Sustainable bioproduction of animal proteins for human consumption and optimizing the use of protein rich by-products from the food industry

Doctoral thesis by

Randi Christel Andreassen

Department of Nutrition

Institute of Basic Medical Sciences

Faculty of Medicine University of Oslo

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Ås, Norway, August 2021

R. Christell Andreassen

Selected abbreviations

MCs	Microcarriers
MuSC	Skeletal muscle satellite cell
FBS	Fetal bovine serum
ECM	Extracellular matrix
GAG	Glycosaminoglycan
PBS	Phosphate-buffered saline
SEC	Size-exclusion chromatography
EPH	Enzymatic protein hydrolysis
RT-PCR	Real time polymerase chain reaction
MYOD	Myoblast determination protein 1
MYOG	Myogenin
PAX7	Paired boxed protein 7
MYF5	Myogenic factor 5
CCND1	Cyclin D1
MKI67	Marker Of Proliferation Ki-67
GF	Growth factor
ATP	Adenosine triphosphate
LDH	Lactate dehydrogenase
NCAM	Neural cell adhesion molecule
LC	Liquid chromatography
TNBS	Trinitrobenzenesulfonic acid
FA	Focal adhesions
SFM	Serum-free medium
TF	transcription factor
PI3K	Phosphatidylinositol 3-kinase
GPI	Glycosylphospatidylinositol
DNA	Deoxyribonucleic acid
ESC	Embryonic stem cell
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
AT	Adipose tissue
FAO	UN Food and Agricultural organization
LC	Liquid chromatography
LC-MS/MS	Tandem mass spectrometry
SEC	Size-exclusion chromatography
FOXO	Forkhead box

Abstract

The global population growth is posing enormous challenges to the sustainability of the current food production system. The average consumption of meat has tripled over the last 50 years, and meat consumption tends to rise as people get richer, threatening to make high-quality meat and animal protein products a limited resource. Conventional meat production is associated with considerable negative environmental impacts. Sustainable development approaches include achieving responsible production, use, and consumption of meat products. Along with meat production, significant quantities of organic waste by-products related to environmental pollution are generated. These by-products contain high-quality proteins and nutrients that are currently underused. Opportunities to extract additional value from by-products and sustainable alternatives to conventional meat production should be examined. A promising alternative to the traditional way of producing animal proteins is cultured meat. This meat bypasses animal production by growing muscle cells under a suitable cell culture medium in bioreactors and can potentially be produced more sustainably and efficiently than conventional meat. Despite a high uncertainty due to the early developmental stage of the technology, life-cycle analyses suggest that cultured meat production requires 90% less land, 75% less water, 95% less eutrophication while contributing to 75% less greenhouse gas emissions. At the same time, cultured meat could provide an opportunity for more responsible waste by-product management. However, there are major technological hurdles to overcome until cultured meat can be served at the dinner table, such as formulating a cost-effective serum-free media (SFM), developing suitable scaffolds necessary for muscle cell attachment, and optimizing large-scale production.

By-products are low-cost, easy to obtain, food-safe ingredients with potential cell-stimulating and biocompatible properties. Therefore, they have excellent potential as ingredients in a tailor-made SFM and as muscle cell scaffolding support. In this project, the aim was to solve challenges in cultured meat technology by investigating the feasibility of implementing by-products in the production process. Bovine skeletal muscle satellite cells (MuSCs) were isolated from *Longissimus thoracis* (beef sirloin). A scaffold-based design was used with microcarriers (MCs) providing permanent support to bovine skeletal muscle satellite cell (MuSC) monocultures in different sized bioreactors. Protein hydrolysates and edible MCs were produced from by-products, characterized, and evaluated for their cell growth-promoting efficiency. Further, long-term and scalable cell expansion parameters were investigated from low volume spinner flasks to higher volume bench-bioreactors. The properties studied confirmed that various by-product hydrolysates and fractions effectively maintained cell growth in SFM. Further, the MCs produced using collagen extracted from turkey tendons, and powdered eggshell membrane (ESM) provided high cell culture expansion efficiency. After more than a month, MuSCs expanded in bench-bioreactors retained their proliferative and migratory capacity after dissociation from MCs, indicating that the stem cell pool was well maintained. These are functional cell qualities essential for achieving the massive quantities of MuSCs required in cultured meat production. Also, low volume spinner flask cultures with less controlled environments were robustly reproducible in bench-bioreactors. Therefore, low volume spinner flasks can be used as higher throughput and scaled-down models to optimize MuSCs expansion on MCs. This work provides a promising start for developing a sustainable SFM for cultured meat production while reducing the problematic reliance on culture media cost drivers such as serum and commercial growth factors. More importantly, it contributes to a more responsible waste by-product management, representing a significantly more effective protein conversion strategy while contributing to a circular economy by reducing waste. Furthermore, it was demonstrated that by-products with minimal processing successfully functions as MCs for bovine MuSCs in spinner flask culture. Finally, this work provides much-needed publicly accessible data on MuSCs growth kinetics, behavior, and development, especially in high volume bioreactors, and is a promising start to optimize cell expansion parameters adapted to muscle cells.

Sammendrag av doktoravhandlingen (summary in Norwegian)

Den globale befolkningsveksten bringer med seg enorme utfordringer for bærekraften til det nåværende matproduksjonssystemet. Det gjennomsnittlige forbruket av kjøtt har tredoblet seg de siste 50 årene og etterspørselen er forventet å stige når velstanden øker. Konvensjonell kjøttproduksjon er forbundet med betydelige negative miljøpåvirkninger, knyttet til klimagassutslipp, avskoging og overbruk av tilgjengelige ressurser. I tillegg produseres det betydelige mengder organiske avfallsprodukter som bidrar vesentlig til miljøforurensning. En del av disse biproduktene inneholder imidlertid proteiner og andre næringsstoffer av høy kvalitet som har potensialet til å bli forvaltet videre. Muligheter for å hente merverdi fra biprodukter og alternativer til konvensjonell kjøttproduksjon bør undersøkes og utvikles. Et lovende alternativ til den nåværende måten å produsere animalske proteiner på er en prosess kalt «dyrket kjøtt». Dyrket kjøtt innebærer å dyrke levende muskelceller i et egnet næringsmedium i bioreaktor tanker, uavhengig av dyreproduksjon. Dermed kan dyrket kjøtt potensielt produseres mer bærekraftig og effektivt enn vanlig kjøtt. Til tross for usikkerheter tilknyttet det tidlige utviklingsstadiet av teknologien, estimerer livssyklusanalyser at dyrket kjøttproduksjon krever 90% mindre landområder, 75% mindre vann, 95% mindre eutrofiering, mens det bidrar til 75% mindre klimagassutslipp. Samtidig kan dyrket kjøtt gi en mulighet for en mer ansvarlig avfallshåndtering. Imidlertid er det store teknologiske hindringer å overvinne før dyrket kjøtt kan serveres ved middagsbordet. For eksempel trengs formulering av et kostnadseffektivt serumfritt næringsmedium, utvikling av egnede mikrobærere som muskelceller kan vokse på og optimalisering av storskala-produksjon.

Biprodukter er rimelige, kommer i massive mengder, er godkjent for bruk i mat og har potensielt cellestimulerende og biokompatible egenskaper. Derfor har de et utmerket potensial som ingredienser i ett skreddersydd serumfritt medium og som spiselige mikrobærere. I dette prosjektet var målet å løse utfordringer forbundet med dyrket kjøtt-teknologien ved å undersøke muligheten for å inkludere biprodukter i produksjonsprosessen. Bovine muskel stamceller ble isolert fra ytrefilet og brukt i forsøkene. Protein hydrolysater og spiselige mikrobærere ble produsert fra biprodukter, karakterisert og evaluert for deres cellevekstfremmende effektivitet. Videre ble skalerbare celleekspansjonsparametere undersøkt fra spinnerflasker med lavt volum til benk-bioreaktorer med større volum. Egenskapene som ble studert bekreftet at forskjellige biprodukt hydrolysater og hydrolysat fraksjoner effektivt kunne opprettholde celleveksten uten serum. Microbærene produsert av kollagen ekstrahert fra kalkunsener og pulverisert eggeskallmembran ga høy cellekulturekspansjonseffektivitet. Etter mer enn en måned med stamcelleekspansjon i benk-bioreaktorer beholdt cellene sin proliferative og migrerende kapasitet etter dissosiasjon fra mikrobærere, noe som indikerer at stamcelle egenskapene var godt vedlikeholdt. Dette er funksjonelle cellekvaliteter som er avgjørende for å oppnå de enorme mengdene stamceller som trengs i dyrket kjøttproduksjon. Spinneflaskekulturer med lavt volum og mindre kontrollert miljø var også robust reproduserbare i benk-bioreaktorer. Derfor kan spinnerflasker brukes som nedskalerte modeller for å optimalisere stamcelleekspansjon på mikrobærere. Dette arbeidet gir en lovende start for å utvikle et bærekraftig serumfritt medium for dyrket kjøttproduksjon, samtidig som den problematiske avhengigheten av høykostnads supplementer i celle medium som serum og kommersielle vekstfaktorer reduseres. Enda viktigere, det bidrar til en mer ansvarlig avfallshåndtering, som representerer en betydelig mer effektiv proteinkonverteringsstrategi, samtidig som det bidrar til en sirkulær økonomi ved å redusere avfall. Videre ble det demonstrert at biprodukter med minimal behandling lykkes som mikrobærere for bovine stamceller. Til slutt gir dette arbeidet tiltrengte offentlig tilgjengelige data om stamceller, cellevekst, kinetikk, atferd og utvikling, spesielt i bioreaktorer med høyt volum, og er en lovende start for å optimalisere celleekspansjonsparametere tilpasset muskelceller.

List of scientific papers

This PhD thesis is based on the following original scientific papers:

Paper I

Screening of by-products from the food industry as growth promoting agents in serum-free media for skeletal muscle cell culture.

R. Christel Andreassen, Mona E. Pedersen, Kenneth A. Kristoffersen, Sissel Beate Rønning.

Food & Function, 2020, 11, 2477-2488.

Paper II

Peptides from food by-products in a simple and effective tailor-made serum-free media for bovine skeletal muscle cell culture.

R. Christel Andreassen, Mona Elisabeth Pedersen, Sileshi Gizachew Wubshet, Rita de Cassia Lemos Lima, Svein Olav Kolset, MJ Post, Sissel Beate Rønning.

Manuscript prepared for submission to *Food Chemistry,* September 2021.

Paper III

Up-scaling and long-term expansion of bovine skeletal muscle stem cells from spinner flasks to lab-bench stirred bioreactors.

Dimitrios Tzimorotas, Nina Solberg, R. Christel Andreassen, Vincent Bodiou, Panagiota Moutsatsou, Mona Elisabeth Pedersen, Sissel Beate Rønning.

Paper submitted to *Journal of Biotechnology*, 2021, August 2021.

Paper IV

Production of edible microcarriers based on by-products from the food industry to facilitate the expansion of bovine skeletal muscle satellite cells for cultured meat production.

R. Christel Andreassen, Sissel Beate Rønning, Nina Therese Solberg, Krister Gjestvang Grønlien, Svein Olav Kolset, Mona Elisabeth Pedersen.

Revised paper prepared for resubmission to *Biomaterials*, September 2021.

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1 Introduction

1.1 Livestock meat and nutritious proteins as a limited resource: a global perspective

It is estimated that the global population will be approximately 10 billion by 2050. The continually growing population accompanied by a rising middle-class and elderly population is followed by an increased food demand, especially for luxury foods such as high-quality animal proteins and meat [1, 2]. A variety of technological advancements have improved agricultural productivity and radically transformed the food system, resulting in decreased food prices and increased accessibility to food [2]. Based on FAO data, the average consumption of dairy and fish has doubled while the meat consumption has tripled over the last 50 years due to population growth and a high demand for protein-based foods [3, 4].

Protein is acknowledged as an integral part of a healthy balanced diet. Animal products have high nutritional value and are excellent sources for quality protein and other essential nutrients not as readily found in plant-derived foods such as zinc, vitamin A, highly bioavailable iron, and vitamin B12 [5]. Meat also contains all essential amino acids, other bioavailable vitamin B types, omega-3 fatty acids, and minerals (e.g., phosphorous, magnesium, and calcium) [6, 7]. There is accumulating evidence that recognizes the importance of protein intake due to the association with an increased muscle protein synthesis, especially in older adults prone to muscle atrophy. [8]. The nutritional value of meat, together with texture and aroma, has made it one of the most demanded foods globally [9]. As a result, the world's livestock sector is rapidly expanding to meet the continuously growing demand for animal protein. The strong tendency towards increased meat consumption is anticipated to continue with an estimated 70% increase in global meat demand by 2050, largely due to the demand predicted in developing regions to fulfill their nutritional needs [10]. Figure 1.1 illustrates worldwide changes in meat consumption between 1961 and 2013.

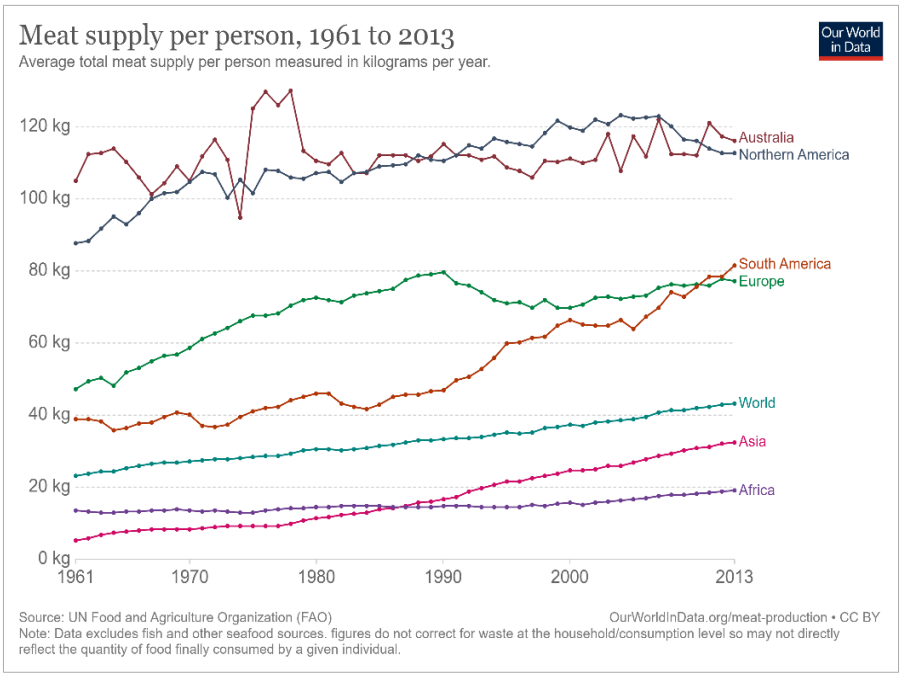

Figure 1.1: Based on FAO data, the average consumption of meat has tripled over the last 50 years and meat consumption tends to rise as people get richer. This chart illustrates a time-series of meat consumption per capita, measured in kg per person per year from 1961 to 2013. The global average (world) meat consumption per capita has increased approximately 20 kg since 1961 to 2014 (from 23.08 kg to 43.22 kg). However, the increase is much higher for some individual countries [10].

The problem is that the livestock meat industry poses enormous challenges to the sustainability of food production and the UN 2030 Agenda for Sustainable Development [11]. Conventional meat production is associated with considerable negative environmental impacts such as depletion of

natural resources, greenhouse gas emission, pollution, and waste management issues [2]. For instance, meat production requires an estimated 70% arable land to grow livestock feed [12]. Consequently, large amounts of foods that humans consume are fed to livestock, e.g., more than 670 million tons of cereal are consumed by livestock animals each year, leaving less food to the growing human population [3]. Livestock meat production also contributes to the global health concern of antibiotic resistance in human pathogens because of the extensive antibiotic use in agriculture [13, 14]. Therefore, reducing conventional meat production is a reasonable bioeconomic approach [15]. However, food production is largely consumer-directed. Most consumers are unwilling to reduce their meat consumption because cultural associations with meat are hard to change, even though plant-based meat alternatives have become more accessible recently [16, 17]. Unfortunately, while plant-based meat alternatives contribute to improved sustainable development, the production resource requirements are significant [5].

Along with meat production, considerable quantities of slaughter by-products related to environmental pollution are generated. These by-products contain high-quality proteins and other nutrients that are currently underused [18]. In the interest of sustainable, economic, and ethical food development, waste by-products require optimal energy recovery. Therefore, opportunities to extract additional value from by-products and alternatives to conventional meat production should be examined and developed [15]. A revolutionary and promising alternative to the traditional way of producing animal proteins is cellular agriculture, specifically cultured meat. In theory, this meat will bypass animal production and can be produced more efficiently with significantly lower energy input and protective environmental impact compared to conventional meat [19, 20]. Cultured meat also provides an opportunity to use the currently available resources provided by the food industry, representing a significantly more effective protein conversion strategy, not diminishing conventional meat production but aiding it [21].

1.2 Cultured meat as a sustainable alternative to conventional meat production

A groundbreaking and sustainable alternative to conventional livestock production is using cell-based technologies to produce meat, seafood, leather, and milk. This meat will bypass animal production and is predicted to be less resource-intensive, produced faster, and more efficiently than conventional meat production [19]. It is estimated that 10.000 kg cultured meat can be generated from a small ~1.0 g muscle tissue biopsy in a matter of days rather than the months required to raise the larger farmed animals [22]. Despite a high uncertainty due to the early developmental stage of the technology, it is predicted that the overall environmental impact, specifically greenhouse gas emissions, water consumption, and land use in cultured meat production, is significantly lower than conventional meat production [23, 24]. More specifically, life-cycle analyses suggest that cultured meat production requires 90% less land, 75% less water, 95% less eutrophication while contributing to 75% less greenhouse gas emissions [24, 25]. For example, it is stipulated that conventional meat production is responsible for approximately 18% of the total CO_2 emissions, and switching to cultured meat can reduce it to less than 1.0 % [23]. Also, cultured meat can potentially be a safer and healthier product than conventional meat, as the production can be performed without antibiotics and a lower risk of potentially fatal infections commonly associated with conventional meat, such as *Salmonella* and *Listeria* [26]. Cultured meat also provides the opportunity to customize the nutritional composition of the meat. It is possible to enhance various nutritious factors, e.g., the amount of omega-3 fatty acids through genetic engineering or supplementation directly in the cell growth medium. Another advantage of cultured meat proteins is their potential to retain the functional properties of the original animal-based meat, giving them the overall characteristics of meat products that are challenging to reproduce in non-animal proteins. In addition to contributing to public health

and more sustainable development, cultured meat will positively impact animal welfare by reducing the number of animals kept in captivity as farmed industrial products.

As cultured meat development progresses and technological challenges are overcome, it is essential to do a comprehensive nutritional analysis and evaluate impacts on human health and the environment against food security concerns. Especially since the production might be energy-intensive and some environmental benefits are dependent on the transition to clean energy sources [20, 23, 24]. Novel food products produced outside a living organism (*in vitro*) from mammalian stem cells will require approval by national food authorities, and governmental regulatory agencies will eventually require regulations. In addition, consumer acceptance of these products will require direct benefits to the consumer such as taste, cost, convenience, and trust [27]. Further, when significant health and environmental benefits are documented, consumer acceptance must be encouraged to avoid the same complications observed when introducing genetically modified food [28]. It is reasonable to assume that livestock products and cultured meat will co-exist side by side when cultured meat is available. However, it is also important to explore the potential conflicts between a revolutionary shift in meat production methods and ethical, social, and political values. As cellular agriculture food production may represent a new era for humanity, considerable implications will follow, such as changing entire industries, livestock biodiversity, relationships between humans, animals, and nature. One example, is historical shifts like the transition to synthetic chemistry in the nineteenth century (e.g., alizarin, vanilla) eradicated entire industries, which changed economies and landscapes while, on the other hand, freeing up land for additional food production [29].

At present, cultured meat development is primarily supported by private investors, which has led to a competition-based system with a severe lack of transparency. Moreover, because of proprietary rights, the current technological progress is largely unknown [5]. Nevertheless, the innovative approach to alternative meat production has gathered increased investments, and there are more than 70 funded cell-based startup companies focusing on cultivated meat. Figure 1.2 shows the geographical distribution of many registered cell-based meat companies worldwide. Ultimately, cell-based technology has the potential to support the global demand for meat while conserving planetary health [25]. However, there are still major technological hurdles until cultured meat can be served at the dinner table (discussed in chapter 1.4).

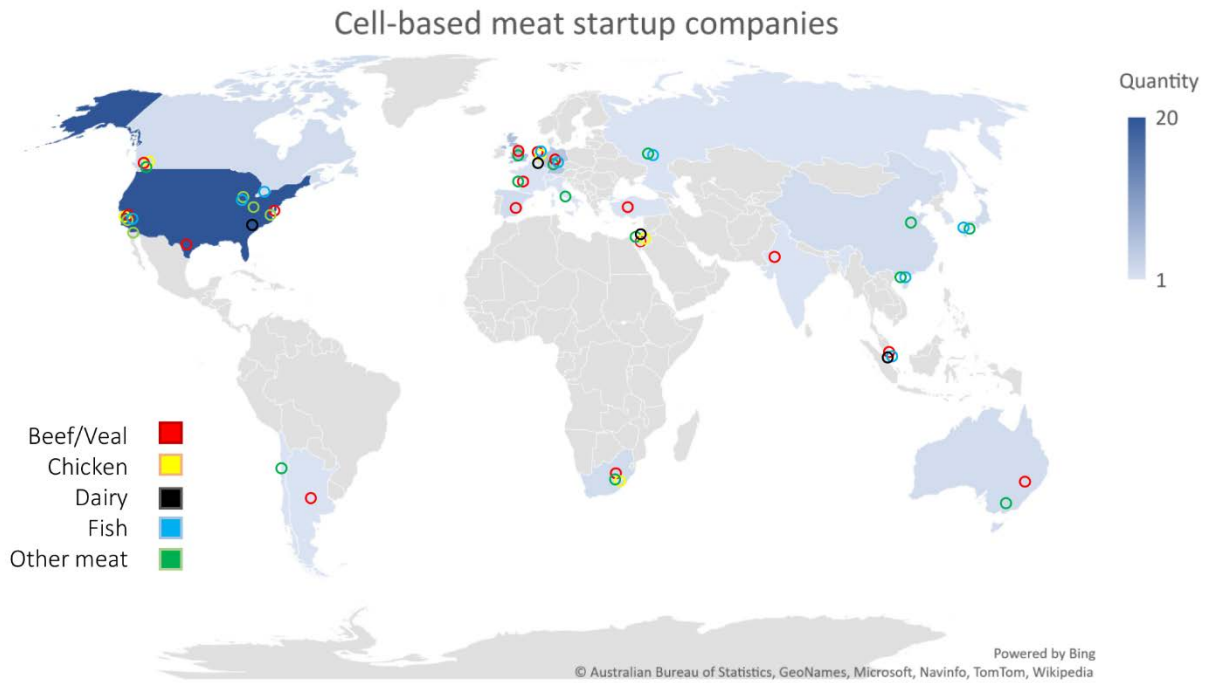

Figure 1.2: Geographical distribution of meat cell-based companies around the world: The blue color intensity on the map indicates the number (quantity) of companies in each country, while the different colored circles indicate the company animal source focus, where the beef/veal and other meat categories are the most prevalent. The information was retrieved from the Good Food Institute alternative protein company database (July 2021). The image was created with Microsoft word filled map.

