

**High nutritional quality of human induced pluripotent stem cells-generated proteins by
advanced scalable peptide hydrogel 3D suspension system**

by

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Abstract

Cell-cultured protein technology has become increasingly attractive due to its sustainability and climate benefit. The aim of this study is to test the nutritional quality of the human induced pluripotent stem cells (hiPSCs)-cultured proteins in advanced scalable 3D peptide hydrogel system for highly efficient production of cell cultured proteins. Previous studies in Dr. Sun's lab demonstrated an advanced PGmatrix3D (PG-3D) scaffolding hydrogel for long-term hiPSCs maintenance with significantly higher cell expansion and superior pluripotency when compared with traditional 2D cell culture or other Matrigel, Vitronectin, or Polyethylene glycol (PEG)-based 3D cell culture systems. In this study, the PGmatrix3D suspension system (PG-3DSUSP), specifically designed for scaling up cell manufacturing, was selected for 3D cultured hiPSC protein production. Results indicate that PG-3DSUSP is a competitive system to the well-established PG-3D system in terms of cell growth performance and cell pluripotency. The nutritional value of hiPSC-generated proteins including amino acid composition and *in vitro* protein digestibility were further evaluated. Amino acid composition of hiPSCs as measured using hydrophilic interaction chromatography liquid chromatography demonstrated a significant higher essential amino acid content (39.0%) when compared with human skeletal muscle protein (31.8%). *In vitro* protein digestibility of hiPSCs as measured by pH drop method showed a significant higher ($78.0 \pm 0.7\%$) when compared with commercial beef protein isolate ($75.7 \pm 0.6\%$). Taken together, this is the first study to report an advanced PG-3DSUSP culture to produce the highly efficient hiPSCs-generated proteins that possess more essential amino acids and demonstrate better digestibility. The hiPSCs-generated proteins with super nutrition quality may be of particular significance as novel alternative proteins in food engineering and industries for future food, beverage, and supplement applications.

Keywords: hiPSCs, PepGel 3D suspension culture system, nutritional quality, amino acid composition, *in vitro* protein digestibility, regenerative agriculture

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Chapter 1 Literature review

1.1 Alternative protein sources for human consumptions

Food proteins contain amino acids (AA), which are essential for the growth and development of all bodily organs, muscles, hormones, and biological fluids (Boye et al., 2012). It is considered high-quality protein to consume animal-based, protein-dense foods such as meat, eggs, and milk, because they meet all of the essential amino acid (EAA) requirements and are easily digested and absorbed (defined by how amino acids are balanced across the small intestine) (Churchward-Venne et al., 2017). The Recommended Dietary Allowance (RDA) of protein is currently 0.8 g per kilogram body weight (BW) of weight for an adult with minimal physical activity (Wu, 2016). However, the growing global population poses a challenge in meeting the protein needs of the most vulnerable (Salter and Lopez-Viso, 2021). Recent evidence suggests that an increase in RDA from 0.8 g to 1.2 g per kg of body weight is needed to compensate for age-related changes in physiological responsiveness to protein intake among adults over 65 (Loveday, 2019).

Globally, the rapid increase in human population has caused food security issues (Chakraborty et al., 2019). As the population continues to grow, proteins will become more of an important part of people's diets. However, a wide range of ecological issues, including desertification, climate change, and overuse of freshwater and fossil fuels, have decreased the availability of viable agricultural land, freshwater, and fossil fuels (Li, 2020). This issue in global food consumption is challenged by the human needs to meet proteins requirements (Post, 2012). Also, it has been shown that high consumption of red meat (animal-based protein sources) increases the risk of all-cause mortality, type-2 diabetes, colorectal cancer, and cardiovascular disease (Libera et al., 2021). Hence, alternative protein refers to alternative sources of protein

that require less intensive production methods than conventional meat. Several alternative protein sources have gained interest from researchers and the food industry in recent years, such as cultured meat, plant-based meat, insect protein, and single-cell protein (Hadi and Brightwell, 2021). The use of alternative protein sources is expected to reduce negative environmental impacts by up to 50%, resulting in significant ecological sustainability (Yusuf and Setiarto, 2022).

1.1.1 Cultured meat

Cultured meat is artificial meat, also called in vitro meat or lab grown meat, produced by in vitro cell cultures of animal cells (Goodwin and Shoulders, 2013). Specifically, cultured meat products are generated from animal cells in a bioreactor, rather than through animal slaughter (Treich, 2021). In the 1930s, Frederick Edwin Smith and Winston Churchill first proposed cultured meat as alternative sources for conventional meat (Zhang et al., 2020). Early research reported that cultured meat is produced via tissue engineering techniques with the utilization of regenerative medicines (Post, 2014). In 2013, Professor Mark Post at Maastricht University created the first hamburger patty with cultured meat generated from cells (Choudhury et al., 2020). Cultured chicken meat has been commercialized and is being produced in the pipeline in Singapore. Researchers from both United States and Europe have been developing cultured meat for several years (Edelman et al., 2005). Cultured meat represents more than a technological revolution, but also a potential economic and societal resolution, with the possibilities to disrupt traditional meat production (Treich, 2021).

In recent years, most of cultured meat technologies are based on using skeletal muscle tissue engineering, stem cells, cell co-cultures, and tissue culture methods are practical, avoiding

livestock problems such as animal harm and environmental pollution (Zhang et al., 2020, Shahini, et al., 2018). Additionally, induced pluripotent stem cells (iPSCs) are totipotent stem cells with the ability to self-renew and differentiate in multiple directions, and they are often used in the medical field (Chen et al., 2020). It is possible to produce meat alternatives using iPSCs that can be further scaled up for production in bioreactors (Genovese et al., 2017, Zhang et al., 2022).

There are three different ways to induce livestock myogenesis *in vitro*: muscle stem cell culture, pluripotent stem cell differentiation, and direct reprogramming using transgenesis (Genovesr et al., 2017, Chio et al., 2021). During the production of cultured meat, a sequential process is used to produce muscle stem cells-based *in vitro* muscle tissues: (1) muscle samples for stem cell collection, (2) muscle tissue dissociation and isolation, (3) primary cell culture, (4) upscaled cell culture, (5) muscle differentiation and maturation, and (6) muscle tissue harvesting and processing (Chio et al., 2021). It is important that the final cultured meats have similar nutritional benefits, texture, and flavor as the real meat, as well as low risks of microbial contamination (Chen et al., 2022).

As an alternative source of protein, cultured meat has recently emerged as a viable solution to some of the challenges associated with traditional livestock production. The benefits of cultured meat production may include reducing bacterial and disease exposure in meat (Chen et al., 2022, Pandurangan and Kim, 2015). Also, cultured meat is an important consideration for ensuring 'naturalness' and a high level of nutritional content, in comparison to conventional meat products (Chen et al., 2022). However, the market application of cultured meats is currently limited by nutritional, technological, sensory, regulatory, and cost limitations, social perception

(e.g., public neophobia and technophobia), and insufficient understanding of the potential health benefits or food safety risks (Broucke et al., 2023).

1.1.2 Plant-based meat

People are increasingly adopting vegetarians and consuming plant-based diets or reducing their consumption of animal-based foods (Safdar et al., 2022). People who are concerned about their health often prefer plant-based, healthy, and sustainable diets, as they are commonly perceived to be natural and nutritious (Sucapane et al., 2021, Safdar et al., 2022). A plant-based meat alternative has been heavily marketed as healthier and more environmentally friendly options since plants are abundant and offer many health benefits (Choudhury et al., 2020). It may be traced back to the 10th century when Asian communities first started using plant-based ingredients, such as fermented soybean cake (e.g., tofu and tempeh), or wheat (e.g., seitan), as meat analogs (Hadi and Brightwell, 2021).

Plant-based meat products are made by processing plant protein using extrusion, silking, conditioning, and other techniques, mimicking the texture of meat (Zhang et al., 2022). The most used ingredients of plant-based meats are isolates, concentrates, or textured proteins derived from soy, pea, and wheat (Wang et al., 2022). There are typically three steps involved in the production of plant-based meats: (i) Plant proteins are isolated, and some of them are hydrolyzed to improve their solubility and crosslinking capacity; (ii) Food adhesives, plant-based fats and flour are mixed with plant proteins to make meat texture; (iii) To form a meat-like texture, plant proteins are stretched, kneaded, trimmed, pressed, folded, and extruded with other ingredients (Rubio et al., 2020). There are already commercially available meat products made from heme-containing proteins found in the roots of soy plants (such as Impossible Foods' plant-based sausage and burgers) and foods made from insect proteins (Hwang et al., 2020). However, it is

challenging to replicate the organoleptic and nutritional profile of real meat due to its more than 1000 fat-and water-soluble components (Choudhury et al., 2020).

