the carrier material is not digestible or competes for absorption in the gut, resulting in a protein appearing less digestible than it is in a natural setting (Schulten et al., 2011).

Endogenous nitrogen losses, such as secretions from the intestines and sloughed intestinal cells, can affect the *in vivo* digestibility data. These losses may result in an overestimation of protein digestibility since the nitrogen lost is not derived from the protein isolate being tested (Grala et al., 1998).

Biological differences between individual animals can introduce variability in digestion, requiring larger sample sizes to account for this inconsistency. Additionally, these studies are resource-intensive, involving significant financial and time commitments, as well as the use of invasive techniques that may cause animal stress. These challenges make *in vivo* assays less practical for large-scale or routine analysis.

2.5.Conclusion

This study comprehensively analysed seventeen protein isolates, highlighting their crude protein content, amino acid digestibility, and DIAAS values. The methods employed proved consistent across batches and aligned well with existing literature for casein, and company-reported data for the studied samples regarding their amino acid content,

affirming the reliability of the approaches used and providing valuable insights into their potential for feed applications.

Crude protein content showed good alignment with reported values, though slight discrepancies were noted, likely due to differences in calibration for the Dumas method. Cross-checking the calibration protocols between labs and accessing the original methods used by AB Agri could help validate these findings.

The digestion method proved effective in distinguishing some differences between protein isolates, even within the same species. Casein stood out as a reliable reference protein, showing high digestibility and strong DIAAS, as well as reproducibility across batches. Among the yeast samples, hydrolysed bioethanol yeast had the highest digestibility thanks to the hydrolysis process making its proteins more accessible. In contrast, dried pot ale yeast had lower digestibility, likely due to residual cell wall components.

For plant-based proteins, rapeseed meal and pea protein concentrate had lower digestibility likely due to the presence of anti-nutritional factors, such as fibre and other complex matrix elements. Soybean meal performed moderately, showing the typical challenges of the *in vitro* digestion to assess coarse samples. DIAAS values provided further insight, highlighting methionine and lysine as common limiting factors in plant proteins, which points to the importance of blending or supplementing these proteins for better nutritional value.

Most of the samples provided by AB Agri were highly digestible isolates, which have been selected for that purpose. The OPA method was able to discriminate between protein isolates only when the amino acid content between samples was very different. Due to its sensitivity to sample matrix effects and its limited reactivity with certain amino acids, the OPA method could not discriminate between protein isolates with a similar amino acid content and digestibility.

This highlights the importance of the amino acid hydrolysis method, which is the golden standard to assess amino acid amounts. The method was optimised to be less dangerous, less expensive and less time-consuming than previous methods and the amino acid composition of casein seems to be coherent with expected values.

Overall, the DIAAS rankings provided a clear picture of protein quality, with Brewer's yeast samples emerging as exceptionally high-quality options, even surpassing casein in some cases. *Clostridium sp* also showed a high lysine content, digestibility and DIAAR, making it valuable in animal feed to meet animal nutrient requirements. These findings show that protein blending and careful processing can significantly enhance the nutritional value of protein sources. The methods used are effective and provide a solid foundation for improving the quality of protein isolates in the future.

Further work is required to assess how sample composition influences detection using the same *in vitro* system. A focus on more complex and biologically relevant matrix should be explored, by evaluating the method's performance on bacteria samples.

3. Focus on novel sources of protein: bacteria

3.1. Introduction

Protein is a macronutrient essential for animal health and nutrition. As global demand for livestock products continues to grow, the need for sustainable, high-quality protein sources for animal feed is increasing (S. W. Kim et al., 2019). Traditional protein sources, such as soy and fishmeal, are often associated with significant environmental impacts and intensive resource use. To address these challenges, novel protein sources are being explored, including bacteria, which offer advantages in terms of sustainability, scalability, and nutritional value (Elahi et al., 2022).

Bacteria hold great promise as an alternative to conventional feed ingredients. These single-cell proteins (SCPs) can be efficiently produced using a variety of substrates, such as industrial by-products and food waste, making them a highly sustainable option for animal feed and supporting a circular economy by valorising low-value streams (Carter & Codabaccus, 2022; Pereira et al., 2022). Additionally, bacteria can be grown in controlled environments, reducing reliance on arable land and helping to minimise environmental impact.

To be used in animal nutrition, bacterial biomass must meet feed-grade quality standards, ensuring consistency, safety, and freedom from contaminants, and production should comply with Hazard Analysis and Critical Control Points (HACCP) principles to mitigate microbiological, chemical, or physical hazards (FAO, 2020).

Bacteria must also satisfy species-specific nutritional requirements, providing adequate protein and digestible amino acids while keeping undesirable components (nucleic acids, residual substrates or anti-nutritional factors) within safe limits. Ensuring these criteria are met is essential for both animal health and performance and for the successful integration of bacteria into commercial feed formulations.

Non-protein nitrogen (NPN) compounds, such as nucleic acids and free amino acids, are prevalent in bacterial biomass and contribute substantially to total nitrogen content without representing true protein (Mæhre et al., 2018). The Dumas combustion method measures total nitrogen and applies a universal nitrogen-to-protein conversion factor (NPCF) of 6.25 to estimate crude protein, but this overestimates actual protein levels in bacteria by 15 to 25% due to NPN inflation. Source-specific NPCFs are recommended for accuracy, preventing misinterpretation of nutritional quality and ensuring reliable DIAAS calculations (Fujihara, 2010). Bacteria have been reported to have a NPCF between 5.1 and 5.6 (Mæhre et al., 2018).

Depending on their metabolic types, bacteria can vary significantly in their protein and their amino acid content, changing their digestibility. As they can use various substrates, their protein yield and amino acid content is affected.

Autotrophs like *Cupriavidus necator* may have a different amino acid composition compared to heterotrophs that grow on organic carbon sources (Jahn et al., 2021). *Cupriavidus necator* can also accumulate polyhydroxybutyrate (PHB), which is a biodegradable polyester mainly produced as an intracellular storage compound when

the bacterium is grown under nutrient-limited conditions with an excess of carbon (Obruca et al., 2017).

PHB has several benefits for industrial applications. This biodegradable polymer, made from renewable sources like sugar and glycerol, breaks down into CO_2 and water. It offers an eco-friendly alternative to polypropylene for packaging, containers, films, and seed coatings. While there is evidence that PHB can impact protein content and protein digestibility in bacterial biomass (Emilia et al., 2024), much of the current knowledge is still in the early stages. Further studies are needed to fully understand its effects on nutrient bioavailability, protein digestibility, and overall feed efficiency in animal systems.

Optimising growth conditions by using carbon and nitrogen sources from agricultural waste and adjusting environmental factors such as pH, oxygen levels, and temperature can enhance the protein quality of bacterial strains such as *Paracoccus pantotrophus*. This soil isolate has been shown to synthesise PHB under nutrient-limited conditions (Ucisik-akkaya, 2009).

One of the main aspects in evaluating the potential of bacteria as a feed source is their nutritional quality, particularly their protein and amino acid content. The crude protein content of bacterial biomass can vary significantly depending on the species and growth conditions. For instance, some bacterial strains have been reported to contain protein levels comparable to or even higher than conventional protein sources like soybean meal or fishmeal (Santillan et al., 2024).

The amino acid composition of bacteria is another critical aspect to consider. Indispensable amino acids, which cannot be synthesised by the animal body and must be obtained through diet, are of particular importance. The balance and availability of these indispensable amino acids determine the overall protein quality and its ability to meet animal nutritional requirements.

To assess the protein quality of novel food sources, including bacteria, various methods are utilised. One of them is the Digestible Indispensable Amino Acid Score (DIAAS), which has been recommended by the Food and Agriculture Organization (FAO) as the preferred method for evaluating protein quality. DIAAS takes into account both the amino acid profile of the protein source and its digestibility, providing a comprehensive measure of protein quality (Wolfe et al., 2016).

In vitro digestion methods have been developed to simulate the human gastrointestinal system and assess the digestibility of proteins. These methods, such as the INFOGEST static *in vitro* simulation of gastrointestinal food digestion, offer a standardised approach to evaluate protein digestibility without the need for animal trials. By combining *in vitro* digestion methods with amino acid analysis, the digestibility of individual amino acids can be determined and the DIAAS values calculated for novel protein sources.

The study described in this chapter evaluates the nutritional quality of various bacteria using the INFOGEST *in vitro* digestion method.

Aims

- To determine the protein quality of various bacteria with the INFOGEST *in vitro* digestion method
- To assess a potential relationship between PHB content in *Cupriavidus necator* and *in vitro* digestibility data with the INFOGEST *in vitro* digestion method

Hypotheses

- Differences in bacterial species influence the digestibility of amino acid
- Bioplastics (PHB) content influences protein content and total amino acid digestibility in *Cupriavidus necator*

3.2. Materials and methods

3.2.1. Materials

All chemicals and reagents were purchased from Merck (Dorset, UK) unless stated otherwise. The samples and a brief description of them are reported in Table 3.1. The bacteria selected represent diverse metabolic types, cell sizes, and PHB contents, and are non-pathogenic, making them promising feed candidates. Factors such as PHB accumulation, cell wall structure, and small cell size may affect digestibility and nutrient availability, warranting further evaluation for practical feed use.

Table 3.1: List of samples and description

List of samples	Description	Batch number[1]
Paracoccus pantotrophus (soil isolate)	CO_2 fixing, non-pathogenic, role in denitrification	1
Methylococcus capsulatus (Calysta)	Bath strain, methanotrophic	1
Cupriavidus necator (with PHB)	Wild type, H16, can produce and store polyhydroxybutyrate (PHB), presence of PHB in significant amounts	2
Cupriavidus necator (without PHB)	Wild type, H16, does not accumulate or contain significant levels of PHB, absence of PHB	2
Brevundimonas diminuta (small bacteria)	Small bacteria	2
Priestia megaterium (large bacteria)	Large bacteria	2
Escherichia coli (gram negative)	Gram negative	2
Bacillus subtilis (gram positive)	Gram positive	2
Thermus thermophilus (thermophile)	Thermophile	2
Psychrobacter frigidicola (psychrophile)	Psychrophile	2
Cupriavidus necator (H_2 limited, 2% PHB)	Wild type, H16, H_2 limited conditions, 2 g PHB/100 g dry cell or 2% PHB	3
Cupriavidus necator (O_2 limited, 25% PHB)	Wild type, H16, O_2 limited conditions, 25 g PHB/100 g dry cell or 25% PHB	3
Cupriavidus necator (CO_2 limited, 28% PHB)	Wild type, H16, CO_2 limited conditions, 28 g PHB/100 g dry cell or 28% PHB	3
Cupriavidus necator (NH_4 limited, 78% PHB)	Wild type, H16, NH_4 limited conditions, 78 g PHB/100 g dry cell or 78% PHB	3
Casein	Pure, high-quality protein from bovine milk (no 10545691, Thermo Scientific, MA, US)	3

[1]The 15 samples were processed in 3 batches: Batch 1 (2 samples digested across 3 days), Batch 2 (8 samples digested across a separate set of 3 days) and Batch 3 (5 samples digested across 4 days)

The samples were a range of bacteria with various characteristics. The Calysta strain of *Methylococcus capsulatus* was provided by Calysta and was commercially available at

the date it was acquired. Other bacteria were generated and provided by PhD students at the Biodiscovery Institute (BDI) (UoN) in April, July, September 2022 and January 2023.

For batch three, *Cupriavidus necator* wild-type strain H16 was cultivated under varying nutrient-limited conditions to produce biomass with differing levels of intracellular polyhydroxybutyrate (PHB). The PHB content was measured at the end of the growth phase and was characterised as follows: under H_2-limited conditions, the PHB content was 2 g PHB/100 g dry cell weight (2% PHB); under O_2-limited conditions, it was 25 g PHB/100 g dry cell weight (25% PHB); under CO_2-limited conditions, it was 28 g PHB/100 g dry cell weight (28% PHB); and under NH_4-limited conditions, the PHB content reached 78 g PHB/100 g dry cell weight (78% PHB).

Since it took several harvests using continuous fermentation to collect enough bacteria for each sample for further analysis, the bacterial cells from each harvest were combined into a single 50-mL tube for each sample. The samples were freeze-dried until constant weight upon handover. They were obtained in powder form after being ground using a pestle and a mortar.

3.2.2. Crude protein content

The crude protein content was determined using an EA 1112 elemental analyser (Thermo Scientific) to obtain the nitrogen content in bacteria, as described in Chapter 2.2.2, using the standard NPCF of 6.25.

3.2.3. *In vitro* digestion method

The bacterial samples were provided in freeze-dried powder form and were used directly without further pre-treatment. The *in vitro* digestion method was performed on bacteria following the INFOGEST static *in vitro* simulation of gastrointestinal food digestion described in Chapter 2.2.3. Three biological replicates were generated through three *in vitro* digestions on different days.

Each bacterium was weighed with 8% protein or 80 mg of protein per digestion sample for batches one and three, and 10% protein or 100 mg of protein per digestion sample for batch two. The protein content was increased to 10% in batch two to improve digestion efficiency and ensure more consistent protein quantification across the samples.

3.2.4. Amino acid analysis

The amino acid content of the bacteria was analysed before and after digestion by LC-MS/MS using a hydrolysis by oven crimp tube digestion. This method is described in Chapter 2.2.5.

3.2.5. Calculation of amino acid digestibility and digestible indispensable amino acid score (DIAAS)

The amount of individual amino acid digestibility and DIAAS were calculated with the equations described in Chapter 2.2.6.

3.2.6. Data analysis

Prior to statistical analysis, data were checked for normality and homogeneity of variance using GraphPad Prism, and the experimental design ensured independence of measurements. Data met the assumptions required for parametric testing.

Genstat (24th Edition) was used for statistical analyses. One-way ANOVA was applied followed by, when appropriate, a Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$. The graphs were produced with Graph Pad Prism 10.3.1.

3.3. Results

The results are presented in four parts: crude protein content of bacterial samples, and amino acid composition and amino acid digestibility with DIAAS values for each bacterial batch. Three batches were studied, with key outcomes including *in vitro* digestibility, DIAAR/DIAAS, and amino acid profiles summarised in the corresponding figures and tables.

3.3.1. Crude protein content

The protein content of *Paracoccus pantotrophus* (49.12 g/100g dry weight) was significantly lower than the protein content of *Methylococcus capsulatus* (75.75 g/100g dry weight) ($p < 0.05$), and both bacteria had a significantly lower protein content than that of casein, as shown in Figure 3.1.

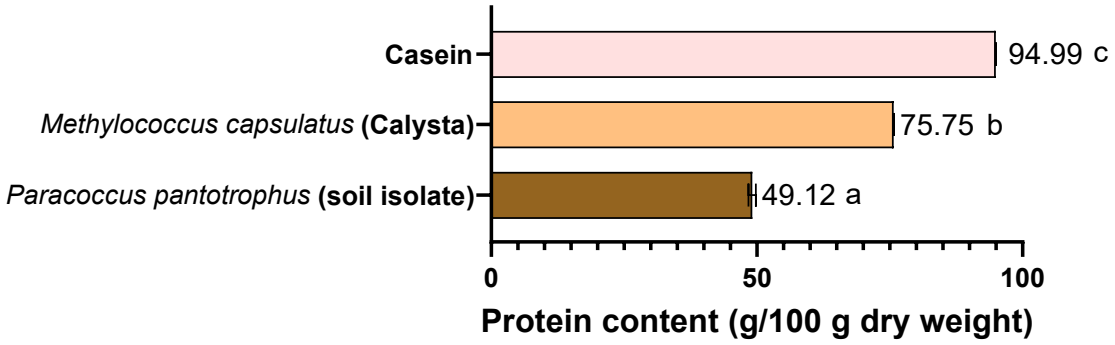


Figure 3.1: Crude protein content (g/100g dry weight) of bacteria in batch one using the Dumas method (N-analyser). Data is shown as mean with SD, n = 2 technical replicates per bacterium. There was a significant effect of bacterial protein source ($p < 0.001$), using a one-way ANOVA. Different letters $^{a, b, c}$ indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

The bacteria in batch two had a protein content ranging from 47.80 g/100g dry weight to 88.21 g/100g dry weight (Figure 3.2).

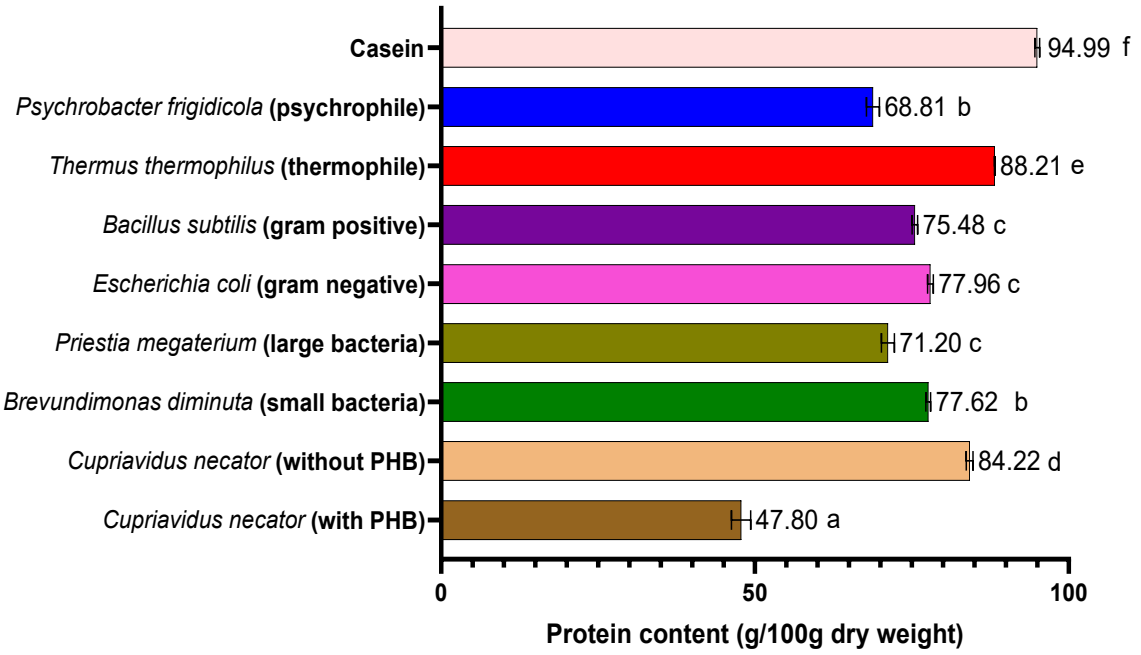


Figure 3.2: Crude protein content (g/100g dry weight) of bacteria in batch two using the Dumas method (N-analyser). Data is shown as mean with SD, n = 2 technical replicates per bacterium. There was a significant effect of bacterial protein source ($p < 0.001$), using a one-way ANOVA. Different letters $^{a, b, c, d, e, f}$ indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

The protein content of casein was significantly higher than that of all the bacterial ($p < 0.05$). The protein content ranged from 47.80 g/100g dry weight for *Cupriavidus necator* (with PHB) to 88.21 g/100g dry weight for *Thermus thermophilus*. *Psychrobacter frigidicola* had a significantly lower protein content than *Thermus thermophilus* ($p < 0.05$). The protein content of *Brevundimonas diminuta* was significantly higher than that of *Priestia megaturium* ($p < 0.05$). *Cupriavidus necator* protein content was significantly higher without PHB than with PHB ($p < 0.05$).

Figure 3.3 shows the crude protein content of bacteria in batch three.

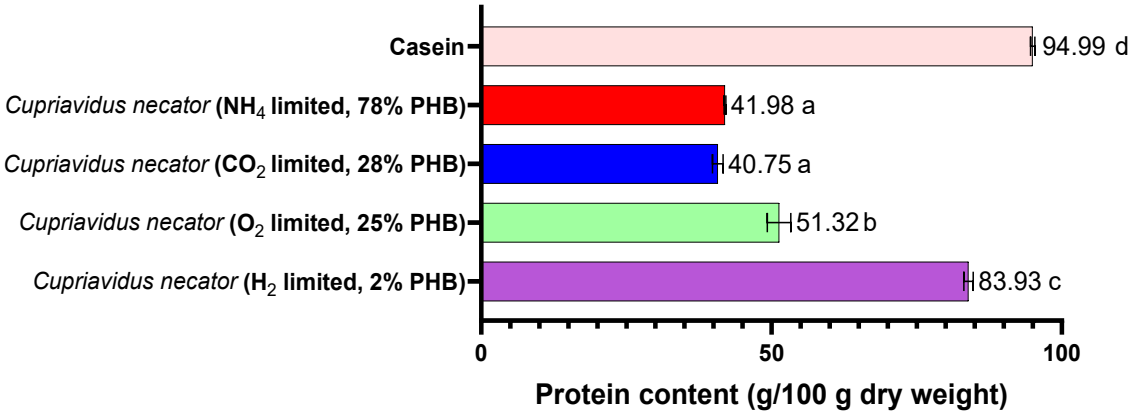


Figure 3.3: Crude protein content (g/100g dry weight) of bacteria in batch three using the Dumas method (N-analyser), data is shown as mean with SD, n = 2 technical replicates per bacterial protein. There was a significant effect of protein feed source ($p < 0.001$), using a one-way ANOVA. Different letters $^{a, b, c, d}$ indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

Casein had a significantly higher protein content than that of all the bacteria. The protein content ranged from 41.98 g/100g dry weight for *Cupriavidus necator* with 78% PHB to 83.93 g/100g dry weight for *Cupriavidus necator* with 2% PHB. The protein content of *Cupriavidus necator* with 25% PHB was significantly higher than that of *Cupriavidus necator* with 28% PHB and 78% PHB, but significantly lower than that of *Cupriavidus necator* with 2% PHB ($p < 0.05$).

The correlation between PHB content and crude protein content in batch three, as shown in Figure 3.4, exhibited a moderate negative correlation ($r = -0.7126$) but not statistical significance was found ($p = 0.2874$).