1.3 Valorization of by-products and opportunities in cultured meat

One way to reduce the environmental impact of conventional meat production is by effectively reutilize food by-products. This also represents an industrial opportunity for the food industry to increase the value of by-products, and at the same time, contribute to the circular economy by reducing waste. Likewise, there are also opportunities for by-product use in the cellular agriculture market to overcome some of their major technological hurdles.

Depending on the species, livestock and fish by-products are composed of various organs and tissues from carcasses, including hides, skin, feathers, eggshells, hoofs, head, lung, tongue, heart, fat, and tissue trimmings. Other typical by-products of meat processing are liquid streams, e.g., blood, cook-out liquids, glue water, brines, and exudates. The amount of by-products generated depends on the animal origin. However, it is estimated that nearly 40-60% of farmed fish and animal's total mass are classified as residual products with food-grade quality. In 2014 on a global scale, approximately 128 and 263 million tons of by-products were generated, respectively [30]. Meat by-products have excellent nutritional value and contain essential amino acids, lipids, carbohydrates, vitamins, and minerals. They also have excellent functional and physicochemical properties such as emulsifying, gelling, mineral binding, or water holding capacity giving them the overall characteristics of meat products that are challenging to reproduce in non-animal proteins [9]. Furthermore, by-products are low-cost, easy to obtain, food-safe ingredients with potential cell-stimulating and biocompatible properties and have excellent potential in aiding cultured meat production as they can be included in the final meat product [30-35]. Figure 1.3 illustrates examples of how by-product derivatives can benefit cultured meat production. Because of these properties, by-product degradation products can be used in many different applications and are already used as food ingredients, animal feed, fertilizer, biofuel, adhesives, and more[36] [9, 37].

In addition, there are opportunities in markets such as pharmaceuticals, biomedical, cosmetics, and the nutraceutical industry. Some examples include the use of processed eggshell membrane in biomedical scaffolds and wound healing and extracted collagen in drug delivery and hydrogels. Further, bioactive peptides can be isolated for blood sugar and blood pressure regulation, removing heavy metals from the bloodstream (chelating), immune modulation, opioid, antioxidant, and antimicrobial properties [30-35]. Interestingly, antioxidant peptides are often multifunctional and may exhibit other bioactivities, such as antihypertensive, anti-inflammatory, or antimicrobial effects, making them attractive substances in food ingredients for enhancing human health [38]. Regarding human health, the discovery of safer health intervention agents such as functional biopeptides is especially attractive because they may prevent serious adverse effects associated with commonly prescribed drugs available today [39].

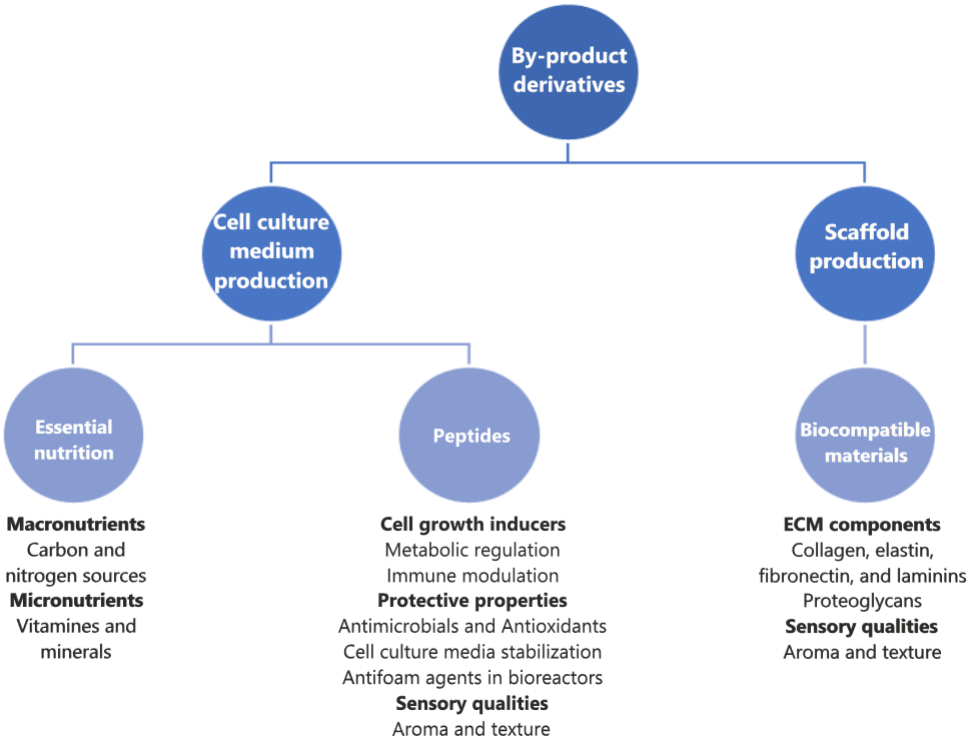

Figure 1.3: Derivatives with various functions can be isolated from by-products. These derivatives can help overcome technological challenges in cultured meat production, such as producing a sustainable cell culture medium (cell nutrition) and scaffolds for cells to attach and colonize.

The idea of using every part of raw materials without waste is not new, but it comes at a cost. One highlighted concern of using by-products from the meat industry is the risk of contamination, or transmittable diseases such as various prion diseases, foot-and-mouth disease, avian influenza or dioxin [40, 41]. This risk is eliminated or largely reduced by meticulous health and safety routines. By-products must be appropriately managed, collected, sorted, and processed efficiently at a low cost to escape incineration without energy recovery [30, 37]. Nevertheless, it can be argued that a zero-waste transition is equally important as the transition to renewable energy sources to ensure sustainable development and escape the worst effects of climate change [20, 42]. Several industrial technologies are established to gain further value from meat by-products, such as protein hydrolysis combined with separation and isolation of various protein degradation products (discussed in chapter 1.5.2).

1.4 Cultured meat technology

The idea of cultured meat was already referenced in literature from the nineteenth century. In 1931 Winston Churchill wrote, "we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing, by growing these parts separately under a suitable medium" [43]. The discovery of muscle stem cells 40 years later made culture meat a real possibility because of their tremendous self-renewal capacity and programmed commitment to form new muscle tissue [44]. Muscle stem cells reside between the basal lamina and sarcolemma (plasma membrane) of skeletal muscle fibers. They normally lay dormant in a quiescent state in the adult muscle, ready to be activated during exercise, injury, or disease. Then they self-renew, grow, and can undergo myogenic differentiation to fuse together and restore damaged muscle. Cultured meat methodology is inspired by techniques used in tissue engineering and regenerative medicine, which uses a combination of stem cells, biomaterials, and growth factors (GFs) to stimulate cell and tissue growth to restore, repair, or replace the function of damaged tissues [45, 46].

In cultured meat production, starter cells (e.g., stem cells) can be harvested and isolated from a tissue biopsy and expanded to large numbers (proliferation) in bioreactor tanks filled with cell culture medium (cell nutrition), typically using scaffolds for three-dimensional (3D) cell support. The cells can then be stimulated to structure into skeletal muscle tissues (differentiation) before the biomass is harvested and assembled into a new meat product [47]. A schematic overview of the process is shown in figure 1.4.

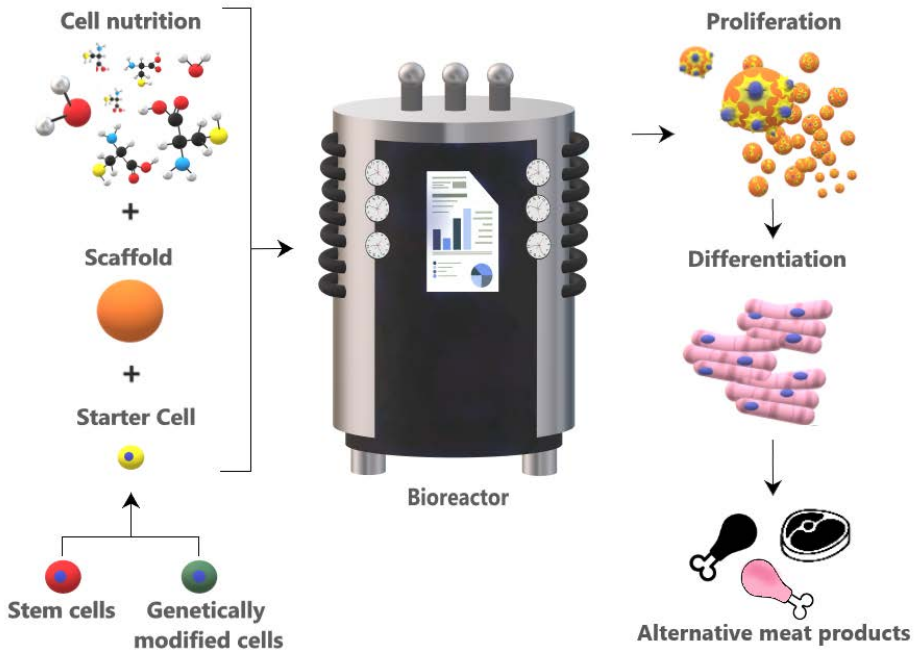

Figure 1.4: During cultured meat processing, the starter cells can be expanded to large numbers in bioreactors together with cell nutrition and scaffolds necessary for cell attachment. The cells are then stimulated to differentiate into skeletal muscle cells/tissues before the biomass is harvested and assembled into a final meat product. Image was created with Microsoft Paint 3D Version 6.2015.4017.0, the “alternative meat products” are icons from Microsoft word.

In theory, the process can produce specific muscle proteins, a muscle cell biomass, or a complete meat copy. Engineering a complete meat copy with muscle, fat, blood vessels, and extracellular matrix (ECM) as natural support is the ultimate goal. However, this is a highly complicated task, especially since one of the main challenges in tissue engineering is the ability to vascularize tissues [48]. Cells require a sufficient supply of nutrients, oxygen, and waste product removal, which

usually is aided by the vascular system at organism level (*in vivo*). A complete meat copy is anticipated to be too large to be maintained by diffusion [49, 50]. Adding further complexity to the engineering of muscle is muscle fiber maturation. Muscle fiber maturation and accumulation of contractile proteins (myosin and actin) require myotube contraction similar to muscle exercise. This is possible to accomplish *in vitro* by mechanical stretching of scaffolds or delivering electrical pulses [51-53]. As an alternative to a complete meat copy, the harvested cell biomass can be made into minced meat, used as protein enhancement and blended into sausages or bread, and more. The first proof-of-concept burger made in 2013 by Professor Mark Post and team members was made with a muscle cell biomass with added coloring (beet juice) and flavorings such as caramel, salt, and egg powder. However, the burger had a production cost of €269, 906. The current challenge is to succeed with a large-scale production that is economically and environmentally friendly. The main technological barriers that must be solved before efficient muscle protein bio-production can be achieved are listed in box 1.

Box 1. Technological challenges

1. Selecting a suitable cell source with high self-renewal capacity while retaining the potential to differentiate into muscle cells.
2. The formulation of cost-effective serum-free cell culture medium (SFM) customized to the different stages of cell development (proliferation or differentiation).
3. Production of food-safe biocompatible scaffolds necessary for muscle cell attachment.
4. Up-scaling of cost-effective cell expansion in bioreactors for industrial production.
5. Attaining optimal functional and nutritional attributes of the animal proteins or cell biomass as ingredients in existing food products or new meat products.

1.4.1 Skeletal muscle satellite cells

In tissue engineering, selecting the most suitable cell source is very important to mimic the characteristics of the native tissue *in vitro*. Cells of tissue-specific phenotypes must be able to replicate and multiply to large numbers while retaining their potential to differentiate into myogenic (muscle) lineages [54]. Therefore, the use of stem cells is a prerequisite for cultured meat production because of the tremendous potential these cells have to multiply. The stem cells traditionally used in large-scale biopharmaceutical production are usually genetically stable cell lines that are gene-edited to have infinite self-renewal (immortalized) and produce the desired protein products. The muscle cells commonly used in cultured meat production, on the other hand, are genetically variable primary cells usually isolated directly from animal tissues, which retain their morphological and functional characteristics from the tissue of origin [55]. Several stem cell types (listed in box 2) have been evaluated for cultured meat production, and the production could be carried out with stem cells from any species, e.g., bovine, porcine, avian, sheep, fish, and more.

Mesenchymal stem cells (MSCs) are multipotent and can differentiate into bone, cartilage, and adipose tissue. In contrast, skeletal muscle satellite cells (MuSCs) are unipotent and function as myogenic precursors that need little input to differentiate into skeletal myotubes [47, 56]. In theory, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have an infinite ability for self-renewal. However, unfortunately, the differentiation stimuli protocols reportedly have varied results, often showing limited differentiation capacity [47]. Compared to ESCs and iPSCs, MSCs and MuSCs have a relatively limited ability for self-renewal after myogenic lineage commitment. The major drawback of using MuSCs is that they are prone to enter a state of stable cell growth arrest (cellular senescence) and become resistant to growth-promoting stimuli, again causing limited proliferation and differentiation potential. Cellular senescence is a significant limitation in stem cell therapy for tissue reconstruction because it results in the inability to expand undifferentiated MuSCs in culture while maintaining the transplantation potential [57]. Likewise, in large-scale cultured meat production, senescence is a limitation because the technology depends on a high self-renewal and differentiation capacity.

Box 2. Suggested stem cell choices

1. Embryonic stem cells (ESCs) are pluripotent, meaning that they can differentiate into any tissue.
2. Induced pluripotent stem cells (iPSCs) that require gene editing.
3. Adult stem cells, e.g., mesenchymal stem cells (MSCs) and skeletal muscle satellite cells (MuSCs).

Nevertheless, MuSCs have been suggested as the most promising choice for cultured meat production because they imitate muscle cell development and progression (myogenesis) with high efficiency [58]. In addition, it is possible to employ cell immortalization techniques to extend self-renewal potential [59]. Generally, the whole meat culture system at an industrial scale could benefit greatly from gene manipulation. However, it might further complicate regulatory concerns and consumer acceptance or induce more mutations over time, resulting in reduced meat quality. Nevertheless, the first proof-of-concept burger was made with MuSCs. At the same time, the same creator's laboratory has made innovative use of small molecular inhibitors to maintain bovine MuSCs stemness and extend the renewal potential through transient inhibition of p38 (a protein associated with aging and impaired muscle regeneration) [60].

The current strategies in cultured meat production predict that both MuSCs and fat cells (adipocytes) are the minimal necessary components of cultured meat [47]. Cell biomasses containing only MuSCs do not have significant amounts of fat, except for a small amount of intracellular and membrane-bound phospholipids. Introducing fat is essential because fat greatly contributes to meat taste and texture. A likely scenario to achieve a favorable fat profile is by blending it into the muscle cell biomass or culturing the different cell types in separate bioreactors and mixing them into a final product after culturing [61]. If adipocytes are being cultured in parallel with MuSCs, developing sustainable biomanufacturing strategies for adipose tissue requires similar considerations as the myogenic lineages [62]. Another possibility is co-culturing adipocytes and MuSCs together, which is necessary when producing a complex meat copy with marbling. However, even though co-culture represents a more realistic tissue environment, it is challenging, mainly due to the different cell culture medium compositions, volume, and duration requirements of the different cell types [63, 64]. In addition, co-culture cell medium and scaffold optimization is needed to allow symmetrical cell culture. It is expected that such optimization implies compromising each cell types performance which negatively effects overall productivity. Reportedly, adipocytes secretions can negatively impact MuSC proliferation and differentiation efficiency [5].

Recently, research conducted with importance to cultured meat has shown that co-cultures of fibroblasts (most common cells of connective tissue in mammalians) and myofibroblasts enhanced myogenesis progression. Also, co- and tri-culture with MuSCs, smooth muscle, and endothelial cells improved ECM deposition and myogenic differentiation [65, 66]. In contrast, the high purity of MuSCs cell culture without fibroblast interference is an important parameter for maintaining cell stemness and self-renewal capacity [67]. This may not be surprising since the native environment of muscle tissues comprises muscle fibers, connective and adipose tissue, and contains several cell types. The different cell types secrete multiple factors, such as GFs, that facilitate the complex cell-to-cell communication involved in cell proliferation, survival, and differentiation, which is critical in tissue development [56, 68].

1.4.2 Myogenesis

Muscle stem cells are quiescent in the adult muscle. In response to injury (e.g., exercise), they typically become activated, proliferate, and undergo myogenic differentiation, then fuse and restore damaged muscle while allowing repopulation of the stem cell niche. There is a complex interplay of extracellular signaling molecules (e.g., GFs and hormones) and intracellular signaling pathways that determine muscle cell myogenesis, such as MuSC activation, inducing proliferation, inhibition or activation of differentiation, and stimulation of protein synthesis [69]. The integration of signals in the muscle environment leads to changes in gene expression, and the process is associated with several extensively studied transcription factors (TFs) and proteins that can be monitored and serve as markers for the different cell developmental stages.

The *Paired boxed 7 (PAX7)* and *forkhead box* (*FOXO*) TFs are highly expressed in quiescent and newly activated satellite cells. M*yogenic factor 5* (MYF5*)* and *myoblast determination protein 1* (*MYOD1*) are expressed in activated, proliferating and early differentiating cells (myoblasts), while myogenin (*MYOG*) is considered a late differentiation marker (myocytes and myotubes) [70]. MuSC activation result in a cell division that produce cells with myogenic commitment and cells programmed to repopulate the quiescent MuSC pool [71]. Once the MuSCs have reached high densities, *PAX7* and *MYF5* expression is repressed while *MYOG* is expressed to drive cell cycle exit and form myocytes. Finally, decreased *MYOD1* indicates the formation of multinucleated myofibers [70]. A simplified overview of myogenesis and associated signaling molecules is shown in figure 1.5. Because the signals in the muscle environment determine muscle cell development, proliferation and differentiation can be controlled by stimulating the signaling pathways relevant to myogenesis, e.g., by switching the ingredients in the cell culture media. Other factors that influence muscle cell signaling and development is the ECM.

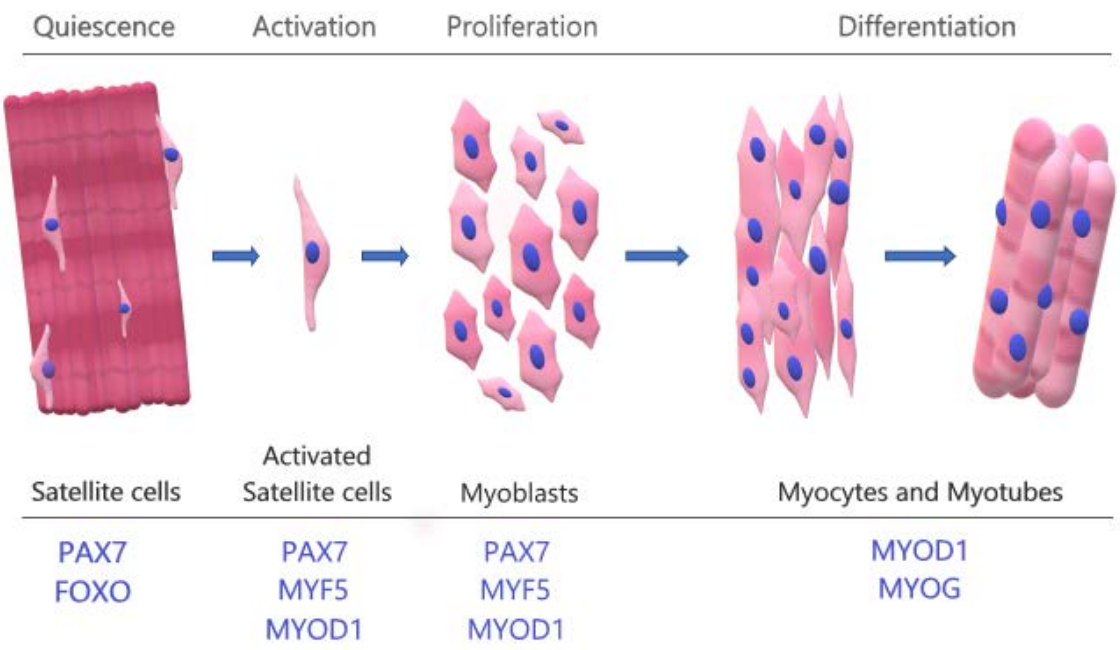

Figure 1.5: A simplified overview of myogenesis and associated transcription factors. Upon muscle injury, quiescent MuSCs residing between the muscle cell membrane (sarcolemma) and basement membrane (basal lamina) in muscle fibers are activated and enter myogenesis. The cell numbers accumulate (proliferation) before they differentiate into skeletal muscle. The different cell stages are associated with various TF expressions (in blue). Image was created with Microsoft Paint 3D Version 6.2015.4017.0.

1.4.3 Extracellular matrix

The ECM is a 3D-network of extracellular macromolecules that provides structural support and growth regulation for cells. It is a major component in mammal tissue, and the amount of ECM material varies between tissues. As a comparison, connective tissues such as cartilage and bone contain 90-98%, while skeletal muscle tissues, which represent the largest body components in humans, the total muscle mass is comprised of 1-10% ECM material [72, 73]. In addition to providing structural support, the transmission of contractile force, and separating different tissues, the ECM is involved in numerous essential biological processes, such as providing connections and interactions for cells, control signal channels, and regulates dynamic behaviors related to cell growth, differentiation, migration, and muscle regeneration [74]. ECM is also actively involved in maintaining the MuSC pool.

Muscle fibers are embedded into the ECM, which generally consists of fibrous protein (collagens, elastin, fibrillin, and fibulin), glycoproteins (fibronectin, laminins, tenascin, and thrombospondin), and proteoglycans with their characteristic negatively charged polysaccharide chains (glycosaminoglycans). Membrane-associated proteoglycans can be glycosylphosphatidylinositol (GPI)-anchored or transmembrane. The syndecans are a family of transmembrane proteoglycans, and there are 4 syndecans in mammals present on different cell types and at different times during development [75]. Collagens have a dominant role in scaffolding and are the main constituents of the structural network of ECM in muscle. There are different types of collagens present in various amounts in muscle depending on the location (I, III, IV, V, XII, XIII, XIV, XV, XVIII, and XXII). The fibril-forming collagen types I and III are the most abundant, accounting for up to 75% of total muscle collagen [73]. The different types have different characteristics and thus are believed to serve different roles in ECM function. For example, type I collagen gives strength and rigidity, while type III forms a looser meshwork and gives elasticity. Aberrant changes in collagen stability are related to many diseases. Crosslinked fibers stabilize the collagen. Otherwise, it would denature at physiological temperature. Denaturation of

collagen is when the protein loses the triple helical structure with the helix-to-coil transformation of the molecule. Preserving the stability of collagen is very important in tissue engineering.

The ECM is constantly synthesized, secreted, and modified largely in response to cellular signaling, which directly influences its function and the interaction and biochemical signals to surrounding cells [76]. As well as serving as reservoirs and modulators of GF and cytokines, the ECM presents polypeptide motifs or ligands that promote cell attachment and spreading through interaction with different cell surface receptors and cell adhesion signaling mechanisms. Cells bind to the ECM through integrins and focal adhesions (FA). Syndecans, for example, is present in the FA of muscle cells and has a functional role in FA assembly [75]. Further, laminin serves as a ligand for receptors such as integrin, and the integrin receptors are the main cell adhesion molecules that regulate binding to ECM proteins. Different ECM components (i.e., fibronectin, collagen VI, and different proteoglycans) may also promote MuSC division. While laminin, glycosaminoglycans (GAGs), and other proteoglycans have been shown to promote MuSC differentiation and cell fusion into myofibers [73]. Because these ongoing bidirectional interactions between cells and the ECM (dynamic reciprocity) are so significant for cell development, biomaterials used as *in vitro* cell scaffolds aim to replicate the native composition and structure of the ECM. While replicating these features is challenging, the muscle development pathways important to modulate for efficiency in culture meat production can largely be controlled by a combination of stimulating cell culture media ingredients and scaffold composition.