1.1.3 Insect protein

It has been proposed that insects are an alternative protein source and contribute to the global food security issue by meeting global demand for food protein (Churchward-Venne et al., 2017). Traditionally, insects have been consumed as part of the human diet in many parts of the world, such as Africa, Asia, and Central and South America (Salter and Lopez-Viso, 2021). The Food and Agriculture Organization of the United Nations (FAO) estimates that over 1900 insect species are consumed worldwide (Hadi and Brightwell, 2021). There are a wide variety of insects that can be consumed for food, including crickets, locusts, grasshoppers, caterpillars, beetles, ants, and fly larvae (Loveday, 2019). Furthermore, insect proteins offer new and accessible business opportunities to developing countries since they are relatively easy, cheap, and fast to farm, which makes them the best option for ensuring food security with an ever-growing global population (Gravel and Doyen, 2020).

An insect protein has many positive characteristics, such as high nutrient content and digestibility, while being an environmentally friendly protein source (Gravel and Doyen, 2020). Currently, there is little knowledge about the bioavailability of insect-derived nutrients. In vitro protein digestibility of insect proteins is currently reported to be between 77% and 98% (Ramos-Elorduy et al., 1997). Tryptophan and lysine are essential amino acids, meaning that the body cannot synthesize them and they must be obtained through dietary intake. (Loveday et al., 2019). In addition to being a rich source of protein, insects are also rich in fat, minerals, vitamins, and fiber (Mintah et al., 2020). However, insects remain one of the most challenging protein sources to introduce to the mainstream market due to low consumer acceptance (de Castro et al., 2018).

First, there is concern that insects may transmit pathogens or viruses from the feed, environment, or the insect rearing techniques, which can pose a risk to human health (Yusuf and Setiarto, 2022). Second, there is no clear legislation to regulate this insect protein, which could deter companies from producing and selling insects as food (de Castro et al., 2018). Currently, the development of insect-based foods is in a transitional phase, and a promising new technique combines protein-processing technology with insects to develop new products (Kim et al., 2019).

1.1.4 Single-cell protein

A single-cell protein (SCP) is derived from the cultivation of microorganisms, such as microalgae, yeast, fungi, and bacteria, and can be used as an alternative to conventional protein sources for human and animal consumption (Pereira et al., 2022). Among them, microalgae have the highest protein content (60–70% w/w) and can be grown on liquid effluents containing organic matter (Bertasini et al., 2022). SCPs will become increasingly important as a protein source, despite providing a relatively small portion of human nutrition (Ritalla et al., 2017). In addition to providing a source of protein, SCPs can also play a role in converting agricultural waste into food and feed for humans and animals, utilizing waste management technologies (Ukaegbu-Obi, 2016).

In general, the production process involves preparing the nutrient media, possibly from waste, cultivating the SCP, including solid state fermentation; separating and concentrating the SCP; drying in some cases; and converting the SCP into ingredients and products (Ritalla et al., 2017). The advantages of SCPs are that they can be produced from residual organic streams, such as food and agricultural wastes and byproducts, which do not require pure substrates (Bertasini et al., 2022). Additionally, SCP is rich in certain essential amino acids, such as lysine

and methionine, which are usually limited in plant and animal foods (Suman et al., 2015).

Although SCP is typically manufactured from food grade substrates, there is hope that processes will be developed to produce SCP from inexpensive waste materials generated by the food and beverage processing industries, as well as from forestry and agriculture sources (Ritalla et al., 2017).

1.2 Cell-cultured protein technology

With the use of cell culture technology, biotechnology can contribute to food security by enabling the growth of foods in bioreactors, thus reducing the need for arable land (Wikandari et al., 2021). As an alternative paradigm to conventional livestock production, cellular agriculture could improve the sustainability of protein production by reducing the environmental and animal welfare impacts (Fish et al., 2020). Cell culture is a widely used in vitro tool for developing cell biology research, drug mechanisms, protein synthesis, and tissue engineering (Kapalcynska et al., 2018). Harrison carried out the first cell culture in 1907 during his research on nerve fibers (Harrison, 1906). Since then, cell culture has been used to observe the growth and differentiation of cells in vitro (Kapalcynska et al., 2018). A cell culture begins with the removal of cells from animals or plants, and then they are cultivated in a culture vessel in an artificial environment. An important aspect of controlling the growth of cells is regulating the culture conditions, including temperature, relative humidity, nutrient levels, pH levels, and carbon dioxide levels (Wikandari et al., 2021).

There are two main cell culture systems: two-dimensional (2D) culture and three-dimensional (3D) cell culture. It has been used for over a century to study the responses of cells to biophysical and biochemical stimulations in two-dimensional (2D) cultures (Duval et al., 2017). Cell cultures are typically performed in two dimensions (2D). However, it reported that

2D cell culture has many limitations, such as inaccurately representing tissue cells in vitro, cell morphology changes, and division methods (Xu et al., 2021, Kapalcynska et al., 2018). New and improved methods that utilize three-dimensional (3D) cell culture techniques suggest that much more advanced experiments can be conducted, yielding valuable insights (Jensen and Teng., 2020). 3D cell culture is a better way to culture cells as it mimics the environment of cells in vivo and provides more accuracy in cell-to-cell interactions, tumor, and drug delivery (Jensen and Teng, 2020). Nonetheless, researchers still 2D cell culture as the preferred method because of its low cost, easy of observation and analysis, and chemical cues (Xu et al., 2021).

1.2.1 2D culture system

Cells growth adjust their environment to influence their differentiation and proliferation. A typical cell from division, through proliferation, to migration, and apoptosis is an accurately controlled series of events. (Haycock, 2011). In 2D cell culture, cells grow as a monolayer, or a plate attached to a plastic surface in a culture flask. Cell growth in 2D monolayers relies on adherence to the flat surface to get the same nutrients and growth factors in the medium (Duval et al., 2017). 2D cell culture has been the primary method to culture cells since the early 1900s (Jensen and Teng, 2020). To date, most cancer research in vitro is used traditional two-dimensional (2D) cell culture (Xu et al., 2019) as one of the most straightforward and low-cost methods of choice (Van Zundert et al., 2020).

However, the disadvantages of 2D cell culture have been recognized. 2D cell culture is a monolayer and an unrealistic condition for cell growth. Cell growth on 2D surfaces result in cells flattening, which shapes the cell and alters its gene expression (Knight and Przyborski, 2015). Cell flattening on 2D cell culture also affects the nuclear shape that leads to difference in gene expression and protein synthesis (Thomas et al, 2002). Also, some important features of cancer

cells that cannot be modeled in 2D cell culture systems (Duval et al., 2017). Therefore, cell growth on 2D surfaces cannot mimic the natural cell environment. Cell growth *in vivo* is surrounded by extracellular matrix (ECM) in a three-dimensional (3D) structure, whereas 2D cell culture cannot provide it (Xu et al., 2019). Also, cells in 2D do not consider the intercellular communication, which affects cell differentiation, proliferation, and cellular functions (Kapalcynska et al., 2018).

1.2.2 3D culture system

To simulate the living environment of the cells more accurately, researchers found that several 3D methods maintained the morphology and behavior of cells growing. 3D cell culture created environments for cells to grow with surrounding in all three dimensions. One of the first three-dimensional cultures was carried out by Hamburg and Salmon in 1970 used a soft agar to support human tumor stem cells growth (Hamburger and Salmon, 1977). Researchers found that similarities between the morphology and behavior of cells growing in 3D cell culture and *in vivo* has been recorded (Kapalcynska et al., 2018).

There are two types of 3D cell culture methods, scaffold techniques and scaffold-free techniques. In scaffold techniques, cells are grown on a hydrogel-based support, a hard polymer material-based support, natural or synthetic, of animal origin (Habanjar et al., 2021). Hydrogels are a class of scaffolds that are frequently used for 3D cell culture. Hydrogels are the network of hydrophilic polymer to be held together by intramolecular and intermolecular physical attractions, and they facilitate nutrients, oxygen, and metabolite transport (Habanjar et al., 2021; Xu et al., 2019). The advantages of hydrogels are that they can be formed under relatively mild conditions, can be modified to possess cell adhesion ligands and other desirable characteristics, and can be easily fabricated for use in soft tissue grafts (Fitzgerald et al., 2015).