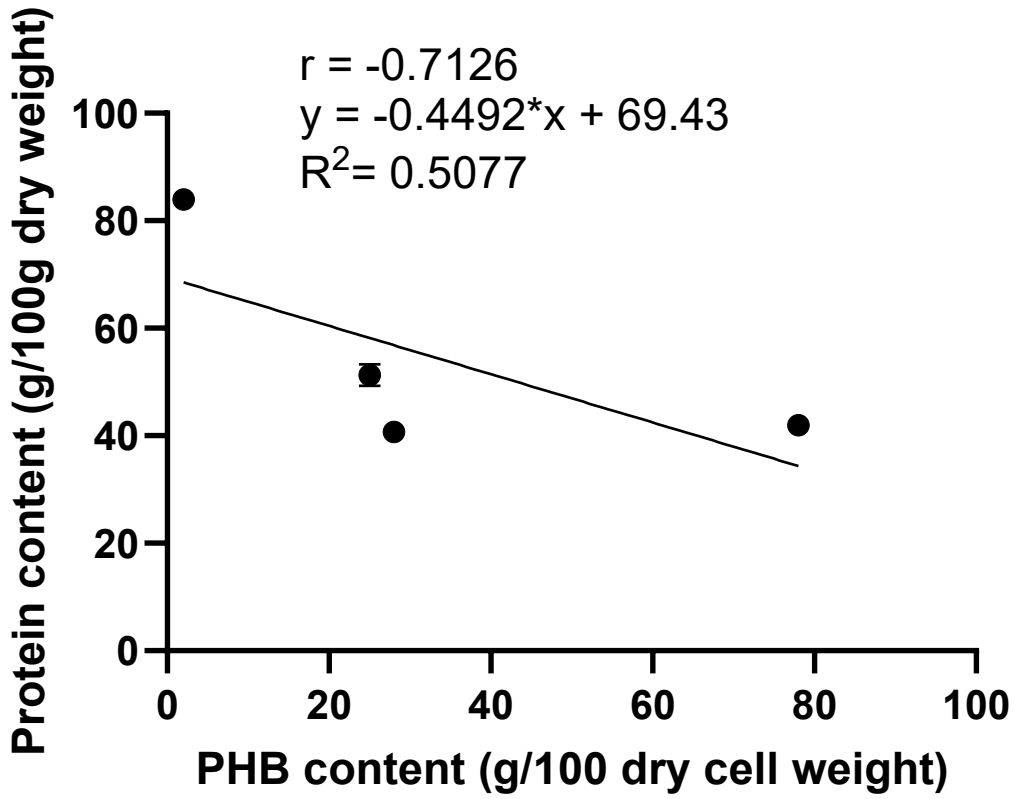


Figure 3.4: Correlation of PHB content with crude Protein content (Dumas method) of bacteria in batch three. Each point represents a *Cupriavidus necator* sample.

The calculated R^2 value (0.5077) suggests that approximately 50.77% of the variation in crude protein content can be attributed to differences in PHB content. However, the correlation was not statistically significant (p = 0.2874).

3.3.2. Amino acid content, amino acid digestibility and DIAAS of bacteria in batch one

The amino acid content of the bacterial in batch one is reported in Table 3.2.

Table 3.2: Amino acid content of bacteria in batch one

Amino acid content (g/100g protein) [1]				
Amino acid	***Paracoccus pantotrophus* (soil isolate)**	***Methylococcus capsulatus* (Calysta)**	**Casein**	**p-value[2]**
Indispensable amino acids				
Lysine	3.95 ± 1.15[a]	4.19 ± 0.80[a]	8.02 ± 0.97[b]	0.036*
Methionine	1.28 ± 0.17[a]	2.23 ± 1.20[b]	3.10 ± 0.18[b]	0.002*
Threonine	3.46 ± 0.02	2.89 ± 0.66	3.78 ± 0.29	0.117
Valine	4.83 ± 0.21[a]	4.13 ± 1.33[a]	6.74 ± 0.60[b]	0.022*
Leucine	5.81 ± 0.10	8.07 ± 3.59	9.73 ± 1.56	0.298
Isoleucine	3.48 ± 0.02	4.59 ± 1.73	4.88 ± 0.40	0.112
Histidine	1.64 ± 0.06[a]	1.31 ± 0.22[a]	3.04 ± 0.28[b]	0.004*
Phenylalanine	3.20 ± 0.11[a]	2.71 ± 0.37[a]	5.30 ± 0.49[b]	0.008*
Dispensable amino acids				
Tyrosine	2.40 ± 0.02	3.21 ± 1.66	4.72 ± 0.30	0.164
Cystine	0.40 ± 0.02	0.50 ± 0.31	0.39 ± 0.15	0.962
Serine	3.19 ± 0.14[a]	4.36 ± 0.89[ab]	5.99 ± 0.81[b]	0.025*
Glycine	4.21 ± 0.19[b]	3.01 ± 0.70[b]	1.95 ± 0.10[a]	0.002*
Glutamic acid	11.98 ± 0.31	11.32 ± 2.70	23.93 ± 4.28	0.029*
Proline	4.60 ± 0.08[a]	3.39 ± 0.64[a]	12.21 ± 1.45[b]	0.004*
Alanine	7.52 ± 0.49[b]	5.67 ± 0.47[b]	2.92 ± 0.31[a]	0.005*
Arginine	5.27 ± 0.05	6.75 ± 4.52	3.73 ± 0.33	0.286
Aspartic acid	7.45 ± 0.54	8.27 ± 2.19	6.94 ± 0.68	0.061

[1]Data is shown as mean with SD, n = 2 technical replicates per bacterial protein

[2]p-value for one-way ANOVA

*Significant difference. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$)

Casein had a significantly higher lysine, valine, histidine, phenylalanine, and proline content than that of *Paracoccus pantotrophus* and *Methylococcus capsulatus* ($p < 0.05$). Methionine content was significantly lower ($p < 0.05$) in *Paracoccus pantotrophus* than in *Methylococcus capsulatus* and casein. Serine content was significantly higher in *Paracoccus pantotrophus* ($p < 0.05$) than in casein, but not in *Methylococcus capsulatus*.

Casein had a significantly lower glycine and alanine content compared to that of *Paracoccus pantotrophus* and *Methylococcus capsulatus* ($p < 0.05$). *Parococcus pantotrophus* had a significantly lower methionine content than that of *Methylococcus capsulatus* and casein.

The amino acid digestibility of bacteria in batch one is reported in Table 3.3.

Table 3.3: Bacterial amino acid *in vitro* digestibility (batch one)

	Digestibility (%) [1]			
Amino acid	***Paracoccus pantotrophus* (soil isolate)**	***Methylococcus capsulatus* (Calysta)**	**Casein**	**p-value[2]**
Indispensable amino acids				
Lysine	63.27 ± 2.13^{a}	73.07 ± 2.18^{b}	96.73 ± 1.63^{c}	<0.001*
Methionine	71.43 ± 4.85^{a}	77.80 ± 6.49^{ab}	96.40 ± 1.80^{b}	0.024*
Threonine	38.03 ± 6.09^{a}	47.47 ± 11.04^{a}	87.47 ± 4.78^{b}	0.009*
Valine	58.77 ± 6.06^{a}	64.97 ± 8.24^{a}	94.50 ± 2.76^{b}	0.013*
Leucine	61.47 ± 3.75^{a}	66.83 ± 10.16^{ab}	96.20 ± 2.09^{b}	0.017*
Isoleucine	55.30 ± 4.90^{a}	73.70 ± 0.80^{b}	93.00 ± 3.83^{c}	<0.001*
Histidine	50.40 ± 2.35^{a}	66.90 ± 4.74^{a}	94.47 ± 3.43^{b}	<0.001*
Phenylalanine	56.50 ± 4.18^{a}	65.00 ± 2.85^{a}	96.23 ± 1.93^{b}	<0.001*
Dispensable amino acids				
Tyrosine	35.30	11.80	86.80 ± 8.36	N/A
Cystine	19.90 ± 14.85	39.70 ± 14.19	79.50 ± 10.50	0.400
Serine	65.20 ± 2.37	73.60 ± 16.49	89.80 ± 5.89	0.277
Glycine	67.95 ± 7.80^{a}	47.05 ± 1.59^{a}	99.63 ± 5.57^{b}	0.007*
Glutamic acid	68.90 ± 3.72^{a}	79.73 ± 1.52^{a}	96.33 ± 1.16^{b}	<0.001*
Proline	66.30 ± 4.45^{a}	73.47 ± 6.28^{a}	97.70 ± 1.18^{b}	0.006*
Alanine	77.90 ± 6.94	83.90 ± 8.82	96.07 ± 3.45	0.193
Arginine	56.70 ± 6.04	62.40 ± 10.96	94.90 ± 4.16	0.066
Aspartic acid	63.23 ± 5.23	73.23 ± 14.05	96.03 ± 2.28	0.090
Total amino acid	60.70 ± 2.59^{a}	68.83 ± 7.90^{a}	95.07 ± 2.45^{b}	0.007*

[1]Data is shown as mean with SEM, n = 3 technical replicates per bacterial protein; for values shown as single measurements, error terms could not be calculated

[2]p-value for one-way ANOVA (values shown as single measurements were excluded)

*Significant difference. Different letters $^{a, b, c}$ indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$)

The crude protein is 49.12 g/100g dry weight for *Parococcus pantotrophus* and 75.75 g/100g dry weight for *Methylococcus capsulatus*. For most amino acids, casein had a significantly higher digestibility than that of *Methylococcus capsulatus* and *Paracoccus pantotrophus* ($p < 0.05$). *Methylococcus capsulatus* had a significantly higher lysine digestibility than that of *Paracoccus pantotrophus* but significantly lower lysine digestibility than that of casein ($p < 0.05$).

The DIAAS of bacteria in batch one varied between 0.37 and 0.72, as reported in Table 3.4.

Table 3.4: DIAAR and DIAAS of bacteria (batch one)

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43		
Casein (Protein reference)	1.41	1.41	1.32	1.31	1.28	1.76	**1.06**[1]	1.46	**1.06**	**Threonine**
Paracoccus pantotrophus (soil isolate)	**0.41**	0.60	0.54	0.44	**0.37**	0.51	0.43	0.66	**0.37/ 0.41**	**SAA/Histidine**
Methylococcus capsulatus (Calysta)	**0.77**	1.81	1.38	0.94	1.18	**0.72**	**0.77**	1.07	**0.72/ 0.77**	**AAA/Histidine /Threonine**

[1]Values in bold represent the lowest DIAAR and the DIAAS

Casein had the highest DIAAS (1.06) with threonine as a limiting amino acid. The DIAAS of *Paracoccus pantotrophus* was 0.41, and SAA were the limiting amino acids. The histidine DIAAR was close to the DIAAS, at 0.41. For *Methylococcus capsulatus*, the limiting amino acid was the aromatic amino acids (0.72), with close DIAAR for histidine and threonine (both 0.77). The DIAAR of *Methylococcus capsulatus* ranged from 0.37 to 0.66 and the DIAAR of *Paracoccus pantotrophus* from 0.72 to 1.81.

3.3.3. Amino acid content, amino acid digestibility and DIAAS of bacteria in batch two

The amino acid content of bacteria in batch two is reported in Table 3.5.

Table 3.5: Amino acid content of bacteria in batch two

Amino acid content (g/100g protein)[1]										
Amino acid	***Cupriavidus necator*** **(with PHB)**	***Cupriavidus necator*** **(without PHB)**	***Brevundimona diminuta*** **(small bacteria)**	***Priestia megaterium*** **(large bacteria)**	***Escherichia coli*** **(gram negative)**	***Bacillus subtilis*** **(gram positive)**	***Thermus thermophilus*** **(thermophile)**	***Psychrobacter frigidicola*** **(psychrophile)**	**Casein**	**p-value[2]**
Indispensable amino acids										
Lysine	5.54 ± 0.06^{ab}	5.46 ± 0.06^{ab}	3.05 ± 1.12^{a}	5.20 ± 0.97^{ab}	4.18 ± 0.54^{a}	5.31 ± 0.52^{ab}	5.17 ± 1.30^{ab}	4.64 ± 0.39^{ab}	8.02 ± 0.97^{b}	0.009*
Methionine	1.86 ± 0.07^{ab}	2.03 ± 0.16^{ab}	1.21 ± 0.46^{a}	1.68 ± 0.43^{a}	1.36 ± 0.21^{a}	1.73 ± 0.30^{a}	1.33 ± 0.40^{a}	1.17 ± 0.14^{a}	3.10 ± 0.18^{b}	0.002*
Threonine	3.63 ± 0.02	3.43 ± 0.22	2.67 ± 1.12	2.82 ± 0.45	2.84 ± 0.40	2.91 ± 0.48	3.70 ± 0.99	2.66 ± 0.25	3.78 ± 0.29	0.347
Valine	4.82 ± 0.20^{ab}	4.55 ± 0.30^{ab}	3.98 ± 0.88^{ab}	3.91 ± 0.66^{ab}	3.60 ± 0.28^{a}	3.89 ± 0.27^{a}	5.27 ± 1.23^{ab}	3.23 ± 0.31^{a}	6.74 ± 0.60^{b}	0.008*
Leucine	6.59 ± 0.35	6.67 ± 0.62	4.95 ± 1.64	5.65 ± 1.17	4.88 ± 0.35	5.76 ± 0.90	9.25 ± 2.94	4.29 ± 0.15	9.73 ± 1.56	0.028*
Isoleucine	3.37 ± 0.10^{ab}	3.26 ± 0.23^{ab}	2.89 ± 0.51^{ab}	3.57 ± 0.57^{ab}	3.04 ± 0.31^{ab}	3.48 ± 0.41^{ab}	2.95 ± 0.90^{ab}	2.77 ± 0.11^{a}	4.88 ± 0.40^{b}	0.031*
Histidine	1.58 ± 0.07^{a}	1.73 ± 0.13^{a}	1.57 ± 0.13^{a}	1.68 ± 0.33^{a}	1.36 ± 0.19^{a}	1.69 ± 0.07^{a}	1.64 ± 0.40^{a}	1.10 ± 0.12^{a}	3.04 ± 0.28^{b}	<0.001*
Phenylalanine	3.56 ± 0.13^{ab}	3.58 ± 0.12^{ab}	3.21 ± 0.71^{ab}	2.93 ± 0.51^{a}	2.77 ± 0.33^{a}	2.96 ± 0.15^{a}	3.85 ± 1.02^{ab}	2.36 ± 0.24^{a}	5.30 ± 0.49^{b}	0.008*
Dispensable amino acids										
Tyrosine	2.78 ± 0.05^{bc}	2.28 ± 0.08^{abc}	1.79 ± 0.49^{abc}	2.30 ± 0.31^{abc}	1.41 ± 0.03^{ab}	2.39 ± 0.27^{bc}	2.94 ± 0.67^{c}	0.89 ± 0.16^{a}	4.72 ± 0.30^{d}	<0.001*
Cystine	0.35 ± 0.07	0.53 ± 0.08	0.40 ± 0.12	0.35 ± 0.08	0.49 ± 0.02	0.38 ± 0.02	0.20 ± 0.12	0.40 ± 0.04	0.39 ± 0.15	0.145
Serine	3.36 ± 0.09^{a}	3.13 ± 0.13^{a}	2.94 ± 0.01^{a}	2.66 ± 0.37^{a}	2.37 ± 0.35^{a}	2.87 ± 0.46^{a}	2.89 ± 0.92^{a}	2.50 ± 0.13^{a}	5.99 ± 0.81^{b}	<0.001*
Glycine	3.73 ± 0.16^{b}	3.05 ± 0.00^{ab}	2.95 ± 0.40^{ab}	2.83 ± 0.42^{ab}	2.34 ± 0.13^{a}	2.56 ± 0.05^{ab}	3.19 ± 0.56^{ab}	3.14 ± 0.25^{ab}	1.95 ± 0.10^{a}	0.006*
Glutamic acid	10.37 ± 0.63^{a}	10.44 ± 0.45^{a}	13.54 ± 2.73^{ab}	13.97 ± 2.97^{ab}	9.37 ± 1.45^{a}	13.30 ± 1.02^{ab}	12.81 ± 3.33^{a}	9.01 ± 0.93^{a}	23.93 ± 4.28^{b}	0.004*
Proline	3.67 ± 0.16^{a}	3.73 ± 0.16^{a}	3.35 ± 0.74^{a}	2.78 ± 0.50^{a}	2.47 ± 0.27^{a}	3.09 ± 0.27^{a}	5.16 ± 1.37^{a}	2.93 ± 0.33^{a}	12.21 ± 1.45^{b}	<0.001*
Alanine	6.69 ± 0.32	6.65 ± 0.71	4.62 ± 3.33	7.14 ± 1.37	4.09 ± 0.82	7.13 ± 0.90	6.70 ± 2.13	5.10 ± 0.48	2.92 ± 0.31	0.141
Arginine	5.86 ± 0.10	6.03 ± 0.33	5.72 ± 0.73	4.36 ± 0.65	4.25 ± 0.45	4.20 ± 0.24	7.48 ± 2.20	3.79 ± 0.61	3.73 ± 0.33	0.019*
Aspartic acid	7.96 ± 0.38	6.82 ± 0.08	6.77 ± 0.38	6.18 ± 0.80	5.88 ± 0.59	6.07 ± 0.46	6.13 ± 1.10	6.37 ± 0.45	6.94 ± 0.68	0.128

[1]Data is shown as mean with SD, n = 2 technical replicates per bacterial protein

[2]p-value for one-way ANOVA

*Significant difference. Different letters [a, b, c, d] indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

Overall, indispensable amino acid contents varied among bacteria, with some strains exhibiting levels comparable to or higher than casein, while others were significantly lower. Among the bacteria, *Cupriavidus necator* (with and without PHB) and *Brevundimonas diminuta* generally had lower amino acid contents compared to other bacterial sources such as *Thermus thermophilus* and *Bacillus subtilis*.

Brevundimona diminuta and *Escherichia coli* had a significantly lower lysine content than that of casein ($p < 0.05$) but were not statistically different from the bacteria. Methionine content of casein was significantly lower than that of *Brevundimona diminuta*, *Priestia megaterium*, *Escherichia coli*, *Bacillus subtilis*, *Thermus thermophilus* and *Psychrobacter frigidicola* ($p < 0.05$).

Indispensable amino acid digestibility varied between 47.13% and 92.53% across bacteria in batch two, as reported in Table 3.6.

Table 3.6: Bacterial amino acid *in vitro* digestibility (batch two)

	Digestibility (%) [1]									
Amino acid	***Cupriavidus necator* (with PHB)**	***Cupriavidus necator* (without PHB)**	***Brevundimonas diminuta* (small bacteria)**	***Priestia megaterium* (large bacteria)**	***Escherichia coli* (gram negative)**	***Bacillus subtilis* (gram positive)**	***Thermus thermophilus* (thermophile)**	***Psychrobacter frigidicola* (psychrophile)**	**Casein**	**p-value[2]**
					Indispensable amino acids					
Lysine	53.00 ± 7.66^{a}	77.10 ± 3.28^{ab}	80.30 ± 5.75^{ab}	80.77 ± 10.64^{ab}	82.73 ± 5.00^{ab}	70.97 ± 12.25^{ab}	72.17 ± 3.50^{ab}	88.87 ± 5.92^{ab}	94.03 ± 0.87^{b}	0.030*
Methionine	71.10 ± 5.06^{ab}	84.97 ± 2.96^{ab}	77.23 ± 4.04^{ab}	91.90 ± 5.39^{b}	80.20 ± 3.10^{ab}	66.70 ± 15.76^{ab}	53.90 ± 4.57^{a}	96.35 ± 0.86^{b}	93.63 ± 1.74^{b}	0.007*
Threonine	59.43 ± 3.94^{ab}	73.97 ± 7.78^{ab}	71.63 ± 2.92^{ab}	90.73 ± 4.87^{b}	79.93 ± 5.69^{ab}	73.87 ± 14.58^{ab}	47.13 ± 5.27^{a}	93.23 ± 4.99^{b}	86.93 ± 4.05^{b}	0.003*
Valine	56.57 ± 10.50	75.60 ± 2.94	76.80 ± 3.99	84.43 ± 11.54	81.70 ± 4.15	69.70 ± 17.58	52.10 ± 7.85	87.87 ± 11.04	88.47 ± 1.42	0.108
Leucine	67.87 ± 7.62	75.03 ± 3.51	80.27 ± 4.24	89.10 ± 5.98	84.67 ± 0.29	80.90 ± 9.82	63.57 ± 3.36	92.53 ± 5.83	92.10 ± 2.00	0.012*
Isoleucine	66.70 ± 8.21	72.93 ± 10.02	84.63 ± 2.92	86.40 ± 4.66	86.47 ± 2.20	76.57 ± 11.73	71.13 ± 4.99	88.93 ± 8.57	87.03 ± 2.99	0.269
Histidine	71.63 ± 7.47	89.37 ± 2.05	81.37 ± 5.06	88.23 ± 6.54	92.03 ± 3.81	79.50 ± 10.26	82.67 ± 3.58	94.03 ± 4.76	95.33 ± 0.79	0.119
Phenylalanine	63.17 ± 4.63^{ab}	75.30 ± 1.83^{ab}	75.43 ± 2.79^{ab}	88.70 ± 7.24^{ab}	84.37 ± 3.18^{ab}	73.70 ± 13.25^{ab}	57.67 ± 5.35^{a}	82.07 ± 7.05^{ab}	94.80 ± 0.49^{b}	0.012*
					Dispensable amino acids					
Tyrosine	24.67 ± 0.41	39.65 ± 2.74	25.00	74.95 ± 20.45	16.20	58.90 ± 33.56	28.80	78.60 ± 12.33	85.20 ± 0.42	0.054
Cystine	51.55 ± 23.80	60.60	95.67 ± 4.33	80.45 ± 15.96	75.90 ± 19.68	79.13 ± 10.61	100.00 ± 0.00	72.57 ± 27.43	68.40	0.401
Serine	35.63 ± 16.93^{ab}	66.60 ± 2.88^{ab}	68.47 ± 8.44^{ab}	91.45 ± 6.98^{ab}	79.90 ± 2.29^{ab}	62.37 ± 20.18^{ab}	21.37 ± 4.46^{a}	20.80	72.40 ± 8.82^{b}	0.004*
Glycine	N/A	28.80	86.10 ± 11.10	100.00 ± 0.00	55.25 ± 36.54	100.00	88.70	100.00	85.90 ± 25.06	0.434
Glutamic acid	63.27 ± 9.39	83.80 ± 2.75	82.83 ± 3.25	69.00 ± 4.87	80.55 ± 3.31	73.70 ± 10.95	69.63 ± 6.47	90.43 ± 5.40	94.20 ± 0.81	0.035*
Proline	60.30 ± 1.55	76.07 ± 8.99	80.60 ± 3.01	89.27 ± 6.15	90.55 ± 2.49	73.45 ± 21.68	70.15 ± 4.94	90.73 ± 3.66	96.13 ± 0.63	0.050
Alanine	49.43 ± 15.48	73.47 ± 5.57	88.63 ± 6.76	62.83 ± 18.64	55.60 ± 23.35	53.20 ± 22.09	58.47 ± 8.56	84.75 ± 7.47	77.60 ± 6.94	0.473
Arginine	56.97 ± 10.63	80.40 ± 3.16	78.43 ± 3.28	77.37 ± 12.12	83.70 ± 5.75	67.73 ± 16.23	66.53 ± 2.66	87.00 ± 7.18	87.37 ± 2.32	0.224
Aspartic acid	63.40 ± 1.31	82.97 ± 4.35	76.30 ± 2.55	82.30 ± 9.94	80.90 ± 5.00	70.60 ± 17.27	65.77 ± 1.62	92.83 ± 5.29	84.03 ± 3.28	0.204
Total amino acid	54.33 ± 8.50	76.07 ± 0.37	77.20 ± 3.87	73.17 ± 6.56	82.13 ± 6.43	68.90 ± 13.94	61.33 ± 4.65	88.47 ± 6.75	90.10 ± 0.95	0.029*

[1]Data is shown as mean with SEM, n = 3 technical replicates per bacterial protein; for values shown as single measurements, error terms could not be calculated

[2]p-value for one-way ANOVA (values shown as single measurements were excluded)

*Significant difference. Different letters $^{a, b}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

Crude protein content ranged from 47.80 g/100g dry weight for *Cupriavidus necator* (with PHB) to 88.21 g/100g dry weight for *Thermus thermophilus*.