1.5 Cell culture media

Mammalian cells are typically grown with a suitable nourishing medium in humified incubators with optimal physiological conditions. The cell medium is identified as the cost driver in cultured meat technology [77]. A *major challenge* is the serum added to the medium. Fetal bovine serum (FBS) typically provides the necessary nutrient supplementation required for efficient cell growth in cell culture. FBS is the supernatant of clotted blood from a 3- to 9- month old bovine fetus mainly collected by cardiac puncture to minimize the risk of microorganism contamination. Together with the fetus, the reproductive tract is removed from the carcass of a slaughtered pregnant cow before the unanesthetized fetus's heart is punctured by inserting a needle between the ribs directly into the heart, and the blood is collected using vacuum extraction or gravity and massage. Depending on the fetus's age and equipment used, the bleeding procedure takes between 2-5 minutes, while the time from the mother's death to the cardiac puncture is around 25-30 minutes. The amount of serum obtained depends on the fetus's size and ranges between 150 ml-550 ml [78].

FBS contains an undefined mixture of stimulating factors required to sustain cell growth and maintenance of most mammalian cells. While FBS has never fully been characterized, typical serum components are listed in table 1.1 [79]. Proteomic and metabolic studies of FBS have revealed 1800 different proteins and more than 4000 metabolites, where the mixture greatly varies from batch to batch. Together with composition variation, the serum may also be contaminated with viruses, microorganisms, or prions that pose a safety risk and interfere with experimental outcomes [80-82]. The use of FBS in research, diagnostics, and the pharmaceutical industry has increased, and serum supply is lower than the demand [83]. Serum is therefore very expensive, and the cost can be up to 95% of the total cost of the cell medium. Reportedly, the market is loosely regulated, resulting in fraudulent batches of FBS reaching scientific communities on several occasions [82].

Table 1.1 Typical components in serum		
Serum proteins:	**Growth factors and cytokines:**	**Hormones:**
Albumin	Epidermal Growth Factor (EGF)	Insulin
Globulins	Fibroblast Growth Factor (FGF)	Glucagon
α1-Antitrypsin	Nerve Growth Factor (NGF)	Corticosteroids
α2-Macroglobulin	Endothelial Cell Growth Factor (ECGF)	Prostaglandins
Transport proteins:	Platelet-derived Growth Factor (PDGF)	Pituitary Glandotropic Factors
Transferrin	Insulin-like Growth Factors (IGFs)	Corticosteroids
Transcortin	Interleukins	Vasopressin
α1- and β1-Lipoprotein	Interferons	Thyroid Hormones
Attachment factors:	Transforming Growth Factors (TGFs)	**Vitamins:**
Fibronectin	**Carbohydrates:**	Vitamin A/Retinol,Retinoic Acid
Laminin	Glucose	Vitamin B group/Thiamine
Enzymes:	Galactose	Riboflavin
Lactate Dehydrogenase	Fructose	Pyridoxine/Pyridoxalphosphate
Alkaline Phosphatase	Mannose	Cobalamin
γ-Glutamyl Transferase	Ribose	Folic Acid
Fatty acids and lipids:	Glycolytic Metabolites	Niacinamide/Nicotinic Acid
Free and protein-bound fatty acids	**Nonprotein nitrogen:**	Panthotenic Acid
Triglycerides	Urea	Biotin
Phospholipids	Purines/Pyrimidines	Vitamin C/Ascorbic Acid
Cholesterol	Polyamines	Vitamin E/α-Tocopherol
Ethanolamine	Creatinine	**Trace elements:**
Phosphatidylethanolamine	Amino Acids	Selenium, Iron and Zinc

In addition to being expensive with quality and reproducibility challenges, there are ethical and biosafety concerns due to the nature of how FBS is harvested. As such, serum supplementation is a limiting factor and cannot support sustainable large-scale cultured meat production. Therefore, extensive research in the last decade has been focused on reducing and replacing FBS with chemically defined media as part of good cell culture practice (GCCP) [84]. Serum replacement approaches include supplementing basal media with growth-enhancing factors, using conditioned media containing cell-secreted products, using additional cell types (e.g., microbes) as GF producers in the cell culture, or gene editing cells so they produce their own GFs in large quantities [5, 59, 85].

1.5.1 Serum-free media

The challenge of producing a SFM is identifying and replacing the specific components from serum that promote cell growth and optimize the medium composition to each cell type. A universal SFM for all cell types is highly unlikely because different cells typically have specific individual requirements. Currently, SFMs are commercially available for many cell types commonly used in medical and industrial applications. These cells are usually genetically modified and transformed into stable cell lines with altered metabolism and extended self-renewal potential. Also, these SFMs are typically proprietary, and the composition is not published for commercial reasons. Thus, making it difficult to transfer knowledge between exiting large-scale cell biotechnology production and cellular agriculture [81]. Furthermore, the commercially available SFMs and serum substitute alternatives adapted to MuSCs are not food-grade, and cost and performance are still an issue [84, 86].

The general approach to developing a SFM includes selecting a basal medium that provides a suitable microenvironment for cells *in vitro*, containing buffering pH substances and essential nutrition, broadly classified into macronutrients (carbon and nitrogen sources) and micronutrients (vitamins and minerals). For mammalian cells, carbon sources include carbohydrates and lipids, while amino acids are considered the most significant nitrogen source [81, 87]. Other essential components added to the basal medium include insulin, which is the most commonly used hormone in cell culture, and recombinantly produced or purified GFs (especially FGF2 and TGFβ). Together with transferrin, a protein that transfers iron into cells, and selenium, a trace element that protects cells against oxidative stress, and serum proteins (e.g., albumin), which also serve cell-protective functions and aid transport of important ligands. While some genetically modified cell lines can be maintained in this relatively simple medium mixture, most cells need many additional supplements for efficient cell adhesion, growth, proliferation, and survival.

In contrast to animals at the organismal level, most mammalian cells lack the biosynthetic capacity to efficiently utilize many nutrition sources, which is important to consider when supplementing medium for mammalian cells [69]. Generally, the metabolic requirements of mammalian cells are reflected by the composition of plasma and intestinal fluids, which contain a wide range of low molecular weight nutrients and macromolecules. The most common components added to the SFM and their purpose are listed in table 1.2 (excludes basal media components) [81]. Which factors to include in the SFM is cell-dependent, and because metabolic activity changes during cell development, different medium compositions are necessary for proliferating and differentiating cells.

Table 1.2 Supplements in SFM

Factors	Example purpose
Hormones	Endocrine cell signaling.
Growth factors	Increase cell proliferation and specific cell functions.
Proteins	Cell adhesion or lipid carriers
Protein hydrolysates	Amino acids and bioactive peptides
Glutamine	Metabolized into other amino acids and precursor for protein synthesis.
Lipids	Constituents of cellular membranes, transport, and signaling.
Antibiotics and Antimycotics	Prevent bacterial and fungal growth.
Attachment factors	Cell adhesion.

It is anticipated that the SFM residues will impact the sensory characteristics of the final cultured meat product, such as appearance, aroma, flavor, and texture (organoleptic properties) [88]. Research conducted with importance to cultured meat and MuSCs has identified a group of potential media components that can have beneficial effects on production while enhancing the nutritional profile or aroma of cultured meat, such as Vitamin K and metmyoglobin, which improves proliferation [89, 90], taurine, creatine, and retinoic acid, which enhance differentiation of myoblasts. Creatine is also found to be beneficial for mass muscle gain *in vivo* [91-93]. The amino acid leucine and its metabolites are known for promoting protein synthesis in myocytes [94, 95]. Moreover, other amino acids such as glutamic acid and asparagine contribute to the umami flavor in meat, which is also necessary for cultured meat products [96]. Other components added to the media purely for their organoleptic properties or enhancement of the final meat products nutritional profile will need a thorough investigation to determine the impact on cell development as it may impact the meat quality.

SFM production aims are to achieve a chemically defined media to ensure reproducibility with a consistent standard. Interestingly, many find that the cell performance decreases with the higher degree of chemical definition [97], most likely representing a gap in functional cell knowledge. Reportedly, most commercially available serum replacements show lower performance and are only

suitable for a limited number of cell lines [47]. Studies conducted with importance for muscle stem cell culture have demonstrated that commercial SFM stimulates proliferation, but not to the extent of serum-containing medium [98]. Others have shown that adding an array of different GFs, improves myogenesis progression and can be sufficient as serum replacement [99]. GFs are essential signaling molecules in tissue engineering and biopharmaceutical production because they regulate many vital cellular processes. GFs bind to cell surface receptors that activates cell signaling pathways that can promote, e.g., cell survival, proliferation, migration, and differentiation. However, GFs are expansive, and the cost can be over 95% of the total SFM composition. Thus, the use of commercial GFs may not be suitable in cultured meat. The Good Food Institute has reported that finding an inexpensive replacement for GFs and a basal media is required for the price of cultured meat to be comparable to the conventional meat market-level [77]. Commercially available basal media are also not designed with cost in mind. They are mainly produced with biomedical research-grade quality, applied in industries where the final product value is exceptionally high (millions of dollars per kg) compared to the food industry market value [69].

It is possible to formulate a cultured meat basal media using inexpensive growth-inducing and nutritive sources. Cost-effective GFs or growth-inducing sources that may be suitable in cultured meat are conditioned media developed using GF producing cells, producing GFs without purification, and producing analogs that mimic GFs, or using small molecule inducers [56, 100]. Small molecular inducers are a group of small organic compounds with bioactive properties and low molecular weight < 900 Da. They can bind specific macromolecules, acting as effectors by altering the activity or function of their target. Reportedly, using small molecules increase fibroblast cell expansion and gives a lower profile of apoptotic and necrotic cells [101]. Critically, more research is needed to determine the appropriate dosage and food safety while using alternative GF sources, especially small molecule inducers [47]. Food safety is a general concern while using derivatives of biomedical research grade in any stage of cellular agriculture production.

In attempts to further reduce the costs, recycling media could be beneficial as many nutrients are still present in the media for some time. Media recycling has been a successful approach in bacterial and algae cultures [47]. However, this could be challenging with regards to GFs, because of their short half-lives. Regardless of the SFM development method for cultured meat, the media must be sustainable, inexpensive, and of food-grade quality. Inexpensive nutritive sources are already available, such as glucose produced via hydrolysis of starch and vitamins and amino acids produced by fermentation. However, other available complex ingredients such as hydrolysates that may serve multiple functions in a cultured meat SFM should be studied as they potentially can reduce the media cost even further [69]. In this context, inexpensive by-product hydrolysates could be an especially attractive option because it contributes to a circular bioeconomy.

1.5.2 Protein hydrolysis and characterization

Protein hydrolysis is a process that involves breaking down proteins into smaller peptides and free amino acids making them more water-soluble. In order to catalyze the reaction, the activation energy can be reduced either by using enzymes at optimal temperatures or by acidic/alkaline conditions at elevated temperatures and high pressure (chemical hydrolysis). Enzymatic protein hydrolysis (EPH) is considered a gentler biotechnological process compared to chemical hydrolysis, and the advantage is a higher product yield without affecting the nutritional quality and no unwanted toxic compounds. EPH is also easier to adapt to produce a product with the desired properties because of enzymatic specificity. The enzyme specificity refers to how different enzymes (such as endo- and exo-peptidases) cleave specific peptide bonds that result in unique digestion patterns with peptides of different amino

acid chain lengths, which impacts the functional and biological properties of the protein hydrolysate (Figure 1.6) [102]. In this way, specific functional proteins and derivatives can be isolated, or bioactive peptides encrypted within the primary structure of proteins can be released from by-products via hydrolysis. Thus, they can exert beneficial effects on physiological functions beyond nutritional value, e.g., cell structuring support and cell-stimulating effects.

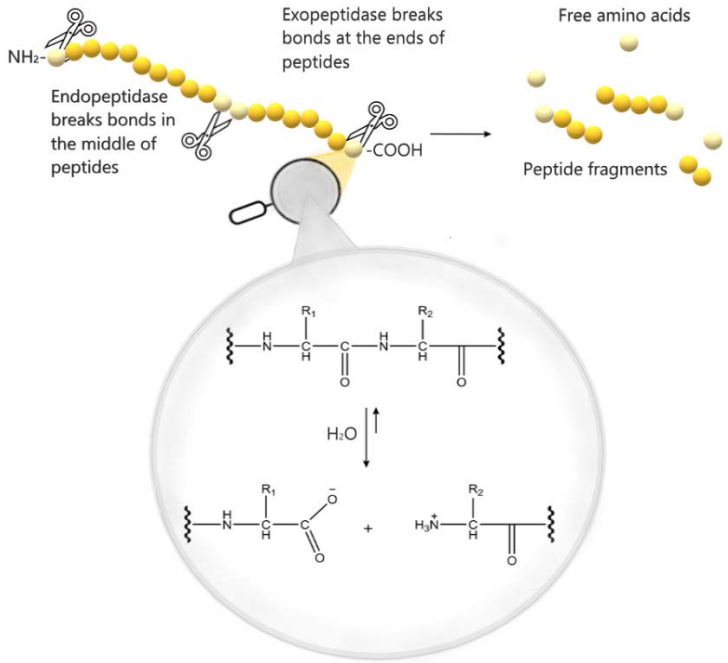

Figure 1.6: Enzymes such as endopeptidases and exopeptidases catalyze protein degradation. Endopeptidases cut bonds in the middle of the molecule, forming smaller peptides. Exopeptidases break bonds from the end of the peptide, releasing free amino acids. Image was created with Microsoft Paint 3D Version 6.2015.4017.0.

Hydrolysate characterization

Research has focused on the generation, separation, purification, and identification of novel peptides from various protein sources. Protein hydrolysates are often characterized with respect to protein, fat and ash content, amino acid composition, hydrophobicity, degree of hydrolysis, molecular size distribution, and flow phenomena of matters. Specific protein degradation products can be isolated, and protein hydrolysates can be fractionated in various ways, e.g., separating peptides according to size or polarity using chromatography. The goal is often to find a degradation product fraction exhibiting the desired properties, such as ACE inhibitory effects aimed at blood pressure regulation. However, it is time-consuming to test all peptides in order to discover bioactivity considering the complex mixtures of protein hydrolysates. Among the modern analytical approaches for the discovery of bioactive constituents in complex mixtures is the use of chromatography-coupled bioassay where eluents of a separation (fractions) are directed to high-throughput bio-screening, an approach used to identify and characterize bioactive peptides from chicken hydrolysates with DPP-IV inhibitory effect for blood sugar regulation [103]. Exciting approaches using in silico tools can predict good or bad precursors for peptides with biological activity and model bioactivity as a function of molecular structures. These bioinformatic tools have been used to design bioactive peptides from dietary derived proteins, and analysis of the meat proteome has shown a high occurrence of bioactive motifs [39, 104-106].

Industrial production aims to solubilize and recover as much protein as possible from the biomass and produce products with a defined composition while tightly controlling and monitoring the process. Many factors must be considered to determine the outcome of a protein hydrolysis process, such as enzyme choice regarding specificity, catalytic efficacy and stability, enzyme to substrate ratio, time, temperature, and pH [107]. One of the main challenges is the large variations in by-product raw material quality and protein, carbohydrate, and fat composition. The by-product composition and enzyme choice significantly impact the nutritional, physicochemical, and bioactive properties of the end hydrolysate product, which affects application opportunities and ultimately decides the valorization of the by-product. Research groups are continuously working to optimize and automize industrial large-scale hydrolysis production by employing fast real-time analytical tools, sorting the by-products going into the process, monitoring the progression of the ongoing hydrolysis, and analyzing the end hydrolysate product. These analytical tools must ensure minimal batch-to-batch variation and high-quality yield at low costs. A schematic overview of a typical large-scale by-product hydrolysis process is shown in figure 1.7.

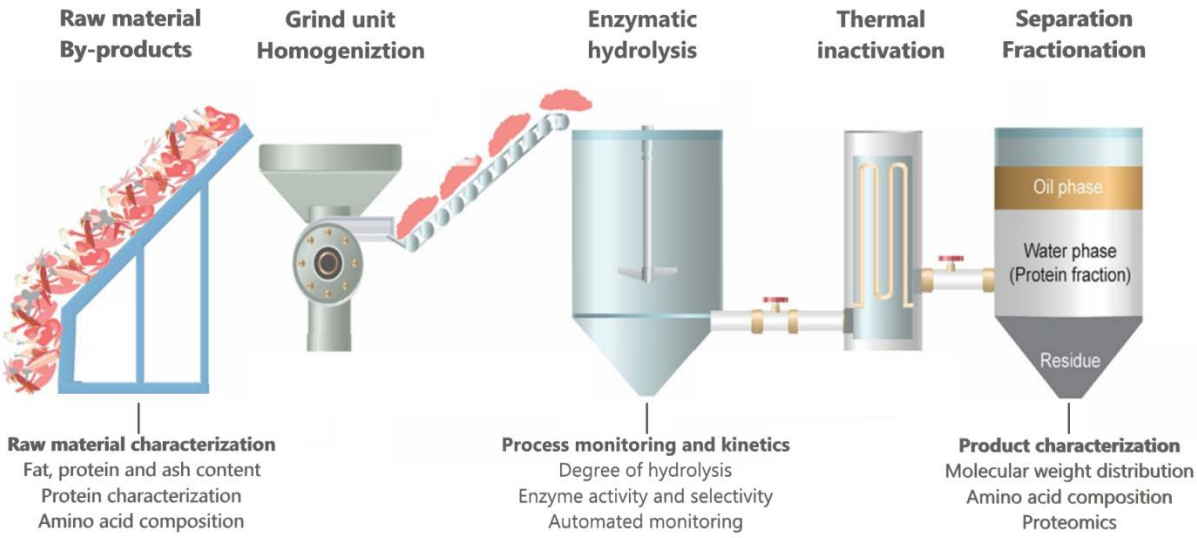

Figure 1.7: A schematic overview of a typical large-scale by-product hydrolysis process. First, the raw materials are sorted, homogenized in a grinder, and hydrolyzed in large tanks. Next, the enzymes are inactivated by heat denaturation before the hydrolysates are fractionated by centrifugation. This image was adapted from Wubshet et al., [107] with permission.

1.5.3 Bioactive peptides and hydrolysates as complex serum replacement

By-product hydrolysates are promising ingredients in SFM for cultured meat. Hydrolysates are low-cost, easy to obtain, food-safe ingredients containing a wide range of low molecular weight nutrients found in common basal media, in combination with potential GF mimicking properties that can promote cell growth or differentiation, antimicrobial properties that abolish the need for antibiotics, and antioxidant properties that protect cells from oxidative stress and can increase the stability of the cell media. Also, the products formed during hydrolysis, such as different peptides and free amino acids, can exhibit sensory characteristics desirable in cultured meat [108, 109]. As such, the complexity of hydrolysates mixtures can potentially serve multiple functions (Figure 1.8), in addition to cell nutrition, that can benefit cultured meat production.

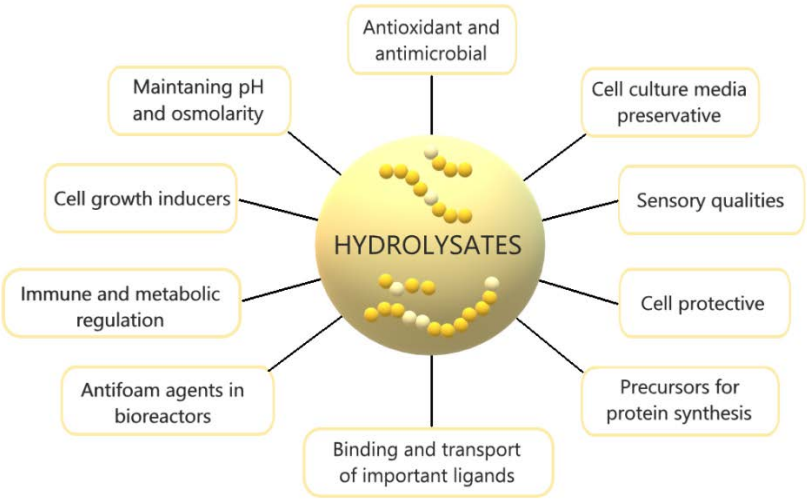

Figure 1.8: Hydrolysates contain components that can serve multiple functions in an efficient SFM. They can exert effects beneficial to cultured meat production such as antioxidant, antimicrobial, growth inducing, and sensory properties and serve an essential nutritive function.

Encrypted within the primary structure of proteins are sequences for potential bioactive peptides that can exert various effects on physiological function, including cell-stimulating effects. Bioactive peptides, usually 2-20 amino acid residues in length, are inactive within the sequence of proteins and can be released by enzymatic hydrolysis [105]. The activities of peptides are determined by the amino acid compositions, sequences, and structures [110]. Hydrolysates are reported to have growth-promoting effects and stimulate insulin-associated signaling pathways in mammalian cell culture [111]. An example is Primatone RL, a protein hydrolysate of meat used as a medium supplement that improves cell growth for many cell lines [112]. There is also a promising cell culture supplement containing lysate of porcine platelets [113]. While the cell growth-promoting mechanisms in hydrolysates are not fully elucidated, they contain analogs for hormones and active domains of specific proteins that stimulate cell signaling, such as GFs. Thus, these peptide sequences can mimic native GFs that bind GF receptors. Other components known to have a beneficial effect on cell growth are serum proteins, e.g., albumin. Albumin is the major protein in serum, accounting for almost 60% of the total protein concentration. The proposed advantages of supplementing albumin in cell culture are related to its cell-protective functions such as maintaining pH, antioxidant properties, binding, and transport of important ligands (lipids, ions, amino acids, bioactive molecules). Interestingly, studies have shown that albumin contributes to the efficiency of bioactive molecules in improving cell growth and survival[114], and hydrolysates have shown promise as substitutes for serum albumin in bovine cell culture [115].

Other culture-protecting ingredients are also valuable for most successful cell culture media. The use of antibiotics and antimycotics in cell culture media is standard for preventing bacterial and fungal infections. However, due to consumer sensitivity and the risk of developing antibiotic-resistant bacteria, antibiotics and antimycotics are not suitable options in cultured meat production. Another issue is related to cell culture media is stability and oxidative stress. Components in cell culture have varying stability and are subject to degradation. Thus, stabilizing additives such as antioxidants are part of the medium formulations. Extensive research has reported good antimicrobial, antifungal, antiviral and antioxidant properties in by-product hydrolysates, making them promising ingredient replacements [38, 116]. For example, the by-products hydrolysates of eggshell membrane (ESM), egg whites, and blood sources have antimicrobial effects and high assurance of highly efficient antioxidant peptides [32, 117-121]. Reportedly, ESM also has anti-inflammatory properties [122]. Another

ingredient is crucial during the up-scaling of cell expansion in bioreactors because agitation and oxygenation (sparging) are required. The culture media must be constantly supplied with appropriate oxygen concentration to ensure efficient carbon metabolism [69]. The resulting gas bubbles and foam created during sparging can physically damage cells. To resolve this problem, a synthetic antifoam is usually added. Synthetic antifoam is most likely unsuitable in cultured meat production, but hydrolysates are suggested to have properties that can replace antifoam [69].