There are different types of hydrogels from natural or synthetic materials. The natural hydrogels found in nature include collagen, Matrigel, and alginate (Worthington et.al, 2015). Some commonly used natural hydrogel materials, such as collagen, are very expensive and lack of repeatability (Habanjar et al., 2021). On the other hand, natural hydrogels are complex because they are usually biocompatible, and difficult to adjust mechanically (Worthington et.al, 2015). There are several synthetic hydrogels including polyethylene glycol (PEG), polylactic acid, and polyethylene oxide (Xu et al., 2021). There is a commonly used material is PEG, which regarded to have greater flexibility in treatment and easier to be produced (Habanjar et al., 2021).

In Dr. Sun's lab, they found a novel scaffolding peptide hydrogel (PepGel) for 3D culture of iPSCs. Peptide hydrogel 3D scaffolding technologies, such as PGmatrix for cell culture, have been reported to grow spheroid-like cells physiologically mimicking the environment of human body that can be used an in vitro tool researching cell activities (Xu et al., 2021). It recently demonstrated that human induced pluripotent stem cells (hiPSCs) can be regenerated for more than 50 generations at 20-30 proliferation folds every 5-6 days with 95% cell viability in PepGel. hiPSCs cultured in PepGel showed superior performance and pluripotent integrity compared to traditional 2D and existing 3D culture methods (Li et al., 2017, Li et al., 2021). Peptide hydrogel systems have gained popularity in 3D cell culture due to their high water content and good biocompatibility. The Peptids can form a solid physical hydrogel under conditions that mimic in vivo, which easy for cell encapsulation and spheroids isolation (Liang et al., 2017).

1.3 Nutritional quality of the protein

A protein can be classified as "quality" in human nutrition based on (1) its relative contribution to the individual's amino acid requirement, and (2) its digestibility (House et al.,

2010). A high nutritional quality of a food protein is defined as contain much essential amino acids and has an acceptable % digestibility. So far, there is no ideal natural protein to maintain our growth. The best quality protein are animal protein sources contain all essential amino acids, but excessive intake of animal protein sources may lead to the risk of chronic diseases (Barrón-Hoyos et al., 2013). There are many different protein products available commercially to meet our daily needs. Before developing a new protein source, researchers need to test its protein content, amino acid compositions, and protein digestibility.

1.3.1 Protein content

Protein can be classified by their structure, biological function, and composition. For the quantification of total protein, the following methods are commonly used: combustion-based carbon to nitrogen ratio (C/N) analysis using isotope ratio mass spectrometry (IRMS) or other elemental analysis techniques, Kjeldahl titration, Dumas methods, biuret tests like Lowry's, Bradford, and bicinchoninic acid tests (Kambhampati et al., 2019).

As a primary source of nitrogen, proteins are essential for the structure and function of the body (Sa et al., 2020). Currently, protein content is used for nitrogen content, and then calculated by the determined nitrogen content by 6.25 as the standard nitrogen-to-protein conversion factor. Kjeldahl and Dumas methods are measure protein content depend on the determination of nitrogen. Kjeldhal method is widely accepted and used throughout the world for nitrogen analysis in a large variety of materials, including foods, beverages, agricultural products, environmental samples, chemicals, and biochemicals (Sáez-Plaza et al., 2013). Kjeldahl method works by converting protein content into ammonium ions by sulfuric acid in the presence of catalysts in 1883 (Thompson et al., 2002). However, the disadvantages of this approach is that its use of concentrated sulfuric acid at high temperatures can be hazardous, as

can be use of some of the catalysts that are available (Mihaljev et al., 2015). Over the past thirty years, there has been a trend towards replacing the Kjeldahl method with instruments that is the Dumas method, which takes advantage of the combustion of the test material and determination of the resulting elemental nitrogen (Thompson et al., 2002).

Due to the experimental complexity and danger of Kjeldahl and Dumas methods, some simple and rapid methods have been developed include biuret method, Lowry method, and UV absorbance method at 280 nm. Biuret method is determined the protein content based on the peptide bonds in food products, and Lowry method is a modified version of the Biuret method that increases its sensitivity and specificity (Subroto et al., 2015). The advantage of the Lowry method over the Biuret method is that it is more sensitive, meaning it can detect lower levels of protein content in samples (Martina and Vojtech, 2015). The principles of ultraviolet 280 nm absorption method is that proteins contain aromatic amino acids, such as tryptophan and tyrosine, which demonstrate strong UV absorption at 280 nm (Mach et al., 1995). Ultraviolet 280 nm absorption methods are frequently applied to measure protein content in various food products, including milk and meat (Nakai and Herreid, 1964). Overall, an important consideration before choosing a method is whether total or specific protein is being measured.

1.3.2 Amino acid compositions

A protein must be hydrolyzed into its basic building blocks, amino acids or short peptides, before it can be used by the human body (Joye, 2019). The term amino acids (AAs) refers to organic compounds that contain both amino and acid groups (Butterworth, 2005, Wu, 2009). In addition to building tissue proteins, AAs also serve as essential substrates for the synthesis of a wide variety of low-molecular-weight compounds (e.g., NO, polyamines, glutathione, creatine, carnitine, carnosine, thyroid hormones, serotonin, melanin, melatonin, and

heme) that are of enormous physiological importance (Wu, 2013). Furthermore, amino acids play a role in regulation of gene expression, which involves the translation of genetic information encoded in a gene as ribonucleic acid (RNA, including messenger, transfer, and ribosomal RNA) into proteins (Wu et al. 2013).

Protein contains 20 amino acids which can be divided into two groups, essential amino acids (EAA) and non-essential amino acids (NEAA). EAA cannot be made by the human body and must be obtained through the diet. There are 9 essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Leucine, isoleucine, and valine are known as branched-chain amino acids (BCAAs), which have aliphatic side chains (a central carbon atom bonded to ≥ 3 carbon atoms). Due to their ability to promote protein synthesis and influence metabolism, BCAAs make up approximately 40% of the body's essential amino acid requirement (Xiao and Guo, 2022). Recent evidence suggests that BCAAs and their derivatives could potentially serve as biomarkers for various diseases, including insulin resistance (IR), type 2 diabetes mellitus (T2DM), cancer, and cardiovascular diseases (CVDs) (Nie et al., 2018). NEAA, can be synthesized by the human body, so they are unnecessary to be acquired from the diet. NEAA that can be produced by the human body, including 11 amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine (Heger, 2013).

In organism, leucine levels indicate several physiological factors, such as how much food is available, how much insulin is required, and whether new muscle mass will be able to be built (Saxton et al., 2016). It has been recognized for more than 30 years that leucine stimulates skeletal muscle protein synthesis (MPS) independently in cells and animals (Pasiakos and McClung, 2011). There is evidence that leucine and insulin have anabolic effects that activate

distinct intracellular signaling pathways, which ultimately converge at the mammalian target of rapamycin (mTOR). This convergence of pathways is responsible for the initiation and elongation of translation, which leads to increased skeletal muscle protein synthesis. (Drummond and Rasmussen, 2008). Among the BCAAs, researchers demonstrated that leucine stimulated muscle protein synthesis in a manner distinct from valine and isoleucine (Escobar et al., 2006, Columbus et al., 2015).

Amino acid composition analysis (AAA) is a traditional used for protein analysis method with wide applications in medicine and food science and is indispensable in protein quantification (Fountoulakis et al., 1998). Since amino acid composition determines protein quality, AAA can be used to guide plant breeding and engineering efforts to improve food, feed, nutraceuticals, and pharmaceuticals (Kambhampati et al., 2019). The main methods for analyzing the composition of amino acids are chemical analysis using high performance liquid chromatography (UHPLC/UPLC or HPLC), gas chromatography (GC), or capillary electrophoresis (CE) followed by detection with absorbance, fluorescence, or mass spectrometry (MS) (Kambhampati et al., 2019). It consists of two steps, hydrolysis of substrate and chromatographic separation and detection of the resulting residues (Fountoulakis et al., 1998). The first step in amino acid analysis is protein hydrolysis, which breaks down peptide bonds to release free amino acids and carboxyl groups (Scott et al., 2014), then further analysis performed with different instruments.