Total amino acid digestibility was highest for casein (90.1%), followed by *Psychrobacter frigidicola* (88.4%) and *Thermus thermophilus* (84.0%), suggesting that these bacteria have digestibility profiles approaching those of the reference protein. Conversely, *Cupriavidus necator* (with PHB) demonstrated the lowest total digestibility (54.3%), indicating the potential impact of PHB accumulation.

Lysine digestibility of *Cupriavidus necator* with PHB was significantly lower than that of casein ($p < 0.05$). *Thermus thermophilus* had a significantly lower methionine, threonine and phenylalanine digestibility than that of *Priestia megaterium*, *Psychrobacter frigidicola* and casein ($p < 0.05$).

For dispensable amino acids, glutamic acid digestibility was relatively higher in *Bacillus subtilis* and *Psychrobacter frigidicola*, while serine digestibility varied widely, with Thermus thermophilus being significantly lower than casein and other bacteria ($p < 0.05$). These results highlight the variability in amino acid digestibility across bacteria and their potential as alternative protein sources.

The DIAAS ranged from 0.34 to 0.69 across bacteria in batch two, as reported in Table 3.7.

Table 3.7: DIAAR and DIAAS of bacteria (batch two)

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43		
Casein (Protein reference)	1.45	1.33	1.36	1.32	**0.93**[1]	1.74	1.06	1.39	**0.93**	**SAA**
Cupriavidus necator (with PHB)	**0.57**	0.70	0.68	**0.52**	**0.56**	**0.56**	0.70	0.63	**0.52/0.56/ 0.57**	**Lysine/SAA/AAA/ Histidine**
Cupriavidus necator (without PHB)	0.77	**0.74**	0.76	**0.74**	0.76	**0.69**	0.82	0.80	**0.69/0.74**	**AAA/Lysine/ Isoleucine**
Brevundimonas diminuta (small bacteria)	0.64	0.76	0.60	**0.43**	**0.47**	0.55	0.62	0.71	**0.43/0.47**	**Lysine/SAA**
Priestia megaterium (large bacteria)	**0.69**	0.96	0.76	0.74	**0.65**	0.83	0.82	0.77	**0.65/0.69**	**SAA/Histidine**
Escherichia coli (gram negative)	0.63	0.82	0.63	0.61	**0.54**	**0.49**	0.73	0.68	**0.49/0.54**	**AAA/SAA**
Bacillus subtilis (gram positive)	0.88	1.12	0.91	0.68	**0.52**	**0.53**	0.71	0.61	**0.52**	**SAA/AAA**
Thermus thermophilus (thermophile)	0.68	0.66	0.89	0.65	**0.34**	0.59	0.56	0.64	**0.34**	**SAA**
Psychrobacter frigidicola (psychrophile)	**0.52**	0.77	0.60	0.72	**0.53**	**0.51**	0.80	0.66	**0.51/0.52/0.53**	**AAA/Histidine/ SAA**

[1]Values in bold represent the lowest DIAAR and the DIAAS

Casein had a DIAAS of 0.93 with SAA as limiting amino acids. *Thermus thermophilus* had a DIAAS of 0.34 with SAA as limiting amino acids. *Brevundimonas diminuta* had lysine as the limiting amino acid with a DIAAS of 0.43 and a DIAAR of 0.47 for sulphur-containing amino acids. For *Escherichia coli*, the limiting amino acids were AAA with 0.49, and a close DIAAR of 0.54 for SAA.

The limiting amino acids were aromatic amino acids for *Psychrobacter frigidicola* with a DIAAS of 0.51, and close DIAAR of 0.52 for histidine and 0.53 for SAA. *Bacillus subtilis* DIAAS was 0.52 and sulphur-containing amino acids were the limiting amino acids, with a close DIAAR of 0.53 for aromatic amino acids. The DIAAS of *Cupriavidus necator* with PHB was also 0.52, with lysine as a limiting amino acid, and close DIAAR of 0.56 for SAA and AAA, and 0.57 for histidine. The limiting amino acids of *Priestia megaterium* were sulphur-containing amino acids with a DIAAS of 0.65 and a close DIAAR of 0.69 with histidine. *Cupriavidus necator* with PHB had a DIAAS of 0.69 and aromatic amino acids as limiting amino, as well as a close DIAAR of 0.74 for both lysine and isoleucine.

3.3.4. Amino acid content, amino acid digestibility and DIAAS of bacteria in batch three

The amino acid content of bacteria in batch three is reported in Table 3.8.

Table 3.8: Amino acid content of bacteria in batch three

	Amino acid content (g/100g protein)[1]					
Amino acid	***Cupriavidus necator*** **(H_2 limited, 2% PHB)**	***Cupriavidus necator*** **(O_2 limited, 25% PHB)**	***Cupriavidus necator*** **(CO_2 limited, 28% PHB)**	***Cupriavidus necator*** **(NH_4 limited, 78% PHB)**	**Casein**	**p-value[2]**
Indispensable amino acids						
Lysine	5.02 ± 0.00^{a}	5.47 ± 0.41^{a}	4.53 ± 0.16^{a}	5.76 ± 0.15^{ab}	8.02 ± 0.97^{b}	0.005*
Methionine	1.55 ± 0.30^{a}	1.55 ± 0.05^{a}	1.89 ± 0.16^{a}	1.90 ± 0.12^{a}	3.10 ± 0.18^{b}	0.002*
Threonine	3.88 ± 0.02	4.11 ± 0.16	4.01 ± 0.23	4.50 ± 0.06	3.78 ± 0.29	0.065
Valine	4.55 ± 0.51^{a}	5.12 ± 0.07^{ab}	5.07 ± 0.36^{ab}	5.54 ± 0.20^{ab}	6.74 ± 0.60^{b}	0.018*
Leucine	6.98 ± 0.05	6.90 ± 0.49	6.67 ± 0.48	7.49 ± 0.07	9.73 ± 1.56	0.046*
Isoleucine	3.49 ± 0.04^{a}	3.78 ± 0.26^{ab}	3.84 ± 0.22^{ab}	3.98 ± 0.31^{ab}	4.88 ± 0.40^{b}	0.024*
Histidine	1.80 ± 0.09^{a}	1.88 ± 0.08^{a}	1.99 ± 0.00^{a}	1.83 ± 0.07^{a}	3.04 ± 0.28^{b}	0.001*
Phenylalanine	3.74 ± 0.11^{a}	3.83 ± 0.07^{a}	3.82 ± 0.14^{a}	4.15 ± 0.04^{a}	5.30 ± 0.49^{b}	0.005*
Dispensable amino acids						
Tyrosine	2.77 ± 0.16^{a}	2.97 ± 0.12^{a}	3.10 ± 0.04^{a}	3.36 ± 0.15^{a}	4.72 ± 0.30^{b}	<0.001*
Cystine	0.65 ± 0.03	0.63 ± 0.13	0.73 ± 0.18	0.70 ± 0.06	0.39 ± 0.15	0.163
Serine	3.30 ± 0.01^{a}	3.65 ± 0.23^{a}	3.64 ± 0.20^{a}	4.19 ± 0.26^{ab}	5.99 ± 0.81^{b}	0.006*
Glycine	3.17 ± 0.43^{b}	4.02 ± 0.25^{bc}	4.96 ± 0.02^{cd}	5.31 ± 0.15^{d}	1.95 ± 0.10^{a}	<0.001*
Glutamic acid	10.12 ± 0.37^{a}	10.46 ± 0.32^{a}	9.80 ± 0.04^{a}	11.20 ± 0.37^{a}	23.93 ± 4.28^{b}	0.003*
Proline	3.69 ± 0.02^{a}	3.81 ± 0.06^{a}	3.63 ± 0.10^{a}	3.97 ± 0.04^{a}	12.21 ± 1.45^{b}	<0.001*
Alanine	9.56 ± 0.31^{b}	8.85 ± 0.28^{b}	8.55 ± 0.72^{b}	9.03 ± 0.34^{b}	2.92 ± 0.31^{a}	<0.001*
Arginine	6.46 ± 0.02^{b}	6.25 ± 0.18^{b}	6.20 ± 0.07^{b}	6.27 ± 0.21^{b}	3.73 ± 0.33^{a}	<0.001*
Aspartic acid	7.99 ± 1.05	8.34 ± 0.47	7.81 ± 0.75	8.69 ± 0.24	6.94 ± 0.68	0.261

[1]Data is shown as mean with SD, n = 2 technical replicates per bacterial protein

[2]p-value for one-way ANOVA

*Significant difference. Different letters $^{a, b}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

For lysine, methionine, histidine, phenylalanine, tyrosine, glutamic acid and proline, casein content was significantly higher than that of the bacteria ($p < 0.05$). However, the casein content of alanine, arginine and glycine was significantly lower than that of the bacteria ($p < 0.05$). *Cupriavidus necator* with 78% PHB had a significantly higher glycine content than that of *Cupriavidus necator* with 2% PHB and 25% PHB and casein ($p < 0.05$).

Table 3.9 shows the amino acid digestibility across bacterial in batch three.

Table 3.9: Bacterial amino acid *in vitro* digestibility (batch three)

	Digestibility (%) [1]					
Amino acid	***Cupriavidus necator* (H_2 limited, 2% PHB)**	***Cupriavidus necator* (O_2 limited, 25% PHB)**	***Cupriavidus necator* (CO_2 limited, 28% PHB)**	***Cupriavidus necator* (NH_4 limited, 78% PHB)**	**Casein**	**p-value[2]**
Indispensable amino acids						
Lysine	79.40 ± 1.28^{ab}	80.02 ± 8.72^{ab}	77.30 ± 8.06^{ab}	65.96 ± 2.95^{a}	96.75 ± 1.63^{b}	0.036*
Methionine	85.09 ± 2.13	84.68 ± 6.46	81.96 ± 3.07	79.72 ± 5.75	96.41 ± 1.80	0.196
Threonine	75.80 ± 0.14	63.83 ± 21.40	80.87 ± 2.53	41.57 ± 6.86	87.45 ± 4.78	0.130
Valine	80.77 ± 3.50^{ab}	78.54 ± 9.42^{ab}	73.18 ± 7.52^{ab}	64.32 ± 1.93^{a}	94.52 ± 2.75^{b}	0.041*
Leucine	84.86 ± 2.86^{ab}	81.14 ± 9.69^{ab}	69.74 ± 2.30^{a}	77.09 ± 1.62^{ab}	96.21 ± 2.09^{b}	0.042*
Isoleucine	88.18 ± 0.50	77.65 ± 11.18	67.97 ± 1.23	67.48 ± 3.07	92.99 ± 3.84	0.025*
Histidine	88.08 ± 4.92	91.77 ± 4.44	73.20	74.29 ± 5.38	94.45 ± 3.42	0.068
Phenylalanine	78.10 ± 1.13	76.78 ± 10.68	74.60 ± 8.39	63.51 ± 4.22	96.26 ± 1.92	0.055
Dispensable amino acids						
Tyrosine	39.50	97.20	77.30	88.70	86.81 ± 8.35	N/A
Cystine	60.70 ± 12.73	87.80 ± 8.33	45.40	45.41 ± 16.42	79.47 ± 16.77	0.249
Serine	92.00	79.79 ± 13.95	62.60	70.66 ± 6.58	89.80 ± 5.89	0.402
Glycine	69.22 ± 10.41	95.90	81.66 ± 1.98	84.00	94.88 ± 4.18	0.150
Glutamic acid	84.90 ± 1.90^{ab}	83.04 ± 7.15^{ab}	74.31 ± 1.52^{a}	72.87 ± 1.36^{a}	96.33 ± 1.14^{b}	0.008*
Proline	83.37 ± 9.99	77.96 ± 11.16	65.50 ± 6.11	78.83 ± 2.31	97.71 ± 1.17	0.126
Alanine	90.62 ± 3.00^{b}	90.98 ± 2.73^{b}	89.90 ± 2.78^{b}	52.03 ± 18.00^{a}	96.05 ± 3.45^{b}	0.012*
Arginine	67.71 ± 9.92	79.06 ± 8.72	74.70 ± 6.26	59.27 ± 13.05	94.92 ± 4.15	0.171
Aspartic acid	74.86 ± 2.25^{a}	95.40	63.93 ± 1.88^{a}	64.67 ± 6.24^{a}	96.02 ± 2.28^{b}	0.002*
Total amino acid	82.09 ± 1.67	70.37 ± 15.56	68.47 ± 4.52	65.54 ± 2.55	95.10 ± 2.45	0.115

[1]Data is shown as mean with SEM, n = 3 technical replicates per bacterial protein; for values shown as single measurements, error terms could not be calculated

[2]p-value for one-way ANOVA (values shown as single measurements were excluded)

[*]Significant difference. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$)

The protein content ranged from 41.98 g/100g dry weight for *Cupriavidus necator* with 78% PHB to 83.93 g/100g dry weight for *Cupriavidus necator* with 2% PHB. The total amino acid digestibility ranged from 65.54% to 82.09% across bacteria.

Indispensable amino acid digestibility exhibited significant variability, with *Cupriavidus necator* (H_2 limited, 2% PHB) showing the highest total amino acid digestibility, approaching casein levels, while *Cupriavidus necator* (NH_4 limited, 78% PHB) had the lowest digestibility. *Cupriavidus necator* with 78% PHB had a significantly lower lysine and valine digestibility than that of casein ($p < 0.05$).

For *Cupriavidus necator* with 28% PHB, leucine digestibility was significantly lower than that of casein ($p < 0.05$). Compared to all other protein sources, alanine digestibility was significantly lower in *Cupriavidus necator* with 78% PHB ($p < 0.05$). Significantly higher aspartic acid digestibility was observed for casein than for *Cupriavidus necator* with 2% PHB, 28% PHB and 78% PHB ($p < 0.05$).

A strong negative correlation was observed between PHB content and total amino acid digestibility in batch three (Figure 3.5) (r = -0.8211).

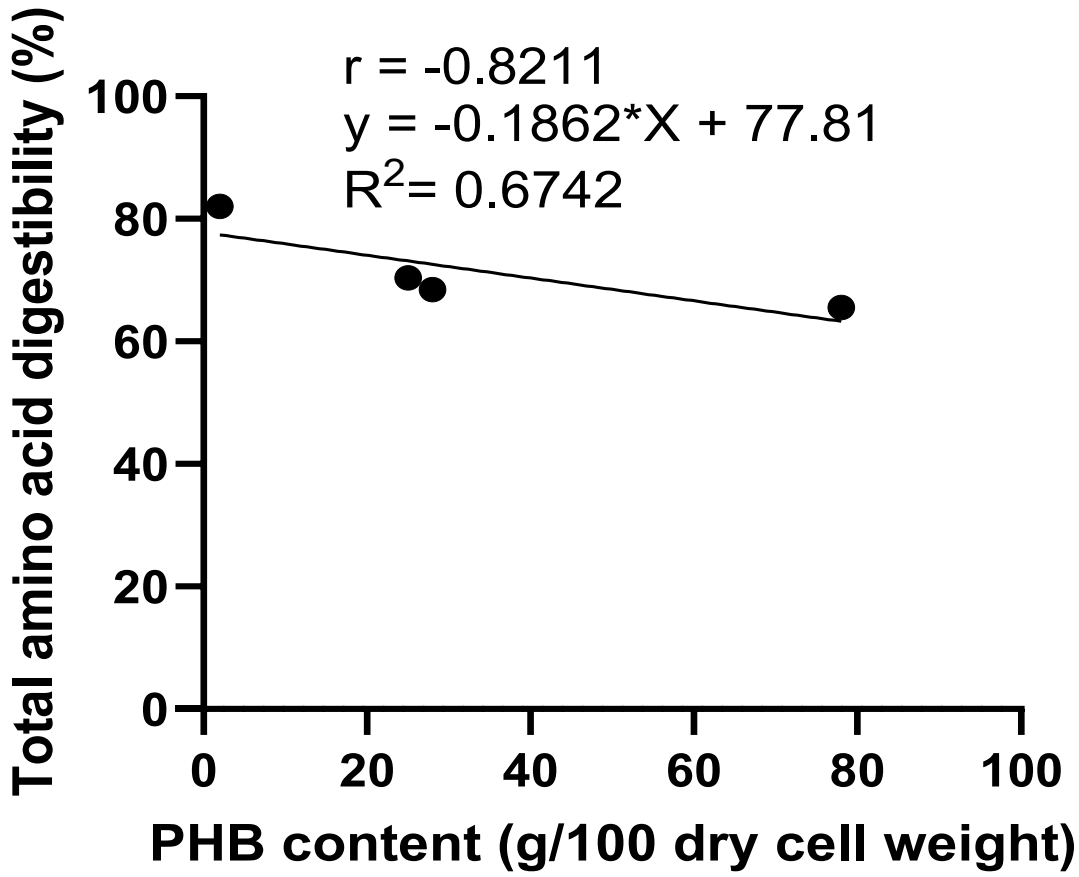


Figure 3.5: Correlation of PHB content with total amino acid digestibility of bacteria in batch three. Each point represents a sample.

According to the R^2 value (0.6742), approximately 67.42% of the variation in total amino acid digestibility can be attributed to differences in PHB content. However, the correlation was not statistically significant (p = 0.1790).

The DIAAS of *Cupriavidus necator* with various PHB contents and substrate limitations ranged from 0.56 to 0.69 (Table 3.10).

Table 3.10: DIAAR and DIAAS of bacteria (batch three)

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43		
Casein (Protein reference)	1.43	1.42	1.42	1.36	1.31	1.77	**1.07**[1]	1.48	**1.07**	**Threonine**
Cupriavidus necator (H_2 limited, 2% PHB)	0.79	0.96	0.90	0.70	**0.63**	0.77	0.95	1.02	**0.63**	**SAA**
Cupriavidus necator (O_2 limited, 25% PHB)	0.86	0.92	0.85	0.77	**0.69**	1.12	0.85	0.93	**0.69**	**SAA**
Cupriavidus necator (CO_2 limited, 28% PHB)	0.73	0.82	0.70	**0.61**	0.70	1.01	1.05	0.86	**0.61**	**Lysine**
Cupriavidus necator (NH_4 limited, 78% PHB)	0.68	0.84	0.87	0.67	0.68	**0.56**	**0.60**	0.83	**0.56/ 0.60**	**AAA/ Threonine**

[1]Values in bold represent the lowest DIAAR and the DIAAS

Casein had a high DIAAS of 1.07 with threonine as a limiting amino acid. The lowest DIAAS was 0.56 for *Cupriavidus necator* with 78% PHB and aromatic amino acids as limiting amino acids, with a close DIAAR of 0.60 for threonine. The DIAAS of *Cupriavidus necator* with 28% PHB was 0.51, and lysine was the limiting amino acids. For *Cupriavidus necator* with 2% PHB and 25% PHB, the limiting amino acids were SAA and the DIAAS were 0.63 and 0.69 respectively.

3.4. Discussion

Hypothesis: Calysta has a promising protein quality.

Methylococcus capsulatus (Calysta) emerged as a promising protein source with a DIAAS value of 0.77. This bacterial protein exhibited moderate to high digestibility for indispensable amino acids, critical for muscle protein synthesis and overall protein quality. The balanced amino acid content of *Methylococcus capsulatus* highlights its potential as a sustainable alternative to conventional protein sources, particularly in animal feed applications.

The high protein quality of Calysta can be attributed to its heterotrophic growth mode, which may favour a more balanced amino acid composition and higher digestibility compared to autotrophic bacteria. Moreover, the absence of bioplastic accumulation in Calysta further contributes to its high digestibility, as no structural barriers are limiting enzymatic accessibility to amino acids during digestion.

Hypothesis: Paracoccus pantotrophus *has a relevant amino acid profile and digestibility for animal feed.*

Paracoccus pantotrophus exhibited moderate digestibility compared to *Methylococcus capsulatus* (Calysta). Despite its reduced digestibility, particularly for indispensable amino acids like lysine and methionine, *Paracoccus pantotrophus* remains an interesting candidate as a protein source due to its balanced amino acid profile for certain amino acids, such as leucine and valine and a could be use combined with other bacterial protein sources.

Hypothesis: bacterial species influence digestibility.

Overall, bacteria show some promise as alternative protein sources. However, their effectiveness as a reliable protein source depends on several key factors, including the bacterial species and growth conditions.

The digestibility of bacteria varied significantly across species, reflecting differences in their amino acid profiles, cell wall structures, and growth conditions. While some bacteria, such as *Methylococcus capsulatus* and *Psychrobacter frigidicola*, demonstrated high digestibility values for indispensable amino acids, others, such as *Cupriavidus necator* with high PHB content, showed reduced digestibility for key amino acids like lysine and valine. These differences highlight the importance of bacterial species in determining the nutritional quality of their proteins.

Cupriavidus necator exhibited lower digestibility compared to *Methylococcus capsulatus*. This might be explained by species-specific characteristics, such as the presence of cell wall components that limit enzymatic access to amino acids, which might impact digestibility (Onyeaka et al., 2022).

To illustrate the structural features that can limit protein digestibility in Gram-negative bacteria, a schematic of the bacterial cell envelope is presented in Figure 3.6, adapted from Silhavy et al., 2010.

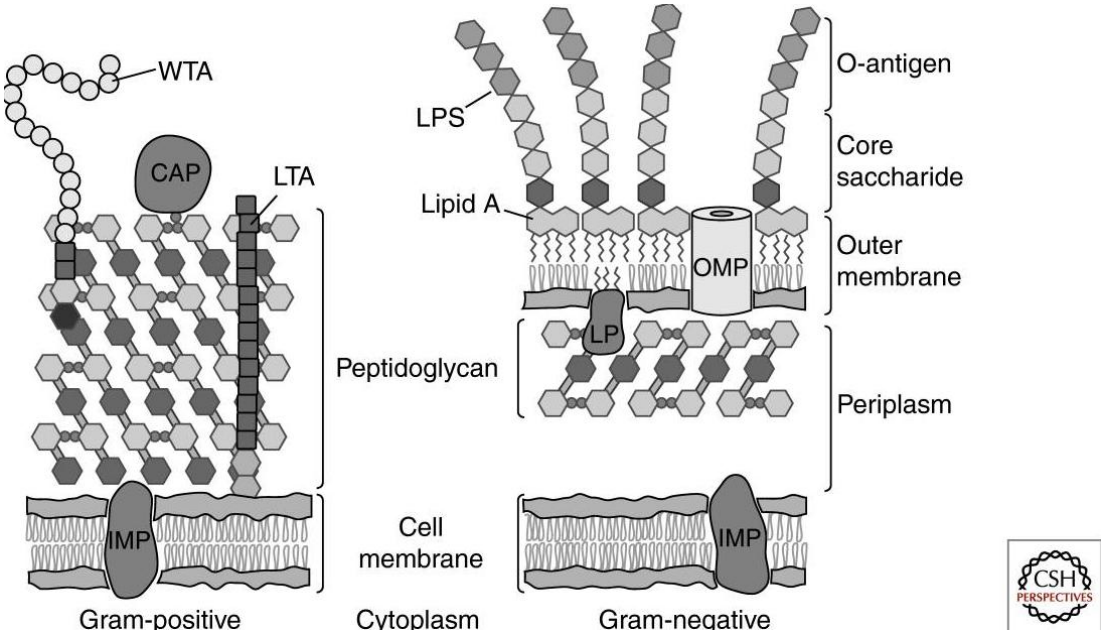


Figure 3.6: Gram-negative bacterial cell envelope illustrating structural barriers to protein digestibility (Silhavy et al., 2010).