The increased research interest for bioactive peptides has rapidly expanded the toolbox to discover peptides with desired properties and makes it less time-consuming to determine good precursors. For example, one efficient approach uses bioinformatics tools to search for homology between amino acid sequences with known cell signaling molecules to find analogs. Considering that the cost of purified GFs is unsuitable for cultured meat, this topic is particularly important to investigate fully. Quantitative structure-activity relationship (QSAR) models can also predict bioactive peptides with antimicrobial, antioxidant, and evaluate flavor characteristics of peptides [105]. As with any alternative serum replacement ingredients, more research is needed to determine food safety and possible cell phenotype alterations that can affect meat quality. The trend in culture media development is largely focused on plant-based options because of risks associated with animal-derived materials, such as the introduction of pathogenic agents. As mentioned previously (chapter 1.5.2), meticulous control, safety routines, and proper maintenance can generally remove or reduce this risk. While the complete elimination of all possible contaminants is unlikely (whatever supplements are added), the anticipated level of control implemented in cultured meat production implies that any issues can easily be detected and handled effectively. At the same time, there are concerns over the batch-to-batch variations within hydrolysate products. An improved, thoroughly controlled processing method that can incorporate seasonal variations in agricultural livestock feed and other variables that impact the quality of the by-products going into the reaction should minimize the variations and ensure production with a consistent standard [97].

The desired goal to identify all components, including bioactive peptides in complex protein hydrolysates, will be challenging. However, once the processing method has reached a sufficient standard and the chemical composition is elucidated, the variation could be at an acceptable level. Meaning, even when the media is not considered completely defined, the SFM can have a low enough variation that does not compromise cultured meat quality. The complete formulation of commercially available SFM is generally not available and cannot be considered completely defined. Reportedly, their formulation can be changed without informing the users, and the same supplement can have different formulations between suppliers [81]. These SFMs are still accepted in large-scale cell culture productions in other industries. Although many companies with a cultured meat focus are working to formulate a SFM, their formulations will most likely be subject to proprietary, resulting in restricted public insight and likely face the same challenges as commercially available SFM.

1.6 Scaling up cell expansion using scaffold-based technology

The accumulated knowledge from stem cell technology and tissue engineering have made cultured meat a possibility. The physical and biochemical features of the native tissue can be mimicked under 3D-cell culture conditions to form mature muscle tissues. The choice of a 3D-cell culture system depends on the production purpose and cell origin. One promising option for cultured meat production is the scaffold-based design where cells grow on the surface of small, solid, and often spherical 3D-shaped scaffolds such as MCs in bioreactors. This form of cell culture permits high-density cell expansion by providing a larger surface area [123].

In order to achieve the massive quantities of MuSCs needed in cultured meat production, bioreactor tanks are an attractive choice. In this context, stirred spinner flasks and bioreactors serve the same purpose by ensuring a stable temperature with efficient nutrient and oxygen flow [124]. When MuSCs are grown on MCs in suspension, gas flow and the agitation system, e.g., an impeller, ensure sufficient mixing of nutrients and gases. Once the MuSCs have reached high densities, new MCs are added to the reactor to provide more available surfaces for the cells to transfer onto, colonize, and continue proliferation. Stirred spinner flasks and bench bioreactors range in size from 0.05-10.0 L. Bioreactors tanks are capable of holding much larger volumes of up to 50 000 L (which is required for profitable full-scale cultured meat production), the up-scaling process is challenging. It entails many complex factors, such as gas inputs (oxygen, nitrogen, and CO_2), agitation, and pH control (figure 1.9)[125].

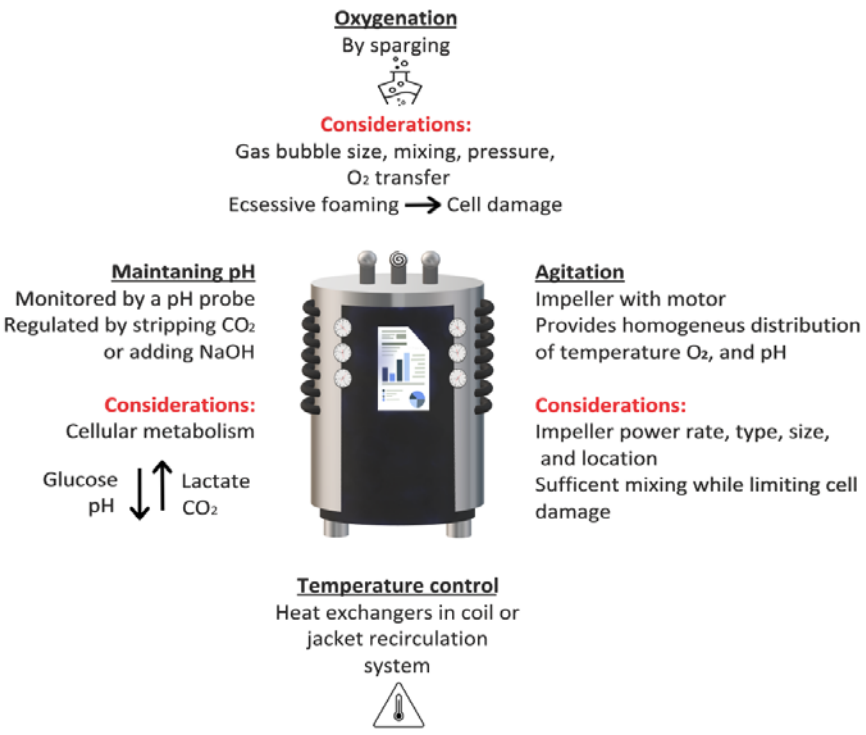

Figure 1.9: Bioreactors provide extensive control over parameters such as temperature, pH, O_2, agitation, pressure, oxygen transfer capacity, and mixing, which are essential to keep cells in the appropriate physical and chemical environment. In addition, monitoring parameters that impact the biological attributes, such as product yield, cell growth, and quality, is critical to maintain performance. However, these parameters will be different at different scales and must be optimized accordingly.

It is important to maintain a homogenous constant temperature in the bioreactor as most mammalian cells have an optimal physiological temperature of 37 °C. Temperature changes can dramatically affect cell viability, and lower temperatures can result in reduced cell metabolism. Mammalian cells also have an optimal physiological pH ranging from 7.0 - 7.4. The cell culture medium contains buffers that naturally maintain optimal pH. However, during cellular metabolism, cells produce CO_2 and water as they convert glucose into lactate. As a result, lactate accumulates, and the environment becomes more acidic. When maintaining pH by the bicarbonate equilibrium, adding CO_2 to increase dissolved CO_2 and adding air to strip the dissolved CO_2 out is insufficient to increase the pH; a basic solution like NaOH may be required to be pumped in the bioreactor. Stirred bioreactors have a significant advantage over static culture vessels because they supply a more homogenous environment which improves overall product quality. However, adequate oxygen supply must be balanced against the damaging effects of hydrodynamic shear stress on cells inflicted by agitation and aeration. Also, monitoring nutrient utilization, waste production, and the cell's developmental phases (quiescence, proliferation, differentiation) is necessary to ensure efficient cell expansion. Although bioreactors are widely used for the large-scale production of biopharmaceuticals using mammalian cells, and many commercial

systems have been developed to facilitate and intensify the process, the technology must be adapted and improved before it is suitable for cultured meat production.

Currently, bioreactor design is a major research focus because no full-scale bioreactor design exists for cultured meat production. Both the proliferation step and the differentiation step will likely require customized designs to maximize energy input and minimize waste production [5]. Also, no studies have been published on expanding bovine MuSCs on a significant bioreactor scale. While research demonstrates that bovine MuSCs and MSCs can be expanded on commercial MCs in spinner flasks, and bovine MuSCs culture is comparable to human MSCs, the tissue culturing techniques are still small-scale, short-term (8-9 days), with limited cell doublings, and rather costly [126, 127]. In addition, the stirred spinner flasks used have limited control over temperature, pH, oxygen supply, and nutrient consumption. Similarly, no full-scale scaffold design for complete meat copies exists yet, and optimization of the MC scaffold design is still required to ensure high efficiency and sustainability.

For MuSCs to proliferate efficiently, they need to attach to a surface. A common method to grow adherent cells is in single-cell monolayers (2D), where cells grow on a flat rigid surface resulting in a small surface to volume ratio [128]. MCs provide a much larger surface area and are composed of a matrix that maintains suspension in cell culture media and allow cell adherence during stirring in a spinner flask or bioreactor system. MCs are typically 100-500 μm in diameter and differ in their physical properties such as porosity, density, size, rigidity, and surface chemistry [129, 130]. Commercial MCs are often made with materials such as glass, diethyl aminoethyl (DEAE)-dextran, polyacrylamide, and polystyrene [130, 131]. However, MCs made from synthetic polymers lack cell recognition sites which negatively affect cell adhesion and growth [132]. These MCs generally need surface treatments such as texturizing, a coating of ECM proteins, or at the most basic level, an ionic charge to attract cells, promote cell adherence, and improve biocompatibility [129, 130]. They also typically require a cell MC dissociation step following cell expansion. While the dissociation process varies (e.g., chemical or mechanical), it often results in a significant cell/tissue yield loss due to incomplete cell detachment or MC aggregation [133, 134]. This will make cultured meat production less efficient and more costly.

The commercially available Cytodex® (Cyt) series are widely used inedible MC, made of a dextran matrix with a diameter of approximately 200 μm. Cyt1 MC has positively charged diethylaminoethyl groups throughout the matrix and has produced good results during the expansion of bovine MuSCs [133]. However, it is anticipated that the material of choice for cultured meat production will be natural polymers. While biological collagen and gelatin are already commonly used in tissue engineering for scaffold production, they often lack the structural stability needed in agitated cell culture and need crosslinking treatments often unsuitable for food production applications [135]. Other natural polymers used as cell culture scaffolds, such as cellulose or chitin and its derivative chitosan [74], tend to cause stomach upset and constipation because of the high amount of insoluble fiber. The commercially available MCs adapted for genetically stable cell lines used in the medical field are not of acceptable food-grade and are too expensive for cellular agriculture applications. However, exciting work is currently ongoing related to edible scaffold production using textured soy protein and salmon gelatin [66, 136], and many techniques have demonstrated potential for producing edible and biodegradable scaffolds in muscle tissue engineering, such as electrospinning [137, 138].

1.7 Microcarriers and biocompatible materials from by-products

MCs can be made of a variety of biomaterials using a large number of manufacturing techniques [139, 140]. These scaffolds intend to mimic the cells natural 3D-environment and must be appropriate for the desired cell type and specific tissue [141]. Some key considerations are important when designing or determining the suitability of a scaffold. First of all, the scaffold architecture is of critical importance and should provide structural support for cells. Another important design criteria is that of biocompatibility [139]. Biocompatibility refers to the potential of a material to induce an appropriate tissue response. It should not be toxic, antigenic, or produce an immunological response when exposed to cells. It is also important to consider food safety, sustainability, and cost in the cultured meat scaffold design.

The MC must provide mechanical structural integrity and support to the developing cells and be strong enough to endure higher temperatures and agitated cell culture. This support can be temporary or permanent. If the structure is permanent, the scaffold constitutes will be a main component of the final meat product and must be edible and should resemble the composition and properties of meat [66]. A biodegradable MC is very attractive in cultured meat production, especially when engineering a complete meat copy. This allows the tissues own cells to take over and potentially produce their own ECM, possibly leaving fibrous encapsulation that can work as a vascularization network. However, this requires co-culture, and the degradation by-products of the scaffold must be non-toxic, and no inedible residues can remain in the meat [142]. Whether the MCs are permanent or biodegradable, the porosity is very important. Pore size has been shown to impact cell attachment and cell viability in the construct [143, 144]. Ideally, cells should be able to adhere to and migrate through the surface, proliferate, and function normally within the scaffold. In this way, the MC can support an even higher cell density per unit of MC bed volume compared to solid MCs by allowing cells to protrude into the polymer interior with the additional benefit of protecting cells against shear stress in agitated cultures [129]. These pores should be interconnected and large enough for cell penetration and migration, and nutrients diffusion, and waste products removal.

The biomaterials in the MC are also involved in interactions with biomolecules and cells during the tissue development process [145]. Because cells can sense the physiological conditions necessary for normal cellular behavior, the matrix surface properties are directly related to biological activity *in vitro*, such as cell attachment, spreading, and growth. *In vivo*, the ECM provides connections and interactions for cells, and regulates cell survival, expansion, morphology, differentiation, and migration [74]. Therefore, the biomaterials should possess polypeptide motifs or ligands that promote a stronger and more rapid cell attachment and spreading through interaction with different cell adhesion signaling mechanisms. For example, the tri-amino acid sequence, arginine-glycine-aspartate (RGD), is a particularly widely studied adhesive peptide that acts as the principal integrin-binding domain present within ECM proteins [146]. The presence of binding domains in ECM proteins such as collagen, fibronectin, and laminins is very important factors to consider for the successful development of biomaterials that can retain cell-collagen binding motifs in tissue engineering and scaffolds [147]. However, different tissues have different ECM compositions and which components that should be included in what quantity depends on the cell type. Highlighting the need to tailor the ECM in scaffolds for specific applications. Thus, replicating these important native features of the ECM in an ECM analogue is very challenging and a major research focus.

The optimal biomaterial to use in the design of MCs for cultured meat production should be capable of mimicking the natural 3D-network that provides structural support and maintains normal cellular behavior in MuSCs, i.e., the ECM [143, 148, 149]. As cultured meat provides the opportunity to customize the nutritional composition of meat, it is beneficial to produce edible MCs

with nutrient-enhancing components that can be included in the final meat product, especially if the scaffold is permanent. Hence, using food-grade biocompatible materials with naturally high cell-stimulating properties and porous structures as scaffolds, combined with previous knowledge of MC dynamics, could be ideal. By-products from the food industry such as carcasses and eggshell membranes (ESMs) are excellent sources for collagen, glycoproteins, and proteoglycans that are all present in the ECM [150]. They also have excellent functional and physicochemical properties, giving them the overall characteristics of meat products that are challenging to reproduce in non-meat proteins [9]. Thus, by-products can favorably contribute to sensory attributes of the meat product, such as taste and texture, while complying with regulations for use as food ingredients and additives. However, different material sources may be more or less suitable in scaffold production, and more research is needed to determine good precursors.

Using protein-based biomaterials, e.g., collagen derived from animal sources, has been explored in tissue engineering. Although collagen is edible, nonantigenic, has good biocompatibility, and works in the tissue-healing process, the proteins degrade fast and have low mechanical strength, resulting in a lack of structural support in agitated culture [74]. Providing mechanical strength to collagen MCs includes selecting a more temperature stable collagen source (e.g., avian collagen), choosing a non-toxic crosslinking method (e.g., UVA-riboflavin crosslinking), and possibly combining collagen with more stable fibrous biomaterials [151]. Processed ESM powder is highly bioactive, anti-inflammatory, regulates cellular functions during wound healing, and is a promising biomaterial for tissue engineering [31, 135, 152, 153]. Previous work has demonstrated that combining collagen with ESM and crosslinking the scaffold improves the mechanical properties of collagen, making them more suitable for agitated cell culture [135, 153].

2 Aims of the project

The main objective of this thesis was to contribute to the field of cultured meat technology and optimize the use of protein rich by-products from the food industry.

Sub-objectives:

- Serum-free medium (SFM):
  Using by-product hydrolysates and fractions to formulate a low-cost SFM with high cell growth-promoting efficiency adapted for bovine MuSC (Paper I and II).

- Up-scaling MuSCs expansion in bioreactors:
  Investigate different cultivation strategies from low volume spinner flasks to higher volume bench-bioreactors and monitor MuSCs development during long-term cultivation in bench-bioreactors (Paper III).

- Production of edible microcarriers (MCs):
  Using by-products to develop low-cost edible MCs with high cell expansion efficiency that can be included in the final cultured meat product (Paper IV).

3 Selected methods and methodological considerations

This section highlights selected methodological approaches used in this project, with emphasis on benefits and challenges. When the applied methods have clear limitations, suggested alternative approaches for future studies are listed. Classical standardized methods with limited effects on evaluation of our results will not be discussed. The detailed descriptions of all methods are found in the original papers and manuscripts.

3.1 Protein hydrolysates

Production

There are many important factors to consider that determine the hydrolysis production outcome. The key EPH-regulating factors can be classified into the following subgroups [107]:

1. Process-specific parameters, such as substrate concentration, enzyme to substrate ratio, time, temperature, and pH.
2. Substrate-specific factors, such as origin, age, feed regimen, and complexity.
3. Protease-specific factors, such as specificity, stability, and sensitivity to inhibitors.

In paper I, two enzymes with different enzyme activity were selected because the choice of by-product material and enzyme affect protein degradation during hydrolysis (Alcalase and Flavourzyme). Furthermore, due to the ESMs highly crosslinked nature, alkaline chemical hydrolysis was selected (NaOH) to ensure a higher degradation. For future optimization, different hydrolysis parameters such as time and enzyme to substrate ratio should be investigated to save time and research materials. Furthermore, optimization might also increase the bioactivity of the hydrolysates, as others have reported a decrease in bioactivity with an increased hydrolysis time [103]. This could be a result of an excessive breakdown of the bioactive peptides. Also, the approach could be significantly improved by using in silico tools to predict good raw material precursors for peptides with biological activity and model bioactivity as a function of molecular structures. Figure 3.1 show paper I and II principal workflow.

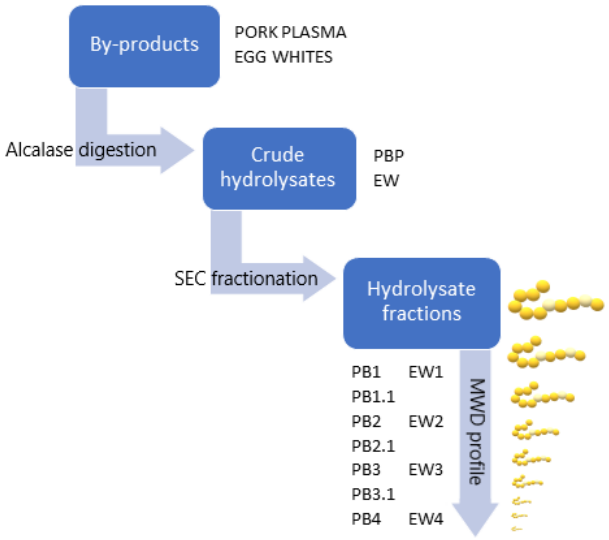

Figure 3.1 Hydrolysate fractionation workflow. By-products were hydrolyzed using Alcalase then fractionated according to size using size exclusion chromatography (SEC). The first fractions eluted from the SEC column shows a molecular weight distribution (MWD) profile with larger peptides, and the later fractions contains constituents with lower and lower MW.

Characterization and fractionation

Liquid chromatography (LC) is based on combining a liquid mobile phase with the sample in question and moving them through a column filled with a material that has specific sites for binding or retention. The aim is to separate or resolve the sample of interest from other compounds in the sample, and the mobile phases selected depends on the nature of samples and separation target. *Size-exclusion chromatography (SEC)* can separate proteins and peptides in a sample according to molecular weight (MW). Larger molecules have shorter retention time, while smaller molecules have longer retention times because they migrate into and through the materials in the column. SEC analysis with UV detection (214 nm) and analytical column filled with porous (145Å) silica-based beads was used to characterize the molecular weight distribution (MWD) in hydrolysates, other crude extracts, and hydrolysate fractions used in paper I and II. A larger preparative column was used to fractionate hydrolysates in Paper II. SEC in combination with UV detection is commonly used for characterization of hydrolysates because peptide bonds absorb strongly at 214 nm [107]. However, it is important to consider that free amino acid content are not very accurately detected at this wavelength, while proteins and peptides are detected by absorption contributions from both peptide bonds and their side-groups resulting in scaling errors [154]. Despite the limitations, this method provides useful information to differentiate the materials used in the bio screening (paper I) and validation of size fractionation in paper II. Also, larger columns (diameter) provide lower separation accuracy. The method is preferred due to cost, speed, and reproducibility and is very accurate when confirmed with an orthogonal method, e.g., sedimentation velocity analytical ultracentrifugation (SV-AUC). After SEC fractionation, the protein hydrolysate fractions are still highly complex. One of the successful analytical approaches to resolve constituents of complex biological matrices is sequential orthogonal chromatographic separations. Reversed-phase chromatography separation, orthogonal to the preceding SEC fractionation could be performed on the most bioactive fractions. In a future comprehensive study, it would be interesting to subject the fractions of interest to LC-MS/MS analyses for identification of bioactive peptides.

Degree of hydrolysis (DH) describes the extent of hydrolysis in the peptide product and is a measurement of the percentage of cleaved peptide bonds in a protein hydrolysate. The trinitrobenzenesulfonic acid (TNBS) method was used in paper I. The TNBS method measures the number of free N-terminals carried out via derivatization of their amino groups and measured by UV-visible spectroscopy detection. The TNBS method have very stable derivatization reagents compared to other techniques based on the same principles such as the OPA method. However, one limitation involves a derivatization reagent known to exhibit different reactivity towards some amino acids. For example, the measurement will not be accurate when the hydrolysate contains high amounts of proline- and cysteine-rich peptides due to incomplete biding [107].

Nitrogen, Carbon and Sulfur was determined using elemental combustion analysis from solid dry powder samples that are converted to N_2, CO_2 and SO_2, the concentrations were measured by gas chromatography. The Kjeldahl method was used to estimate total nitrogen content with a conversion factor of 6.25 [155]. It is important to consider that this conversion factor is not optimal and can result in 15-20% deviation. Historically, the conversion factor was set to 6.25 assuming that all nitrogen in food is derived from proteins [156]. Optimally, the conversion factor should be customized to each raw material origin, different proteins need different correction factors because they have different amino acid sequences.

3.2 Cell culture

Isolated Skeletal muscle satellite cells (MuSCs) were used throughout the experimental design of this project. *In vitro* cell culture is one of the most common model organisms used in science. It is a relatively easy and low-cost way to obtain a simplified picture of essential cell functional information that may be generalized to more complex systems. While it is very important to consider the differences in function and localization when comparing cultured cells with differentiated cells in complex tissues, this project primarily aims to resolve the mass production of cells for their edible qualities, which entails manipulating cell doublings rates. Nevertheless, general good cell culture practice is essential to ensure reproducible results. This implies monitoring the cell culture consistency in parameters such as cell doubling time and morphology and avoiding microbial infections. Primary cell characteristics may change with each passage, especially when optimum culture conditions are not maintained. In this instance, other parameters such as changes in cell viability, morphology, and muscle-specific markers were closely monitored. All experiments were performed with cells of similar cell viability and confluency and in the second or third cell culture passage to ensure reproducibility. Generally, primary cells are less genetically robust and more challenging to work with than immortalized cell lines. However, primary cells represent the native cells of tissues better than genetically altered cell lines because they are directly derived from the muscle.

Skeletal muscle satellite cell isolation and identification

MuSCs were isolated from *Longissimus thoracis* (beef sirloin) using an established protocol with documented muscle cell purity and reproducibility. After the isolation, it is crucial to verify cell specificity because of possible fibroblasts contamination. This was performed using different antibodies during immunostaining: NCAM (muscle cell marker) and TE-7 (fibroblast marker) [157]. Our in-house primary cell culture isolation of MuSCs usually has a purity of 90% (MuSCs vs. fibroblast contamination) [158]. Upon thawing, these cells were conditioned to low glucose and low FBS cell culture media concentrations. However, cells isolated from different individual animal donors might present different behaviors, which were closely monitored, and no significant discrepancies were found. This was also relevant in paper II and III, where some experiments were performed in a different location using different bovine MuSCs: MosaMeat (MM-MuSCs). The biggest difference in the isolation protocol were the purification step and medium type. At MosaMeat, after isolation MuSCs were purified by fluorescence-activated cell sorting (FACS), by staining cells with NCAM, Integrin β1 (CD29), platelet endothelial cell adhesion molecule-1 (CD31), and marker of hematopoietic cells (CD45). Then the MuSCs were sorted by gating for the CD31/CD45-, CD29+/NCAM1+ population. Upon thawing the cells were maintained in MosaMeat patented serum-free medium (MM-SFM).