1.3.3 Protein digestibility

Dietary essential amino acids are provided mostly as intact dietary proteins, which require digestion to release their component amino acids and peptides (Butts et al., 2012). A protein digestibility is essentially a measure of its proteolysis susceptibility. It can be used as an

indicator of its bioavailability (Duodu et al., 2003). It is possible that a proteins with high digestibility would have a better nutritional value than a protein with low digestibility because it would provide more amino acids for absorption during proteolysis process (Duodu et al., 2003). Previously, protein quality was measured in growth experiments with rats using parameters such as Protein Efficiency Ratio (PER) and Net Protein Utilization (NPU). A PER compares the growth responses of young rats fed a small amount of a test protein with those of control rats fed a similar amount of casein, and the NPU is the outcome of digestibility (the extent of digestion and absorption) and biological value (the ratio of digestion to absorption) (Schaafsma, 2012). In a living organism, the processes of digestion and absorption are intricate, highly interdependent, and tightly regulated, and they have evolved to effectively release nutrients required for growth, maintenance, and reproduction (Butts et al., 2012). In addition, the gut microbiota and antinutritional factors have different effects on the digestive tract, making simulation of their effects particularly challenging (Butts et al., 2012). Thus, *in vitro* methods are useful to guide selection of proteins for these *in vivo* models while reducing the cost of finding suitable, alternative proteins protein sources (De Bhowmick et al., 2022).

In vitro digestibility methods began as simple one-step incubations with pepsin or other proteases, such as papain, pronase, and rennin (Boisen et al., 2000). Although single enzyme methods were effective in comparing treatment effects on a specific food, they yielded lower digestible protein values compared to those measured *in vivo* (faecal nitrogen digestions) (Butts et al., 2012). Subsequently, researchers have developed multi-enzyme procedures that measure pH drops following a 10-minute digestion period (Hsu et al., 1997). The pH drop method is an *in vitro* procedure to measure protein digestibility via multienzyme systems. The pH drop three-enzyme (trypsin-chymotrypsin-peptidase) method is widely used *in vitro* protein digestibility that

determines the pH after 10 min of reaction (Hsu et al., 1997). It was based on the principle that hydrolysis of amino acid carboxyl groups causes deionization and the subsequent release of free protons, which measures the release of H⁺ ions by broken peptide bonds and the generation of free carboxyl groups (PH-drop assays) (Mendes et al., 2016). This *in vitro* method utilizing a multienzyme system was found to be highly correlated with *in vivo* apparent digestibility (Hsu et al., 1997).

The multienzyme system include trypsin, chymotrypsin, and peptidases. However, peptidases had been discontinued, so researchers have identified proteases as substitution. Additionally, a four-enzyme (trypsin-chymotrypsin-peptidase-pronate) method has previously been employed to measure protein digestibility, but the results may had been overestimated (Tavano et al., 2016). These assays utilize different enzyme sequences in an attempt to mimic the human digestion process. In addition, a titrimetric method that takes advantage of pronase was incorporated as an additional hydrolysis step (Tavano et al., 2016).

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Chapter 2 - Experiment

2.1 Abstract

Cell-cultured protein technology has become increasingly attractive due to its sustainability and climate benefit. The aim of this study is to test the nutritional quality of the human induced pluripotent stem cells (hiPSCs)-cultured proteins in advanced scalable 3D peptide hydrogel system for highly efficient production of cell cultured proteins. Previous studies in Dr. Sun's lab demonstrated an advanced PGmatrix3D (PG-3D) scaffolding hydrogel for long-term hiPSCs maintenance with significantly higher cell expansion and superior pluripotency when compared with traditional 2D cell culture or other Matrigel, Vitronectin, or Polyethylene glycol (PEG)-based 3D cell culture systems. In this study, the PGmatrix3D suspension system (PG-3DSUSP), specifically designed for scaling up cell manufacturing, was selected for 3D cultured hiPSC protein production. Results indicate that PG-3DSUSP is a competitive system to the well-established PG-3D system in terms of cell growth performance and cell pluripotency. The nutritional value of hiPSC-generated proteins including amino acid composition and *in vitro* protein digestibility were further evaluated. Amino acid composition of hiPSCs as measured using hydrophilic interaction chromatography liquid chromatography demonstrated a significant higher essential amino acid content (39.0%) when compared with human skeletal muscle protein (31.8%). *In vitro* protein digestibility of hiPSCs as measured by pH drop method showed a significant higher ($78.0 \pm 0.7\%$) when compared with commercial beef protein isolate ($75.7 \pm 0.6\%$). Taken together, this is the first study to report an advanced PG-3DSUSP culture to produce the highly efficient hiPSCs-generated proteins that possess more essential amino acids and demonstrate better digestibility. The hiPSCs-generated proteins with super nutrition quality

may be of particular significance as novel alternative proteins in food engineering and industries for future food, beverage, and supplement applications.

Keywords: hiPSCs, PepGel 3D suspension culture system, nutritional quality, amino acid composition, *in vitro* protein digestibility, regenerative agriculture

2.2 Introduction

As the most important component of animal and human tissues, proteins are large macromolecules consisting of mainly 20 amino acids (AAs) (Wu, 2016). The body requires complete proteins in adequate quantities for the synthesis of tissue proteins and maintenance of normal metabolic functions (Davies and Jakeman, 2020). Generally, AAs can be divided into essential amino acids (EAA) and non-essential amino acids (NEAA). EAA are particularly important nutrients since that cannot be synthesized in humans and therefore must be provided in the diet from dietary protein sources (Atherton et al., 2010, Davies and Jakeman, 2020). Dietary protein sources mainly come from plant-based and animal-based food products. Plant-based proteins have become more popular due to their sustainability and lower production costs (Gorissen et al., 2018). However, plant-based proteins are sometimes incomplete by missing or reducing specific EAA such as lysine, methionine, and tryptophan (Gilbert et al., 2011). Although animal-based products (i.e., eggs, milk, meat, fish, and poultry, etc.) are provided complete proteins with all EAA (Hoffman and Falvo, 2004), the global production of animal proteins may face sustainability challenges. Animal agriculture usually produces 1 kg of high-quality animal proteins by feeding 6 kg plant proteins to livestock, which impacts land and water resources, as well as greenhouse gas emissions (Hertzler et al., 2020). Also, high animal-based

sources, particularly red meat, are associated with risk of many chronic diseases, such as cancer, cardiovascular diseases, diabetes, and obesity (Libera et al., 2021).

The Recommended Dietary Allowance (RDA) of proteins (0.8 g/kg body weight) can meet for adults with minimum physical activity per day, but special groups (e.g., infants, children, pregnant) need more proteins to meet growth needs (Wu, 2016, Deer and Volpi, 2015, Jinno et al. 2020). High protein diets (defined as exceeding the current RDA) are heavily promoted by athletes as “the gold standard” for building muscle mass and/or losing body fat (Delimaris, 2013). Athletes are recommended to consume 1.6-2.4 g/kg each day during weight loss (Hector and Phillips, 2018). However, a wide range of ecological issues, including desertification, climate change, and overuse of freshwater and fossil fuels, have decreased the availability of viable agricultural land, freshwater, and fossil fuels (Li, 2020). This issue in global food consumption is challenged by the human protein needs (Post, 2012). Around 800 million people are chronically undernourished due to the challenges humanity faces in ensuring food security and sustaining the environment (Li, 2020). A sustainable solution to food or protein insecurity, especially the provision of high-quality food proteins from alternative resources for humans, appears urgently needed in the face of these nutritional deficiencies (Wu et al., 2014).

In recent years, a modern biotechnology approach that accelerates food production to achieve ‘zero hunger’ goals set by the United Nations is the application of cell culture, marker-assisted selection, and genetic engineering (Wikandari et al., 2021). Cell culture is a widely *in vitro* tool used for mechanisms of disease, drug action, tissue engineering, and protein production (Kapałczyńska et al., 2018). Cell culture technology allows cells to grow in bioreactors, reducing land use for agriculture (Wikandari et al., 2021). Over the few decades, regenerative agriculture has become an emerging research field to produce protein products using muscle stem cells

(MSCs) and tissues in cell culture system (Stephens, et al., 2018). However, there are some challenges of MSCs for protein production, such as maintaining their stem integrity after a few expansion generations, and grand scaling up (Bodiou et al., 2020). Unlike MSCs, pluripotent stem cells (PSCs) or induced pluripotent stem cells (iPSCs) are good candidates for protein regeneration which can be derived from blood stem cells using high-efficient method and indefinitely renewable (Zhu and Huangfu, 2013). Here, the lab of Sun's found a good source, human-induced pluripotent stem cells, would be a high-quality material for protein production. Human pluripotent stem cells (hPSCs) consist of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESC), which can be differentiated into almost all types of somatic cells (Zhu and Huangfu, 2013). hiPSCs are expressed by differentiated human somatic cells via transcription factors and surface markers such as Oct4 (Takahashi et al., 2007, Ho et al., 2012). Transcriptional regulation is usually maintained by these cells through a set of unique transcription factors (Umei and Tohyama, 2022).