The diagram highlights the outer membrane, including the lipopolysaccharide (LPS) layer, the periplasmic peptidoglycan, and the inner membrane. This layered structure creates a physical barrier that may restrict enzymatic access to intracellular proteins, contributing to the lower digestibility observed for certain bacterial protein sources, such as *Cupriavidus necator*, compared with others like *Methylococcus capsulatus*.

Thermus thermophilus exhibited high protein content but lower amino acid digestibility, which is likely due to its robust cell wall structures adapted to extreme environments (Quintela et al., 1999). In contrast, *Psychrobacter frigidicola* showed modest protein quality, which might be due to its susceptibility to unfold at higher

temperatures than its optimal activity temperature. This enables enzyme accessibility to the protein during digestion (Nowak & Otzen, 2023).

Digestibility values for indispensable amino acids, including methionine and lysine, were inconsistent across bacterial species, indicating potential challenges in meeting dietary amino acid requirements with certain bacteria. In contrast, resistance to specific temperatures, high-cell density and cell wall compositions constitute interesting characteristics for feed formulation.

Interestingly, several bacterial species, such as *Paracoccus pantotrophus*, *Methylococcus capsulatus*, and *Psychrobacter frigidicola*, had histidine as one of their low DIAAR, and was the limiting amino acid for *Clostridium sp* (Chapter 2). This common limitation might arise from the complexity and energy costs associated with histidine biosynthesis, which involves multiple enzymatic steps, making it energetically expensive for bacteria to produce histidine *de novo* (Alifano et al., 1996).

These outcomes suggest that careful selection of bacterial species is crucial when considering their application as protein sources. Species with high digestibility values for indispensable amino acids and balanced amino acid profiles are more likely to meet nutritional demands effectively.

While this study provides valuable insights into the digestibility and protein quality of bacteria, certain limitations must be acknowledged. Batch variability in the production of bacteria may influence the generalisability of the findings. For instance, casein showed notable variability between batches in terms of DIAAS, changing from

threonine as the limiting amino acid in batch one to sulphur amino acids (SAA) as the limiting amino acid in batch two.

These differences signify that even a highly standardised reference protein like casein is susceptible to batch-specific conditions, such as differences in raw material sourcing, handling, enzyme activity, and simulated fluids, during analysis. Such variability underscores the importance of standardising experimental conditions and highlights the need for caution when interpreting results across batches.

Another limitation is the use of 6.25 for the nitrogen-to-protein conversion factor (NPCF) which might be overestimating the protein content of the bacterial protein sources. The presence of a significant amount of non-protein nitrogen (NPN) compounds, such as nucleic acids, contribute to the total nitrogen detected during protein analysis, increasing the crude protein content when using the Dumas method. The current gap in understanding of appropriate NPCFs for bacterial protein emphasises the need for future studies.

Hypothesis: bioplastic content impacts the bacterial protein content and total amino acid digestibility.

The accumulation of polyhydroxybutyrate (PHB) in bacterial cells was a key factor influencing both protein content and digestibility. *Cupriavidus necator*, under different growth conditions, showed an inverse relationship trend between PHB content and total amino acid digestibility. This means that PHB negatively impacts amino acid availability, although the correlation was not statistically significant.

PHB granules may encapsulate or physically obstruct proteins within bacterial cells, reducing their accessibility to digestive enzymes (Koning et al., 2023). An inverse

relationship between PHB content and total amino acid digestibility is expected, though not necessarily significant.

Polyhydroxybutyrate (PHB) is stored as intracellular granules within the bacterial cytoplasm, typically 0.2 to 0.5 µm in diameter, composed of amorphous PHB surrounded by a phospholipid monolayer and associated proteins (phasin, depolymerase) (Jendrossek & Pfeiffer, 2014). These granules physically encapsulate or sterically block nearby proteins, reducing accessibility to proteolytic enzymes during digestion and lowering amino acid release (Milne & Subramaniam, 2009). PHB biosynthesis does not chemically alter the cell wall composition but occurs concurrently with growth arrest under nutrient limitation, potentially increasing cell wall rigidity or thickness in some Gram-negative species due to reduced membrane turnover (Sharma et al., 2016).

Interestingly, it has been shown that PHB accumulation is observed in other species than *Cupriavidus necator*, which could further dilute protein content and impact digestibility (Bhagowati et al., 2015; Narancic et al., 2016). These productions illustrate how environmental or growth conditions influence PHB synthesis. These findings underscore the need to optimise growth conditions to mitigate PHB synthesis, particularly for species intended for use as protein sources.

These results imply that controlling PHB accumulation during bacterial growth is critical for improving the nutritional quality of bacterial proteins. Strategies such as optimising growth conditions, meaning limiting PHB-promoting nutrients, or employing genetic modifications to reduce PHB synthesis, could enhance amino acid digestibility and overall protein quality.

3.5.Conclusion

The exploration of bacteria as alternative protein sources highlights their potential to address the increasing global demand for sustainable and scalable protein production. This study evaluated the digestibility, amino acid content, and protein quality of multiple bacterial species, revealing both strengths and limitations.

Bacteria, such as *Methylococcus capsulatus* (Calysta), were highly digestible, with DIAAS values comparable to casein, making them relevant candidates for commercial applications like aquafeeds and livestock nutrition. However, other species, such as *Paracoccus pantotrophus* and *Cupriavidus necator* with high PHB content, showed reduced digestibility for certain indispensable amino acids, highlighting the variability in protein quality among bacterial species.

The findings also revealed challenges, including the accumulation of polyhydroxybutyrate (PHB), which negatively impacts digestibility and amino acid availability. Furthermore, batch variability underscores the importance of standardised production and analytical methods to ensure consistency in protein quality assessments.

The results presented in this chapter indicate that bacteria have the potential to serve as viable and sustainable protein sources, particularly if their production is optimised for higher digestibility and balanced amino acid profiles.

However, several issues need to be addressed to ensure their effectiveness. These include managing PHB accumulation, improving indispensable amino acid content, investigating the NPCF for each bacterium and enhancing digestibility through enzyme treatments or feed formulation strategies. Despite these challenges, bacteria

offer unique advantages including scalability, the ability to thrive on minimal resources, and the potential for customisation to improve their nutritional profiles.

In conclusion, bacteria show significant promise as sustainable protein sources for animal feed, particularly when their production is tailored to enhance digestibility and amino acid profiles. While bacteria like *Methylococcus capsulatus* stand out, continued research and optimisation are needed to address challenges such as PHB accumulation and batch variability. This study provides a foundation for advancing bacteria as viable alternatives to traditional protein sources in animal feed and informs strategies to refine their production and application.

To improve the robustness and applicability of the *in vitro* approach, further research should investigate its correspondence with *in vivo* outcomes using selected protein sources. Establishing this relationship could help strengthen the reliability and predictive capacity of the *in vitro* approach.

4. Comparison between *in vitro* data and *in vivo* values from a chicken trial

4.1. Introduction

With a more and more pressing demand for poultry products (Pesti & Choct, 2023a), the poultry industry strives to improve feed formulations to enhance bird performance and reduce costs. Understanding the protein quality of feed ingredients is paramount for feed optimisation. This involves *in vivo* or *in vitro* testing methods. While *in vivo* methods are prominent for their accuracy, they are often expensive, laborious and time-consuming (Zaefarian et al., 2021).

Alternatively, *in vitro* methods provide a more efficient approach for evaluating a large number of samples, making them great tools in research and quality control contexts (Hirsch & Schildknecht, 2019). These techniques are designed to reproduce the physiological and chemical characteristics of the digestive system, allowing information on digestibility.

One widely used *in vitro* method for rapid evaluation of protein samples is the pepsin digestibility test (Ofori-Anti et al., 2008; Thomas et al., 2004; Utterback, 2015). This quick and simple method is preferred in research and quality control. However, the pepsin digestibility test has limitations, particularly with samples containing fats or carbohydrates, which are common in practical feed formulations. Pepsin is a proteolytic enzyme that only breaks down proteins, implying that it does not digest fats or carbohydrates (Heda et al., 2025).

To tackle these challenges, multi-enzyme tests have been developed (Bryan & Classen, 2020b). These tests involve a combination of enzymes, either in single or

multiple stages, to more accurately replicate the animal digestive process. The reliability of *in vitro* methods in predicting *in vitro* outcomes needs to be validated to ensure their application. No consensus has yet been reached regarding the *in vitro* chicken digestion method (Michels et al., 2023).

The chicken and human digestive systems share a few similarities. Both systems follow a similar pattern during digestion, having a mouth, an oesophagus, a stomach, a small intestine and a large intestine. The digestive systems are comprised of similar enzymes and digestive fluids to break down food and absorb nutrients. Both chickens and humans rely on peristalsis, or the muscle contraction involved in the moving of food through the digestive tract (Hodgkiss, 1986; Patel & Thavamani, 2025).

The established INFOGEST consensus *in vitro* digestion method mimicking the human digestive system is a valuable tool for studying feed ingredients. This results from the fact that it is a standardised and reproducible protocol which can provide detailed digestive information on how different feed ingredients break down during digestion. This non-invasive and cost-effective method supports nutritional optimisation and can be applied to a wide range of feed ingredients (Ariëns et al., 2021).

With the use of the INFOGEST method, several values can be generated alongside the digestibility data, such as the Digestible Indispensable Amino Acid Score (DIAAS). Given the fact that this score takes into account the amino acid profile and digestibility of a protein source, it provides a nuanced understanding of protein quality in nutrition (Komatsu et al., 2023; Sousa et al., 2023).

This score is used in this study because, while the amino acid requirements of a human child between 6 months and 3 years old differ from those of a broiler chicken,

there are similarities in their protein needs. Both have high requirements for indispensable amino acids for growth and development, making the DIAAS a useful tool for evaluating protein quality in both contexts.

The use of the DIAAS based on the human reference pattern also enables comparisons with other plant-based protein values generated by the same *in vitro* digestion method. This could help find benefits in protein sources unthought for poultry.

Soybean meal represents the main source of protein in poultry diets. Overall, soybean meal accounts for more than 70% of the protein source used in compound feeds for poultry and other livestock (Safalaoh, 2002). This is mainly due to its high protein content and excellent amino acid profile, particularly rich in lysine (Fernandez et al., 1994b). Certain soya-based engineered proteins available on the market are meant to have improved protein digestibility, such as AlphaSoy 530.

According to the company supplier, AlphaSoy 530 can increase the digestibility of protein and amino acids by 3%, has a higher fast-digestible protein content, and has up to 16% higher net energy compared to other soya products as well as ANF reduced to safe levels (*AlphaSoy 530 | Advanced Protein Nutrition | Piglets | Special Ingredients*, 2025). AlphaSoy 530 is tailored for optimal health and performance in antibiotic-free feeding systems, addressing the specific nutritional needs of young animals. Antibiotic-free feeding systems are livestock or poultry production systems in which no antibiotics are added to the feed or water for growth promotion or disease prevention.

Processing significantly influences protein digestibility and amino acid availability in plant-based feed ingredients. Soybean meal undergoes basic solvent extraction and heat treatment, which inactivates most trypsin inhibitors but can leave residual fibre and phytate (phytic acid), an anti-nutritional factor (ANF) that binds minerals and amino acids, reducing protein bioavailability (Kuenz et al., 2022). To obtain AlphaSoy 530 from the soybean meal, the ingredient feed goes through additional thermo-mechanical and enzymatic treatments to further degrade fibre, hydrolyse phytate, and minimise ANFs, resulting in higher fast-digestible protein fractions.

High-temperature treatments can trigger the Maillard reaction, where reducing sugars react with free amino groups (especially lysine), forming advanced glycation end-products that resist enzymatic digestion (Hofmann et al., 2020). This may reduce lysine availability despite high crude digestibility, particularly in heat-treated soy products (González-Vega et al., 2011). Controlled processing is therefore essential to balance ANF reduction with preservation of amino acid integrity.

Other protein sources involve oilseeds such as sunflower. Sunflower expeller, derived from sunflower seeds, usually contains 28 to 36% protein, depending on the de-hulling process (Ibagon et al., 2021a). While lower in lysine compared to soybean meal, it has been shown to be rich in methionine and cysteine, making it a valuable partial replacement in feed formulations. Sunflower meal is particularly beneficial for ruminants and can replace a significant portion of soybean meal in poultry diets when supplemented with lysine (Yaqoob et al., 2022). Processing steps such as dehulling and oil extraction influence the nutritional value of sunflower products. Partially

dehulled sunflower meal and sunflower expeller differ in amino acid digestibility (Ibagon et al., 2021b).

Since only a few studies have investigated the correlation between the INFOGEST *in vitro* method and *in vivo* methods in poultry, and these studies cover limited feed types and conditions (Bryan & Classen, 2020c), the study in this chapter aimed to investigate the potential relationship between laboratory digestibility values from an established digestion method and *in vivo* digestibility data from a chicken trial, focusing on the three protein sources mentioned: soybean meal, AlphaSoy and sunflower expeller. The three protein sources were chosen to test the system's robustness, using two soy-based feeds and one alternative protein source.

Lower inclusion levels such as 15 or 20 % were considered insufficient to meet the chicken protein requirements. Therefore, inclusion levels of 30, 45, and 60 % were selected to reflect the range commonly used in commercial poultry diets, covering low, standard, and high protein concentrations, and allowing evaluation of the INFOGEST *in vitro* method across practical feeding scenarios and varying protein concentrations.

Aims

- To determine the protein quality of soybean meal, AlphaSoy and sunflower expeller as feed ingredients with the INFOGEST *in vitro* method
- To determine the protein quality of soybean meal, AlphaSoy and sunflower expeller as formulated feeds at three various inclusion levels (30, 45 and 60%) with the INFOGEST *in vitro* method

- To assess a potential relationship between *in vitro* digestibility data from the INFOGEST in vitro digestion method and *in vivo* digestibility data from a chicken trial using soybean meal, AlphaSoy and sunflower expeller

Hypothesis

There is a relationship between digestibility values obtained from laboratory digestion experiments and *in vivo* digestibility measurements from a chicken trial, using three protein sources (soybean meal, AlphaSoy and sunflower expeller) at three different inclusion levels (30%, 45% and 60%).

4.2. Materials and methods

4.2.1. Materials

All chemicals and reagents were purchased from Merck (Dorset, UK) unless stated otherwise. The samples and a brief description of them are reported in Table 4.1.

Table 4.1: List of samples and description

Sample code	Sample type	Description	Batch number[1]
Soybean meal	Feed ingredient	Co-product of soybean oil extraction, heat-treated	1
AlphaSoy	Feed ingredient	Soya protein treated in special thermo-mechanical and enzyme treatment process	1
Sunflower expeller	Feed ingredient	Sunflower protein with 10% fat from dehulled kernels	1
Soybean meal 30%	Formulated feed	Feed including 30% of soybean meal	2
Soybean meal 45%	Formulated feed	Feed including 45% of soybean meal	2
Soybean meal 60%	Formulated feed	Feed including 45% of soybean meal	2
AlphaSoy 30%	Formulated feed	Feed including 30% of AlphaSoy	2
AlphaSoy 45%	Formulated feed	Feed including 45% of AlphaSoy	2
AlphaSoy 60%	Formulated feed	Feed including 60% of AlphaSoy	2
Sunflower expeller 30%	Formulated feed	Feed including 30% of sunflower expeller	2
Sunflower expeller 45%	Formulated feed	Feed including 45% of sunflower expeller	2
Sunflower expeller 60%	Formulated feed	Feed including 60% of sunflower expeller	2
Casein (Protein reference)	Protein reference	Pure, high-quality protein from bovine milk (no 10545691, Thermo Scientific, MA, US)	1, 2
Protein Free Cookie (PFC)	Blank	Blank digestion control	1, 2

[1]The 12 samples were processed in two batches: Batch 1 (feed ingredients digested across 4 days) and Batch 2 (formulated feeds digested across a separate set of 4 days)

The feed ingredients were provided by AB Agri (UK) in November and December 2023. They consisted of soybean meal, AlphaSoy and sunflower expeller and were

commercially available at the date they were obtained. They were minimally processed and obtained in powder form.

When obtained, soybean meal was less homogenous than the other samples, therefore it was blended for 5 minutes at intensity 8 with a Thermomix (TM6, Vorwerk, Germany) prior to analysis in the lab. The formulated feeds were prepared at Nottingham Trent University's Poultry Unit, as described in Chapter 4.2.7.2., and 500 g of each formulated feed was transferred to the Sutton Bonington campus of the University of Nottingham for analysis.

Casein was used as a protein reference for digestibility as it is highly digestible, as shown in Chapter 2.2. A 'protein-free cookie' was prepared and included as a blank digestion control to account for endogenous amino acids present in the reagents, primarily originating from the enzymes.

The protein-free cookie ensured accurate simulation of background digestion in the presence of food. The recipe for the protein-free cookie included the following ingredients for 100 g of protein-free cookie: 41.3 g purified corn starch, 15.7 g sucrose, 4.9 g cellulose, 0.7 g baking powder, 0.5 g ground ginger, and 36.9 g margarine.

The ingredients were thoroughly mixed with a spatula and spread evenly on a baking tray lined with non-stick baking paper. The cookie dough was baked at 175 °C for 30 minutes. After baking, the mixture was processed using the kneading function of a Thermomix for 1 minute to achieve a uniform consistency. The protein-free cookie was stored at −20 °C until further analysis.

4.2.2. Crude protein content

A sample of each feed ingredient and each formulated feed was freeze-dried until a constant weight and the crude protein content was determined using an EA 1112 elemental analyser (Thermo Scientific) to obtain the nitrogen content for each feed ingredient, as described in Chapter 2.2.2, with 6.25 as the NPCF.

4.2.3. In vitro digestion method

The *in vitro* digestion method was performed on each feed ingredient and formulated feed following the INFOGEST static *in vitro* simulation of gastrointestinal food digestion described in Chapter 2.2.3. Four biological replicates were generated through four *in vitro* digestions on different days. Each sample was weighed with 8% of protein or 80 mg of protein per digestion sample.

4.2.4. OPA method

Once the *in vitro* digestions were completed, the degree of hydrolysis was estimated on the absorbable fractions of the feed ingredients and formulated feeds using the o-phthaldialdehyde (OPA) assay described in Chapter 2.2.4 with two adjustments.

One was that the serial dilution of L-glutamic acid stock solution (100 mmol/L) was prepared in Di-sodiumtetraborate-decahydrate solution (Borate-solution, 0.1 mol/L) instead of water. Borate solution typically has a pH between 9.0 and 10.5. The OPA assay performed better in alkaline conditions as it improved reaction kinetics and facilitated amine deprotonation.

The second adjustment was the incubation time of the microplate after adding the OPA solution. To ensure that the reaction equilibrium was reached and the

fluorescence intensity stabilised, the incubation time was 30 minutes in the dark instead of 10 minutes.

4.2.5. Amino acid analysis

The amino acid content of the samples was analysed before and after digestion on feed ingredients, formulated feeds and ileal digesta, before and after digestion, by LC-MS/MS using hydrolysis by oven crimp tube digestion. This method is described in Chapter 2.2.5.

4.2.6. Calculation of amino acid digestibility and digestible indispensable amino acid score (DIAAS)

The amount of individual amino acid digestibility and DIAAS were calculated with the equations described in Chapter 2.2.6.

4.2.7. Broiler chicken trial

4.2.8.1 Ethical approval

This trial was conducted at Nottingham Trent University's (NTU) Poultry Unit and received approval from the internal ethical review committee. A Home Office licence would have been required if the trial involved regulated procedures as defined by the Animals (Scientific Procedures) Act (1968), such as causing pain, suffering, distress, or lasting harm to the birds beyond mild, temporary effects. As the committee determined that no 'regulated procedures' were involved, the study was carried out under DEFRA regulations and did not require a Home Office licence.

4.2.8.2 Experimental design

The study employed a completely randomized design with nine dietary treatments, resulting from the combination of three protein sources (soybean meal, AlphaSoy, sunflower expeller) and three inclusion levels (30, 45, 60 %). Each treatment was allocated to five replicate pens, with four birds per pen, allowing assessment of digestibility across different protein sources and protein concentrations under controlled housing and feeding conditions.

4.2.8.3 Nutrient targets

The experimental diets were formulated to meet or exceed the nutrient requirements of broiler chickens according to NRC 1994 recommendations. Protein and amino acid levels were adjusted according to the inclusion of each protein source (soybean meal, AlphaSoy, sunflower expeller) to achieve target crude protein contents corresponding to 30%, 45%, and 60% inclusion. Energy, fat, and essential vitamins and minerals were maintained consistently across all diets to isolate the effects of protein source and level on digestibility. Titanium dioxide (5 g/kg) was included as an inert marker for digestibility measurements.

4.2.8.4 Feed formulation

Birds were given a commercially formulated chick starter diet (mash) from the day of hatch (day 0) until day 17. They were then fed formulated feeds prepared for this trial until day 20. The formulated feeds were prepared at the Poultry Unit on the Brackenhurst Campus, NTU. They were prepared with three protein sources provided by AB Agri: soybean meal, AlphaSoy and sunflower expeller.

They were directly shipped to the Brackenhurst Campus in bags of 20 kg. For each protein source, three diets were prepared, each with a different inclusion level. The three inclusion levels were 300 g/kg sample or 30%, 450 g/kg sample or 45%, and 600 g/kg sample or 60%. In total, nine feeds were formulated. All the ingredients are reported in Table 4.2.

Table 4.2: Ingredients of the formulated feeds used in the broiler chicken trial

	% (w/w) of ingredient		
Ingredient	**Inclusion level 30%**	**Inclusion level 45%**	**Inclusion level 60%**
Protein source[1]	300	450	600
Soya oil	50	50	50
Vitamin/mineral premix	50	50	50
Titanium dioxide	5	5	5
Glucose	297.5	222.5	147.5
Corn starch	297.5	222.5	147.5

[1] Soybean meal, AlphaSoy or sunflower expeller

For each formulated feed, 12 kg was prepared and mixed using a ribbon mixer (400 L capacity, Gardner, Gloucester). The protein sources were homogenised by mixing before analysis. Dry materials were mixed for 5 minutes before the addition of the soya oil, and then for a further 20 minutes to ensure homogeneity. Titanium dioxide was added to all diets at 5 g/kg inclusion as an inert marker for digestibility measures. A sample of each formulated feed was taken immediately post-mixing for analysis before being split into bags of 2 kg. Sealed bags of formulated feeds were stored in a cool dry room until needed.