Determining cell growth and viability

While assessing consistent cell growth and viability prior to experimental application is important, these parameters were essential to analyze in all papers and manuscripts included in this dissertation. A combination of parameters such as cell nuclei count (paper II) and content of DNA, ATP, and lactate dehydrogenase (LDH) (paper I, II, III, and IV) was measured to determine cell growth, metabolic activity, and cytotoxicity, respectively. Typically, most commercially available assays are based on either colorimetric, luminescent, or fluorescent signal intensity detection of the desired markers by spectroscopy. Spectroscopy measures the absorption and emission of light and other radiation, which depends on the emitted wavelength.

Due to the complex nature of cell biological functions (e.g., metabolism) in combination with cell culture heterogeneity (where cells are constantly in different stages of the cell cycle), many different

factors should be measured to fully assess and compare the portion of healthy proliferating cells and apoptotic or necrotic cells within a population. It is important to remember that these factors are surrogates for the intended cellular function and thus an indirect measure of cell growth and viability. For example, while senescent cells are in growth arrest, they are still metabolically active. Moreover, many assays can be influenced by changes in culture conditions such as pH [159]. Nevertheless, these factors provide reliable insight into cell performance and quality. It is also essential to consider that in contrast to automized cell nucleic count imaging, spectroscopic assays fail to provide information concerning morphology, and therefore cell developmental information. Another limitation concerning the techniques used to assess cell growth and viability is that the applications are single parameter endpoint analysis. While multiple timepoints were selected in papers II, III and IV, the endpoint assays imply using multiple samplings with different cell plates, contributing to experimental variation. Experimental variation, pH influence, and validation of assay function can be resolved by using different controls, such as optimized culture conditions with untreated cells, positive, negative, and plate background or media background controls. To get an even more holistic characterization of the cell health status, multiple parameters could be measured at once in real-time on live cells using fully automated compact microscopes with cameras inside an incubator. Where the incorporated software analyze the images using an algorithm to calculate, e.g., cell confluence over time [160].

Measuring gene expression of specific proliferation markers is also helpful to assess cell growth potential. In papers II, III and IV, RT-PCR was used to determine proliferation markers cyclin D1 (CCND1) or ki-67 (MKI67) mRNA expression. Both markers are widely studied and associated with highly proliferative cells [161]. Such mRNA expression data together with cell growth assays and measuring, e.g., glucose consumption and lactate production (paper IV) give a more substantial evidence basis. While mRNA content is a good indicator for the presence of functional proteins, this should be validated using protein expression analysis techniques, such as western blotting.

Determining cell development and morphology

Myogenesis is associated with several extensively studied transcription factors (TFs) and proteins that can be monitored and serve as markers for the different cell developmental stages. In this project, *PAX7*, *MYF5*, *MYOD1*, and *MYOG* mRNA expression were measured by RT-PCR to assess MuSC progression in papers II, III, and IV. While mRNA expression of muscle cell development markers follow distinctive patterns, the mechanisms regulating myogenesis in MuSCs is complex. For example, mRNA expression of *MYF5* and *MYOD1* can be detected in quiescent MuSCs, but due to post-transcriptional regulation prevents their translation into functional proteins [70]. As mentioned earlier presence of activated proteins can be validated by using western blotting. In papers II-IV, immunostaining of neural cell adhesion molecule (NCAM), a muscle specific marker, and Desmin, a marker for myogenic differentiation, were visualized contributing to a more robust evaluation of cell phenotype [162, 163].

During immunostaining in papers II-IV, other proteins were also visualized, this time to assess cell morphology. For example, α-tubulin and actin are both cytoskeleton constituents and provide detailed information on cell morphology. Cytoskeleton and nuclei visualization can also indirectly aid the assessment of cell developmental progress, as myocytes and myotubes have distinct characteristics such as multinucleated larger cell filaments and highly oriented cell alignment. However, another complexity level is added when investigating 3D objects or using biocompatible biomaterials for visualization, discussed in section 3.4.

Cell adhesion

In paper IV, the cell attachment on MCs was investigated using RT-PCR to amplify mRNA expression of integrin-subunit β5 (*ITGβ5*), the actin-binding protein vinculin (*VCL*) that is distributed throughout FA, and syndecan-4 (*SDC4*), a co-receptor for matrix proteins. This gave valuable information on different specific cell receptor interactions with the different biomaterials. However, the assessment of cell biding and the presence of binding domains in ECM derivates on biomaterials are extremely complex. Therefore, optimally more cell adhesion factors should be assessed (on gene and protein level) together with, for example, visualization of focal adhesion rafts. Nevertheless, paper IV shows the proof of concept for waste by-products in producing efficient, sustainable, and entirely edible MCs. The future perspectives outline the plans to optimize MC production before a more comprehensive investigation on cell adhesion.

3.3 Bioreactors

Glucose is a major carbon source for cell biosynthesis and initially high levels can improve cell growth in early cell expansion. However, high glucose concentration can also lead to a shift in cell metabolism, where MuSCs are directed towards more inefficient cell metabolism via glycolysis rather than mitochondrial oxidative phosphorylation [164, 165]. To avoid this in the current system MuSCs were adapted to low-glucose medium (~1g/l). Other important cell parameters to consider are the accumulation of lactate and lactate dehydrogenase (LDH). Lactate is a by-product of cell metabolism that contributes to lowered pH in bioreactor systems, typically inducing adverse effects on cell viability. LDH is a crucial enzyme in anaerobic respiration and catalyzes the interconversion of pyruvate to L-lactate during glycolysis. In bioprocessing, the release of LDH is used as a marker for cell lysis and cytotoxicity. This parameter must be accounted for during the long-term experiment as it might affect the calculation of growth rates and biomass yields [166].

The effects of gas inputs were not investigated in this work (paper III). During our initial bioreactors experiments, one of the main challenges was that sparging led to excessive foaming and MC-cell losses. This is when MCs attach to gas-medium interfaces [167, 168], and travel with the bubbles to the surface, then the bubbles burst and damage the cells attached to the MCs. This can be prevented by using surfactants. However, these are often toxic to the cells or can inhibit cell attachment to the MCs [168]. We avoided foam formation by using overhead gases instead of sparging, and all lab-bench bioreactor experiments were performed using headspace aeration. During the industrial scale-up sparging will be necessary to supply cells with sufficient oxygen. However, MuSCs are shown to adapt to hypoxic conditions quickly, and the oxygen levels can control myogenesis and muscle regeneration. Hypoxia (3-6% O2) is shown to promote myogenesis [169], while anoxia (oxygen levels below 1% O2) appears to damage cells [169, 170].

The commercial Cytodex© MC series has previously produced good results during the expansion of bovine MuSCs in low volume spinner flasks. Specifically, Cytodex©1 (Cyt1) is reportedly more suitable than Cytodex©3 (Cyt3) [133]. Our preliminary screening of Cyt1 and Cyt3 was in line with Verbruggen et al., and the current work shows that more cells attach when using Cyt1 in spinner flask cell culture. Both MC types are based on a cross-linked dextran matrix with similar dimensions. The difference is that Cyt1 has positively charged diethylaminoethyl groups throughout the matrix, while Cyt3 is coated with a denatured collagen layer. However, Cyt1 is not a porous MC and requires a dissociation step after cell expansion reducing biomass yield, questioning the suitability in cultured meat production. Nevertheless, Cyt1 was used to expand cells in this study because it enabled easy data comparison between laboratories working with bovine MuSC expansion.

3.4 Edible Microcarriers

Production process

Collagen was extracted from industrially produced turkey (*Meleagris gallopavo*) rest raw material in a process involving pepsin hydrolysis. The Isolation provides collagen with high purity, high thermal stability, and a high degree of biocompatibility [171]. The collagen was solubilized in acetic acid to form spherical MCs. The dissolved solution was dripped into liquid nitrogen with a syringe then freeze-dried.

The simple nature of the cryo-technique used to produce collagen-based MCs do not offer high finetuning options. As a result, the collagen-based MCs had an inappropriately large size with a pore size that may be considered too small. There are available techniques that can control the production process more precisely while reducing the size of collagen-based MCs, such as wet spinning [172]. In addition, the pore size of the edible MCs might not facilitate sufficient space for myofiber formation. However, cell migration inside the MCs was not successfully analyzed during this work. Research has demonstrated that the freezing rate essentially determines the pore size of collagen-GAG scaffolds, and the collagen-GAG suspensions frozen quickly in liquid nitrogen have smaller pores and more regular shapes [173-175]. Hence, it is possible to produce collagen MCs with larger pores and uniform shapes by controlling the freezing temperature. Likewise, the simple ESM MC production process, involving milling and shifting the powder using sieves provides few finetuning options, especially concerning shape and pore size. The ESM MCs had oval disk shapes. While it is recognized that spherical particles provide good hydrodynamic properties and minimal stress on cells in agitated culture [129], the effects of shape were not investigated in this study. Further investigation of complete morphological parameters could be necessary after production optimization.

One of the major challenges of using protein-based biomaterials (e.g., collagen), is that the proteins degrade fast and have low mechanical strength, resulting in a lack of structural support in agitated culture [74]. This problem was solved by selecting a more temperature stable collagen source (avian collagen) and using a non-toxic crosslinking method (UVA-riboflavin crosslinking). The denaturation temperature of collagen is related to the physiological temperature in different species. Among the most used collagen sources, the denaturation temperatures range from 36.3 °C (calf skin) to 44.0 °C (chicken kneel bone) [176, 177]. In this study, turkey tendons were used as collagen source. Turkeys have an average body temperature of 41.1 °C, which indicate excellent thermal stability. Crosslinking the protein can enhance the chemical stability, thermal stability, swelling properties, mechanical properties, and pore size of scaffolds. Physical and chemical crosslinking methods have advantages and limitations. The main limitation of physical crosslinking is restricted fine-tuning of the physicochemical properties, including mechanical strength, swelling abilities and water holding capacities [178]. Chemical crosslinking of collagen allows tunability of the physicochemical properties. However, the main limitation of chemical crosslinking is the potential toxicity from the crosslinking agents, making the constructs less biocompatible. Also, the kinetics of the reaction is hard to control. Photochemical crosslinking is another type of crosslinking that requires a photosensitive compound and absorption of optical radiation. This type of crosslinking has advantages over chemical crosslinking. The UVA-riboflavin crosslinking induces more covalent bonds to strengthen and keep the collagen structure more stable in agitated cell culture medium at 37°C, where the material is exposed to shear force and therefore more prone to degradation. In addition, further strengthening of the material was achieved by combining collagen with a naturally more crosslinked stable fibrous biomaterial (ESM) [151].

Characterization

When characterizing MCs, it is crucial to consider that MCs are hydrated before *in vitro* use, and the wet state makes them swell, changing their morphology considerably. Scanning electron microscopy (SEM) was used on dehydrated MCs to investigate pore size and texture. However, MC dimensions were calculated using software and an extensive microscopy data set of MCs in a hydrated state, analyzed during and after cell culture. Also, mechanical properties were measured using a texture analyzer on hydrated MCs, and the actual swelling capacities were measured.

Using the texture analyzer to measure mechanical properties such as hardness (maximum strain set at 50%) was useful to differentiate between crosslinked and non-crosslinked materials. Further analysis using the TNBS method and Young's modulus is currently being performed to assess the ratio of available crosslinking points (primary functional amino groups) and elasticity of MCs before and after crosslinking, which can give further insight into crosslinking density. While the rough estimate of pore size and distribution assessed using SEM was useful, pore size should be investigated in more detail because it is important for the seeding and migration of cells. A more holistic characterization of the MCs properties should be done after production optimalization (e.g., surface chemistry, quantitative size distribution, biodegradation profiles).

Cell-microcarrier analysis

Challenges involving the analysis of cell parameters are mentioned previously in chapter 3.2. However, one very challenging aspect of analyzing cell efficiency on complex biomaterials is the material's interference while using classical molecular biology techniques. Firstly, most imaging modalities do not provide sufficient 3D information, and immunostaining with antibodies often results in unspecific binding introducing significant background noise. This is due to the high protein content in the biomaterials. Cytoskeleton constituents and cell nuclei were visualized in cells on MCs in paper IV, where the unspecific binding was a considerable problem. Another aspect that makes analysis involving MCs difficult is RNA and protein isolation. To achieve sufficient RNA quantities with high purity, RNA isolation was customized using Precellys® beads to homogenize hard tissues (paper IV). Researchers are working to optimize an efficient MC-Cell analysis platform. One of many promising approaches is a customized high throughput Flow Cytometry screening that implements the 3D issues and preserves cells attached to the MCs during physical handling [179].

4 Ethical considerations

In the course of this Ph.D. project, ethical approval was not required. The muscle cells used were extracted from beef sirloin (Nortura AS, Rudshøgda, Norway). Thus, in compliance with Norwegian law regulations concerning experimental use of animals FOR-2015-06-18-761 §2a, ethical approval was not necessary when samples are collected from slaughter animals/non-experimental agriculture and aquaculture. This regulation was also confirmed by direct communication with the Norwegian food safety authority (Mattilsynet).

5 Summary of main results

In this project, the focus was to contribute to cultured meat technology and investigate the possible feasibility of implementing by-products from the food industry. Hydrolysates and edible MCs were produced, characterized, and evaluated for their efficiency to promote cell growth. Further, long-term and scalable cell expansion parameters were investigated from low volume to higher volume bioreactors. A scaffold-based design was used with edible MCs or inedible commercial MC providing permanent support to monocultures of bovine MuSCs in spinner flasks or bench-bioreactors. A summary of the main subtasks, achievements, and considerations are sectioned into three parts: 5.1 Serum-free media, 5.2 Up-scaling cell expansion, and 5.3 Edible MCs. Overall, data from this project show that by-product hydrolysates promote cell growth, turkey collagen and ESM are suitable materials for MC production, and low volume spinner flask cultures with less controlled environments were robustly reproducible in bench-bioreactors.

5.1 Serum-free media

In this work, by-product materials were evaluated for their potential as growth-promoting agents in a SFM adapted for MuSC. The study resulted in one paper (published in *Food & function*) and one manuscript (papers I and II). Table 5.1 provides a summary of the main subtasks, achievements, and considerations of this work.

Table 5.1 Summary of subtasks, achieved results and considerations in SFM production

Subtask	Achievements	Considerations
Generating bioactive substrates from food by-products using chemical and enzymatic hydrolysis.	9 hydrolysates were produced using two different enzymes (Alcalase and Flavourzyme) or NaOH on 6 types of raw materials.	In order to save time and materials, different hydrolysis parameters such as time and enzyme to substrate ratio should be optimized.
Characterize by-product hydrolysates and crude extracts.	Hydrolysates with high protein content and distinct molecular size distribution patterns were produced. The Choice of by-product material and enzyme affected protein degradation during hydrolysis.	SEC analysis underestimates free amino acid content and overestimates larger peptides. Other properties, such as ash, fat, and amino acid content, should be investigated to get a more holistic characterization.
Perform screening of crude hydrolysates and extracts for their efficiency in promoting cell growth and viability.	Substrates rich in small peptides increased cell growth depending on the combination of enzyme and raw material. Pork plasma and egg white hydrolysates generated with Alcalase and yeast extract had the most promising effect on cell growth and viability.	The substrates tested contain a complex mixture of peptides. Some can promote cell growth, and some might inhibit cell growth. Thus, purification is needed to isolate the growth-promoting agents. A screening time of 3 days may not sufficiently reflect long-term effects.
Fractionate and purify selected by-product hydrolysates.	Pork plasma and egg white hydrolysates produced with Alcalase were separated according to size into 4-8 different fractions using SEC.	Because of fraction size distribution overlap, the growth-promoting fractions could be purified even further to isolate peptides of interest.

Characterize by-product hydrolysate fractions.	Sufficient size separation of hydrolysates was achieved, showing distinct molecular size distribution patterns. The fractions showed differences in metabolic compositions and peptide fingerprints which was dependent on both the material origin and fractionation.	Glycerol and other residues form processing were detected in low MW fractions. The fractions may need more refinement because of their highly complex nature and processing residue contamination. A more comprehensive characterization would be beneficial, such as LC-MS/MS.
Screening cell growth-promoting effects of by-product hydrolysate fractions.	Fractionated by-product hydrolysates have a significantly more potent effect on cell proliferation than crude hydrolysate extracts.	The growth-promoting effect of many hydrolysate fractions was similar, probably due to separation overlaps and/or processing residues accumulated in low MW fractions.
Investigating growth promoting effects of selected hydrolysate fractions together with a commercial serum replacement (Ultroser G) – Tailor made SFM.	Hydrolysate fractions maintained a higher cell growth efficiency than Ultroser G and crude hydrolysate extracts. However, none of the SFM formulations improved cell growth like FBS. Indicating that some important growth-promoting factors are either missing or in nonsufficient concentrations in the SFM.	A long-term cultivation should be performed by passaging MuSCs in the fraction-based SFM over 2-3 weeks to confirm short-term culture effects.
Investigating the hydrolysate fractions effect on cell development.	Fraction-based media increased expression of the myogenic transcription factors. The proliferation marker *CCND1* was also upregulated in media containing fractions. Indicating that MuSCs retained their myogenic and proliferation potential.	Protein expression should be analyzed to verify the translation of transcripts to functional proteins. In addition, other markers for late differentiation (*MYOG*) would be beneficial to analyze for a more conclusive myoblast phenotyping.
Investigating the hydrolysate fractions effect on cell signaling.	Decreased insulin-like growth factor receptor in fraction-based SFM may indicate a lowered ligand binding and subsequently lower presence of growth factors such as IGF-I and IGF-II. No differences were found in activation of AKT, S6RP, or ERK protein expressions.	Other signaling pathways should be examined further, e.g., Notch/HES-1. It may be beneficial to do proteomics comparing the different SFM, then confirm positive protein expressions via western blotting.
Investigating cell differentiation in SFM cultivation	MuSCs were able to expand to confluency in fraction-based SFM and differentiate on stimuli.	MuSCs are prone to become resilient to growth stimuli and enter cellular senescence. Thus, this experiment should be repeated after long-term passaging in fraction-based SFM.

5.2 Up-scaling cell expansion

This work aimed to up-scale long-term cultivation of bovine MuSCs expansion in bioreactors without compromising cell quality. Different cultivation strategies were examined (cell expansion volumes, seeding density, temperature, and growth medium composition) from low volume spinner flasks to higher volume bench-bioreactors. The study resulted in one paper (paper III), submitted to *Journal of Biotechnology*. Table 5.2 provides a summary of the main subtasks, achievements, and considerations of this work.

Table 5.2 Summary of subtasks, achieved results and considerations in up-scaling cell expansion

Subtask	Achievements	Considerations
Scaling up long-term MuSC culture expansion from spinner flasks to bench-bioreactors.	Successful long-term (38 days) up-scaling of MuSCs were achieved using a bench-bioreactor and commercial MCs. The MuSC culture had a high purity where 80-90% of cells on MCs were NCAM positive. Also, the cells retained their proliferative and migratory capacity after dissociation from MCs.	Adverse effect due to sparging led to significant foaming and cell-MC loss. Overhead gases were used instead. However, the lack of sparging is especially problematic at higher cell densities. A nontoxic antifoam solution should be developed. Further, MuSCs ability to differentiate after MC dissociation should be investigated.
Investigate cell growth and viability during long-term up-scaling.	MuSCs continuously proliferated with a peak at 24-27 days, indicated by increased quantities of DNA, cell nuclei count, and gene expression of *CCND1*. The metabolic activity was consistent, indicated by glucose consumption, lactate production, and stable ATP levels.	The cell growth kinetics resembled the growth curves in spinner flasks. However, the growth rate was slightly slower compared to cell culturing in 2D monolayers.
Investigate cell development during long-term up-scaling.	Increased expression of *PAX7* and decreased *MYOG* indicated that MuSCs retained their stemness after 38 days of expansion. This is essential to maintain cell self-renewal.	For a more holistic verification of cell phenotype, other MuSC and myoblast-specific markers can be detected, such as *FOXO*, *MYF5*, and *MHC*. Protein expression should preferably be analyzed to verify the presence of functional proteins.
Investigate cell morphology during long-term up-scaling.	The MuSCs proliferating on MCs consistently displayed a mono-nucleated spread cell morphology throughout the experiments, visualized by a-tubulin, actin, and NCAM protein expression. However, the distribution of cells on the MCs was not uniform. Some MCs were covered with cells others were empty.	The accuracy of empty MCs and reduced biomass yield due to the cell-MC dissociation step questions the suitability of using this commercial MC in cultured meat production. Cell distribution on MCs might be improved by intermittent agitation when fresh MCs are added to the system.
Examine different spinner flask and bioreactor cultivation parameters.	Glucose and lactate trends were consistent regardless of cell source and volume of spinner flask used. The endpoint cell numbers were dramatically affected by the initial seeding density and temperature. Spinner flask cultures were shown to be reproducible in bench-bioreactors	Cell culture parameter set 1 and 2 had more than one variable, making it more challenging to predict non-impacting parameters. Therefore, a one parameter elimination method should be implemented.

5.3 Edible microcarriers

In this work, we focused on the feasibility of using by-products to develop sustainable and entirely edible MCs that can be included in the final cultured meat product to reduce profit loss and maximize cell expansion with high recovery of bovine MuSCs. Three different edible MCs were produced (i.e., collagen, hybrid, and ESM MCs) and tested in addition to commercial Cytodex©1 (Cyt1) MCs in spinner flask culture for 8 days. The study resulted in one paper (paper IV), a revised version will be resubmitted to *Biomaterials*. Table 5.3 provides a summary of the main subtasks, achievements, and considerations of this work.

Table 5.3 Summary of subtasks, achieved results and considerations in edible MC production

Subtask	Achievements	Considerations
Generating edible MCs using collagen isolated from turkey tendons and ESM by-product materials.	Using simple techniques three different MCs were produced. The surface crosslinking and ESM inclusion in collagen increased the mechanical hardness and made the collagen-based MCs more resilient to degradation.	The production technique of collagen-based MCs must be optimized to achieve smaller MC sizes and larger pores. Such techniques are available, e.g., wet spinning.
Characterization of edible MCs.	All edible MCs had a natural interconnected porous structure which is beneficial for cell adherence. The ESM MC size was more comparable to the Cyt1 MCs.	The MC categories had very different parameters (e.g., density, shape, surface area), challenging a direct performance comparison. Further investigation of MCs physical parameters (e.g., surface chemistry) after production optimization is needed.
Examine muscle cell growth and viability in cells on edible MCs.	MuSCs successively attached and covered the entire surface of all MCs while expressing high cell proliferation (DNA, nuclei count, and *MKI67* expression), metabolic activity (ATP, glucose, and lactate), and low cell cytotoxicity (LDH). Thus, demonstrating that both turkey collagen and ESM are suitable biocompatible materials for MuSC.	Because the MC categories parameters were different, the different MCs were compared to their own effectiveness over time. A comparison of cell expansion performance between MC categories should be made with caution. Thus, the surface areas should be quantified, and MCs 3D morphology effects on cell growth should be investigated.
Investigating the muscle cell development on edible MCs.	Different MuSC markers (*PAX7*, *MYF5*, and *MYOD1*) indicated activated and proliferating cells on all MC. Reduced *PAX7* expression may indicate a loss of stemness. Maintaining the stem cell pool is essential for extended self-renewal required for high-density cell expansion.	Total and phosphorylated protein expression should be analyzed to verify the translation of transcripts to functional proteins. In addition, a marker for late differentiation (*MYOG*) would be beneficial to validate cell phenotyping.
Investigating cell morphology on MCs.	The MuSCs proliferating on Cyt1 and ESM MCs displayed a spread mono-nucleated morphology after 8 days. MuSCs on collagen and hybrid MCs showed a more rounded morphology.	The antibodies used showed unspecific binding to the collagen-based MCs resulting in significant background noise and fewer successful immunostaining experiments.
Investigating the muscle cell adhesion on edible MCs.	Relative gene expression of different cell adhesion markers (*VCL*, *ITGβ5*, and *SDC4*) indicated that MuSCs had specific interactions with the different biomaterials.	The MCs surface chemistry should be characterized.