Over more than a decade, hiPSCs have been cultured on two-dimensional (2D) monolayer system with specifically designed coating materials in unnatural cell environments (Villa et al., 2010). However, hiPSCs in 2D cell culture lack an appropriate stem cell niche, which leads to poor maintenance of pluripotency and unwanted differentiation (Li et al., 2021). Due to the absence of physiological properties of real tissues, 2D cell culture is a simplified and unrealistic condition for cell growth (Xu et al. 2019). Considering the limitations of 2D culture methods, three-dimensional (3D) cell culture system mimics the complexity of the extracellular matrix (ECM) and the physiological relevance of an *in vivo* (Huang et al., 2013). Several 3D cell culture platforms have been developed, including hydrogels, scaffolds, and decellularized tissues (Xu et al., 2019). However, most gel solutions are limited by physiological conditions such as pH or

temperature (Huang et al., 2013). Also, encapsulation and isolation of cells are two critical steps for in vitro 3D cell culture to introduce 3D spatial support and recover embedded cells from scaffold matrix (Huang et al., 2013). It is very simple to culture and harvest cells using PepGel procedures that can all be safely performed at either room temperature or 37 °C in neutral pH. To maintain the 3D spheroids of hiPSCs, easily operate procedure of cell culture, and scalable lab-scale manufacturing for protein production, we found a novel scaffolding peptide hydrogel (PepGel) for 3D cell culture of hiPSCs (Li et al., 2021). Comparing to traditional 2D cell culture, hydrogel 3D scaffolding to grow spheroid-like cells physiologically mimicking the environment of human body that can be used as an in vitro tool researching cell activities (Xu et al., 2021). Additionally, it allows for easy and safe cell isolation for further physiological and pathophysiological studies (Huang et al., 2013). Overall, 3D platforms develop to enable a broader range of processes, they are likely to become an increasingly attractive alternative for 2D cell culture (Duval et al., 2017). A novel scaffolding peptide PGmatrix 3D hydrogel (PG-3D) has been developed to generate the 3D hiPSC spheroids with superior pluripotency (Li et al., 2021), but with the limitation of scaling up. Alternatively, we select the scalable lab-scale PGmatrix3D suspension system (PG-3DSUSP) for hiPSC-generated protein production in this study. Then the nutritional value of hiPSCs-generated proteins was characterized. This study is first time to explore the nutritional quality of hiPSCs-generated protein including amino acid composition and protein digestibility.

2.3 Materials and methods

2.3.1 Materials

hiPSCs derived from human fibroblast was purchased from Applied Stemcell (Milpitas, CA, USA). PGmatrix-3D Suspension (PG-3DSUSP) and PG- 3D hydrogel and PGworks were the product of PepGel LLC (Manhattan, KS, USA). hiPSC colony pellets were lyophilized using a FreeZone 6 L Console Freeze Dryer (LabConco, Kansas City, MO, USA). Trypsin (T7409 Trypsin from porcine pancreas Type II-S, lyophilized power, 1,000-2,000 units/mg dry solid), chymotrypsin (C4129 α -Chymotrypsin from bovine pancreas C4129 Type II, lyophilized power, ≥ 40 units/mg protein), and protease (P0029 Protease from *Bacillus* sp.) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Beef Protein Isolate was purchased from Bulk Supplements (Henderson, NV, USA). Human skeletal muscle proteins from Gorissen et al., (2018) were obtained from the m. vastus lateralis from ten volunteers.

2.3.2 Methods

2.2.1 hiPSCs 3D physiological colony culture in PGmatrix3D Suspension

hiPSC 3D physiological colony (spheroids) culture was performed following the PGmatrix3D-Suspension (PG-3DSUSP) using guide (PepGel LLC, Manhattan, KS). mTeSR1 medium (StemCell Technologies) supplemented with PGgrow (PepGel LLC) at ratio of 1000:1 (v/v, mTeSR1: PGgrow) was used to maintain the suspension cell culturing. Briefly, hiPSCs cell suspension with cell density of $1.5-2 \times 10^5$ cell/mL was mixed with PG-3DSUSP solution and hydrogelation trigger at ratios of 2:1:0.03 (v/v) (cell suspension: PG-3DSUSP: trigger). The mixture with final cell density of $1-1.5 \times 10^5$ cell/mL was then transferred into a 6-well plate (3.94 mL/well) and incubated at 37 °C for cell culture. To feed the cells, 2-3 mL of complete

culture medium per well were added 6-well plate at day 1, day 3, and day 4, respectively, and pipetted gently to distribute the fresh medium uniformly into the 3D suspension culture system. Axio Vert A1 microscope (Carl Zeiss Microscopy, Germany) was used to monitor the hiPSC spheroids morphology and its size evaluation.

2.3.2.2 hiPSC spheroids harvesting

The hiPSC colonies were harvested on Day 5 of suspension culture, which was also following the PG-3DSUSP using guide (PepGel LLC). Briefly, the 3D suspension culture system was mechanically disrupted thoroughly by pipetting, then the mixture was transferred to a 50 mL conical centrifuge tube. Then the mixture was centrifuged at 700g for 5min by using swing bucket centrifuge; the supernatant was discarded, and then 3D colonies pellet was collected from the tube bottom. Then the hiPSCs spheroids pellet were lyophilized by Labconco FreeZone 6 L console freeze dryer for further characterization.

2.3.3 Amino acid profile analysis

Approximately 3 mg of hiPSCs was hydrolyzed with 6M HCl for 24h at 110°C. Then the resulting of amino acids were quantified with hydrophilic interaction chromatography coupled tandem mass spectrometry (HILIC-MS/MS) using a Shimadzu Nexera X2 UHPLC system connected to a SCIEX QTRAP 6500+ triple quadrupole-linear ion trap mass spectrometer, equipped with an IonDrive™ Turbo V electrospray ionization source, as described previously (Kambhampati et al., 2019). Positive ion mode was used for all amino acids, except cysteic acid was used in negative mode. Sample was injected on the Infinity Lab Poroshell 120 Z-HILIC column (2.7 µm, 100 × 2.1 mm; Agilent Technologies, Santa Clara, CA, USA) and amino acids were eluted with a gradient of ammonium formate in water (A) and acetonitrile:water (90:10), pH 3.0, at a final concentration of 20 mM ammonium formate (B) with a constant flow of 0.25 mL/min,

followed by 50% B over 6min, then 100% B over 30s, followed by 6.5 min to re-equilibrate the column. Electrospray ionization source parameters were as follows: ion spray voltage, 4.5 kV (ESI + and ESI-); ion source temperature: 400 °C; source gas 1: 45; source gas 2: 40; and curtain gas: 35.

2.3.4 *In vitro* protein digestion

The pH-drop procedure of Hsu et al. (1997) was adopted and applied in this study. Briefly, 62.5 mg of hiPSCs proteins were dispersed in 10 mL of distilled water at 37 °C for 1h. Ten milliliters (10 mL) of a multi-enzyme solution were prepared, containing 16 mg of trypsin (T7409 Trypsin from porcine pancreas Type II-S, lyophilized power, 1,000-2,000 units/mg dry solid), 31 mg of chymotrypsin (C4129 α -Chymotrypsin from bovine pancreas C4129 Type II, lyophilized power, ≥ 40 units/mg protein), and 13 mg of protease (P0029 Protease from *Bacillus* sp.) Protease from *Bacillus* sp. to replace the discontinued peptidase. The multi-enzyme solution was prepared fresh on the day of analysis and kept 37 °C, which its pH was adjusted to about 8.0 as described above (Tinus et al., 2012).

Upon rehydrated, 1 mL of the multi-enzyme solution was added to the 10 mL protein solution. The pH variation was recorded after 10 min reaction. The pH at 10 min of digestion ($\Delta\text{pH}_{10\text{min}}$) was used to estimate protein digestibility by the equation below:

$$Y=65.66+18.10\Delta\text{pH}_{10\text{min}}.$$

Where $\Delta\text{pH}_{10\text{min}} = \text{pH}_{\text{initial}} - \text{pH}_{\text{final}}$, $\text{pH}_{\text{initial}}$ is the pH after stabilizing approximately 8.0, and pH_{final} is the pH of the solution 10 min after enzyme reaction.)