4.2.8.5 Subjects and husbandry

Ross 308 male chicks were sourced from PD Hook Cote hatchery in Oxford, from a flock aged 40 to 48 weeks. The 180 chicks were feather-sexed on the day of hatching and transported by NTU personnel to minimise travel stress. The NTU personnel conduced the chicken trial. A surplus of chicks was ordered to ensure that only healthy individuals were allocated to the trial pens, in accordance with the 3Rs principle by reducing unnecessary use of animals and refining the selection process to minimize suffering.

Day-old chicks were housed in rearing pens in groups of 10 and fed a commercially formulated broiler mash diet. Feed and water were provided *ad libitum*, with steps taken to encourage the chicks to eat and drink as soon as possible. Feed was supplied via 30 cm troughs in each pen, while water was provided using nipple drinker lines with two nipples per pen.

The lighting schedule began with 23 hours of light and 1 hour of darkness on day one, with an additional hour of darkness added daily until day six. From day seven onwards, a six-hour dark period (22:00–24:00 and 02:00–06:00) was maintained for the remainder of the study.

The temperature was initially set at 32 °C on the day of hatching and reduced by approximately 1 °C per day. Health checks were carried out twice daily, and heating and ventilation systems were adjusted based on the chicks' behaviour to maintain appropriate room temperature and humidity while preventing ammonia build-up.

On day 17, the chicks were weighed and randomly allocated to mesh-sided pens lined with wood shavings, with four birds per pen. Each formulated feed was fed to five pens, randomly allocated.

4.2.8.6 Sample collection

On day 20, all birds were individually fed a fresh diet for at least 30 minutes to ensure their gut was filled before culling. The birds were then sequentially culled by cervical dislocation. This is an approved method for small poultry that ensures rapid unconsciousness and minimal distress. Other methods, such as gas or anaesthetic overdose, were considered but cervical dislocation was selected as the most practical and humane approach for this study, in accordance with DEFRA and ASPA guidelines.

The ileal digesta, defined as the portion of the small intestine from Meckel's diverticulum to the ileocecal junction, was then removed and collected in pots. The pots were covered with para-film and put in the freezer at −20 °C until frozen. They were then put into the freeze-dryer (Lyotrap freeze dryer, LTE Scientific, UK) and freeze-dried for five days to a constant weight. As titanium dioxide (TiO_2) was included in the diet as an indigestible marker, all birds and associated materials were excluded from the human food chain.

4.2.8. Apparent ileal digestibility

4.2.8.1 Sample preparation

Ileal digesta samples pots were transferred to the Sutton Bonington Campus of the University of Nottingham (UoN). For each pen (comprising four birds), the samples were pooled into a single composite sample, giving five samples per formulated feed

tested. These composite samples were then ground with a pestle and mortar to produce a fine powder. They were then stored in a desiccator until further analysis.

4.2.8.2 Crude protein content

For each ileal digesta, the crude protein content was determined using an EA 1112 elemental analyser (Thermo Scientific) to obtain the nitrogen content, as described in Chapter 2.2.2.

4.2.8.3 Assessment of feed digestibility using titanium dioxide

For both ileal digesta and formulated feeds, the titanium dioxide (TiO_2) was determined using a method adapted from (Short et al., 1996). Two solutions were required. The first one was a titanium solution (0.5 mg/mL), prepared by weighing 250 mg of TiO_2 into a 600 mL beaker and adding 100 mL of concentrated sulphuric acid (98%, w/w).

The mixture was placed on a hot plate in a fume cupboard and heated until it began to simmer. It was left to heat for approximately 2 hours until the solid dissolved, then allowed to cool on a heat-resistant surface. Subsequently, 200 mL of distilled water was poured into a 500 mL volumetric flask, which was placed in a bucket of cold water. The sulphuric acid and TiO_2 solution was then added slowly, followed by an additional 100 mL of concentrated sulphuric acid.

Finally, the solution was diluted to 500 mL with distilled water. The second solution was a 7.4 M sulphuric acid solution. For this, 400 mL of distilled water was poured into a 1 L bottle and placed in a bucket of cold water in a fume cupboard. Then, 400 mL of concentrated sulphuric acid was carefully added, and the solution was diluted to 1 L with distilled water.

Porcelain crucibles (60 mm, FSE) were used to weigh out 0.2 g of each sample and incinerated to ash overnight in a muffle furnace (Carbolite, Derbyshire). The 7.4 M sulphuric acid solution was then dispensed into each crucible (10 mL) and they were placed on a hotplate until dissolved, which was for approximately 1 hour at 250 °C. The samples were left to cool in the fumehood.

For each sample, 25 ml of distilled water was transferred to a beaker. The crucible content was added to the beaker and rinsed twice with distilled water. The solution was poured into a 100 ml volumetric flask through a quarter-folded paper (Whatman 541, hardened ashless) with a funnel. The beaker was then rinsed with 25 ml distilled water which was also added through the funnel.

After removing the funnel, 10 ml of 30 % hydrogen peroxide (v/v) was added and the solution made up to 100 ml using distilled water. The flask was mixed, giving a yellow-to-orange colour. Alongside the samples, a calibration curve of TiO_2 standards was prepared, as described in Table 4.3, using 100 ml volumetric flasks.

Table 4.3: Standard curve for feed digestibility using TiO2

		Volume (mL)		
No of standard	**0.5 mg/mL TiO_2**	**Concentrated sulphuric acid (98%, w,w)**	**Distilled water**	**Hydrogen peroxide (30%, v/v)**
0	0	10	10	10
1	1	9	10	10
2	2	8	10	10
3	3	7	10	10
4	4	6	10	10
5	5	5	10	10
6	6	4	10	10
7	7	3	10	10
8	8	2	10	10
9	9	1	10	10
10	10	0	10	10

The absorbance of each sample and standard was then measured at 410 nm using a UV-spectrophotometer (Unicam Helios Gamma, Thermo Scientific) which was zeroed on the blank calibration setting. The following equation was used to calculate the amount of titanium dioxide in mg/mg of sample:

Equation 3.1: $\mathrm{TiO_2}\ (mg/mg\ sample) = \left(\frac{Absorbance - b}{a}\right) \times mass\ sample\ (mg)$

Where b is the intercept of the standard curve and a is the gradient.

4.2.8.4 Apparent ileal digestibility calculations

The apparent ileal digestibility (%) was calculated using the following equation as used in (Pedersen et al., 2021):

Equation 3.2: $AID\ (\%) = \left(1 - \frac{T_d \times N_i}{T_i \times N_d}\right) \times 100$

Where T_d is the titanium amount in the diet, N_i is the nutrient (nitrogen or amino acid) amount in the ileal digesta, T_i is the titanium amount in the ileal digesta and N_d is the nutrient (nitrogen or amino acid) amount in the diet.

4.2.9. Data analysis

Prior to statistical analysis, data were checked for normality and homogeneity of variance using GraphPad Prism, and the experimental design ensured independence of measurements. Data met the assumptions required for parametric testing.

Genstat (24th Edition) was used for statistical analyses. One-way and two-way ANOVA were applied followed by, when appropriate, a Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$. The graphs were produced with Graph Pad Prism 10.3.1.

4.3. Results

The results are presented for feed ingredients, formulated feeds, and ileal digesta. Crude protein content, amino acid composition, *in vitro* digestibility, degree of hydrolysis, DIAAR, and DIAAS are reported for feed ingredients and formulated feeds. *In vivo* data from a chicken trial, including apparent ileal amino acid digestibility, are presented alongside correlations and Bland-Altman analyses comparing *in vitro* and *in vivo* digestibility. Key findings are summarised in the corresponding figures and tables.

4.3.1. Crude protein content

There was a significant difference ($p < 0.001$) (Figure 4.1) in the crude protein content (g/100g dry weight) between the feed ingredients.

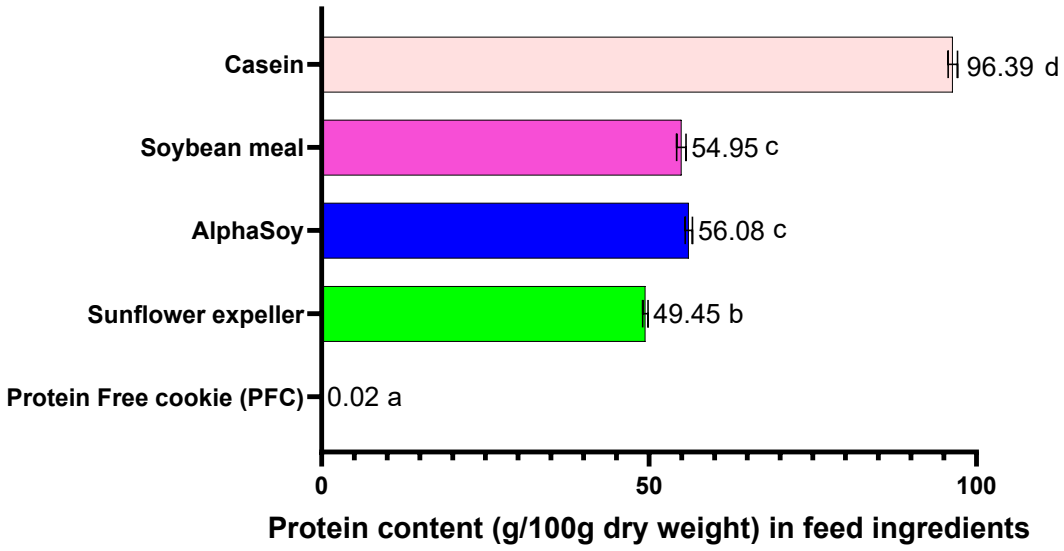


Figure 4.1: Crude protein content (g/100g dry weight) of feed ingredients using the Dumas method (N-analyser). Data is shown as mean with SEM, n = 3 technical replicates per feed ingredient. There was a significant effect of protein feed source

(p < 0.001), using a one-way ANOVA. Different letters [a, b, c, d] indicate a significant difference, determined using a Bonferroni post-hoc test (p < 0.05).

Sunflower expeller protein content was significantly lower (49.45 g/100g dry weight) than the crude protein content of soybean meal and AlphaSoy (p < 0.05), which were 54.95 g/100g dry weight and 56.08 g/100 g dry weight respectively. The PFC was almost free of protein (0.02 g/100g of dry weight).

The crude protein content in formulated feeds is reported in Figure 4.2.

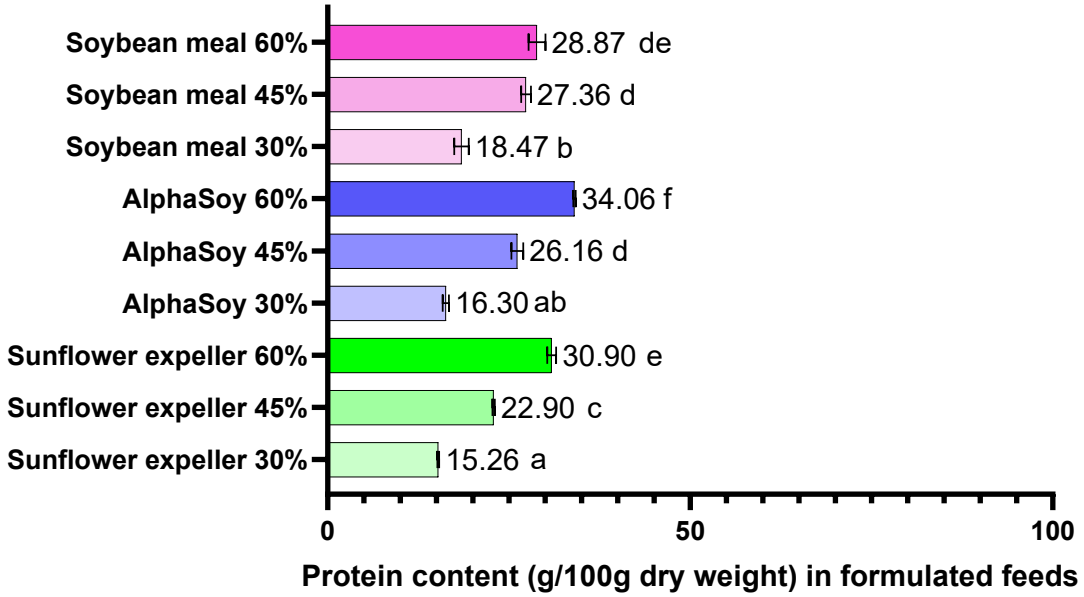


Figure 4.2: Crude protein content (g/100g dry weight) of formulated feeds using the Dumas method (N-analyser). Data is shown as mean with SEM, n = 3 technical replicates per formulated feed. There was a significant effect of protein feed source and inclusion level (p < 0.001), using a two-way ANOVA. Different letters [a, b, c, d, e, f] indicate a significant difference, determined using a Bonferroni post-hoc test (p < 0.05).

Sunflower expeller 30% had the lowest protein content (15.26 g/100g dry weight) and AlphaSoy 60% had the highest protein content (34.06 g/100g dry weight). For all formulated feeds, protein content significantly increased with higher inclusion levels ($p < 0.001$), except for soybean meal 45% and soybean meal 60%.

For ileal digesta (Figure 4.3), crude protein content varied between 11.35 g/100g dry weight for sunflower expeller 30% and 19.91 g/100g dry weight for soybean meal 60%.

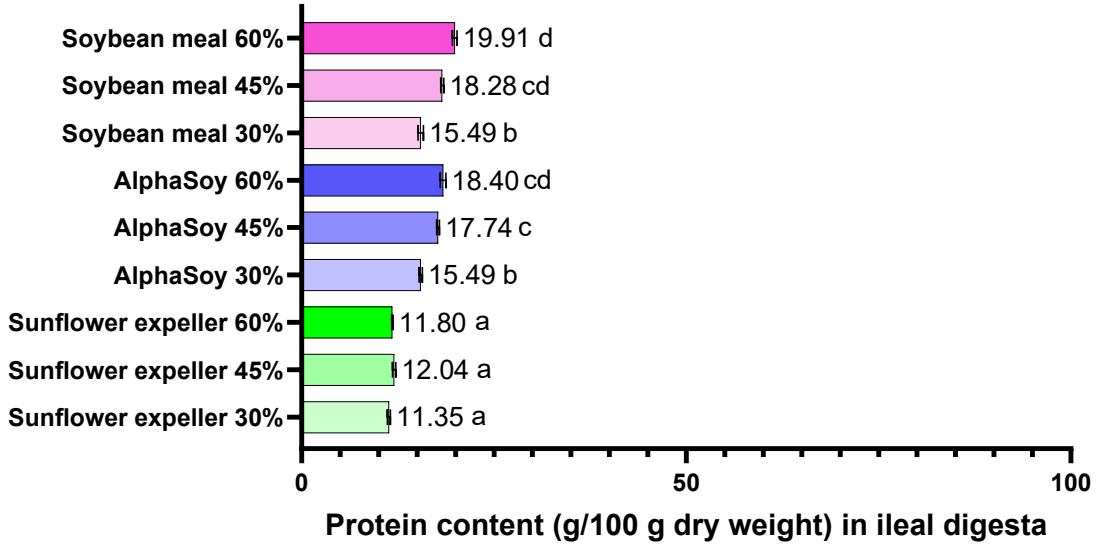


Figure 4.3: Crude protein content (g/100g dry weight) of ileal digesta using the Dumas method (N-analyser). Data is shown as mean with SEM, n = 5 biological replicates per protein source. There was a significant effect of protein feed source and inclusion level ($p < 0.001$), using a two-way ANOVA. Different letters $^{a, b, c, d}$ indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

Sunflower expeller protein content was significantly lower than the protein content of soybean meal and AlphaSoy ($p < 0.05$), for all inclusion levels. However, there was no significant effect of inclusion level for sunflower expeller.

AlphaSoy 45% and AlphaSoy 60% had a significantly higher digesta protein content than AlphaSoy 30% ($p < 0.05$). The digesta crude protein content of soybean meal 30% was significantly lower than that of soybean meal 45% and soybean meal 60% ($p < 0.05$).

4.3.2. Degree of hydrolysis

As shown in Figure 4.4, the degree of hydrolysis (%) was similar for all feed ingredients, with values ranging narrowly between 26.66 and 28.18%.

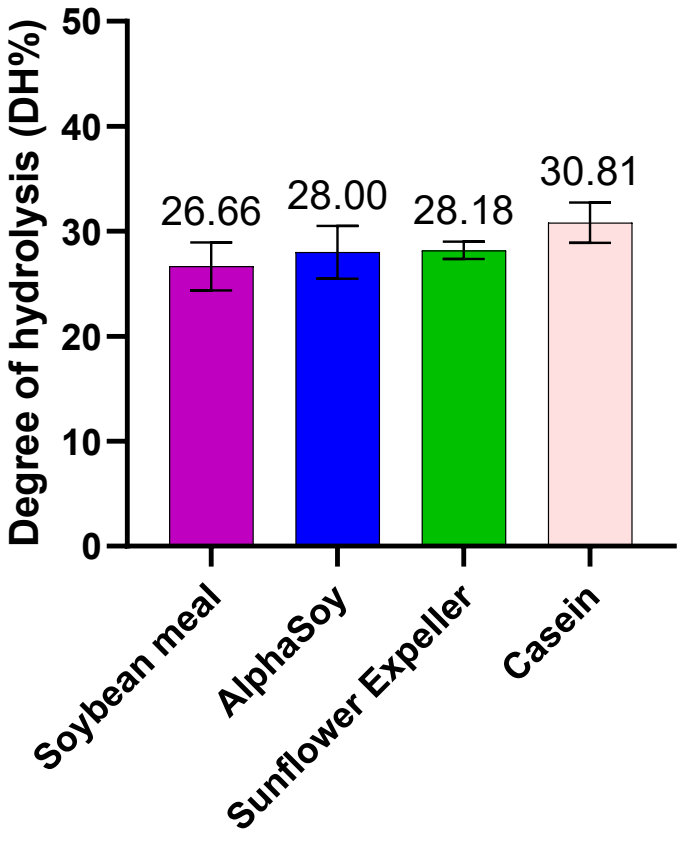


Figure 4.4: Degree of hydrolysis (%) of feed ingredients. Data is shown as mean with SEM, n = 4 technical replicates per feed ingredients. No significant difference in the degree of hydrolysis was determined between the feed ingredients ($p = 0.537$).

The degree of hydrolysis of soybean meal was significantly lower than the degree of hydrolysis of AlphaSoy and sunflower expeller ($p < 0.05$), as shown in Figure 4.5, indicating that soybean meal was less digestible, leading to larger peptides in its absorbable fraction.

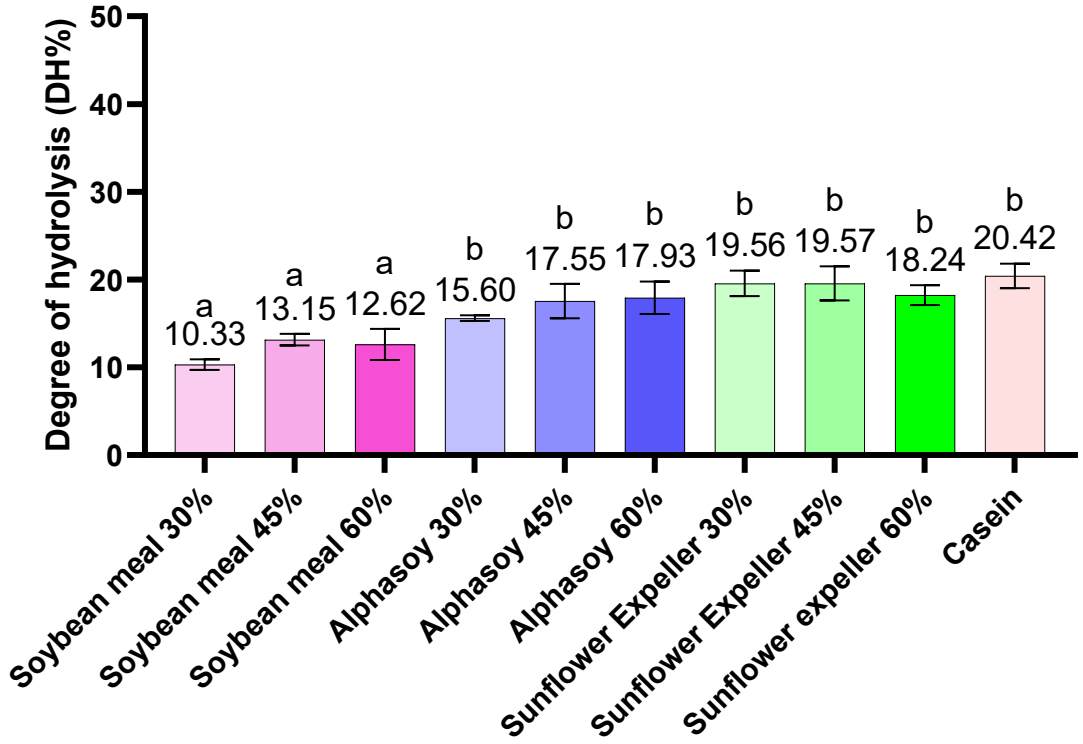


Figure 4.5: Degree of hydrolysis (%) of formulated feeds. Data is shown as mean with SEM, n = 4 technical replicates per formulated feed. There was a significant effect of protein feed source ($p < 0.001$), using a one-way ANOVA. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

4.3.3. Amino acid content, amino acid digestibility and DIAAS of feed ingredients

The amino content of the feed ingredients is reported in Table 4.4.