6 Discussion

The challenges of producing cultured meat are multifaceted. First of all, the system is currently competition-based and deeply entangled in proprietary rights, which has resulted in a severe lack of transparency. Thus, making it difficult to transfer knowledge between alternative meat companies and other relevant industries, most likely slowing development down. Hence, there is very little available data to build on. Secondly, every input to the process must be sustainable and low-cost to compete with food industry market values and satisfy sustainable developmental goals. Cutting costs is a significant barrier to overcome, especially since the technologies cultured meat is based on (e.g., biomedical and pharmaceutical industry) have an exceptionally high end-product value. Therefore, this project provides much needed publicly accessible data on bovine MuSCs cell growth kinetics, behavior, and development. More specifically, we investigated three major challenges currently hindering cultured meat production: sustainable serum-free media (SFM) development, up-scaling cell expansion, and edible microcarrier (MC) production.

By-product hydrolysates as growth-promoting agents in a SFM adapted for bovine MuSCs

The challenges of producing SFM are complex. Critically, we lack the complete functional cell knowledge to identify the exact components in serum detrimental for highly efficient cell growth, making it difficult to meet the desired goal of a completely defined media that ensures reproducibility with a consistent standard. The SFM must be affordable, food-grade, provide sufficient cell growth, and maintain satellite cell myogenicity to meet cultured meat's massive cell quantity requirement.

Do animal-based hydrolysates promote cell growth?
Reportedly, hydrolysates can improve cell growth [112, 113]. Like FBS, it is not fully understood which components of hydrolysates are responsible for the growth-promoting effects. Unlike FBS, the tested materials are food-grade and low-cost with no ethical challenges of use. Two examples of animal-based hydrolysates that increase cell growth for many cell lines are Primatone RL and a promising cell culture supplement containing lysate of porcine platelets [112, 113]. Our work demonstrated that various by-product crude hydrolysate extracts (CE) significantly increased cell growth and viability (paper I). CE contains a wide range of components, from proteins to peptides and simple amino acids [103]. Although some components promote cell growth, others could inhibit cell growth. Therefore, a targeted study of bioactive constituents usually requires multiple purification and fractionation steps. Fractionating hydrolysates might improve bioactivity. This was demonstrated when poultry hydrolysates were fractionated, and one of the fractions significantly improved glucose uptake [103]. When using fractionated pork plasma and egg white hydrolysates in our study (paper II), the growth-promoting effect was even more pronounced than using CE.

Interestingly, the hydrolysate fractions with low molecular weight (MW) enhanced cell growth more than larger MW fractions. However, this was dependent on the SFM formulation. More specifically, there was no significant difference between fractions when combined with a commercial serum replacement (Ultroser G). Importantly, fraction supplementation achieved greater cell growth efficiency than Ultroser G, which contains commercial GFs and hormones. Likewise, Kolkmann et al., demonstrated that commercial SFMs could sustain cell expansion of bovine MuSCs, although not as efficiently as media containing FBS [98]. Although they are promising ingredients (paper II), none of the medium additives we tested increased cell growth to the same level as the medium containing serum (FBS). Previous experiments expanding MuSCs *in vitro* have been challenging because of a rapid transcription factor (TF) shift towards myogenic commitment that initiates myogenesis, resulting in reduced self-renewal [180]. This could be the case when using hydrolysate fractions compared to FBS

(at least after 48h). Another explanation is that non-myogenic cells overgrow MuSCs more rapidly in the FBS-containing medium after 72 h. However, this was not investigated at the endpoint of this study (6 days).

Interestingly, the low MW pork plasma fraction showed similar upregulation of proliferation marker cyclin D1 (*CCND1*) as FBS (paper II). Previous studies have shown that the mRNA and protein expression level of proliferation marker cyclin D1 (*CCND1*) rapidly declines during myogenesis, and no expression is detected in myotubes [181-184]. In paper II, the expression of the different myogenic transcription factors (*PAX7*, *MYF5*, and *MYOD1*) and proliferation marker cyclin D1 (*CCND1*) indicated that MuSCs were activated, proliferating, and retained their myogenic potential in fraction-based SFM. Considering the massive number of cells required in cultured meat products, these are essential cell functional qualities to maintain. However, conclusive cell phenotyping requires multiple time-point analyses and validation of translation of functional activated proteins. It is difficult to determine which FBS components are required for successful and highly efficient MuSC culture, and it appears that some factors are detrimental. Due to fraction overlaps and accumulation of processing residues in the low MW fractions detected by NMR (glycerol, citrate, and formate), more refinement of the hydrolysate mixtures is probably necessary to improve the bioactivity. Nevertheless, the results indicated that the adapted SFM is more suitable for expanding bovine MuSCs by adding a single hydrolysate fraction. This work provides a promising start for developing a SFM for cultured meat production while reducing the problematic reliance on cost drivers such as FBS and GFs.

What are the growth promoting components or mechanisms of by-product hydrolysates?
Hydrolysates contain a wide range of low MW nutrients, and because of their high amino acid content, they are a major nitrogen source. Nitrogen is essential in synthesizing proteins, enzymes, nucleic acids, vitamins, GFs, and hormones [185]. Thus, hydrolysates serve a nutritive function that contributes to cell growth by aiding the cell to produce its own growth-inducing molecules. Hydrolysates also contain larger proteins and peptides. However, most mammalian cells lack the biosynthetic capacity to efficiently utilize larger proteins and peptides as nutritive sources [69]. Encrypted within the primary structure of proteins are sequences for potential bioactive peptides that can stimulate effects on physiological functions beyond nutritional value, e.g., active domains of specific proteins that stimulate cell signaling such as GFs. These peptides are inactive within the sequence of intact proteins and can be released by enzymatic hydrolysis [105]. Thus, these peptide sequences could mimic native GFs that bind GF receptors, regulating essential cell signaling pathways that promote, e.g., cell survival, proliferation, migration, or differentiation [111]. Considering that the cost of purified GFs is unsuitable for cultured meat, this topic is crucial to investigate thoroughly.

Unfortunately, due to the complexity of the hydrolysate mixtures, we did not identify the exact growth-promoting components in the fractionated hydrolysates or the cell signaling pathways regulated by fraction hydrolysate stimulation. While the cell growth-promoting mechanisms in hydrolysates are not fully elucidated, they are shown to stimulate insulin-associated signaling pathways in mammalian cell culture [111]. When we investigated cell signaling after hydrolysate fraction stimulation, a down-regulation of insulin-like growth factor receptor (*IGF1R*) mRNA expression was detected for most fractions tested, while the expression was unchanged by supplementation with a low MW pork plasma fraction. In contrast, the *IGF1R* expression was up-regulated when using a cell medium containing FBS. Decreased insulin-like growth factor receptor expression may indicate a lowered ligand binding and subsequently lower presence of growth factors such as IGF-I and IGF-II. Reportedly, depletion of *IGF1R* in muscle only transiently impairs muscle growth, suggesting that alternative pathways are involved in the muscle development progression, for example, via insulin receptors (IRs) [186].

The insulin-like growth receptor signaling pathway is essential in skeletal muscle development and promotes multiple biological processes [187]. Upon ligand binding, *IGF1R* becomes autophosphorylated and induces the PI3K/Akt/mTOR/S6 pathway, fundamental to skeletal muscle development and cell cycle progression [188]. Crosstalk between PI3K/Akt/mTOR and other signaling pathways is also critical. For example, the RAS/MAPK/ERK and the PI3K/Akt/mTOR signaling pathways operate together in regulating several essential cellular functions, including protein synthesis and cell growth [189]. The nonsignificant difference in Akt, S6RP, ERK protein activation together with increased *SDC4*, *MYOD1*, and *CCND1* mRNA expression indicates that the low MW pork plasma and egg white fractions could regulate other signaling pathways (e.g., Notch/HES-1). Thus, more research is needed to elucidate which cell signaling pathways are stimulated by hydrolysate fractions. Because animal-derived proteins contain high amounts of branched-chain amino acids leucine, isoleucine, and valine, it could also be interesting to investigate the activation of the nutrient-and-energy sensitive rapamycin complex 1 (mTORC1). The mTORC1 pathways mediates, e.g., cell growth, cell proliferation, protein synthesis, and inhibition of autophagy [190].

Because the present work practiced a "one factor at the time" exploration and did not passage the cells in long-term cell culture (more than 2 weeks), it is unlikely that the fraction-based SFM is optimal. The one factor at the time approach is very time-consuming. A better-suited approach for dealing with multi-factor problems could be using bioinformatics tools. The increased research interest for bioactive peptides has rapidly expanded the toolbox for discovering peptides with desired properties making it less time-consuming to determine good precursors. Specifically, precursors with high occurrence of bioactive motifs. For example, one efficient approach uses bioinformatics tools to search for homology between amino acid sequences with known cell signaling molecules to find analogs. In silico tools are already used to predict bioactive peptides with antimicrobial and antioxidant properties and evaluate flavor characteristics of peptides [105]. This approach would greatly benefit SFM development. After refinement of the hydrolysate fractions improves the bioactivity, a more thorough NMR and LC-MS/MS analysis coupled with multivariable analysis is necessary to identify cell growth-promoting compounds in more detail.

Do by-product animal origin and enzyme choice matter?

The enzyme specificity results in unique digestion patterns with peptides of different amino acid chain lengths that impacts the functional and biological properties of the protein hydrolysates [102]. At the same time, the activities of the peptides are also determined by the amino acid compositions, and the amino acid compositions are determined by the by-product raw material compositions. Hence, the raw material composition significantly impacts the end hydrolysate product's nutritional, physicochemical, and bioactive properties [110]. Thus, both matter.

In paper I, the 11 distinct molecular weight distribution (MWD) profiles obtained from SEC analysis complied with previous research demonstrating that the choice of by-product material and enzyme affect protein degradation differently during hydrolysis [191-193]. Interestingly, neither intact lyophilized pork plasma nor pork plasma hydrolysates generated with Flavourzyme had similar cell growth effects as pork plasma digested with Alcalase. Further, the MWD profiles show that Flavourzyme digested raw material contained 2-fold more of the smallest molecules (free amino acid size) and 10-25% fewer small peptides than hydrolysates of the same by-product material produced with Alcalase. This indicates that small- to mid- sized peptides may improve cell growth. Among the analytical approaches for discovering bioactive constituents in complex mixtures are chromatography-coupled bioassays where fractions are directed to high-throughput bio-screening, an approach used to identify and characterize bioactive peptides from chicken hydrolysates [194]. In a previous study, a

low MW hydrolysate fraction from chicken-carcass significantly improved cellular glucose uptake in bovine MuSCs [103]. This was a direct result of a targeted fractionation of the most bioactive peptides.

Interestingly, the variations in by-product composition affect the peptide size distribution and amino acid composition of the fractionated hydrolysates [195]. While the work in paper II did not identify the cell growth-promoting compounds in the hydrolysate fractions, the growth-promoting effects are probably due to a combination of the compound composition and MW. Even though the screening panel was relatively limited, a pattern was observed where Alcalase by-product digestion increased cell growth more than Flavourzyme digestion of the same raw material. To study the correlation between enzyme choice, MWD, raw material composition, and cell growth effects, a large panel of hydrolysates digested with many different enzymes coupled with NMR and multivariable analysis should be performed. To further improve the chances of finding potent bioactive molecules, bioinformatics tools should be employed before this comprehensive study to estimate the occurrence of bioactive motifs [39, 104-106]. Also, for future optimization, different hydrolysis parameters such as time and enzyme to substrate ratio should be investigated to save time and research materials. This optimization might also increase the bioactivity of the hydrolysates, as others have reported a decrease in bioactivity with an increased hydrolysis time [103]. This could be a result of an excessive breakdown of the bioactive peptides.

Can hydrolysates be produced with a consistent and reproducible standard?
Once the most bioactive fraction of the complex protein hydrolysates is elucidated, the fraction can be reproduced with a sufficiently consistent standard. Meaning, even when the media is not considered completely defined, the SFM can have a low enough variation that does not compromise cultured meat quality. Reportedly, commercial SFM formulations also show variations and are still accepted in large-scale cell culture productions in other industries (e.g., pharmaceutical and biomedical) [81]. Research groups are continuously optimizing and automizing industrial large-scale hydrolysis production by employing fast real-time analytical tools, sorting the by-products, monitoring the hydrolysis progression, and characterizing the end hydrolysate product. These analytical tools should eventually ensure minimal batch-to-batch variation and high-quality yield at low costs.

Long-term and scalable cell expansion of bovine MuSCs

In order to achieve the massive quantities of MuSCs needed in cultured meat production, bioreactor tanks are usually used together with MCs. As a scale reference, there were billions of cells in a bite of the first cultured meat burger [125]. However, the up-scaling process is challenging and entails many factors, such as optimized cell nutrients, gas inputs (oxygen, nitrogen, and CO2), agitation, temperature, and pH control [125]. Currently, no full-scale bioreactor design exists for cultured meat production. Thus, bioreactor design and their impact on MuSCs behavior and function is a major research focus.

What do we know about long-term bovine MuSCs expansion?
Compared to immortalized cell lines, non-transformed MuSCs have a relatively limited ability for self-renewal. As mentioned previously, expanding MuSCs *in vitro* tend to show a rapid shift towards myogenic commitment initiating differentiation, resulting in reduced self-renewal [180]. MuSCs are also prone to enter cellular senescence and become resistant to growth-promoting stimuli, causing limited proliferation and differentiation potential. This is a significant limitation in stem cell therapy for muscle tissue reconstruction because they cannot expand undifferentiated MuSCs in culture while maintaining the transplantation potential [57]. Likewise, loss of PAX7 expression, spontaneous differentiation, and senescence resulting in loss of self-renewal potential would be detrimental in large-scale cultured meat production due to the requirement for massive amounts of cells and the

ability to initiate differentiation on timely stimuli. MuSCs proliferation behavior and function is a major research focus because an extended self-renewal ability would significantly decrease the amount of starting material (e.g., biopsy) required to initiate the process. The extended self-renewal issue could be resolved by gene manipulation, but due to food and governmental regulation uncertainties, many groups are focusing on non-transformed cells. However, different colonies isolated from adult muscle are predisposed to different fates. Ono et al., show that the slow-dividing MuSCs population retains long-term self-renewal ability in adult muscle [196]. Therefore, cell colonies derived from the slow-dividing MuSCs have extended self-renewal capacity. In contrast, cells derived from the fast-dividing population rapidly undergo myogenic differentiation after a few cell divisions and eventually become exhausted. Ding et al., showed that bovine satellite cell maintenance depends on cell purity (little interference from, e.g., fibroblast-like cells) and p38 MAPK signaling [60].

For bovine MuSCs expansion in bioreactors there is little data. Reportedly, MuSCs and mesenchymal stem cells can successfully be expanded on commercial MCs in low volume spinner flask culture for up to 9 days and the cultivation of bovine MuSCs is comparable to cultivation of human mesenchymal stem cells [126, 127]. However, tissue culture techniques are still small scale and rather costly. The work form paper III showed that after over a month in the bench-bioreactor, MuSCs retained their stemness on MCs. Showing increased expression of the satellite cell marker *PAX7* and proliferation marker *CCND1* with reduced expression of differentiation marker *MYOG*, indicating a large number of cells with stem cell-like characteristics ($PAX7^{+}MYOD^{-}$) that replenish the stem cell pool throughout the experiments. *PAX7* is a muscle marker highly expressed in newly activated MuSCs and is critical for activation, survival, and normal regenerative function in adult skeletal muscle cells [180, 197]. Knock-down of *PAX7* results in cell growth arrest and loss of *MYF5* expression [198-200]. Further, *PAX7* inhibits differentiation by inhibiting activation of *MYOG* through *MYOD1* [201]. As mentioned previously *CCND1* expression is not detectable in myotubes [182]. While gene expression alone is not enough to validate cell phenotype, visualization of cell nuclei, actin, and α-tubulin showed no visible fused multinucleated myofibers. This, together with the MuSCs continued ability to proliferate and migrate in 2D-culture after enzymatic dissociation from MCs, further validates the MuSCs proliferative and myogenic potential after long-term bench-bioreactor cell expansion.

How the bovine MuSCs retained their stemness in this study without p38 inhibitors is unknown. Currently, there are no publicly available large-scale long-term bovine MuSCs expansion data for comparison. However, research has demonstrated that culturing MuSCs in Ultroser G (a commercial serum replacement) extends the duration of viable cells and increases fractional satellite cell characteristics in primary cell cultures [202, 203]. The cells in this study were adapted to a culture medium containing Ultroser G, lower serum and glucose concentrations. Furuichi et al., showed that high glucose concentrations slow the proliferation of mice MuSCs, possibly due to hyperglycemia, negatively affecting the regenerative capability [204]. Also, high cell purity of MuSCs is important to retain cell stemness [67]. Specifically, maintaining high purity without fibroblast interference can sustain MuSC stemness. Our in-house primary cell culture isolation of MuSCs has a purity of ∼90% [158]. Likewise, after 38 days of expansion, ∼90% of cells still expressed the muscle specific marker NCAM, demonstrating low contamination of fibroblast-like cells in the current system. Together with optimized cell nutrition, cell purity could explain the extended self-renewal capacity. Although serum replacements might provide advantages for optimal cell growth, the high cost and commercial copyright protection are likely not suitable for large-scale cultured meat production [205]. Further, reliance on FBS hinders the relevance of the findings to cultured meat production. However, this work provides much needed data on bovine MuSCs cell growth kinetics, behavior, and development in long-term high volume bioreactor cell culture.

What are the critical parameters for upscaling MuSCs?

Efficient bioprocessing requires control of nutrients during expansion. Glucose is a significant carbon source for cell biosynthesis, and high initial glucose levels can help improve cell growth during early cell expansion. However, this might lead to a shift in cell metabolism, where MuSCs inefficiently generate energy via glycolysis rather than mitochondrial oxidative phosphorylation [164, 165]. In paper III, glucose was consumed, and lactate concentrations slowly increased in predictable patterns. Accumulation of lactate typically induces adverse effects on cell viability. These effects have been reported at a concentration of 2.0-3.6 g/L [206, 207]. None of our experiments exceeded this limit. Nevertheless, the seeding density and temperature had a significant impact on glucose consumption and lactate production.

The effects of gas inputs were not investigated in this work. During our initial bioreactor expansion experiments (paper III), one of the main challenges was that sparging led to excessive foaming and MC losses. This is a well-known phenomenon where the MCs, regardless of cells being present or not, attach to gas-medium interfaces and travel up to the surface where the bubbles burst and damage the attached cells [167, 168]. This can be prevented by using surfactants (antifoam). However, these are often toxic and can inhibit cell attachment to the MCs [168]. By using overhead gases instead of sparging, we avoided foam formation, and the bioreactor experiments were performed using headspace aeration. However, MuSCs are shown to adapt to hypoxic conditions quickly, and the oxygen levels can control myogenesis and muscle regeneration. Hypoxia (3-6% O2) is shown to promote myogenesis, while anoxia (oxygen levels below 1% O_2) appears to damage cells [169, 170]. Therefore, it is essential to supply cells with sufficient oxygen levels, especially during industrial scale-up as the dissolved oxygen becomes an issue at much higher cell densities.

Temperature is another factor that can influence cell proliferation during expansion, and the average body temperature of an adult cow is 38°C. Clark et al., demonstrated that satellite cells from different origins had different sensitivity to temperatures in terms of proliferation, i.e., pectoralis major MuSCs were most proliferative at temperatures of 43°C. At the same time, biceps femoris MuSC proliferated faster between 33°C and 39°C [208]. Ongoing results in our lab has shown that bovine MuSCs dramatically lost ability to expand at temperatures of 35°C and 41°C. This underlines the importance of keeping the temperature homogenous throughout the bioreactor system as it affects the homogeneity and proliferative capacity of MuSCs. Furthermore, the cell numbers reached at the end of our experiments were dramatically affected by the initial seeding density and the temperature in the spinner flasks, with an approximately 7-fold difference in the endpoint cell numbers. Likewise, the cell growth in higher volume bench-bioreactor showed a 6-fold difference in final cell density depending on seeding density and temperature.

Interestingly, these effects were not dependent on MuSCs source. The data suggest that seeding density and temperature are more critical than oxygen as MuSCs can quickly adapt to hypoxic conditions. If the conditions were not fully optimized, the difference between cultivation strategies significantly increased cell expansion variability. However, spinner flask cultures with fewer control options (e.g., pH, dissolved O2, evaporation) were shown to be robustly reproducible in bench-bioreactors. Therefore, low volume spinner flasks can be used as higher throughput and scaled-down models to optimize MuSCs expansion on MCs. This work provides a promising start to optimize cell expansion parameters adapted to muscle cells.

By-product materials in developing low-cost edible MCs with high cell expansion efficiency

Extensive research exists on biocompatible biomaterial scaffolds mainly for medical use, such as stem cell therapy for muscle tissue reconstruction [137, 209]. However, optimizing the MC scaffold design adapted to MuSCs and engineering tissues for human consumption is still required to ensure high efficiency, low cost, and sustainability.

Can animal-based by-products be used to produce functional MCs?
This work demonstrated that by-products materials with minimal processing successfully function as MCs for bovine MuSCs in spinner flask culture. Eggshell membrane (ESM) MC particles were produced by grinding and collagen-based MCs using a simple cryo-technique. The MCs provided mechanical structural integrity and support to the cells and were strong enough to endure higher temperatures in agitated cell culture. ESM is a meshwork of fibers naturally stabilized by extensive crosslinking and mainly consists of structural proteins such as collagen and high amounts of a structural group of proteins, the cysteine-rich ESM proteins (CREMP) [210]. Such increased crosslinking results in a very resilient biomaterial that holds up exceptionally well in agitated cell culture [211, 212]. Collagen proteins are known to degrade fast and have low mechanical strength, resulting in a lack of structural support in agitated culture [74]. In this study (paper IV), turkey tendons were used as a collagen source. The previous characterization of this collagen emphasizes superior thermal stability compared to collagen from other species [171]. Furthermore, the non-toxic UVA-riboflavin crosslinking increased covalent bonds on the collagen-based MC construct and strengthened the mechanical properties, further hindering degradation in agitated cell culture at 37°C [172]. Also, combining collagen with ESM further improves the mechanical properties of collagen, making them even more suitable for agitated cell culture [135, 153].

However, the size and pore size of collagen-based MCs need optimization. The appropriate size of MCs is important because a smaller size means more room for a higher quantity of suspended MCs per liter culture medium providing more surface area, and the interior of larger constructs can become nutrient-limited [129]. Considering the average myofiber is quite large, the pore size of our edible MCs (~15 µm) might not facilitate sufficient space for myofiber formation, and pore directionality is also important for myotube alignment [137]. Many available techniques can efficiently control the production process while reducing the size of collagen-based MCs, such as wet spinning or custom-designed apparatus employing a droplet air-jet [172, 213]. Also, the pore size can be adjusted by regulating the freezing temperature [173-175]. The *ideal size* of smooth MCs is typically 100–300 µm [214], and porous MCs for injectable scaffold delivery for tissue regeneration are typically 200-400 µm with a pore size of 30-80 µm in diameter [215, 216].