2.3.5 Statistical analysis

The protein digestibility results were analyzed in triplicate and were expressed as means $\pm$ SD. The results were used to Student's t-test comparing results ($p \leq 0.05$). Statistical analysis was conducted using JASP statistical system, version 0.16.3 (JASP Team, 2022).

2.4 Results and Discussion

The hiPSC Production System

Peptide based PGmatrix 3D hydrogel (PGmatrix-hiPSC(PG-3D)) was determined to be the superior 3D culture for generating physiological hiPSC colonies in previous study (Li et al., 2021), but with limitation of scalable production due to the operability and economic consideration. Alternatively, a novel and scalable peptide based PGmatrix3D suspension culture (PG-3DSUSP) system was used for cultured hiPSC protein production. **Figure 1** presents the scheme diagram of PG-3DSUSP culture workflow. Both 3D systems require the cell encapsulation step at Day 0. For cell feeding, fresh medium was added to the suspension culture to feed cells until day 5 for harvesting, while replacing medium during cell maintenance is required for PG3D hydrogel. At day 5, the mixture of PG-3DSUSP and hiPSC spheroids was ready for centrifuge to isolate cells from hydrogel. PG-3D hydrogel is stronger scaffold, requiring gel disruption and gel dilution with 15-20 times of DPBS to reduce the gel viscosity for cell isolation, during which air bubble and cell loss could be a major issue, especially for the scale up process (Li et al., 2021). By contrast, PG-3DSUSP culture has the feasibility to scale up cell manufacturing in an easily operable and a cost-effective manner.

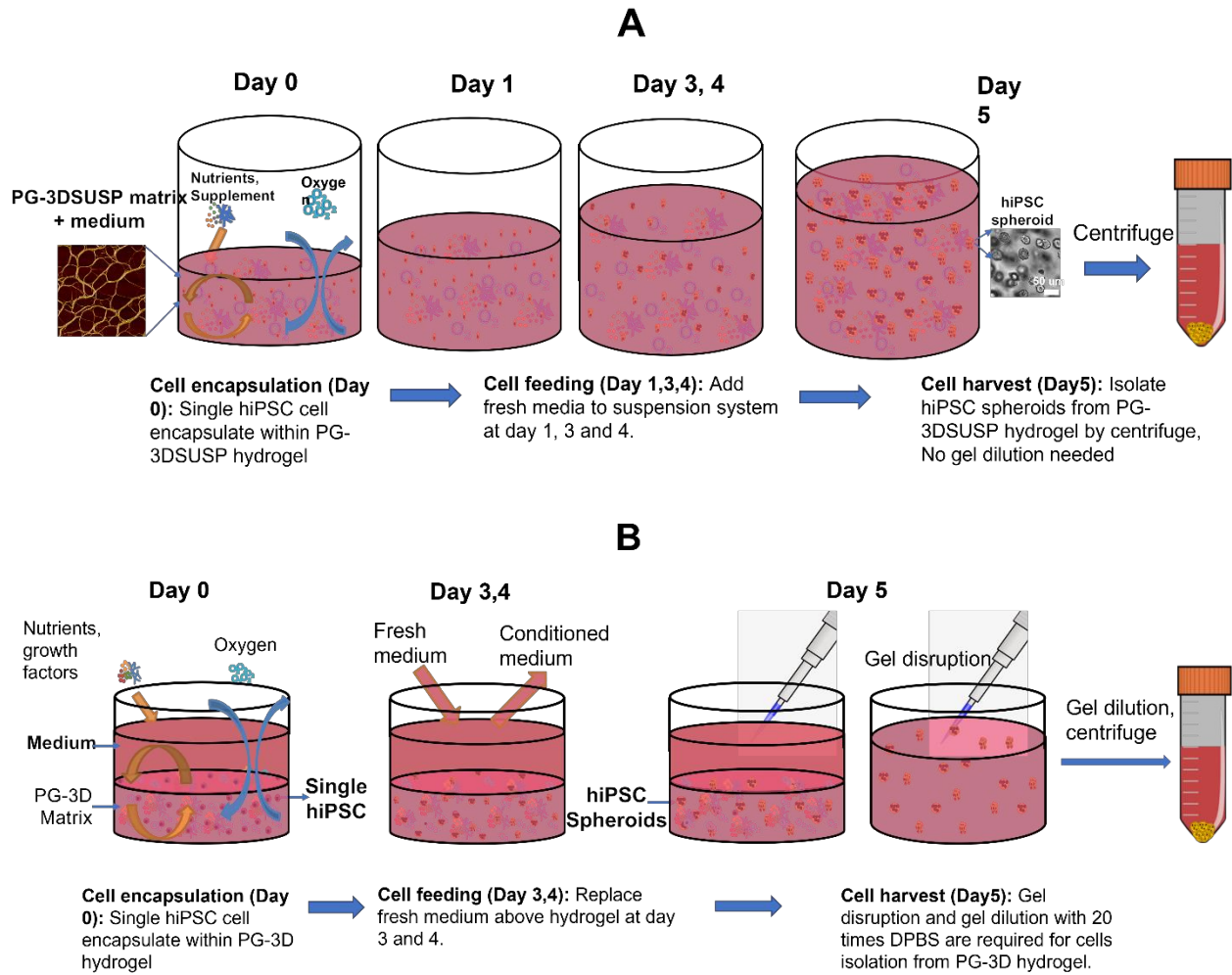


Figure 1(A) Schematic diagram of PG-3DSUSP culture workflow using a well plate. (B) Schematic diagram of PG-3D culture workflow using a well plate.

hiPSCs growth within PG-3DSUSP: Single hiPSCs cell or small clusters were encapsulated within PG-3DSUSP system at seeding density of 1×10^5 cell/mL at day 0. The cell expansion in 3D manner was observed; the developed hiPSC colonies has diameter of 20-30 μm by day 3 of culturing, compared to the cell size of day 0 with 10 μm (Figure 2 A, B). At day 5, the size of hiPSC colonies reached up to 30-60 μm (Figure 2 C). TrypLE was used to trypsinize these 3D colonies into single cells or small cluster cells for proliferation and viability

measurements. Results showed that with total seeding cells of 2.4×10^6 per 6-well plate, cells proliferated to $3.6\text{-}4.8 \times 10^7$ of hiPSCs ($5\text{-}7 \times 10^5$ colonies) by day 5. The overall cell expansion fold across multiple passages was well maintained in the range of 15-20 folds with viability of 95-99% in 5 days. PG-3DSUSP culture system presents comparable cell growth performance to PG-3D culture in terms of cell morphology and growth rate. Both 3D culture system generated the hiPSC colonies with size of 30-50 μm (**Figure 2 D**).

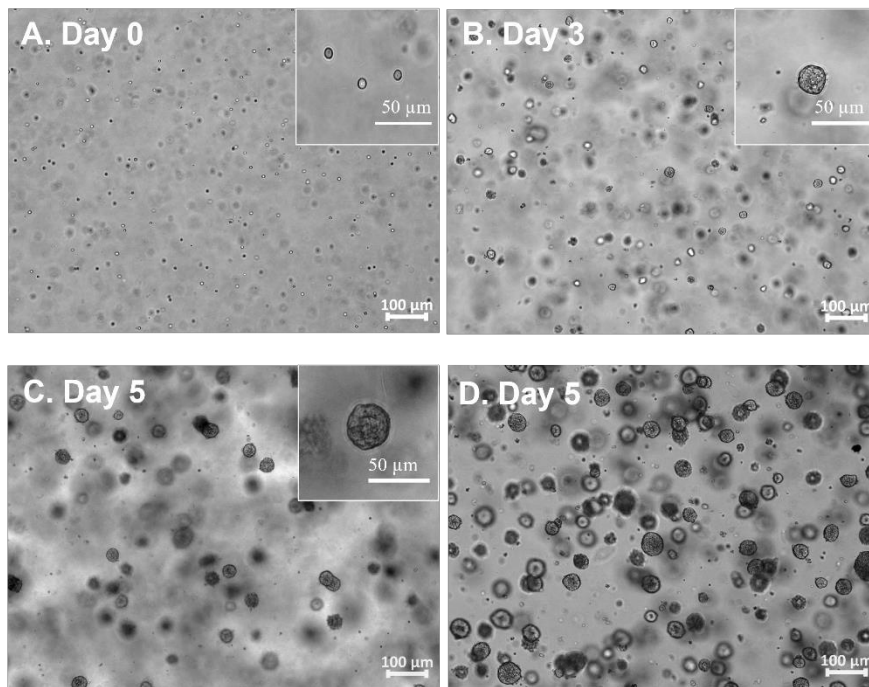


Figure 2 Morphology of hiPSC grown in PG-3DSUSP system from day 0 to day 5 (A, B, C); hiPSC grown in PGmatrix 3D hydrogel at day 5 (D)

Besides the stable long-term cell maintenance, PG-3D hydrogel also proved to generate hiPSC colonies (spheroids) with superior pluripotency compared with that from traditional 2D culture or other Matrigel, Vitronectin, or Polyethylene glycol (PEG)-based 3D cell culture method. PG-3DSUSP culture system was proved to be a competitive culture system to the well-

established PG-3D hydrogel, but with the distinctive advantages of easily cultured workflow and great potential of scalable cell production.