Table 4.4: Amino acid content of feed ingredients

Amino acid content (g/100g protein)[1]					
Amino acid	**Soybean Meal**	**AlphaSoy**	**Sunflower expeller**	**Casein**	**p-value[2]**
Indispensable amino acids					
Lysine	5.37 ± 0.18[a]	4.98 ± 0.34[a]	3.64 ± 0.13[a]	7.27 ± 0.58[b]	<0.001*
Methionine	1.25 ± 0.03[a]	1.24 ± 0.14[a]	2.43 ± 0.16[b]	3.00 ± 0.25[b]	<0.001*
Threonine	3.31 ± 0.12	3.33 ± 0.20	3.10 ± 0.15	3.77 ± 0.13	0.081
Valine	4.29 ± 0.09[a]	4.28 ± 0.16[a]	4.37 ± 0.32[a]	6.07 ± 0.20[b]	<0.001*
Leucine	6.54 ± 0.26	6.38 ± 0.40	5.80 ± 0.25	7.69 ± 0.74	0.095
Isoleucine	3.73 ± 0.04	3.59 ± 0.15	3.55 ± 0.07	4.57 ± 0.60	0.149
Histidine	1.97 ± 0.10	1.83 ± 0.13	1.92 ± 0.05	2.42 ± 0.42	0.325
Phenylalanine	4.38 ± 0.07	4.27 ± 0.22	3.98 ± 0.12	4.79 ± 0.39	0.182
Dispensable amino acids					
Tyrosine	2.88 ± 0.10[a]	2.77 ± 0.04[a]	2.37 ± 0.10[a]	4.38 ± 0.20[b]	<0.001*
Cystine	1.27 ± 0.04[b]	1.25 ± 0.10[b]	1.50 ± 0.05[b]	0.29 ± 0.02[a]	<0.001*
Serine	4.95 ± 0.10	5.11 ± 0.48	4.27 ± 0.21	5.72 ± 0.58	0.154
Glycine	2.89 ± 0.07	2.79 ± 0.06	3.47 ± 0.12	2.64 ± 0.52	0.229
Glutamic acid	17.02 ± 0.20[a]	16.55 ± 0.80[a]	18.61 ± 0.56[ab]	20.86 ± 0.75[b]	0.005*
Proline	5.08 ± 0.07[a]	5.03 ± 0.17[a]	4.27 ± 0.11[a]	11.19 ± 0.72[b]	<0.001*
Alanine	4.62 ± 0.20 [b]	4.45 ± 0.39[ab]	4.49 ± 0.29[ab]	3.08 ± 0.26[a]	0.019*
Arginine	6.27 ± 0.14[bc]	5.94 ± 0.30[b]	7.30 ± 0.34[c]	3.69 ± 0.16[a]	<0.001*
Aspartic acid	10.04 ± 0.25[b]	10.31 ± 0.54[b]	8.71 ± 0.28[ab]	7.25 ± 0.26[a]	0.001*

[1]Data is shown as mean with SEM, n = 3 technical replicates per feed ingredient

[2]p-value for one-way ANOVA

*Significant difference. Different letters [a, b, c] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$)

Many indispensable amino acids were higher in casein than the plant proteins. Sunflower expeller had a significantly higher methionine content than soybean meal

and AlphaSoy ($p < 0.05$). There was no significant difference in amino acid content between soybean meal and Alphasoy.

The amino acid digestibility of the feed ingredients is reported in Table 4.5.

Table 4.5: Feed ingredients amino acid *in vitro* digestibility

	Digestibility (%)[1]				
Amino acid	**Soybean meal**	**AlphaSoy**	**Sunflower expeller**	**Casein**	**p-value[2]**
Indispensable amino acids					
Lysine	95.06 ± 2.85	96.80 ± 6.41	97.67 ± 2.33	100.00 ± 0.00	0.554
Methionine	99.04 ± 0.96	98.17 ± 2.15	98.57 ± 1.43	100.00 ± 0.00	0.551
Threonine	94.02 ± 4.00	98.08 ± 3.85	99.29 ± 0.71	100.00 ± 0.00	0.153
Valine	95.93 ± 2.34	97.43 ± 5.15	98.81 ± 1.19	100.00 ± 0.00	0.060
Leucine	94.65 ± 3.16	98.02 ± 3.96	98.53 ± 1.47	100.00 ± 0.00	0.338
Isoleucine	95.97 ± 2.37	98.03 ± 3.94	99.11 ± 0.89	99.05 ± 0.95	0.553
Histidine	96.46 ± 2.15	98.16 ± 3.69	98.51 ± 1.49	100.00 ± 0.00	0.495
Phenylalanine	96.74 ± 1.92	98.89 ± 2.21	100.00 ± 0.00	100.00 ± 0.00	0.211
Tyrosine	95.06 ± 2.85	96.80 ± 6.41	97.67 ± 2.33	100.00 ± 0.00	0.096
Dispensable amino acids					
Cystine	95.52 ± 2.78	95.88 ± 8.23	94.98 ± 3.34	100.00 ± 0.00	0.604
Serine	95.42 ± 2.20	97.87 ± 4.26	97.53 ± 1.44	98.90 ± 1.10	0.585
Glycine	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	N/A
Glutamic acid	97.17 ± 1.65	98.42 ± 3.16	100.00 ± 0.00	100.00 ± 0.00	0.317
Proline	98.77 ± 0.79	99.14 ± 1.72	100.00 ± 0.00	100.00 ± 0.00	0.412
Alanine	92.58 ± 1.17	96.66 ± 2.44	95.78 ± 2.30	100.00 ± 0.00	0.056
Arginine	90.58 ± 2.67	95.53 ± 6.19	95.95 ± 2.13	100.00 ± 0.00	0.084
Aspartic acid	94.06 ± 3.51	96.94 ± 6.11	98.51 ± 1.49	100.00 ± 0.00	0.413
Total amino acid	95.22 ± 2.19	97.67 ± 4.20	98.37 ± 0.97	99.89 ± 0.11	0.286

[1]Data is shown as mean with SEM, n = 4 technical replicates per feed ingredient

[2]p-value for one-way ANOVA

The crude protein content was 54.95 g/100g dry weight, 56.08 g/100g dry weight and 49.45 g/100g dry weight for soybean meal, AlphaSoy and sunflower expeller

respectively. For all the feed ingredients, indispensable amino acid digestibility was above 95% and there was no significant difference in amino acid digestibility. The DIAAR and DIAAS of the feed ingredients are reported in Table 4.6, varying from 0.62 to 0.89 for the feed ingredients.

Table 4.6: DIAAR and DIAAS of feed ingredients

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)	Expected LAA
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43			
Casein (Reference protein)	1.33	1.41	1.26	1.27	**1.22**[1]	1.76	**1.22**	1.41	**1.22**	**SAA/Threonine**	SAA
Sunflower expeller	0.94	1.09	0.86	**0.62**	1.42	1.21	0.98	0.99	**0.62**	**Lysine**	Lysine
AlphaSoy	0.90	1.10	0.95	**0.85**	**0.90**	1.35	1.06	0.97	**0.85**	**Lysine/SAA**	SAA
Soybean Meal	0.95	1.12	0.94	**0.89**	**0.91**	1.34	1.00	0.96	**0.89/0.91**	**Lysine/SAA**	SAA

[1]Values in bold represent the lowest DIAAR and the DIAAS

Casein had the highest DIAAS (1.22), as expected. Limiting amino acids for casein were SAA and threonine. Lysine was the limiting amino acid for Sunflower expeller, with a DIAAS of 0.62. AlphaSoy had a DIAAS of 0.85 with lysine as a limiting amino acid and close DIAAR of 0.90 with histidine and sulphur-containing amino acids (SAA). The lowest DIAAR of soybean Meal was 0.89 for lysine and 0.91 for SAA.

4.3.4. Amino acid content, amino acid digestibility and DIAAS of formulated feeds

The amino acid content of formulated feeds is reported in Table 4.7.

Table 4.7: Amino acid content of formulated feeds

	Amino acid content (g/100g protein)[1]									
Amino acid	**Soybean meal 30%**	**Soybean meal 45%**	**Soybean meal 60%**	**AlphaSoy 30%**	**AlphaSoy 45%**	**AlphaSoy 60%**	**Sunflower expeller 30%**	**Sunflower expeller 45%**	**Sunflower expeller 60%**	**p-value[2]**
Indispensable amino acids										
Lysine	5.13 ± 0.63^{b}	5.53 ± 0.27^{b}	6.20 ± 0.23^{b}	7.00 ± 0.63^{b}	6.21 ± 0.26^{b}	5.81 ± 0.02^{b}	4.66 ± 0.10^{a}	4.46 ± 0.23^{a}	3.96 ± 0.16^{a}	0.044*
Methionine	0.53 ± 0.19	1.18 ± 0.07	1.14 ± 0.02	0.77 ± 0.05	1.13 ± 0.12	1.17 ± 0.05	1.58 ± 0.05	2.11 ± 0.06	2.11 ± 0.14	0.677
Threonine	3.68 ± 0.50	3.58 ± 0.13	4.05 ± 0.05	4.76 ± 0.20	4.18 ± 0.30	3.67 ± 0.10	4.41 ± 0.25	3.90 ± 0.12	3.56 ± 0.17	0.059
Valine	4.09 ± 0.43	4.48 ± 0.35	4.99 ± 0.23	5.22 ± 0.14	4.79 ± 0.09	4.71 ± 0.23	5.04 ± 0.06	5.06 ± 0.22	4.92 ± 0.18	0.094
Leucine	7.81 ± 0.78	7.70 ± 0.40	7.78 ± 0.31	9.64 ± 0.18	8.09 ± 0.23	7.70 ± 0.58	8.17 ± 0.17	7.11 ± 0.16	6.40 ± 0.19	0.140
Isoleucine	5.06 ± 0.39^{a}	4.19 ± 0.07^{a}	4.92 ± 0.06^{a}	6.35 ± 0.09^{b}	4.94 ± 0.03^{b}	4.53 ± 0.10^{b}	5.97 ± 0.19^{a}	4.97 ± 0.15^{a}	4.10 ± 0.13^{a}	<0.001*
Histidine	2.60 ± 0.24^{a}	2.10 ± 0.06^{a}	2.29 ± 0.05^{a}	3.16 ± 0.06^{b}	2.43 ± 0.02^{b}	2.25 ± 0.10^{b}	2.84 ± 0.04^{b}	2.57 ± 0.07^{b}	2.36 ± 0.05^{b}	0.031*
Phenylalanine	5.22 ± 0.49^{a}	4.93 ± 0.12^{a}	5.29 ± 0.05^{a}	6.60 ± 0.24^{b}	5.48 ± 0.18^{b}	4.99 ± 0.11^{b}	5.95 ± 0.04^{ab}	5.26 ± 0.06^{ab}	4.63 ± 0.11^{ab}	0.007*
Dispensable amino acids										
Tyrosine	3.49 ± 0.34	3.38 ± 0.06	3.71 ± 0.14	4.68 ± 0.31	3.51 ± 0.15	3.26 ± 0.04	3.90 ± 0.05	3.18 ± 0.13	3.11 ± 0.40	0.026*
Cystine	0.92 ± 0.22	0.83 ± 0.00	1.25 ± 0.05	1.47 ± 0.17	1.37 ± 0.03	1.34 ± 0.06	1.34 ± 0.03	1.46 ± 0.06	1.38 ± 0.13	0.119
Serine	2.87 ± 1.20	5.36 ± 0.12	5.27 ± 0.13	3.51 ± 0.39	4.59 ± 0.20	5.20 ± 0.29	2.18 ± 0.43	3.16 ± 0.02	3.87 ± 0.40	0.561
Glycine	5.91 ± 0.07^{a}	4.49 ± 0.22^{a}	4.41 ± 0.03^{a}	6.73 ± 0.17^{a}	4.81 ± 0.11^{a}	4.04 ± 0.15^{a}	7.45 ± 0.02^{b}	5.49 ± 0.17^{b}	4.47 ± 0.11^{b}	<0.001*
Glutamic acid	16.75 ± 2.02^{a}	17.82 ± 0.61^{a}	19.76 ± 0.23^{a}	22.52 ± 1.47^{b}	20.02 ± 0.47^{b}	19.17 ± 0.50^{b}	21.19 ± 0.26^{b}	21.25 ± 0.25^{b}	20.42 ± 0.82^{b}	0.043*
Proline	4.13 ± 0.47	5.16 ± 0.28	5.52 ± 0.06	5.62 ± 0.55	5.33 ± 0.24	5.62 ± 0.26	4.12 ± 0.20	4.42 ± 0.10	4.39 ± 0.25	0.174
Alanine	2.00 ± 1.00	3.51 ± 0.14	3.70 ± 0.11	1.89 ± 0.54	3.09 ± 0.19	3.73 ± 0.28	1.24 ± 0.55	2.69 ± 0.14	3.84 ± 0.14	0.796
Arginine	6.15 ± 0.63^{a}	6.72 ± 0.15^{a}	7.18 ± 0.05^{a}	8.13 ± 0.48^{a}	6.99 ± 0.30^{a}	6.71 ± 0.25^{a}	8.30 ± 0.06^{b}	8.18 ± 0.15^{b}	7.88 ± 0.26^{b}	0.017*
Aspartic acid	10.37 ± 1.06	10.93 ± 0.73	11.68 ± 0.44	13.67 ± 0.78	12.39 ± 0.72	11.38 ± 0.06	10.49 ± 0.08	9.95 ± 0.12	9.23 ± 0.23	0.074

[1]Data is shown as mean with SEM, n = 3 technical replicates per formulated feed

[2]p-value for two-way ANOVA (sample type x inclusion level)

*Significant difference. Different letters [a, b] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

Significant differences were observed between the samples, depending on both the sample type and inclusion level (two-way ANOVA), for lysine, isoleucine, histidine, phenylalanine, tyrosine, glycine, glutamic acid, and arginine content ($p < 0.05$). Lysine content was significantly lower for the sunflower expeller than for all other formulated feeds ($p < 0.05$). AlphaSoy had a significantly higher isoleucine content ($p < 0.05$) than that of sunflower expeller and AlphaSoy.

Histidine and glutamic acid content were significantly lower for soybean meal compared to that of AlphaSoy and sunflower expeller. AlphaSoy had a significantly higher phenylalanine content ($p < 0.05$) than soybean meal, but not sunflower expeller. Glycine and arginine content were significantly higher in sunflower expeller than in soybean meal and AlphaSoy ($p < 0.05$).

The amino acid digestibility values for all the formulated feeds are reported in Table 4.8.

Table 4.8: Formulated feeds amino acid *in vitro* digestibility

	Digestibility (%)										
Amino acid	**Soybean meal 30%**	**Soybean meal 45%**	**Soybean meal 60%**	**AlphaSoy 30%**	**AlphaSoy 45%**	**AlphaSoy 60%**	**Sunflower expeller 30%**	**Sunflower expeller 45%**	**Sunflower expeller 60%**	**Casein**	**p-value**
					Indispensable amino acids						
Lysine	67.21 ± 12.02^{a}	72.79 ± 6.77^{a}	68.56 ± 5.10^{a}	82.89 ± 6.40^{ab}	86.17 ± 6.60^{ab}	93.77 ± 5.40^{ab}	93.01 ± 5.50^{b}	91.37 ± 7.90^{b}	97.04 ± 3.00^{b}	100.00 ± 0.00^{b}	0.003*
Methionine	69.65 ± 12.81^{a}	78.63 ± 10.27^{a}	76.45 ± 5.10^{a}	90.62 ± 5.50^{b}	91.40 ± 7.20^{b}	96.65 ± 3.30^{b}	94.08 ± 5.20^{b}	93.00 ± 7.00^{b}	99.02 ± 1.00^{b}	100.00 ± 0.00^{b}	<0.001*
Threonine	75.50 ± 7.21^{a}	79.06 ± 4.25^{a}	79.39 ± 8.00^{a}	90.57 ± 3.60^{b}	93.25 ± 5.70^{b}	96.86 ± 3.10^{b}	96.68 ± 2.50^{b}	91.46 ± 8.50^{b}	97.60 ± 1.50^{b}	100.00 ± 0.00^{b}	<0.001*
Valine	68.74 ± 14.70^{a}	47.83 ± 13.18^{a}	69.46 ± 10.80^{a}	83.54 ± 7.40^{b}	84.32 ± 7.20^{b}	86.62 ± 8.00^{b}	96.94 ± 2.70^{b}	90.17 ± 7.20^{b}	98.72 ± 1.30^{b}	100.00 ± 0.00^{a}	<0.001*
Leucine	62.27 ± 5.31^{a}	55.23 ± 17.25^{a}	67.62 ± 3.60^{a}	74.76 ± 15.10^{b}	85.45 ± 8.90^{b}	95.55 ± 3.00^{b}	100.00 ± 0.00^{b}	92.67 ± 7.20^{b}	97.68 ± 2.30^{b}	100.00 ± 0.00^{b}	<0.001*
Isoleucine	59.70 ± 10.45^{a}	64.79 ± 1.93^{a}	69.27 ± 3.00^{a}	89.18 ± 6.20^{b}	91.77 ± 5.60^{b}	96.74 ± 2.50^{b}	100.00 ± 0.00^{b}	92.10 ± 7.90^{b}	98.91 ± 1.10^{b}	100.00 ± 0.00^{b}	<0.001*
Histidine	67.98 ± 11.26^{a}	78.71 ± 6.26^{a}	80.43 ± 5.40^{a}	90.78 ± 3.50^{b}	92.75 ± 7.20^{b}	97.46 ± 1.50^{b}	99.10 ± 0.90^{b}	94.51 ± 5.50^{b}	99.72 ± 0.30^{b}	100.00 ± 0.00^{b}	<0.001*
Phenylalanine	66.2 ± 11.15^{a}	75.33 ± 8.05^{a}	69.01 ± 7.60^{a}	87.08 ± 4.60^{b}	92.81 ± 6.10^{b}	97.43 ± 2.60^{b}	100.0 ± 0.00^{b}	94.40 ± 5.60^{b}	99.49 ± 0.50^{b}	100.00 ± 0.00^{b}	<0.001*
					Dispensable amino acids						
Tyrosine	80.0 2 ± 14.13	73.21 ± 12.23	76.93 ± 10.40	95.66 ± 2.60	83.53 ± 16.50	97.95 ± 2.10	100.0 ± 0.00	92.29 ± 7.70	100.0 ± 0.00	100.00 ± 0.00	0.113
Cystine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	100.00 ± 0.00	N/A
Serine	53.08 ± 16.46^{a}	69.66 ± 12.64^{a}	72.33 ± 7.30^{a}	82.17 ± 7.30^{ab}	81.27 ± 11.90^{ab}	92.69 ± 5.20^{ab}	96.65 ± 2.90^{b}	83.43 ± 16.60^{b}	97.44 ± 2.60^{b}	100.00 ± 0.00^{b}	0.004*
Glycine	85.93 ± 10.27	87.50 ± 7.48	89.69 ± 8.90	87.22 ± 8.70	88.42 ± 11.60	96.86 ± 3.10	77.99 ± 22.00	93.45 ± 6.60	96.48 ± 3.50	100.00 ± 0.00	0.723
Glutamic acid	62.27 ± 6.23^{a}	71.56 ± 3.68^{a}	67.85 ± 3.10^{a}	78.69 ± 4.70^{b}	85.33 ± 6.60^{b}	91.06 ± 4.90^{b}	96.85 ± 2.00^{c}	94.35 ± 5.70^{c}	98.43 ± 1.60^{c}	100.00 ± 0.00^{c}	<0.001*
Proline	69.45 ± 2.35^{a}	68.75 ± 3.92^{a}	64.51 ± 2.60^{a}	83.92 ± 3.40^{b}	86.96 ± 6.20^{b}	91.13 ± 3.80^{b}	96.04 ± 4.00^{bc}	91.41 ± 6.80^{bc}	98.47 ± 1.30^{bc}	100.00 ± 0.00^{c}	<0.001*
Alanine	70.47 ± 10.06^{a}	72.07 ± 7.26^{a}	71.02 ± 7.00^{a}	87.82 ± 4.60^{b}	94.05 ± 4.10^{b}	90.99 ± 7.60^{b}	98.94 ± 1.10^{b}	92.87 ± 7.10^{b}	98.41 ± 1.60^{b}	100.00 ± 0.00^{b}	<0.001*
Arginine	62.06 ± 10.87^{a}	69.06 ± 7.67^{a}	67.19 ± 6.70^{a}	81.70 ± 7.70^{b}	87.28 ± 6.00^{b}	86.74 ± 5.80^{b}	91.71 ± 7.50^{b}	92.55 ± 6.30^{b}	98.74 ± 1.30^{b}	100.00 ± 0.00^{b}	<0.001*
Aspartic acid	54.42 ± 7.94^{a}	62.72 ± 4.71^{a}	66.82 ± 8.70^{a}	71.42 ± 5.80^{b}	86.63 ± 5.30^{b}	84.62 ± 5.50^{b}	93.04 ± 2.60^{b}	90.72 ± 9.30^{b}	99.47 ± 0.50^{b}	100.00 ± 0.00^{b}	<0.001*
Total amino acid	60.80 ± 7.32^{a}	67.33 ± 4.81^{a}	67.52 ± 4.70^{a}	80.66 ± 5.00^{b}	86.54 ± 5.90^{b}	90.21 ± 3.70^{b}	94.55 ± 2.10^{b}	91.53 ± 7.30^{b}	97.58 ± 1.30^{b}	100.00 ± 0.00^{b}	<0.001*

[1]Data is shown as mean with SEM, n = 3 technical replicates per formulated feed

[2]p-value for one-way ANOVA (most cystine values were not reported due to values below the limit of detection)

*Significant difference. Different letters $^{a, b, c}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

The crude protein content varied from 15.26 g/100g dry weight to 34.06 g/100g dry weight across all formulated feeds and inclusion levels. The total amino acid digestibility values ranged between 60.80% and 100.00%. For most amino acids, the digestibility in soybean meal was significantly lower compared to that of AlphaSoy and sunflower expeller ($p < 0.05$).

The DIAAS of formulated feeds varied between 0.32 and 0.80, as reported in Table 4.9.

Table 4.9: DIAAR and DIAAS of formulated feeds

Sample	Histidine	Isoleucine	Leucine	Lysine	Sulphur-containing Amino Acids (SAA)	Aromatic Amino Acids (AAA)	Threonine	Valine	DIAAS	Limiting amino acid (LAA)	Expected LAA
Reference pattern for a 6-month to 3-year-old human (based on human milk)	20	32	66	57	27	52	31	43			
Casein (Reference protein)	1.38	1.48	1.31	1.32	**1.26**[1]	1.83	**1.26**	1.46	**1.26**	**SAA/Threonine**	SAA
Soybean meal 30%	0.88	0.94	0.74	0.61	**0.32**	1.20	0.90	0.65	**0.32**	**SAA**	SAA
Soybean meal 45%	0.83	0.85	0.64	0.71	**0.50**	1.19	0.91	0.50	**0.50**	**SAA**	SAA
Soybean meal 60%	0.92	1.06	0.80	0.75	**0.58**	1.25	1.04	0.81	**0.58**	**SAA**	SAA
AlphaSoy 30%	1.43	1.77	1.09	1.02	**0.59**	1.97	1.39	1.01	**0.59**	**SAA**	SAA
AlphaSoy 45%	1.13	1.42	1.05	0.94	**0.70**	1.54	1.26	0.94	**0.70**	**SAA**	SAA
AlphaSoy 60%	1.10	1.37	1.11	0.96	**0.80**	1.55	1.15	0.95	**0.80**	**SAA**	SAA
Sunflower expeller 30%	1.41	1.87	1.24	**0.76**	**0.79**	1.89	1.38	1.14	**0.76/0.79**	**Lysine/SAA**	Lysine
Sunflower expeller 45%	1.21	1.43	1.00	**0.72**	1.06	1.52	1.15	1.06	**0.72**	**Lysine**	Lysine
Sunflower expeller 60%	1.18	1.27	0.95	**0.67**	1.15	1.48	1.12	1.13	**0.67**	**Lysine**	Lysine

The soybean meal DIAAS were 0.32, 0.50 and 0.58 for 30%, 45% and 60% inclusion levels respectively, and the limiting amino acids were the sulphur-containing amino acids. The limiting amino acids for AlphaSoy were the SAA, and the DIAAS were 0.59 at an inclusion level of 30%, 0.70 at an inclusion level of 45% and 0.80 at an inclusion level of 60%.