Currently, there are few published works under the topic of scaffolds/MCs for human consumption. Using edible MCs with nutrient-enhancing components that can be included in the final meat product provides another opportunity to customize the nutritional composition and positively impact the sensory characteristics of cultured meat. Exciting work related to edible scaffold production includes using textured soy protein, salmon gelatin, and mixtures of salmon gelatin and alginate [66, 136, 217]. Like our biomaterials, they are edible, and the scaffolds can be tailored to various sizes and shapes, making them suitable candidates for engineering tissues for human consumption. Importantly, Enrione et al., use of salmon skin to extract gelatin is an excellent example of by-product valorization, contributing to a circular economy [136]. Interestingly, their edible scaffold pore sizes were very different compared to our MCs. The 6 mm soy scaffold has pores with 200–600 µm in diameter, the 3 mm salmon alginate scaffold has 200 µm in diameter pores. This is also very different from the typical porous MCs used in muscle tissue regeneration. Critically, more research is required to

determine the appropriate size of the pores and MCs for MuSCs intended for human consumption. It is anticipated that both the proliferation step and the differentiation step will likely require customized bioreactor designs to maximize energy input and minimize waste production [5]. As the size difference of proliferating and differentiating MuSCs are significant, a customization of MCs might also be required. Unlike our work, their scaffolds were tested in static cell culture. While the soy scaffold was tested with primary cells, using bioreactors with SFM and non-transformed MuSCs is necessary to translate the results from cultured cell lines in static flasks [136, 217]. Reliance on FBS and antibiotics hinders the relevance of the findings to cultured meat production. Nevertheless, our work provides proof of concept that edible MCs can easily be produced from animal-based by-product materials to support efficient cell expansion in low-volume spinner flasks.

Are animal-based by-products biomaterials biocompatible with bovine MuSCs?
The optimal biomaterial should be capable of mimicking the natural 3D-network that provides structural support and maintains normal cellular behavior, i.e., the ECM [143, 148, 149]. The ECM consists of fibrous protein (e.g., collagens), glycoproteins, and proteoglycans with glycosaminoglycans (GAGs). In ECM, the major collagen types are I and III [218]. Likewise, the fibril-forming collagen types I and III accounts for up to 75% of total muscle collagen [73]. Collagen type I has been considered a gold standard for tissue engineering because of its low antigenicity and high ability to form fibrils, while I and III are reportedly the most important types in wound healing [147, 218]. The turkey collagen used in this work was identified as collagen type I and III [171], typical for tendons and ligaments [219, 220]. Like the ECM, the ESM mainly consists of structural proteins such as collagen (III and V), glycoproteins, and proteoglycans, including GAGs.

Reportedly, processed ESM powder is highly bioactive, anti-inflammatory, regulates cellular functions during wound healing, and is a promising biomaterial for tissue engineering [31, 135, 152, 153]. Ruff and co-workers have demonstrated that the anti-inflammatory properties of ESM hydrolysate are retained after *in vitro* digestion [221]. Also, clinical trials demonstrate that oral intake of enzymatic ESM hydrolysate can reduce pain in joints and reduced stiffness [222]. A recent study has shown ESM powder to ameliorate intestinal inflammation by facilitating restitution of epithelial injury and alleviating microbial dysbiosis in mice model [150]. Ongoing research in our lab has demonstrated that oral intake of ESM influence molecular markers of aging in skeletal muscle. Reportedly, feeding trials in young versus elderly mice demonstrated that supplementing EMS in the feed positively impacted on skeletal muscle (unpublished data). Thus, collagen and ESM might be optimal in cultured meat production because they are bioactive, edible, nonantigenic, have good biocompatibility with naturally high cell-stimulating properties, porous structures, and work in tissue healing [74].

In vivo, the ECM provides connections and interactions for cells and regulates cell survival, expansion, morphology, differentiation, and migration [74]. Because cells can sense the physiological conditions necessary for normal cellular behavior, the matrix surface properties are directly related to biological activity *in vitro* (e.g., cell attachment and growth). Thus, matrix stiffness and protein coating significantly affect proliferation in MuSCs [223]. Native collagen is preferred in tissue engineering applications for being a good matrix in cell proliferation [224]. The results from the turkey collagen characterization indicated that the native structure of collagen was preserved [171]. In paper IV, the SEM analysis revealed that the edible MCs had interconnected porous structures. It is established that rough surfaces promote cell attachment, and it is a standard procedure to manipulate smooth MC surfaces to achieve a more complex structure [225].

The biocompatibility of the collagen and ESM MCs was confirmed by measuring DNA, ATP, glucose, lactate, and LDH. Indicating high cell proliferation, viability, and metabolic activity combined with low cytotoxicity. High cell culture expansion efficiency was also demonstrated by examining the relative gene expression of proliferation marker *MKI67* and cell distribution on the edible MCs. Visualization of cell nuclei revealed that MuSCs successively attached, rapidly proliferated, and covered the entire surface. Further, investigating the cell development and adhesion by measuring relative gene expression of different muscle markers (*PAX7*, *MYF5*, and *MYOD1*) and cell adhesion markers (*VCL*, *ITGb5*, and *SDC4*) indicated that MuSCs retained their early myogenic potential and had specific interactions with the different biomaterials. Altogether, our work provides evidence that turkey collagen and ESM are suitable biomaterials for MuSCs.

Animal-based materials in the production of cultured meat

Can animal-based by-products be used in cultured meat production?

A current trend in cultured meat development is largely focused on using plant-based or recombinantly produced proteins as process ingredients. A product without animal-derived components may make it suitable to a broader consumer base (e.g., vegans, vegetarians, certain religious groups). Another reason is the potential risks associated with animal-derived materials, such as the introduction of pathogenic agents. While safety routines and proper maintenance generally can remove or reduce this risk to the same level as in other food production, cultured meat is not necessarily intended for people who are already willing to reduce their meat intake, and animal-derived components in the process could be accepted. In fact, the advantages of using animal-based by-products in the process are compelling. They generally contain the excellent nutritional value of real meat with functional properties giving them the overall characteristics of meat products that are challenging to reproduce in non-animal proteins. This may benefit cultured meat in multiple ways, such as providing cells with macro- and micro- nutrients, act as growth or differentiation inducers, serve cell protective functions, serve as ECM mimicking scaffolding, and contribute to sensory qualities in the final meat product. As long as there are massive amounts of waste by-products available, we propose producing high-quality proteins for human consumption by using low-value food by-products that otherwise could be an environmental burden, adding another level of sustainability to cultured meat production. Importantly, this contributes to more responsible waste management and represents an even more effective protein conversion strategy while contributing to a circular economy by reducing waste. At the same time consumer acceptance must be encouraged to avoid complications observed when introducing other novel foods such as GMOs [28].

In this project, a cost analysis was not performed. However, industrial production of animal-based by-product hydrolysates is already used as enrichment, natural preservatives, and active packaging for food products [226]. This implies that the cost is competitive, and the use is approved by national food authorities. Complete serum-free systems exist for non-transformed rat MuSCs, but they rely on expensive GFs [99]. The use of recombinant proteins and commercial GFs is likely not suitable in cultured meat due to the cost. Stout et al., demonstrated that recombinant proteins are the main cost drivers of their promising cultured meat SFM [227]. While plant-based proteins offer sustainability and environmental benefits, they generally contain a lower essential amino acid content, and recreating the functional properties of animal proteins in plants-based proteins requires significant resources [5, 228]. Logically it may make sense to feed cultured meat cells the same as livestock animals. However, replicating the digestive system of e.g., a cow is exceptionally challenging. Nevertheless, soy hydrolysates are shown to mimic the autocrine growth factor effects of conditioned media for hamster epithelial suspension cell culture in the short-term (96h) [229]. Further, our and other research groups have demonstrated the excellent cell growth-promoting potential of yeast

extracts in SFM [230-233]. A comprehensive evaluation of which serum replacement ingredients mots benefit cultured meat production should be performed independent of animal or plant origin. This study should incorporate cost, overall sustainability, sensory properties, and cell growth-promoting efficiency. It is highly likely that multiple ingredients with different origins may be required for efficient cell expansion of MuSCs.

7 Concluding remarks and future perspectives

In this section, the major conclusions of this work are presented, along with a brief discussion on future studies that could be pursued.

1. **We demonstrated that by-products with food grade quality are promising ingredients in a tailor-made SFM.**

We demonstrated that low-cost by-product hydrolysates have the potential to replace serum in muscle cell culture. Further, fractionated by-product hydrolysates have a more potent effect on cell growth and viability than crude hydrolysate extracts and the commercial serum replacer Ultroser G. However, due to the complexity of the hydrolysate mixtures, we did not identify the exact growth-promoting components in the fractions. A solution to this could be to further purify and fractionate the low molecular weight fractions to possibly achieve higher bioactivity and identify the growth-promoting mechnasims. MuSCs are prone to enter cellular senescence and become resistant to growth-promoting stimuli causing limited proliferation and differentiation potential. Thus, long-term culture and passaging in the fraction-based medium are required. As with any alternative serum replacement ingredients, more research is needed to determine food safety and possible cell phenotype alterations that can affect cultured meat quality. Finally, a media cost analysis should be performed. Nevertheless, this work provides a promising start for developing a SFM for cultured meat production while reducing the problematic reliance on medium cost-drivers such as commercial serum and growth factors.

2. **We demonstrated successful long-term expansion of MuSCs in bench-bioreactors and that low volume spinner flasks can be used as higher throughput and scaled-down models to optimize MuSCs expansion on MCs.**

Successful long-term up-scaling of bovine MuSCs was achieved in higher volume bench-bioreactors. The MuSC culture had a 90% high purity, and the cells retained their proliferative and migratory capacity after dissociation from MCs (paper III). By investigating different cultivation strategies from low volume spinner flasks to higher volume bench-bioreactors and monitor MuSCs development, we discovered that low volume spinner flask cultures with less controlled environments were robustly reproducible in bench-bioreactors. Therefore, low volume spinner flasks can be used as higher throughput and scaled-down models to optimize MuSCs expansion on MCs. This work provides much-needed publicly accessible data on MuSCs growth kinetics, behavior, and development, especially in high volume bioreactors, and is a promising start to optimize cell expansion parameters adapted to muscle cells. A one-parameter elimination method should be implemented to determine the critical cell culture parameters in bioreactor systems. Further, the reliance on non-optimal commercial MCs and FBS hinders the relevance of the findings to cultured meat production. Finally, optimal edible MC and the sustainable SFM should be combined to upscale the expansion of bovine MuSCs.

3. **We demonstrated proof-of-concept that edible MCs can easily be produced from by-product materials and support efficient cell expansion.**

Using simple techniques, three different edible MCs were produced from turkey collagen and ESM by-products (paper IV). The proof-of-concept MCs were resilient to degradation, had characteristics beneficial for cell adherence, and provided highly efficient cell expansion of bovine MuSCs in spinner flask culture. Consequently, by-products are excellent biomaterials for use in the production of edible MCs. However, the collagen-based MC size and pore size needs optimization. In addition, the individual morphologies should be optimized to ensure a higher bead uniformity which decreases cell expansion variability. Finally, further investigation of physical parameters (e.g., surface chemistry, crosslinking density, local Young's modulus) after production optimization is also required.

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ORIGINAL PAPERS AND MANUSCRIPTS

Paper I

I

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Screening of by-products from the food industry as growth promoting agents in serum-free media for skeletal muscle cell culture†

R. Christel Andreassen, [a,b] Mona E. Pedersen, [a] Kenneth A. Kristoffersen [a] and Sissel Beate Rønning *[a]

The most significant cost driver for efficient bio-production of edible animal proteins is the cell culture media, where growth factors account for up to 96% of the total cost. The culture media must be serum-free, affordable, contain only food-grade ingredients, be efficient to promote cell growth and available in massive quantities. The commercially available serum substitutes are expensive and not necessarily food-grade. Identifying inexpensive food-safe alternatives to serum is crucial. By-products from food production are available in massive quantities, contain potential factors that can promote growth and are promising ingredients for serum replacement. The main goal of this study was to explore if food-grade by-product materials can be used as growth promoting agents in skeletal muscle cell culture to develop a tailor-made serum free media. Different by-products, including chicken carcass, cod backbone, eggshell membrane, egg white powder and pork plasma were enzymatically or chemically hydrolyzed. The hydrolysates in addition to lyophilized pork plasma and yeast extract were further characterized by size-exclusion chromatography, elemental combustion analysis and degree of hydrolysis. The materials were used as supplement to or replacement of commercial serum and further evaluated for their effect on metabolic activity, cell proliferation and cell cytotoxicity in muscle cells cultured *in vitro*. Our results indicate that none of the materials were cytotoxic to the skeletal muscle cells. Hydrolysates rich in peptides with approximately 2–15 amino acids in length were shown to improve cell growth and metabolic activity. Of all the materials tested pork plasma hydrolysates and yeast extract were the most promising. Pork plasma hydrolysates increased metabolic activity by 110% and cell proliferation with 48% when cultured in serum-free conditions for 3 days compared with control cells cultured with full serum conditions. Most interestingly, this response was dependent on both material and choice of enzyme used. We suggest that these materials have the potential to replace serum during cultivation and as such be included in a tailor-made serum-free media.

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Introduction

The world's livestock sector is developing at an extraordinary rate to meet the growing demand for high-value animal protein. Livestock products are an excellent protein source with high nutritional value and an important source of essential micronutrients. However, the increasing animal production is not sustainable. The industry is compelled to look for alternative and more environmentally friendly ways to produce animal proteins. A groundbreaking new technology and promising alternative to traditional meat production is cultured meat. This meat will bypass animal production and can in theory be produced faster and more efficiently than conventional meat.[1] Cultured meat is made by harvesting a small biopsy of skeletal muscle cells from a living mammal, these cells are multiplied and grown to produce muscle tissue (*i.e.*, meat). A major challenge with this technology is the serum required for cell growth. In cell culture, fetal bovine serum (FBS) typically provide the necessary nutrient supplementation in culture media. FBS is the supernatant of clotted blood from a bovine fetus mainly collected by cardiac puncture. This serum contains an undefined cocktail of stimulating factors required to sustain cell growth and maintenance of most mammalian cells. Serum is expensive, the cost can be up to 95% of the total cost of the cell media, and the supply is lower than the demand.[2] In addition to being expensive, there are biosafety and ethical issues due to the nature of how FBS is

[a]*Nofima – Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 210, N-1431 Ås, Norway. E-mail: sissel.ronning@nofima.no*
[b]*Department of Nutrition, Institute of Basic Medical Sciences, Faculty of Medicine University of Oslo, Oslo, Norway*

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harvested. As such, serum supplementation is a limiting factor and cannot support sustainable large-scale protein production intended for human consumption.

Extensive research the last decade has been focused on the reduction or replacement of FBS as part of good cell culture practice (GCCP).[3] The challenge of producing a serum-free media (SFM) is to identify and replace the specific components in serum that promote cell growth and determine the optimal media composition to each cell type. Today, defined serum replacements are available for cell types commonly used in medical and industrial applications. These culture media compositions are not published for commercial reasons. However, typical components of serum include serum proteins (Albumin, Globulins), transport proteins (Transferrin, Transcortin, α1- and β1-Lipoprotein), attachment factors (Fibronectin, Laminin), enzymes, hormones (Insulin, Glucagon, Corticosteroids, Prostaglandins), growth factors and cytokines (EGF, FGF, NGF, ECCGF, PDGF, IGFs, Interleukins, Interferons, TGFs), fatty acids and lipids, vitamins, trace elements, carbohydrates, and nonprotein nitrogen.[4] To the best of our knowledge the commercially available serum substitute alternatives for primary skeletal muscle cells are not food-grade and the cost is still an issue.[3,5] Therefore, finding an inexpensive food-safe replacement for serum is of great interest.

By-products from food production are available in massive quantities and are promising ingredients for serum replacement. It is estimated that nearly 40–60% of farmed fish and animals total mass are classified as residual products with food-grade quality, including carcasses, blood and skin.[6] These residues have excellent nutritional value and contain proteins and other essential nutrients with potential bioactive properties.[7] Bioactive peptides can be released from by-products *via* hydrolysis and can exert beneficial effects on physiological functions beyond nutritional value, including cell growth regulation and promoting high cell culture performance.[6] Protein hydrolysates are a mixture of peptides with varying length and free amino acids. Hydrolysis is a process that involves breaking down proteins into smaller and more water-soluble peptides. Such hydrolysis can be performed using chemical or enzymatic processes.[8] Protein hydrolysates are reported to have growth promoting effects in mammalian cell culture.[9] Studies demonstrate that protein hydrolysates of eggshell membrane (ESM) regulate cellular functions and have anti-inflammatory properties.[8] Results from several other studies have indicated that peptides from blood sources have a wide range of bioactive effects, including blood sugar regulation, lowering blood pressure, as well as antioxidant and antimicrobial properties.[10–14] For many decades yeast extract have been used in microbiology as a stimulator of bacterial growth,[15] and the benefits of yeast extract in enhancing protein production and cell growth in some cell types are also documented.[16,17]

Based on the current knowledge, our goal was to investigate if hydrolyzed proteins from food-grade by-product materials and yeast extract could be used as growth promoting agents in skeletal muscle cell culture.

Results and discussion

Nine by-product hydrolysates produced by enzymatic or chemical hydrolysis, in addition to lyophilized pork plasma and yeast extract were characterized for their content of total nitrogen, molecular weight distribution (MWD) and degree of hydrolysis (DH) in dry material mass. Residual components such as lipids, sugars or ashes were not analyzed. The processed materials were further assessed for their effect on cell metabolic activity, proliferation, cytotoxicity and evaluated for their suitability to support cell growth during serum starvation in skeletal muscle cells.

Choice of by-product material and enzyme affected protein degradation during hydrolysis

The total nitrogen content (Fig. 1A) ranged from 52–95%. The degree of hydrolysis (DH) ranged between 20–60% (Fig. 1B), except chemically hydrolyzed ESM at 11%. The latter is probably due to the extra processing steps performed to remove salt compounds after chemical hydrolysis (*i.e.*, dialysis). The dialysis cutoff was set to 100–500 Da and all molecules of smaller sizes were eliminated. The DH of this material is therefore not directly comparable with the other crude hydrolysates. The choice of peptidase and the combination of exopeptidases and endopeptidases influence the DH. Alcalase is a nonspecific serine-type protease derived from *Bacillus licheniformis* and contains manly endopeptidases. Flavourzyme contains an exopeptidase-endopeptidase enzyme mix with mainly exopeptidases produced from *Aspergillus orizae*.[18] The differences in enzyme activity and selectivity under the reaction conditions used can explain the observed differences in DH of the same raw material (Fig. 1B).[19] This could be substrate dependent, for example, egg white powder digested with Alcalase or Flavourzyme showed approximately the same DH percentage.

In the current study, the results from the size exclusion chromatography (SEC) analysis with UV detection (214 nm) demonstrated that the 11 different materials tested had distinct MWD profiles (Fig. 1C). Peptide bonds absorb strongly at this wavelength, SEC in combination with UV detection is therefore one of the more commonly used instrumental setups in the study of food-grade hydrolysates.[20] There are however some limitations to this setup which influence how well the detected MWD profile reflect the actual MWD profile. Free amino acids are not well detected at this wavelength, while proteins and peptides are detected by absorption contributions from both peptide bonds and their side-groups resulting in scaling errors.[21] Despite these limitations, this method provides useful information to differentiate the materials used in this bio screening. The chromatograms were sectioned into four different size ranges (F1, F2, F3 and F4) and the relative area under the curve for each size rage was calculated (see Fig. 1C and ESI Fig. S-1†). Hydrolysates produced using Flavourzyme generally contain larger fractions of free amino acids (F4) (2-fold or greater) compared to Alcalase digestion of the same raw material (ESI Table 1†). These results are consistent with previously published studies of hydrolysates prepared using

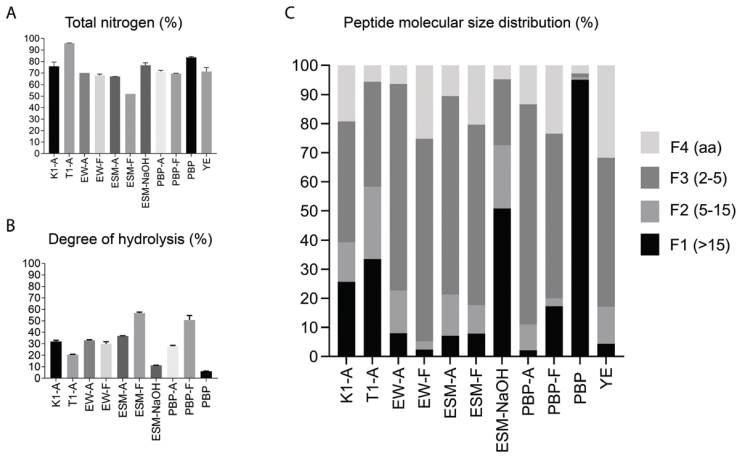

Fig. 1 Chemical characterization of tested materials and hydrolysates including, chicken carcass (K), cod backbone (T), egg whites (EW), eggshell membrane (ESM), pork blood plasma (PBP) and Yeast extract (YE). A: Total nitrogen content was analyzed with elemental combustion analysis and estimated by using the Kjeldhal method with a conversion factor of 6.25. B: Degree of hydrolysis was determined by the TNBS method. C: Size exclusion chromatography (SEC) at 214 nm (UV) was used to determine the molecular weight distribution (MWD) profile of the different materials and hydrolyzed by-products. The raw materials were either lyophilized, or hydrolysed with enzymes (Alcalase (A) or Flavourzyme (F)) or NaOH. The different fractions (F1–4) display the percentage of peptides with approximate amino acid length; >15 (F1), 5–15 (F2), and 2–5 (F3), in addition to free amino acids (aa)(F4).

these enzymes (*i.e.*, Alcalase and Flavourzyme).[18,22,23] Enzymatically hydrolyzed pork plasma had a very different MWD compared to the intact pork plasma powder, the latter predominantly consisted of peptides larger than 15 amino acids (95%, F1). The pork plasma hydrolysates produced in this study mainly contained peptides with approximately 2–5 amino acids in length (F3), this fraction was greater for plasma digested with Alcalase (75.7%) then with Flavourzyme (56.6%). The SEC results show that hydrolysates produced by enzymatic hydrolysis contained mainly large fractions of short peptides and less than 20% larger peptides (>15 amino acids) with two exceptions (chicken carcass and cod backbone digested with Alcalase).

Serum starvation dramatically impacted cell metabolism and proliferation

Serum starvation can cause cellular stress as serum is necessary for optimal cell growth.[24] Skeletal muscle cells cultured for 48 hours in either serum-free or serum-reduced media conditions (serum starvation) showed decreased metabolic activity and cell proliferation (Fig. 2). The metabolic activity was reduced by 18.4% in serum-reduced conditions, while a 52.2% reduction was observed in serum-free media compared to control cells cultivated in normal serum conditions. Likewise, reducing or removing serum decreased cell proliferation by 17.3% and 46%, respectively.