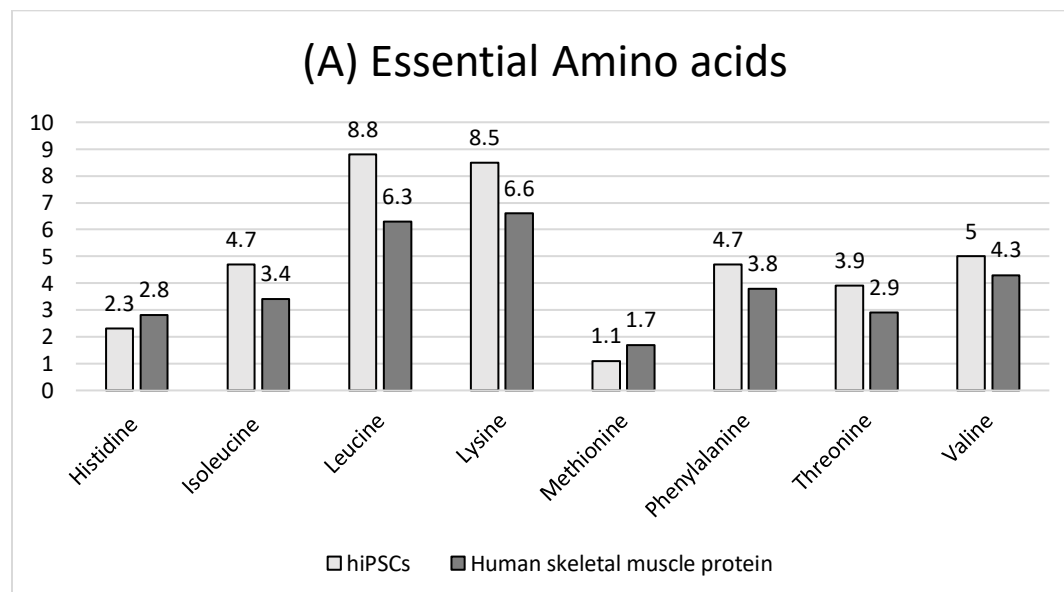
Some studies intended to culture hiPSCs in 2D culture system with specifically designed coating materials, resulting in flat and stretched morphologies of hiPSCs (Li et al., 2021). However, a 2D cell culture system does not mimic the environment of human body, which affected cell processes of hiPSCs such as proliferation, apoptosis, differentiation, gene expression, and drug sensitivities (Liu et al., 2010). Moreover, 2D cell-cultured cells undergo cytoskeletal rearrangements and acquire artificial polarity, resulting in aberrant gene and protein expression (Nath and Devi, 2016).

Hydrogels (natural-based and synthetic-based) have been considered to provide extracellular matrix (ECM) like scaffold for various stem cells due to their 3D nature and high-water content, which may potentially replace to 2D cell culture (Duval et al., 2017, Nicodemus et al. 2008). We have tried to provide more realistic biochemical and biomechanical microenvironments for hiPSCs. It is reported that high-efficient physiological formation of hiPSC spheroids with stable genetic integrity was developed within PGmatrix-hiPSC 3D hydrogel (PG-3D) (Li et al., 2021). PG-3DSUSP is a novel technology for suspension culture, specifically designed for scaling up cell manufacturing. Under the PG-3DSUSP setting, cell growth performance remained consistent through multiple passages with cell increase to 15-20-fold and cell viability of 95%-99%. In addition, hiPSCs from PG-3DSUSP showed similar or higher stemness gene expression levels in comparison to that from PG-3D. Overall, PG-3DSUSP presents similar growth performance in terms of growth rate and gene integrity compared with well-established PG-3D hydrogel system. The superior advantages of PG-3DSUSP are of easy

operability, cost effectiveness, and great potential to scale up for cell culturing and protein production from cultured cells.

hiPSC Nutritional Properties

Amino acid composition of hiPSCs versus human skeletal muscle protein are shown in **Figure 3**. Essential amino acid content of hiPSCs (39.0%) were significantly higher than that of human skeletal muscle protein (31.8%) (Figure 3A). Among those, the content of isoleucine (4.7%), leucine (8.8%), lysine (8.5%), phenylalanine (4.7%), threonine (3.9%), and valine (5.0%) of hiPSCs were higher than human skeletal muscle proteins, and the content of histidine (2.3%) and methionine (1.1%) were lower than human skeletal muscle protein. Total non-essential amino acids of hiPSCs (46.1%) was also significantly higher than human skeletal muscle protein (29.0%) (Figure 3B). *In vitro* protein digestibility of hiPSCs ($78.0 \pm 0.7\%$) was significantly higher than commercial beef protein isolate ($75.7 \pm 0.6\%$, $p < 0.015$) (Figure 4).



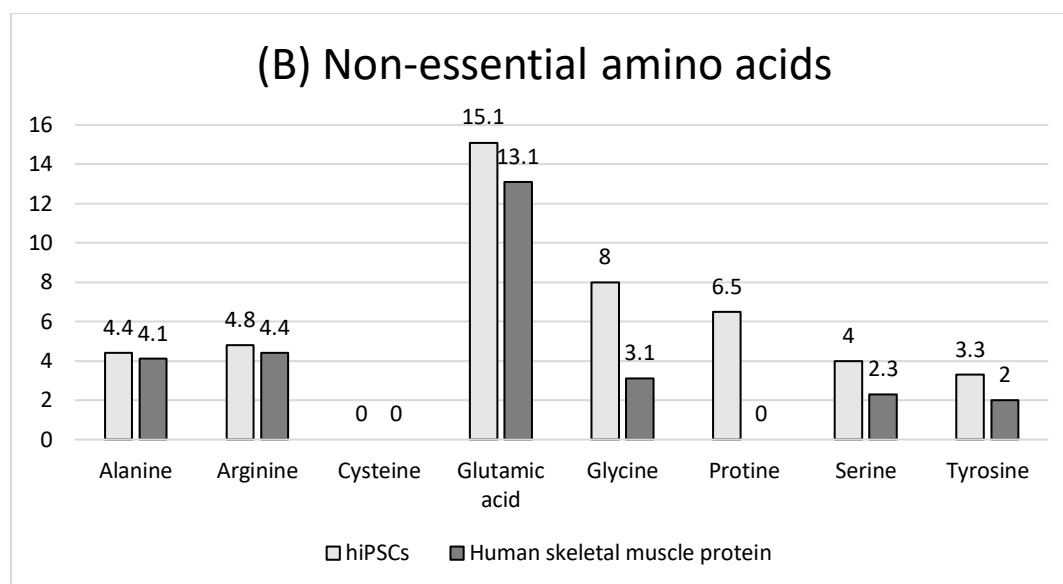


Figure 3 Amino acid content of hiPSCs and human skeletal muscle protein

The nutritional value of hiPSC-generated proteins including amino acid composition and *in vitro* protein digestibility were measured. The total EAA of hiPSCs (39.0%) were higher than human skeletal muscle protein (31.8%). Human skeletal muscle proteins from Gorissen et al., (2018) were obtained from the m. vastus lateralis from ten volunteers. We chose human skeletal muscle protein for comparison with hiPSCs because it is reasonable that they both come from the human. Also, when focusing on muscle protein synthesis, we included human skeletal muscle protein as a reference protein with an 'ideal' amino acid composition. We observed that leucine content (8.8%) was highest in EAA. It has been proposed that leucine is a key mechanism translating diet quality into the muscle protein synthesis response to meals (Norton et al. 2009; Gorissen et al., 2018). It has been shown that leucine stimulates Sestrin2 to translocate to the lysosomal membrane, which activates mTORC1, and results in muscle protein synthesis (Saxton et al., 2016). Hence, leucine contents are an important factor in modulating muscle protein syntheses after protein ingestion (Gorissen et al., 2018). The content of lysine (8.4%) was also

higher than human skeletal muscle (6.6%). Lysine and methionine are necessary amino acids for making carnitine, which plays a vital role in the metabolism of fatty acids and energy production. However, histidine content (2.3%) and methionine content (1.1%) were lower than human skeletal muscle protein. Histidine content of hiPSCs reached the WHO/FAO/UNU requirements (WHO/FAO/UNU Expert Consultation 2007).