Sunflower expeller 30% had a DIAAS of 0.76 with lysine as a limiting amino acid, and a close DIAAR of 0.79 for SAA. Lysine was the limiting amino acid for both Sunflower expeller 45% and 60%, with DIAAS of 0.72 and 0.62 respectively.

4.3.5. Apparent ileal digestibility

The apparent ileal digestibility (%) based on crude protein is reported in Figure 4.6.

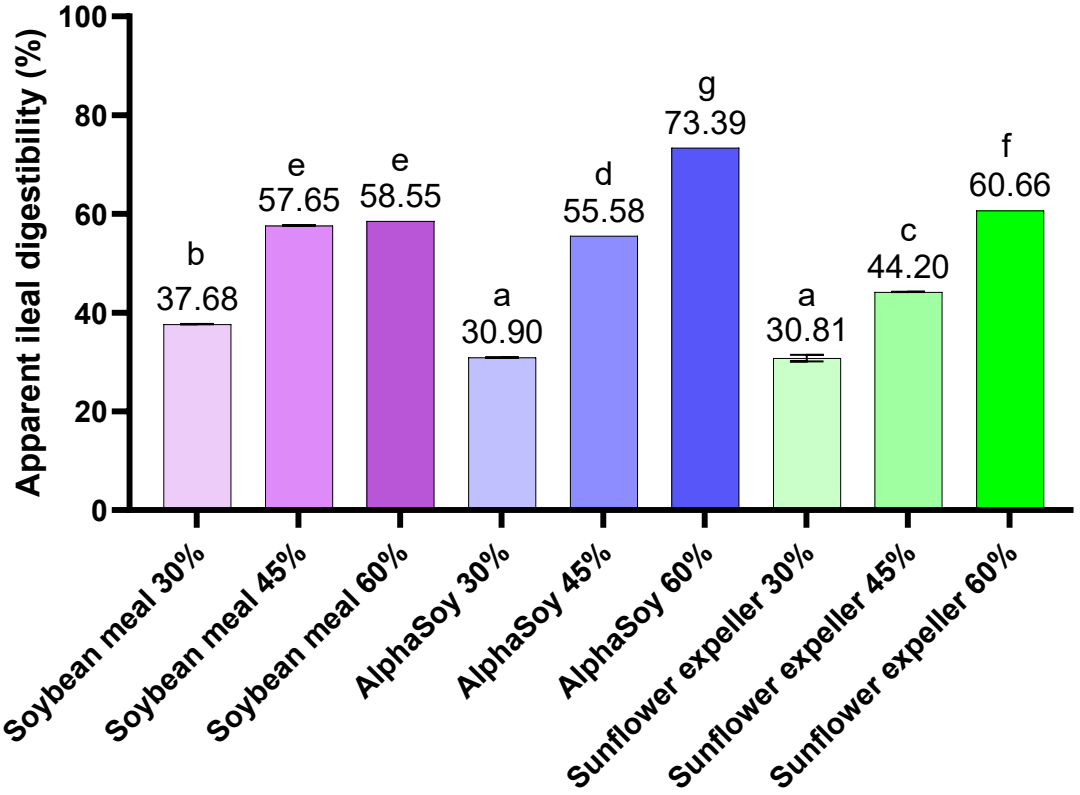


Figure 4.6: Apparent ileal digestibility (%) comparison of ileal digesta based on crude protein from the formulated feeds. Data is shown as mean with SEM, n = 5 biological replicates per protein source. There was a significant effect of protein feed source and inclusion level ($p < 0.001$), using a two-way ANOVA. Different letters [a, b, c, d, e, f, g] indicate a significant difference, determined using a Bonferroni post-hoc test ($p < 0.05$).

No environmental anomalies occurred during the chicken trial. One bird died whilst on the experimental diets. The crude protein content varied from 15.26 g/100g dry weight to 34.06 g/100g dry weight across all formulated feeds and inclusion levels. Digestibility values ranged from 30.81% with sunflower expeller (inclusion level 30%)

to 73.39% with AlphaSoy (inclusion level 60%). The digestibility value significantly increased as the inclusion level increased for all protein sources, except for soybean meal 45% and soybean meal 60% ($p < 0.05$).

At an inclusion level of 30% and 45%, soybean meal had the highest digestibility compared to the digestibility of AlphaSoy and sunflower expeller. The digestibility of AlphaSoy 60% was significantly higher than the digestibility of soybean meal 60% and sunflower expeller 60%.

4.3.6. Ileal (*in vivo*) amino acid digestibility

The ileal (*in vivo*) amino acid digestibility is reported in Table 4.10.

Table 4.10: Ileal (*in vivo*) amino acid digestibility

	Digestibility (%)[1]									
Amino acid	**Soybean meal 30%**	**Soybean meal 45%**	**Soybean meal 60%**	**AlphaSoy 30%**	**AlphaSoy 45%**	**AlphaSoy 60%**	**Sunflower expeller 30%**	**Sunflower expeller 45%**	**Sunflower expeller 60%**	**p-value[2]**
Indispensable amino acids										
Lysine	89.94 ± 0.27^{bc}	90.91 ± 0.48^{bc}	89.19 ± 0.30^{b}	90.54 ± 0.36^{bc}	90.85 ± 0.33^{bc}	91.09 ± 0.49^{c}	87.05 ± 0.56^{a}	89.73 ± 0.37^{bc}	91.04 ± 0.21^{bc}	<0.001*
Methionine	75.81 ± 0.93^{a}	90.00 ± 0.68^{bcd}	86.95 ± 0.83^{b}	77.25 ± 1.41^{a}	87.87 ± 0.57^{bc}	89.79 ± 0.41^{bcd}	86.34 ± 0.67^{b}	91.45 ± 0.37^{cd}	93.34 ± 0.31^{d}	<0.001*
Threonine	85.74 ± 0.39^{abc}	86.85 ± 0.66^{bc}	85.07 ± 0.49^{ab}	85.95 ± 0.52^{bc}	87.17 ± 0.41^{bc}	85.71 ± 0.51^{abc}	83.47 ± 0.56^{a}	85.72 ± 0.39^{abc}	87.67 ± 0.25^{c}	<0.001*
Valine	86.25 ± 0.77^{ab}	88.98 ± 0.80^{bc}	86.55 ± 0.39^{ab}	87.11 ± 0.63^{ab}	89.03 ± 0.56^{bc}	89.02 ± 0.48^{bc}	85.44 ± 0.82^{a}	88.98 ± 0.47^{bc}	90.72 ± 0.40^{c}	0.002*
Leucine	89.67 ± 0.24^{abc}	89.78 ± 0.69^{abc}	87.51 ± 0.70^{a}	90.03 ± 0.61^{abc}	89.87 ± 0.37^{abc}	90.62 ± 0.52^{c}	87.93 ± 0.64^{ab}	89.39 ± 0.45^{abc}	90.40 ± 0.32^{bc}	0.001*
Isoleucine	91.37 ± 0.83	89.73 ± 0.67	88.37 ± 1.29	91.59 ± 0.69	91.57 ± 0.57	89.39 ± 0.74	90.31 ± 0.59	91.12 ± 0.45	91.21 ± 0.56	0.094
Histidine	89.91 ± 1.16^{ab}	88.48 ± 1.09^{ab}	85.00 ± 1.28^{a}	89.53 ± 0.90^{ab}	90.23 ± 0.58^{ab}	86.28 ± 0.63^{ab}	87.23 ± 1.36^{ab}	89.13 ± 1.16^{ab}	90.42 ± 1.09^{b}	0.006*
Phenylalanine	90.89 ± 0.30	90.32 ± 0.73	89.67 ± 1.08	90.91 ± 0.55	91.57 ± 0.45	91.52 ± 0.50	89.46 ± 0.87	91.21 ± 0.51	91.91 ± 0.60	0.121
Dispensable amino acids										
Tyrosine	91.34 ± 0.79	91.84 ± 0.46	89.38 ± 1.05	92.18 ± 0.47	92.43 ± 0.19	89.45 ± 0.86	88.94 ± 0.90	90.00 ± 0.71	91.66 ± 0.76	0.003*
Cystine	47.44 ± 7.38	41.91 ± 11.17	59.81 ± 9.22	55.99 ± 8.11	59.85 ± 7.34	73.95 ± 3.56	55.81 ± 6.52	61.53 ± 6.03	66.66 ± 5.43	0.894
Serine	77.94 ± 1.78^{bc}	88.16 ± 1.65^{cd}	85.12 ± 2.00^{bcd}	76.18 ± 3.08^{bc}	83.74 ± 0.50^{bcd}	90.50 ± 1.29^{d}	55.24 ± 5.16^{a}	73.89 ± 2.49^{b}	88.97 ± 1.80^{bcd}	0.004*
Glycine	93.00 ± 0.39	91.89 ± 0.40	88.39 ± 0.51	92.07 ± 0.34	91.54 ± 0.39	88.87 ± 0.05	88.02 ± 0.76	87.22 ± 0.93	86.13 ± 1.08	0.238
Glutamic acid	90.82 ± 0.22^{ab}	90.89 ± 0.74^{ab}	89.36 ± 0.83^{a}	91.20 ± 0.69^{ab}	90.91 ± 0.33^{ab}	92.26 ± 0.66^{bc}	92.10 ± 0.54^{bc}	94.08 ± 0.40^{cd}	95.17 ± 0.26^{d}	0.003*
Proline	84.59 ± 0.71^{ab}	87.92 ± 1.06^{bcd}	85.31 ± 0.92^{abcd}	85.18 ± 1.12^{abc}	86.92 ± 0.47^{bcd}	89.47 ± 1.17^{cd}	81.75 ± 1.23^{a}	86.91 ± 0.86^{bcd}	89.55 ± 0.48^{d}	0.003*
Alanine	75.90 ± 1.33^{c}	87.24 ± 0.89^{def}	83.78 ± 0.85^{def}	67.08 ± 1.59^{b}	83.31 ± 0.97^{de}	87.56 ± 0.72^{ef}	46.04 ± 2.43^{a}	81.13 ± 1.29^{cd}	89.83 ± 0.55^{f}	<0.001*
Arginine	91.81 ± 0.18^{b}	92.09 ± 0.59^{b}	89.59 ± 0.61^{a}	92.67 ± 0.52^{b}	92.75 ± 0.30^{b}	92.99 ± 0.39^{b}	93.51 ± 0.23^{bc}	95.01 ± 0.26^{cd}	95.95 ± 0.13^{d}	<0.001*
Aspartic acid	86.20 ± 0.14^{ab}	86.76 ± 1.13^{ab}	83.67 ± 1.22^{a}	86.36 ± 1.01^{ab}	86.70 ± 0.46^{ab}	87.34 ± 0.91^{ab}	87.27 ± 0.79^{ab}	89.46 ± 0.75^{bc}	91.37 ± 0.36^{c}	0.004*
Total amino acid	88.31 ± 0.20^{abc}	89.17 ± 0.73^{abcd}	87.00 ± 0.72^{a}	88.47 ± 0.66^{abc}	89.07 ± 0.37^{abcd}	89.82 ± 0.56^{cd}	87.15 ± 0.50^{ab}	89.60 ± 0.35^{bcd}	91.27 ± 0.18^{d}	<0.001*

[1]Data is shown as mean with SEM, n = 5 biological replicates per protein source

[2]p-value for two-way ANOVA (sample type x inclusion level)

Significant difference. Different letters $^{a, b, c, d}$ indicate a significant difference determined using a Bonferroni post-hoc test ($p < 0.05$)

There was a significant protein feed source x inclusion level interaction ($p < 0.001$). For all protein sources and inclusion levels, the total amino acid digestibility was between 87.00% and 91.27%.

For soybean meal, methionine and alanine digestibility were significantly lower at the 30% inclusion level than at the inclusion levels of 45% and 60% ($p < 0.05$).

For AlphaSoy, methionine digestibility was significantly lower at the 30% inclusion level compared to the 45% and 60% inclusion levels ($p < 0.05$). Serine digestibility was significantly higher for AlphaSoy 60% ($p < 0.05$) compared to that of AlphaSoy 30%, but not AlphaSoy 45%. Alanine digestibility, however, was significantly lower at the 60% inclusion level compared to the 30% inclusion level ($p < 0.05$), but not significantly different from the 45% inclusion level.

For sunflower expeller, the total amino acid digestibility was significantly higher at the 60% inclusion level with 91.27% than at the 30% inclusion level with 87.15% ($p < 0.05$), but not significantly different from the 45% inclusion level (89.60%). Lysine, methionine, valine, serine, proline and aspartic acid digestibility were also significantly higher at the 60% inclusion level ($p < 0.05$) than at the 30% inclusion level, but not significantly different from the 45% inclusion level.

Threonine, glutamic acid and arginine digestibility in sunflower expeller were significantly higher at the 60% inclusion level than at the 30% inclusion level, but not significantly different from the 45% inclusion level ($p < 0.05$). Alanine digestibility for sunflower expeller increased significantly with higher inclusion levels ($p < 0.05$).

4.3.7. Correlation and Bland-Altman analysis of *in vitro* and *in vivo* digestibility data

The total amino acid, lysine, methionine and threonine digestibility correlation between *in vivo* and *in vitro* methods across all formulated feeds and inclusion levels is reported in Figure 4.7.

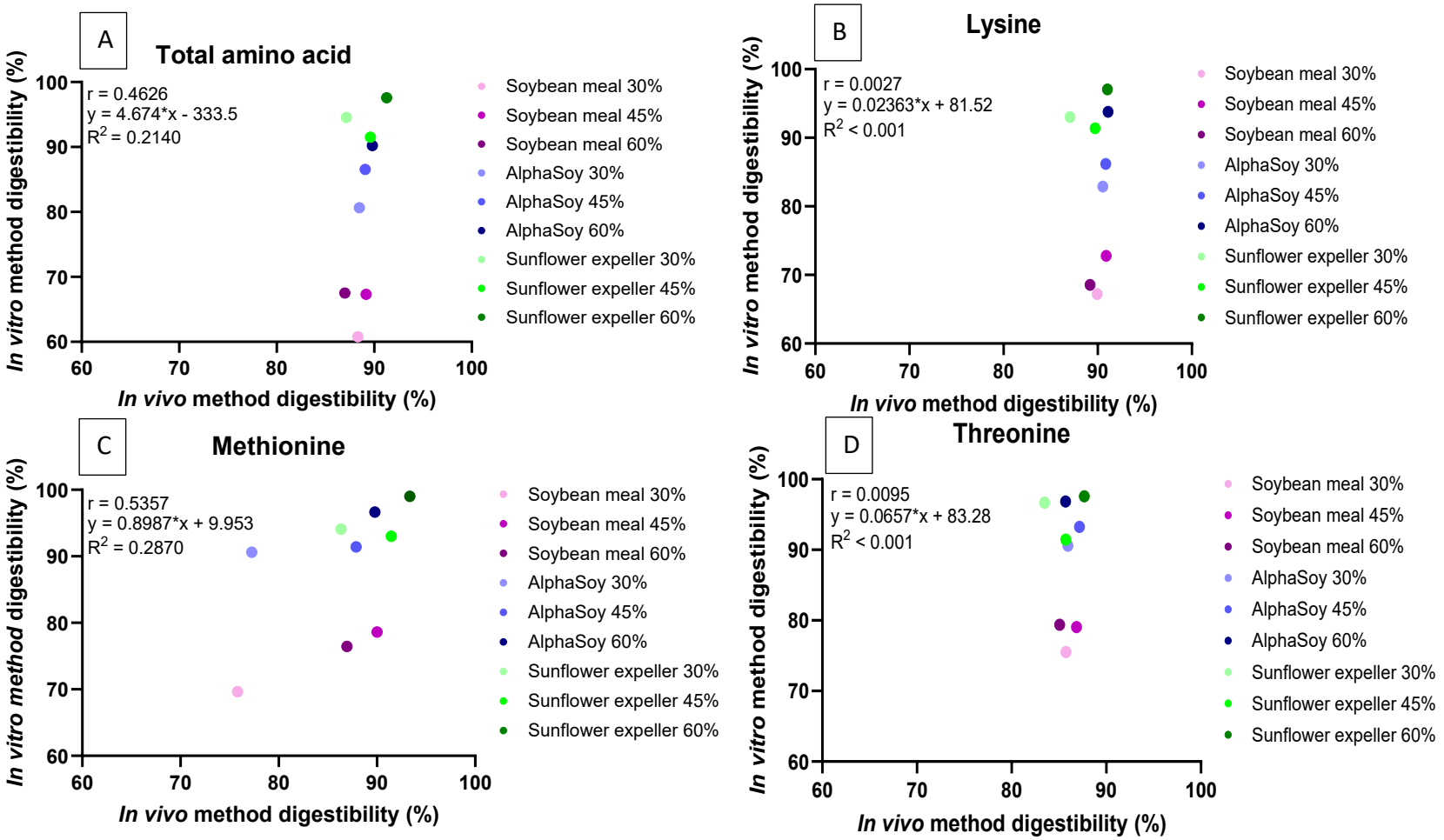


Figure 4.7: Digestibility (%) correlation between in vivo or in vitro method for total amino acid, lysine, methionine and threonine. Each point represents a sample. A. Total amino acid digestibility correlation. B. Lysine digestibility correlation. C. Methionine digestibility correlation. D. Threonine digestibility correlation.

In vitro digestibility values tended to be lower than *in vivo* digestibility values for soybean meal. No specific trend was observed for Alpha Soy in terms of digestibility values between *in vitro* and *in vivo* data. For sunflower expeller, *in vitro* digestibility values tended to be higher than *in vivo* digestibility values.

Total amino acid (TAA) digestibility exhibited a moderate positive correlation ($r = 0.4626$, $R^2 = 0.2140$), indicating that approximately 21% of the variation in *in vitro* measurements was explained by *in vivo* data, suggesting moderate predictive potential. However, the correlation was not statistically significant ($p = 0.2099$).

Methionine showed a similar moderate correlation ($r = 0.5357$, $R^2 = 0.2870$), with *in vitro* methods capturing around 29% of the variation. Similarly, this correlation was not statistically significant ($p = 0.1371$). In contrast, lysine ($r = 0.0027$) and threonine ($r = 0.0095$) digestibility showed negligible correlations with $R^2 < 0.001$.

Figure 4.8 shows the Bland-Altman analysis for digestibility differences between *in vitro* and *in vivo* methods for total amino acids (TAA), lysine, methionine, and threonine, across all formulated feeds and inclusion levels.

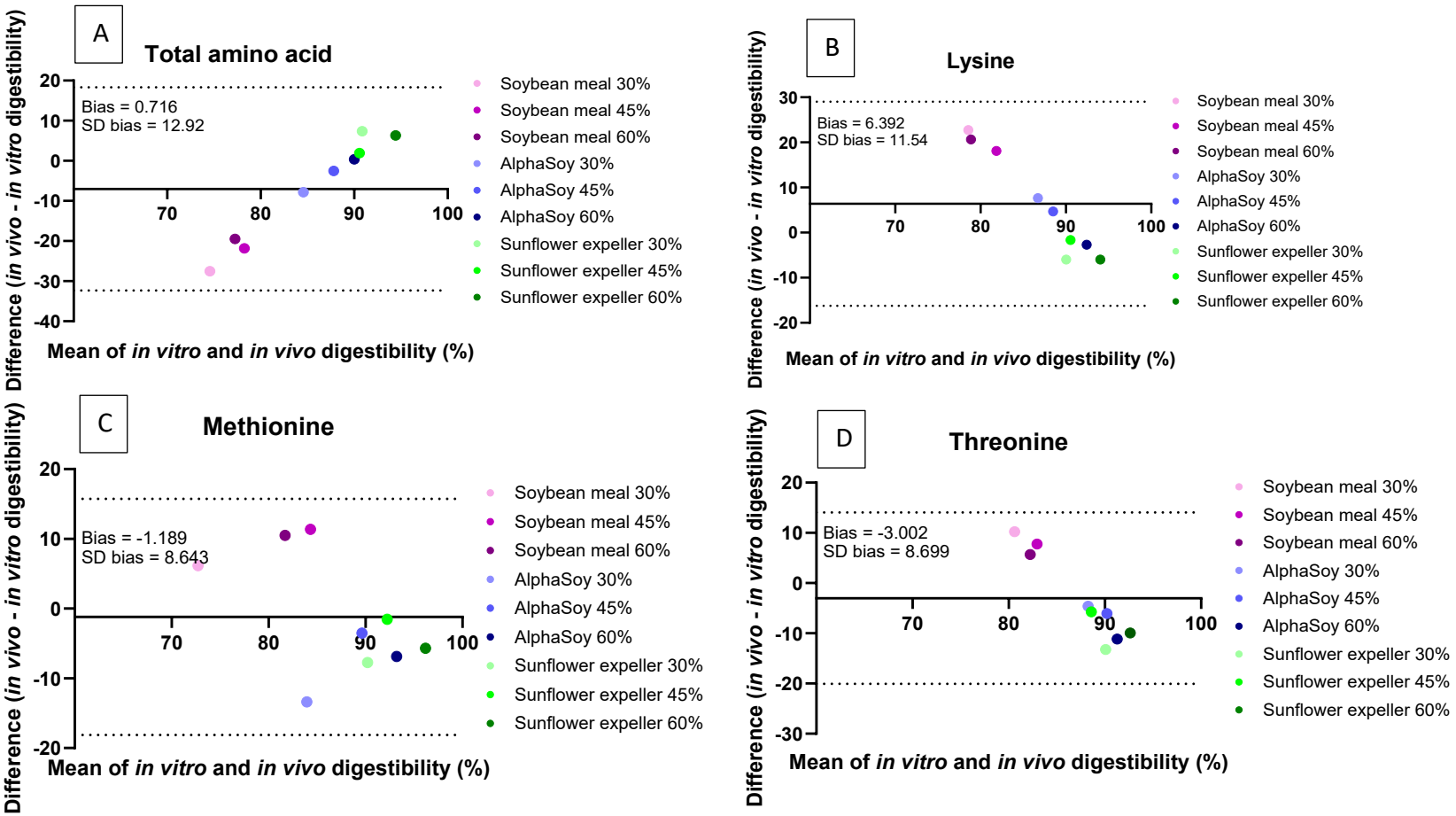


Figure 4.8: Digestibility (%) Bland-Altman analysis between in vivo or in vitro method for total amino acid, lysine, methionine and threonine. Each point represents a sample. A. Total amino acid digestibility correlation. B. Lysine digestibility correlation. C. Methionine digestibility correlation. D. Threonine digestibility correlation.

For TAA, the mean bias was small (+0.716%), with reasonable agreement and some variability (SD = 12.92). Lysine showed a larger positive bias (+6.392%), indicating overestimation by *in vitro* methods. Methionine exhibited a small negative bias (-1.189%) with low variability (SD = 8.643%), while threonine showed a negative bias (-3.002%) with moderate variability (SD = 8.699%).

Table 4.11 summarises the Bland-Altman analysis results for digestibility (%) of indispensable amino acids and total amino acids (TAA).

Table 4.11: Bland-Altman analysis results for digestibility (%) of total amino acid and key amino acids

Digestibility type	Bias (Mean difference)	95% limits of agreement	Slope	p-value[1]
Indispensable amino acids				
Total amino acid	0.72	12.92	1.80	< 0.0001
Lysine	6.39	11.54	-1.95	< 0.0001
Methionine	-1.20	8.64	-0.65	0.13
Threonine	-3.00	8.70	1.91	< 0.0001
Valine	7.32	16.01	-1.90	< 0.0001
Leucine	8.20	16.77	-1.96	< 0.0001
Isoleucine	5.80	15.41	0.10	< 0.0001
Histidine	-0.59	11.09	-1.88	0.0002
Phenylalanine	3.98	12.99	-1.89	< 0.0001
Total amino acid	0.72	12.92	1.80	< 0.0001

[1]p-value for Bland-Altman regression

The mean bias for TAA was small (+0.72%), with *in vitro* values showing general agreement with *in vivo* values, but with proportional bias present ($p < 0.0001$). Lysine exhibited the largest positive bias (+6.39%), indicating a consistent overestimation by

in vitro methods, with significant proportional bias ($p < 0.0001$). Methionine had a small negative bias (-1.20%) and no significant proportional bias ($p = 0.1340$), suggesting a closer agreement between methods.

Threonine showed a negative bias (-3.00%) with significant proportional bias ($p < 0.0001$), indicating systematic underestimation by *in vitro* methods at higher digestibility values. For valine, the mean bias was positive (+7.32%), and significant proportional bias was observed ($p < 0.0001$), suggesting variability at higher digestibility values.

Similarly, leucine exhibited a large positive bias (+8.20%), with significant proportional bias ($p < 0.0001$), confirming consistent overestimation by *in vitro* methods across samples. Isoleucine digestibility showed a smaller positive bias (+5.80%) compared to leucine and valine, but the proportional bias was still significant ($p < 0.0001$).

Histidine had a negligible negative bias (-0.59%) compared to the other amino acids, indicating close agreement between methods. However, significant proportional bias was detected ($p = 0.0002$), suggesting some variability across the range of values. Phenylalanine showed a positive bias (+3.98%), with significant proportional bias ($p < 0.0001$), indicating that *in vitro* methods consistently overestimated digestibility.

4.4. Discussion

Hypothesis: all the feed ingredients are highly digestible.

Using the INFOGEST *in vitro* digestion method, feed ingredients showed a high degree of hydrolysis, high amino acid digestibility, and DIAAS above 0.60, highlighting their

suitability for poultry nutrition. Soybean meal and AlphaSoy appeared as good protein quality sources. The sunflower expeller showed a lower DIAAS than the DIAAS of Soybean meal and AlphaSoy, which might be due to its lower protein content and indicates that it could be used as a complementary protein source, valuable due to its high methionine content.

In formulated feeds, soybean meal had a lower DIAAS than sunflower expeller and AlphaSoy. This lower value does not necessarily indicate inferior nutritional value but limitations of the score. DIAAS essentially is a ratio and allows relative comparisons between various protein sources, but not fully capture the increased digestibility of heat-treated and processed plant-based feeds (Craddock et al., 2021).

Differences in geographic origin, processing conditions and anti-nutritional factor content can cause soybean meal to have more varied (and sometimes lower) digestibility values of amino acids, reducing its effective amino acid supply relative to other protein sources (K. Wang et al., 2023). Information on the provenance of the soybean meal and other ingredients used in this study was not communicated, so potential variability due to source cannot be assessed.

Despite the high lysine digestibility (above 95%) across the feed ingredients, it was the limiting amino acid for all three protein sources. The Maillard reaction, which occurs between amino acids and reducing sugars under high heat, results in the formation of advanced glycation end-products that can reduce the availability of lysine for digestion. Other limiting amino acids are the sulphur-containing amino acids for AlphaSoy and soybean meal, in agreement with literature (Fernandez et al., 1994b).

Hypothesis: processing influences digestibility.

For formulated feeds, soybean meal laboratory data exhibited lower values in degree of hydrolysis, digestibility and DIAAS, compared to the ones for AlphaSoy and sunflower expeller, regardless of its inclusion level. This difference can be explained by the minimal processing of soybean meal compared to the other protein sources, involving only basic extraction and heat treatment.

Sunflower expeller has a supplementary step, which is mechanical pressing. AlphaSoy has the highest refinement as it goes through a specific thermo-mechanical and enzyme treatment process to improve the digestibility and availability of amino acids.

In addition to processing methods, it is important to consider the fibre and phytate contents of these ingredients. Phytate, also called phytic acid or inositol hexaphosphate (IP6), is a compound found in plant seeds, particularly in grains, legumes, nuts and seed. In these plant foods, phytate serves as the main storage form of phosphorus. Phosphorus plays a paramount role in the growth and development of young plants. Plant-based diets rich in fibre typically have a rich level of phytate as well.

Soybean meal theoretically contains higher levels of fibre and phytate compared to AlphaSoy, as the latter undergoes additional processing to reduce these anti-nutritional factors. Phytate can bind to minerals and amino acids, reducing their bioavailability, which may impact the overall nutritional quality of soybean meal.

Sunflower expeller, on the other hand, tends to have lower phytate levels than soybean meal, but its fibre content varies depending on the amount of dehulling, which could contribute to its relatively better digestibility and amino acid

accessibility. These differences in fibre and phytate contents may therefore play a role in the observed differences in digestibility and protein quality across these protein sources (Guggenbuhl et al., 2012).

Balancing amino acid profiles in formulated feeds is crucial and could be achieved here by supplementing a sunflower expeller-based diet with a protein source rich in lysine. Methionine supplementation might also be required in soya-based diets (Macelline et al., 2021).

Hypothesis: the relationship between in vitro *and* in vivo *digestibility is complex, varying by amino acid, and influenced by limitations of the* in vitro *method.*

There is a difference between *in vitro* and *in vivo* digestibility values, and the direction of this difference depends on the amino acid. For total amino acids (TAA), *in vitro* values tend to be slightly higher than *in vivo* values, but the difference is small. For individual amino acids, the *in vitro* method consistently overestimates lysine, leucine, valine, isoleucine, and phenylalanine digestibility, while it underestimates methionine, threonine, and histidine digestibility.

The comparison of *in vitro* and *in vivo* digestibility methods highlights the inherent challenges in using *in vitro* data, which is designed to simulate human digestion, to accurately predict *in vivo* outcomes observed in chickens. For TAA and methionine, moderate correlations and smaller biases suggest some degree of alignment between the methods, though the significant proportional bias for TAA highlights limitations in predictive reliability.

For lysine, leucine, and valine, large positive biases indicate consistent overestimation by in vitro methods, which may compromise their reliability for these

amino acids. Conversely, methionine and histidine exhibit smaller biases, suggesting closer agreement with *in vivo* data. However, threonine showed systematic underestimation at higher digestibility values, reflected in its negative bias and significant proportional bias.

The overestimation of lysine, leucine, and valine digestibility and the underestimation of threonine and methionine highlight specific limitations of *in vitro* methods in accounting for interactions between feed components and digestive enzymes. These interactions, particularly in the chicken gut, differ significantly from those in humans (N. K. Sharma et al., 2022). The methods also lack the enzymatic diversity and microbial activity present *in vivo*, particularly in the ileum. Additionally, while Bland-Altman analysis provides valuable insights into bias and agreement, the variability observed suggests that further refinement of *in vitro* methods is needed to reduce inconsistencies.

Due to the similarity of *in vitro* digestibility values across the formulated feeds and inclusion levels, most of the data formed clusters when plotted. The narrow range of data makes it harder to determine whether a real relationship exists between variables. A broader range of digestibility values would allow for a more robust correlation or comparison, enabling clearer conclusions about the predictive utility of *in vitro* methods.

The comparison of *in vitro* and *in vivo* digestibility data relied on the use of means, despite the number of replicates differing between methods. This disparity may have influenced the observed results. The *in vivo* means, derived from a larger number of replicates, likely better captured variability and provided more precise estimates. In

contrast, *in vitro* means, based on fewer replicates, may have been less reliable, potentially contributing to biases in comparisons.

This discrepancy is particularly important when interpreting Bland-Altman plots and correlations, as unequal replicates can lead to unbalanced estimations of agreement and variability. Future studies should consider standardising replicate numbers or employing statistical methods that account for such disparities to improve the robustness of comparisons.

From a biological perspective, the *in vivo* broiler chicken trial diet was only done in the span of three days, which is a very short time to observe clear differences in the ileal digesta. This short duration may have limited the ability to capture meaningful differences in *in vivo* digestibility across formulated feeds and inclusion levels (An & Kong, 2023).

As the study strictly focused on digestibility, indicators of growth performance and feed efficiency such as FCR and AME Apparent Metabolisable Energy (AME) were not calculated. The Poultry Unit only measured the body weight gain, which indicated that the birds grew during the study.

For sunflower expeller, increasing inclusion levels consistently improved digestibility, a trend that was not observed for soybean meal or AlphaSoy. This suggests that the composition and structural characteristics of the feed may influence the accuracy of *in vitro* predictions.

Sunflower expeller may show greater improvements in digestibility at higher inclusion levels due to its physical and chemical properties. Its moderate residual oil content and processing method can increase the accessibility of amino acids to

digestive enzymes, resulting in more efficient enzyme-substrate interactions. This is supported by studies reporting higher ileal digestibility of amino acids in sunflower expellers compared with sunflower meals (Ibagon et al., 2021c). In contrast, the more consistent digestibility observed across inclusion levels for soybean meal and AlphaSoy may reflect differences in nutrient availability or processing effects that *in vitro* methods fail to fully capture.

Additionally, while Bland-Altman analysis provides valuable insights into bias and agreement, the variability observed suggests that further refinement of *in vitro* methods is needed to reduce inconsistencies. Incorporating additional enzymatic steps or simulating microbial fermentation for all indispensable amino acids could improve the ability of *in vitro* methods to reflect *in vivo* conditions, particularly for amino acids like lysine, threonine, and leucine.

This proves the limitations of the *in vitro* technique sensitivity to capture the complexity of *in vivo* digestibility, particularly when accounting for animal-specific physiological factors.

4.5.Conclusion

This chapter highlights the complex relationship between *in vitro* and *in vivo* digestibility values for various protein sources and amino acids. The findings confirm that *in vitro* methods provide a rapid and cost-effective means of estimating digestibility, but they exhibit limitations in replicating *in vivo* dynamics, particularly those specific to broiler chickens. The differences between *in vitro* and *in vivo* results, as well as the observed biases, demonstrate the inherent challenges of using human digestion models (INFOGEST) to predict poultry digestibility outcomes.

The Bland-Altman analysis provided valuable insights into the direction and magnitude of these biases, demonstrating that proportional bias was significant for most amino acids. The clustering of digestibility data and the narrow range of values observed across protein sources and inclusion levels further complicated the interpretation of correlations. This underscores the importance of expanding the dataset to include a broader range of digestibility values, which could enhance the robustness of future comparisons. Standardising replicate numbers could also enhance the reliability of future studies between *in vitro* and *in vivo* data.

The study revealed protein source-specific trends. Sunflower expeller demonstrated consistently higher digestibility with increasing inclusion levels, unlike soybean meal and AlphaSoy, whose digestibility patterns were less predictable. These results highlight the influence of feed composition and processing methods on the predictive accuracy of *in vitro* models.

Despite these challenges, the findings underscore the potential of *in vitro* methods as screening tools, particularly for initial evaluations of protein quality. Refining these methods by incorporating additional enzymatic steps, pH variations in the digestive system specific to chickens, or microbial fermentation may improve their ability to mimic *in vivo* conditions. This would be especially beneficial for applications involving novel protein sources, where rapid and scalable assessments are crucial.

This study highlights the potential of *in vitro* methods as a cost-effective and practical alternative for initial assessments of digestibility on plant-based proteins. By identifying their strengths and limitations, this research contributes to ongoing efforts to refine these methods and enhance their predictive capacity. With the

growing global demand for sustainable protein sources, improving these methodologies will be essential for advancing the development and assessment of alternative proteins.

5. Discussion

Prior to the work conducted in this thesis, protein sources for poultry feed had already been a well-studied area in animal nutrition. Global poultry meat production rose from 133 Mt in 2020 to 149.7 Mt in 2024 (FAO, 2024), with projections to supply 45 % of meat protein by 2034 (OECD-FAO Agricultural Outlook 2025-2034, 2025). This demand creates a protein gap that conventional sources struggle to fill sustainably. Researchers had extensively explored protein digestibility through *in vivo* chicken trials, as well as traditional protein sources like soybean meal, and adapted *in vitro* techniques for poultry feeds (Bryan et al., 2018; Bryan & Classen, 2020d; Recoules et al., 2017). However, there is currently no widely standardised *in vitro* protocol for ranking plant-based, yeast- and bacterial protein sources against a reference protein for poultry feed, a limitation this thesis aims to address. EU Regulation 2021/1372 authorises insect PAPs, therefore microbial proteins could be the potential next step (EFSA, 2025).

In addition, by-products from plant-based extractions and other industrial processes were recognised as cost-effective and sustainable feed ingredients for animal feed. Alternative proteins, including bacteria, insects, algae, and fungi, are gaining attention for their potential to improve sustainability through lower land use, reduced greenhouse gas emissions, and efficient feed conversion (Nadar et al., 2024; Sillman et al., 2020).

This thesis tackles a key challenge in poultry nutrition: the need for sustainable, high-quality protein sources to meet the rising global demand for poultry products. By examining traditional protein sources through both *in vitro* and *in vivo* methods, this

thesis makes an important contribution to understanding protein digestibility and quality in animal feed. Moreover, the findings on the digestibility of bacterial protein sources offer a strong basis for developing more sustainable and nutritionally effective poultry feeds.

The first hypothesis of this thesis was that methodology would allow for comparative ranking between various protein isolates for animal feed. The results support this hypothesis as significant variation has been observed between different soya-based, sunflower-based, rapeseed-based, yeast-based, bacterial-based and pea-based protein isolates.

Casein, a highly digestible protein, served as an effective reference standard, reinforcing the reliability of the *in vitro* method. This method demonstrated an overall agreement with published data from *in vivo* trials, particularly in its ability to rank protein sources and identify limiting amino acids. DIAAS proved to be a valuable tool for identifying limiting amino acids and evaluating the overall nutritional adequacy of feed ingredients.

The identification of lysine and methionine as limiting amino acids in many samples underscores the need for targeted supplementation or the strategic combination of protein sources to ensure optimal poultry growth and health. Conventional sources like soybean meal exhibited superior digestibility and amino acid balance, which aligns with their current widespread use in poultry nutrition.

Processing techniques, including enzymatic treatment, fermentation, and heat treatment, are critical for improving protein quality by reducing anti-nutritional factors such as phytates, tannins, and protease inhibitors, thereby enhancing amino

acid bioavailability. Enzymatic treatments specifically target the hydrolysis of protein into smaller peptides and free amino acids, which are easier to digest and absorb (He et al., 2025). Fermentation, on the other hand, involves microbial activity that not only reduces anti-nutritional factors but also introduces beneficial metabolites, such as organic acids, which can improve gut health and nutrient uptake (Sawant et al., 2025). Heat-treatment, when applied appropriately, denatures anti-nutritional proteins and enhances the digestibility of complex feed ingredients, though excessive heating may reduce amino acid availability through Maillard reactions and protein denaturation (Loveday, 2023; Zhang et al., 2023).

For instance, yeast-based protein sources demonstrated significant improvements in digestibility following enzymatic hydrolysis and fermentation. These treatments increased the availability of indispensable amino acids, such as lysine and methionine, which are often limiting in plant-based protein sources (Galili & Amir, 2013).

The first hypothesis in this section was that differences in bacterial species would influence amino acid digestibility. The results support this hypothesis, showing that amino acid digestibility varied significantly among the bacterial species tested. These differences likely reflect variations in amino acid composition, cell wall structure, and growth conditions.

Histidine was consistently among the lower-digestibility amino acids across several species, possibly due to the energy required for its biosynthesis (Stepansky & Leustek, 2006). *Methylococcus capsulatus* (Calysta) emerged as a potential protein source, with digestibility and DIAAS values comparable to casein, making it a promising

candidate for livestock and aquafeeds. Its high nutritional profile is attributed to its heterotrophic growth mode, which supports balanced amino acid composition (Longshaw et al., 2025).

Paracoccus pantotrophus, while moderately digestible, offered a balanced amino acid profile for leucine and valine. Optimising its growth conditions, such as using agricultural waste as carbon and nitrogen sources, could improve its protein quality and digestibility (Bachmann et al., 2023). Species like *Thermus thermophilus* exhibited lower digestibility due to rigid cell wall structures (Farci et al., 2018), whereas *Psychrobacter frigidicola* showed higher protein degradation during digestion, likely because proteins from cold-adapted bacteria are more flexible and less stable, making them easier to break down (Siddiqui & Cavicchioli, 2006).

The second hypothesis in the second result section of the study was that bioplastics (PHB) content would impact protein content and total amino acid digestibility in *Cupriavidus necator*. The results of this study indicated that *Cupriavidus necator* samples with higher PHB content tended to have lower protein content and total amino acid digestibility, indicating PHB accumulation may reduce protein quality (P. K. Sharma et al., 2016).

Further studies would be required to directly measure this effect. This research highlights the potential of bacteria to promote sustainable, circular poultry production by reducing dependence on soybean and lowering environmental impacts.

The third hypothesis of this thesis was that there is a potential relationship between digestibility values obtained from laboratory digestion experiments and *in vivo*

digestibility measurements from a chicken trial, using three protein sources (soybean meal, AlphaSoy and Sunflower expeller) at three different inclusion levels (30%, 45% and 60%).

Using the INFOGEST *in vitro* digestion model, feed ingredients demonstrated high digestibility, with DIAAS values above 0.60, confirming their potential suitability for poultry diets (Colombino et al., 2023; Janocha et al., 2022; Senkoylu & Dale, 1999). Soybean meal and AlphaSoy were identified as good protein sources. Sunflower expeller showed potential as a complementary protein source, particularly due to its high methionine content.

The study of soybean meal and AlphaSoy further highlighted the influence of enzymatic and heat treatment in improving digestibility by reducing anti-nutritional factors like fibre and phytate. However, the potential Maillard reaction during high-heat processing might have reduced lysine availability, proving the difficulty of balancing processing benefits and nutritional losses (Almeida et al., 2013b).

The comparison between *in vitro* and *in vivo* digestibility revealed challenges in using *in vitro* methods to predict *in vivo* outcomes. *In vitro* methods overestimated lysine, leucine, and valine digestibility while underestimating methionine and threonine. Variability in replicates, short trial durations, and differences in feed composition complicated these comparisons. The findings establish a foundation for comparing formulated feeds using a standardised *in vitro* model while highlighting the importance of tailored strategies to optimise poultry feed formulations and enhance the accuracy of predictive models in poultry nutrition research (Pesti & Choct, 2023b). Further research is required to confirm the hypothesis.

The use of *in vivo* animal studies in agricultural research has traditionally been the standard for evaluating feed digestibility and protein quality. However, ethical considerations, public concern, and regulatory pressures increasingly emphasise the need to reduce animal testing. In the UK, the Animals (Scientific Procedures) Act 1986 and the principles of the 3Rs—Replacement, Reduction, and Refinement—encourage researchers to minimise the use of animals whenever possible. Testing only 9 formulated feeds *in vivo* required 180 birds, whereas testing all 42 isolates directly in broilers would have required approximately 840 animals, representing a reduction of around 78 %. This approach aligns with the NC3Rs principles of Reduction in animal research.

In this context, standardised *in vitro* digestion models, such as the INFOGEST model used in this study, offer a practical alternative. These models can provide reproducible and controlled assessments of protein digestibility and amino acid availability, while avoiding the ethical and logistical constraints associated with live animal trials. Although *in vitro* methods cannot fully replicate the complexity of the gastrointestinal tract, they allow rapid, comparative evaluation of multiple feed ingredients and formulations, reducing reliance on animal experimentation and supporting more sustainable and ethical agricultural research.

By highlighting the ethical and practical benefits of *in vitro* models, this study aligns with UK policy and demonstrates the potential for standardised laboratory methods to complement, and in some cases reduce, the need for *in vivo* trials.

Main conclusions from this thesis

- The INFOGEST static *in vitro* digestion model demonstrated reproducible performance, reliably evaluating protein digestibility and quality across a wide range of feed ingredients against a reference protein. DIAAS effectively identified limiting amino acids such as lysine and methionine, providing a critical tool for formulating nutritionally balanced poultry diets.
- The comparison between *in vitro* and *in vivo* methods revealed biases for specific amino acids; in vitro methods tended to overestimate lysine and leucine digestibility while underestimating methionine and threonine.
- Processing techniques like enzymatic treatment and fermentation significantly enhanced digestibility by increasing amino acid availability, especially for yeast- and bacterial proteins.
- *Methylococcus capsulatus* (Calysta) showed DIAAS values comparable to casein, highlighting its potential as a sustainable and high-quality replacement for traditional feed ingredients.
- Bacteria, such as those derived from *Paracoccus pantotrophus* and *Psychrobacter frigidicola*, showed species-specific variations in digestibility and amino acid profiles, underlining the importance of selecting species with optimal nutritional properties.
- Higher PHB content in *Cupriavidus necator* reduced protein accessibility and total amino acid digestibility, confirming its impact on protein quality.

Potential Future Work

Future work should be considered to improve knowledge in this area, this could include:

- Amino acid analysis on samples to measure tryptophan, using an adapted method from the hydrolysis by oven crimp tube digestion to quantify tryptophan digestibility
- Study of fibre content in protein sources to better understand its impact on protein digestibility
- Exploration of additional novel protein sources for animal feed, such as algae and insect-based proteins, using the INFOGEST *in vitro* method.
- Investigation of NPCF for bacteria and other and alternative protein sources, and use of relevant NPCF for all protein sources
- Long-term feeding trials to validate the nutritional and performance benefits of novel protein sources in commercial poultry production, upon ethical approval.
- Refinement of *in vitro* models to incorporate exact pH phases, temperature and transit time in chicken digestive system to create a first basis of a standardised chicken digestive system.
- Investigation of the economic feasibility, scalability and environmental impact of producing novel protein sources for commercial use.

Main conclusion from this thesis

This thesis demonstrates that the standardised INFOGEST *in vitro* digestion model is an effective tool for assessing protein quality across a diverse range of poultry feed ingredients, including traditional plant-based feeds, alternative proteins, and bacterial sources. Soybean meal and AlphaSoy exhibited high digestibility and DIAAS values, confirming their suitability for inclusion in poultry diets, while sunflower expeller may serve as a complementary source due to its high methionine content. Microbial proteins, such as *Cupriavidus necator* and *Paracoccus pantotrophus*, showed promising amino acid profiles, although factors such as PHB accumulation in *C. necator* may reduce protein accessibility, suggesting that optimisation of microbial protein composition is important for practical application.

While direct correlation with *in vivo* broiler data was unconclusive, the *in vitro* results provide valuable insight into protein composition and digestibility patterns that can guide further research and feed formulation. Overall, this work highlights the potential to expand feed formulation strategies based on standardised digestibility assessments, offering insight into nutritional performance and the development of alternative protein sources for poultry.

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