In vitro protein digestibility of hiPSCs and commercial beef protein isolate are showed in **Figure 4**. The digestibility of human protein sources are unknown. Studies have shown that animal-based proteins digest more easily than plant proteins (Shaghaghian et al., 2022). Compare with other animal-based sources, beef protein is a high quality sources. In this study, we compared protein digestibility of hiPSCs with commercial beef protein isolate as control group. A high correlation was found between the pH of a protein suspension immediately after 10 min digestion in the multienzyme solution and *in vivo* apparent digestibility (Hsu et al., 1997), particularly when the protein sources were analyzed by plant or animal origin (Butts et al., 2012). Additionally, as *in vivo* methods are expensive, time-consuming, and involve ethical issues, they are not suited to studying multiple samples required to understand ingredient interactions or processing effects, which are commonly assessed using *in vitro* techniques (Rieder et al., 2021). The pH drop three-enzyme (trypsin-chymotrypsin-peptidase) method is widely used in *in vitro* protein digestibility that determines the pH after 10 min of reaction (Hsu et al., 1997). Protease used to replace the discontinued peptidase to build the pH vs. digestibility calibration (Tinus et al., 2012). It was based on the principle that hydrolysis by amino acid carboxyl groups deionize and released free protons (Mendes et al., 2016). This *in vitro* method using multienzyme system was highly correlated with *in vivo* apparent digestibility (Hsu et al., 1997). It improved the prediction of *in vivo* protein digestibility, was reproducible, and predicted *in vivo* (rat fecal)

digestibility accurately (Butts et al., 2012). hiPSCs-generated protein showed higher protein digestibility than animal protein. Intestinal protein availability can be estimated from protein digestibility which reflects the efficiency of protein utilization in the diet (Ketnawa and Ogawa, 2021). It is well known that the protein digestibility of animal protein is higher than of plant protein (Sa et al. 2020). There is a possibility that an infant or elderly may have insufficient digestion of animal proteins due to low secretion of digestive juices or enzymes in the immature state or a malfunctioning GI tract (Lee et al.2021), so that they may need higher protein digestibility to meet their needs. hiPSCs has a higher *in vitro* protein digestibility than animal protein which applied for food products in infants and elderly in the future. Clearly, *in vitro* methods are not mimic the complexity of human digestion. In the future, it will be necessary to investigate in animal models or *in vivo* whether the hiPSCs-generated protein could be edible or has any potential side effects.

This study deconstructed advanced culture hiPSCs in advanced 3D peptide hydrogel (PG-3DSUSP) system is applicable method for lab-scale cell cultured protein production, and cultured hiPSCs exhibited improved nutritional attributes in terms of amino acid composition and *in vitro* protein digestibility for the first time.

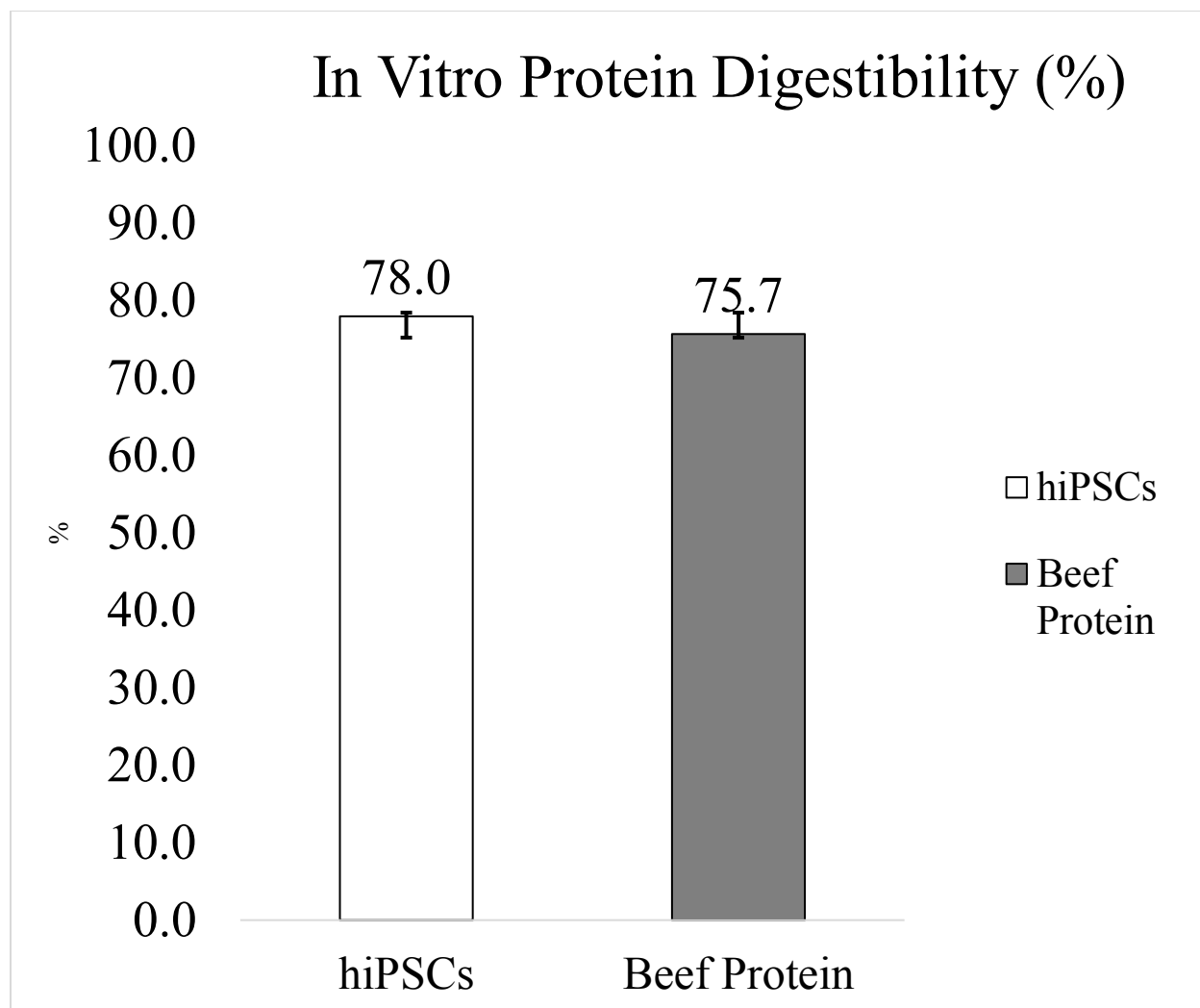


Figure 4 *In vitro* Protein Digestibility of hiPSCs and commercial beef protein isolate (Results are shown as mean $\pm$ SEM). *: p value < 0.05

2.5 Conclusion

Scalable lab-scale PG-3DSUSP hydrogel presents similar hiPSC growth performance to the well-established PG-3D hydrogel culture system, with the consistent hiPSC growth fold expansion of 15-20 in 5 days and viability of 95-99%. PG-3DSUSP is the superior 3D suspension culture hydrogel but with the distinctive advantages of great potential for large scale cell manufacturing. This study also characterized the lab-scale cell cultured protein production

and nutritional quality including the amino acid composition and *in vitro* protein digestibility of hiPSCs-generated protein. hiPSCs-generated protein has a high essential amino acid (EAA) and *in vitro* protein digestibility in 3D PepGel cell culture system. Leucine content was highest in EAA, which is a key factor translating diet quality into the muscle protein synthesis response to meals. A highly efficient production of hiPSCs-generated protein by the advanced 3D hydrogel with more essential amino acids and better digestibility appears of particular significance as novel alternative proteins in food engineering and industries for future food, beverage, and supplement application.

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