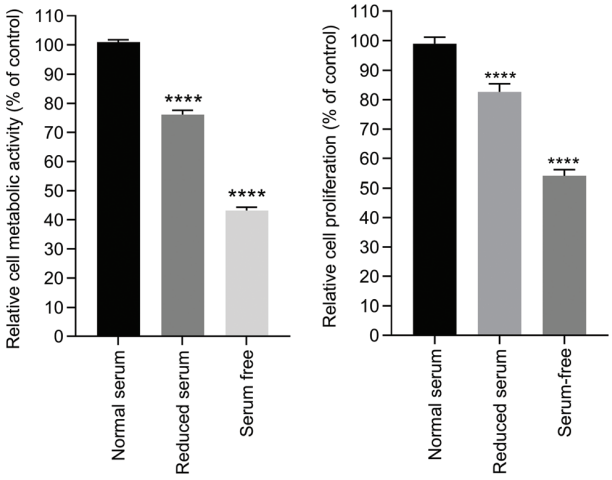

Fig. 2 Cell metabolic activity (ATP) and proliferation (DNA) were analyzed in bovine skeletal muscle cells using CellTiter-Glo ®Luminescent assay (Promega) and CyQuant™ Assay (Invitrogen), respectively. The muscle cells were cultivated for 48 hours in normal serum conditions (2% FBS and 2% Ultroser G), reduced serum (1% FBS and 1% Ultroser G) or serum-free conditions (0% FBS and 0% Ultroser G). The results are presented as mean ± SEM (n = 66 independent cell experiments seeded out in triplicates). Asterisk indicate significant differences compared to control (cells grown in normal serum conditions). ****$p < 0.0001$ determined by one-way ANOVA using Dunnett's multiple comparison test.

Supplementation with by-product hydrolysates and yeast extract to cell culture media enhanced cell growth when serum was present and restored cell growth when serum was reduced and depleted

By-product supplementation to cell culture media promoted muscle cell metabolic activity and proliferation (Fig. 3 and 4). This effect was dose-dependent, with an upper limit of 1 mg mL^{-1}, while media supplementation with 10 mg mL^{-1} nearly depleted cell growth and was highly cytotoxic to the cells. Interestingly, none of the materials were harmful to the skeletal muscle cells below the upper limit. Hydrolysates rich in small peptides with approximately 2–15 amino acids in

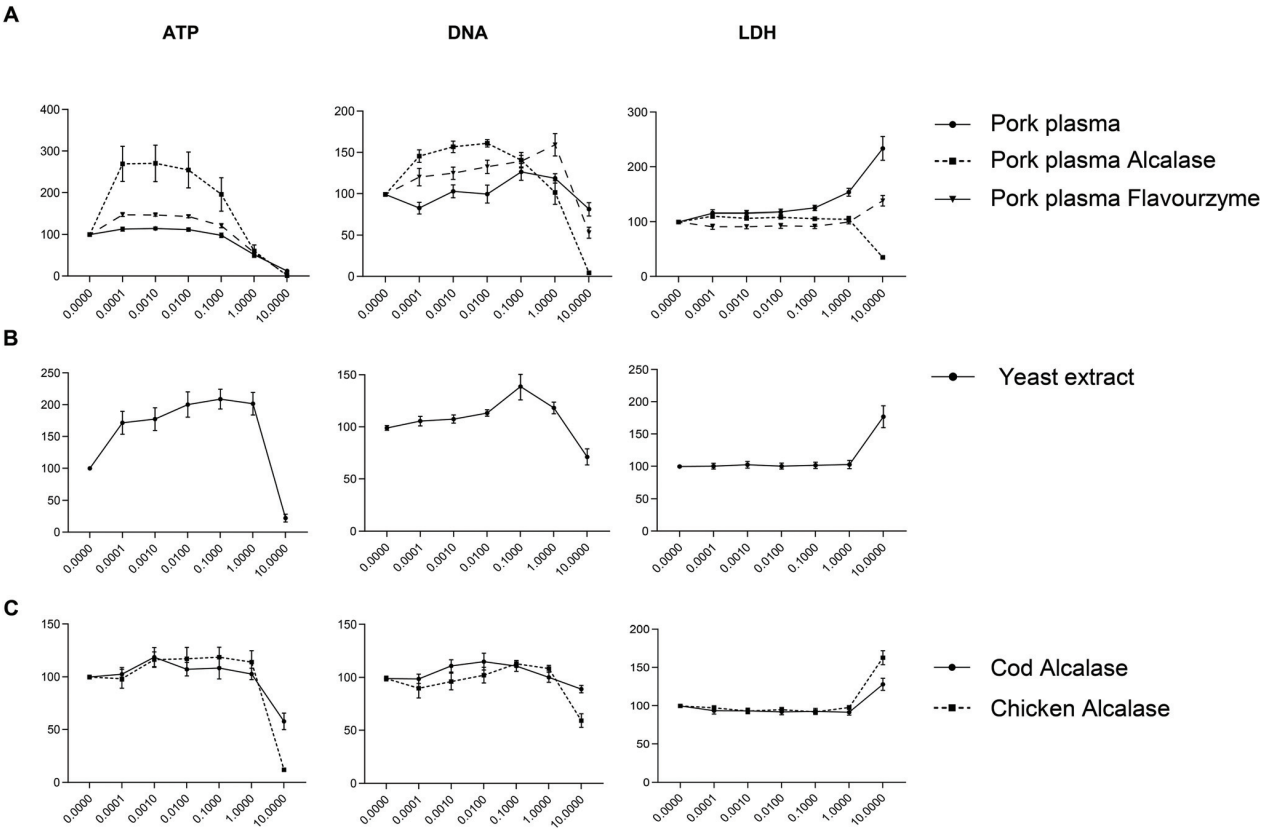

Fig. 3 Supplementation of hydrolysates from pork plasma by-products (A), yeast extract (B), chicken and cod by-products (C) to cell culture media enhanced cell growth in normal serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded out at density 3000 cells per well in 96-well plates and cultivated in media with normal serum conditions supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

length increased cell growth depending on the combination of enzyme and raw material. Of all the materials tested in this study, pork plasma hydrolysates and yeast extract were the most promising (Fig. 3A and B). Hydrolysates from pork plasma generated with Alcalase enzyme had the most potent effect on cell growth, with more than 150% increase in metabolic activity and 50% increase in cell proliferation compared to cells grown in normal serum conditions (Fig. 3A). To our knowledge, this is the first report of pork plasma by-product hydrolysates as a promoter of cell growth. However, a previous study reports a promising animal cell culture supplement containing lysate of porcine platelets.[25] Plasma contains hormones, antibodies, antigens, nutrients and proteins. The main plasma proteins are Albumins (50%), Globulins (alpha 15%, beta 15% and gamma 15%) and Fibrin (5%).[26–28] Fibrin plasma proteins have previously been shown to contain specific sequences that interacts with growth factors (GFs) and enzymatic cleavage of fibrin(ogens) can promote cell proliferation of both endothelial cells and fibroblasts.[29,30] In addition, fibrin(ogens) participate in cell-matrix interactions, forming a provisional matrix that is suggested to act as a reservoir for secreted GFs.[30,31] Interestingly, in this study neither intact pork plasma itself nor pork plasma hydrolysates generated with Flavourzyme had similar positive effects compared with pork plasma digested with Alcalase (Fig. 3A). In the presented experiments, yeast extract enhanced cell metabolic activity more than 100% and cell proliferation with almost 50% (Fig. 3B). Yeast extract is the water-soluble portion of autolyzed yeast. The MWD profile of the yeast extract used in this study shows that it consists of a large fraction of small peptides (63.9%), 31.7% free amino acids and less than 5% (4.4%) large peptides (>15 amino acids), based on the relative area. Like FBS, it is not fully understood which components of yeast extract are responsible for the growth-promoting effects.[16] Unlike FBS, yeast extract is food-grade with no ethical challenges of use. Cod backbone and chicken carcass hydrolysates did not influence cell activity in normal serum conditions (Fig. 3C), these hydrolysates contained 23.7–29.7% less frac-

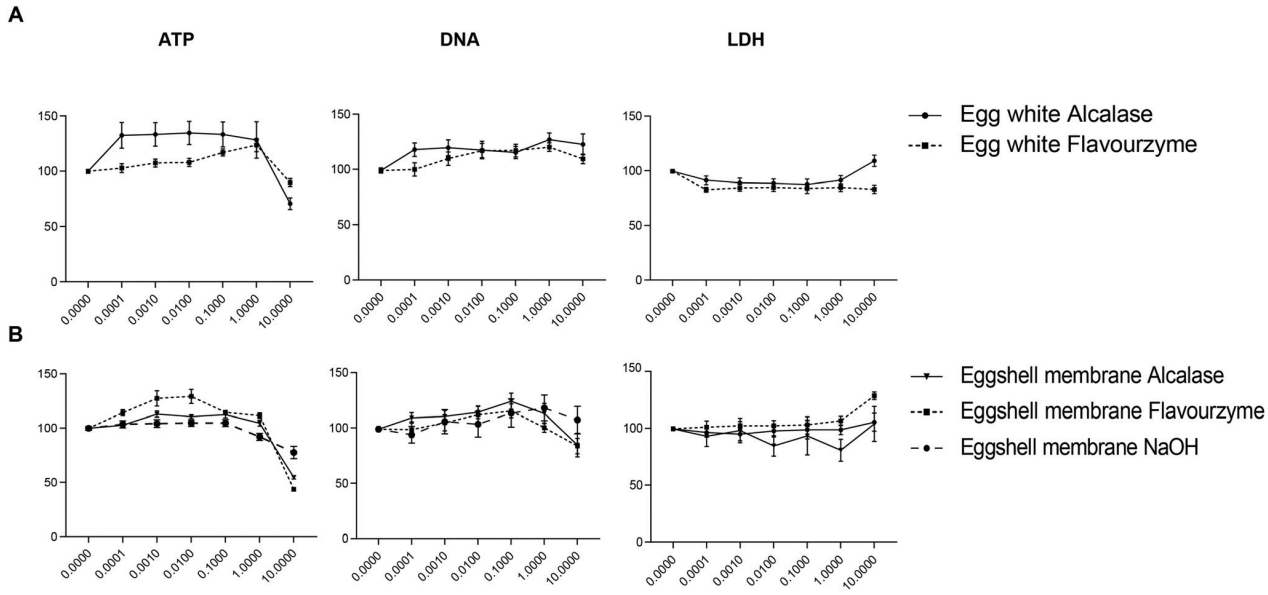

Fig. 4 Supplementation of hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) to cell culture media enhanced cell growth in normal serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded out at density 3000 cells per well in 96-well plates and cultivated in media with normal serum conditions supplemented with protein hydrolysates, extract in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

tions of small peptides and 23.6–31.4% more large peptides compared to pork plasma hydrolyzed with Alcalase. A similar pattern was observed with pork plasma and egg white powder, where treatment with Alcalase increased metabolic activity more compared with Flavourzyme digestion (Fig. 4A). Interestingly, the opposite trend was observed with eggshell membrane, where Flavourzyme hydrolysis increased the metabolic activity more compared with Alcalase and NaOH digestion (Fig. 4B). Previous reports show that animal derived hydrolysate mixtures of low molecular weight compounds (peptones) improved cell growth to a higher degree then a defined mixture of amino acids.[9,32] The SEC analysis data (ESI Table 2†) show that Flavourzyme digested raw material contained 2-fold larger fractions of free amino acids and 10–25% fewer small peptides (2–15 amino acids in length) compared to hydrolysates of the same by-product material produced with Alcalase. Bioactive peptides share common features, such as peptides of 2–20 amino acids in length.[7] The physiological functions of hydrolysates can be regulated by the amino acid sequence composition and length, which is dependent on by-product origin and protein degradation during hydrolysis. This demonstrate that not only the source of by-product, but also the choice of enzyme is important when generating hydrolysates with cell growth enhancing capabilities.

When serum was reduced by 50%, both pork plasma hydrolysates (Fig. 5A), yeast extract (Fig. 5B) and chicken carcass hydrolysate (Fig. 5C) digested with Alcalase were able to restore cellular function and enhance cell growth. In contrast, cod backbone hydrolysates were not able to restore cell function compared to cells grown in normal serum conditions (Fig. 5C). Likewise, egg white and eggshell membrane digested with Alcalase also restored cell function (Fig. 6A and B). Pork plasma and yeast extract were the only materials that were able to completely restore cell function in serum-free conditions. Plasma digested with Alcalase and yeast extract enhanced cell metabolic activity by over 100% and 50%, respectively (Fig. 7A and B). This was dose dependent and high concentrations (1 mg mL^{-1} and 10 mg mL^{-1}) was cytotoxic to the cells. Egg white hydrolysate restored cell metabolic activity to 86% (Fig. 8A), while the other hydrolysates had little or no effect on cellular function (Fig. 7 and 8).

The results showed that pork plasma hydrolysates and yeast extract can recover cell growth (both metabolism and proliferation) in reduced and serum-free conditions. It is possible that additional effects could be obtained by mixing different protein hydrolysates as the results presented in Fig. 7 suggest that pork plasma digested with Alcalase and yeast extract can replace serum during bovine muscle cell cultivation. In this study, the complete chemical profile of the tested materials was not analyzed, and the molecular mechanisms of the observed effects are not yet elucidated. The dose-response curves (Fig. 3–8) reach a plateau, which may be explained by the complex nature of the crude hydrolysates and materials that were used. The materials tested contain a mixture of peptides, some of which could inhibit cell growth as well as peptides that can promote cell growth.[33]

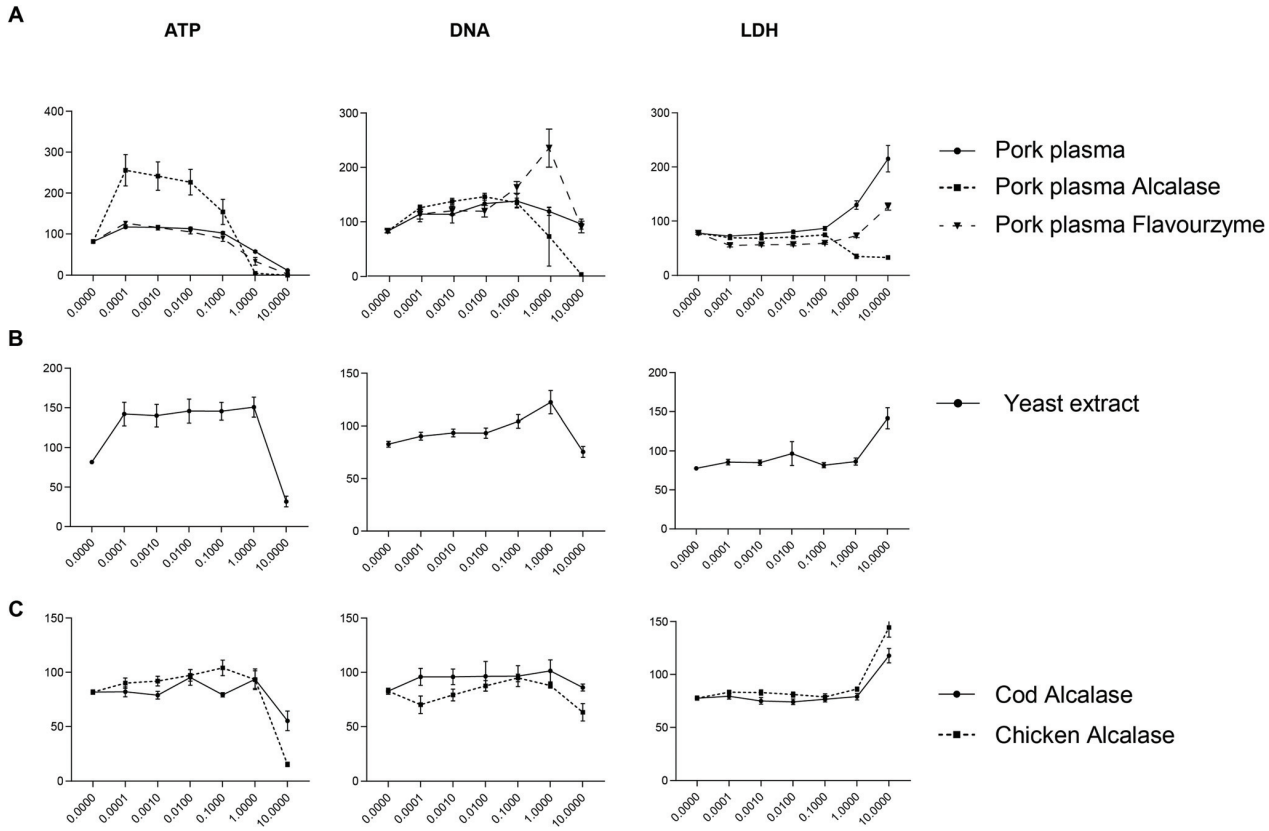

Fig. 5 By-product hydrolysates from pork plasma by-products (A), yeast extract (B), chicken and cod by-products (C) recover and enhance muscle cell metabolic activity and proliferation in reduced serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in DMEM media with 2% FBS and 2% Ultroser G (normal serum conditions). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with reduced serum conditions (1% FBS and 1% Ultroser G) supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

According to an analysis performed by the Good Food Institute, growth factors account for over 99% of the total cell culture medium cost.[34] An optimal media should be formulated with reduced raw materials costs without compromising the cellular yield. None of the materials tested in our study were cytotoxic to the skeletal muscle cells. In fact, the hydrolysates rich in peptides with approximately 2–15 amino acids in length improved cellular growth and metabolic activity. Most importantly, the materials tested are food-grade, inexpensive, easy to produce and presents a higher ethical quality compared to FBS. Since by-products are available in massive quantities, ingredients from such material will undoubtedly reduce the cost of cell culture media which is the most significant cost driver for cultured meat production. Secondly, this represents an industrial opportunity for the food industry to increase the value of by-products and at the same time contribute to the circular economy by reducing waste. Finally, discovery of bioactive peptides can create new business opportunities for other markets such as the pharmaceutical and nutraceutical industry. Research groups are continuously working to optimise big scale hydrolysis production to ensure minimal batch to batch variation and high-quality yield while reducing costs. Among the modern analytical approaches for discovery of bioactive constituents in complex mixtures is the use of chromatography-coupled bioassay where eluents of a separation (fractions) are directed to high-throughput bio-screening, an approach used to identify and characterize bioactive peptides from chicken hydrolysates.[35] Further studies are necessary to evaluate the chemical profile and elucidate the effects of specific hydrolysate fractions and yeast extract on cellular response in bovine muscle cells to optimize and formulate a tailor-made serum-free media for bovine muscle cells.

Materials and methods

Raw-material and chemicals

The cod backbone was provided by Sjømat AS (Oslo, Norway), and the mechanical chicken carcass residue was provided by Nortura (Hærland, Norway). Industrially made and patented

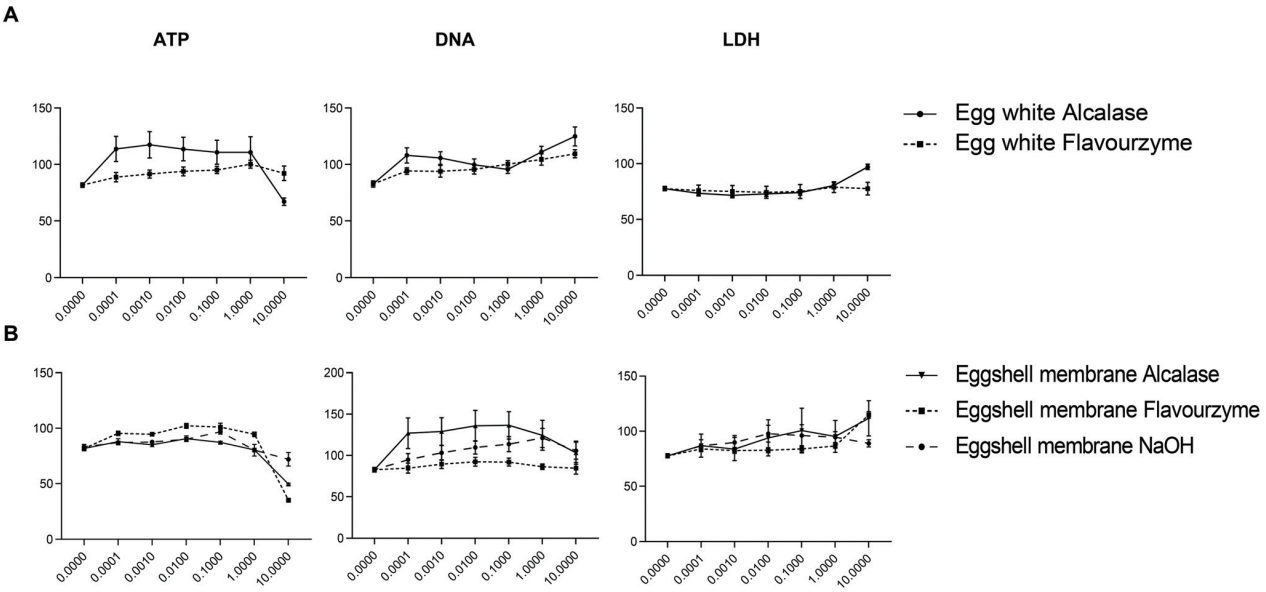

Fig. 6 By-product hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) recover and enhance muscle cell metabolic activity and proliferation in reduced serum conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in DMEM media with 2% FBS and 2% Ultroser G (normal serum conditions). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with reduced serum conditions (1% FBS and 1% Ultroser G) supplemented with protein hydrolysates in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

avian egg white powder and eggshell membrane were provided by Norilia (Oslo, Norway), and the whole pork blood was collected at the time of slaughter from a commercial abattoir Flesland (Hvalstad), Norway. Yeast extract was purchased from Duchefa Biochemies B.V (Haarlem, The Netherlands). The two food-grade enzymes used in this study were Alcalase and Flavourzyme (*Aspergillus oryzae*) purchased from Novozymes A/S (Bagsværd, Denmark). Ultroser G serum substitute was purchased from Pall Corporation (Port Washington, NY, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin solution 10 000 units per mL (P/S), Amphotericin B, and 0.05% trypsin/EDTA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Entactin-Collagen-Laminin (ECL) was purchased form Millipore Sigma (Burlington, MA, USA) and Collagenase from Sigma Aldrich (Merck KGaA, St Lois, MO, USA). All other reagents were from Sigma Chemicals Co. (St Lois, MO, USA) unless otherwise noted.

Preparation of protein hydrolysates

A total of nine different hydrolysates were produced with either Alcalase, Flavourzyme or by chemical hydrolysis with sodium hydroxide (NaOH). Cod and chicken by-products were hydrolyzed according to previously published protocol by G. Wubshet *et al.*[36] In short, samples were homogenized using a food processor. Hydrolysis was carried out in a Reactor-Radley™ jacketed vessel (Radleys, Saffron Walden, Essex, United Kingdom) under stirring (300 rpm, Radley Torque Value 200) at 50 °C, run for 60 minutes and terminated by heat inactivation (95 °C for 15 minutes). The mixture was centrifuged for 15 min at 4600*g*, resulting in a water phase supernatant, a fat phase and solid residue. The water phase supernatant was lyophilized to produce a hydrolysate protein powder. The eggshell membrane (ESM) was produced and harvested using a patented process by Biovotec and washed with 0.1 M hydrochloric acid (HCL) for 10 minutes under 300 rpm stirring and washed twice with dH_2O before it was freeze dried and milled to a powder with an average particle size of 0.25 nm (Helium-Neon Laser Optical System, Sympatic Inc., Clausthal-Zellerfeld, Germany). 10 g ESM powder was mixed with 200 mL dH_2O in a 0.2 L pyrex flasks under stirring (300 rpm) at 50 °C and run for approximately 12 hours. When the temperature reached 50 °C, 2% enzyme (Alcalase or Flavourzyme) or 5% w/v NaOH was added to initiate the reaction. Enzymatic hydrolysis was inactivated by heating (95 °C) for 15 min. Only the NaOH hydrolyzed ESM reaction mixture was dialyzed (Spectrum laboratories, Inc. Biotech CE tubing MWCO 100–500 Da, Repligen Europe B.V, The Netherlands) for five days with two to three daily water changes (dH2O) and inactivated by pH neutralizing with HCL. The hydrolysate mixtures were centrifugated at 4600*g* for 15 minutes before the water phase supernatant was vacuum filtrated and lyophilized.

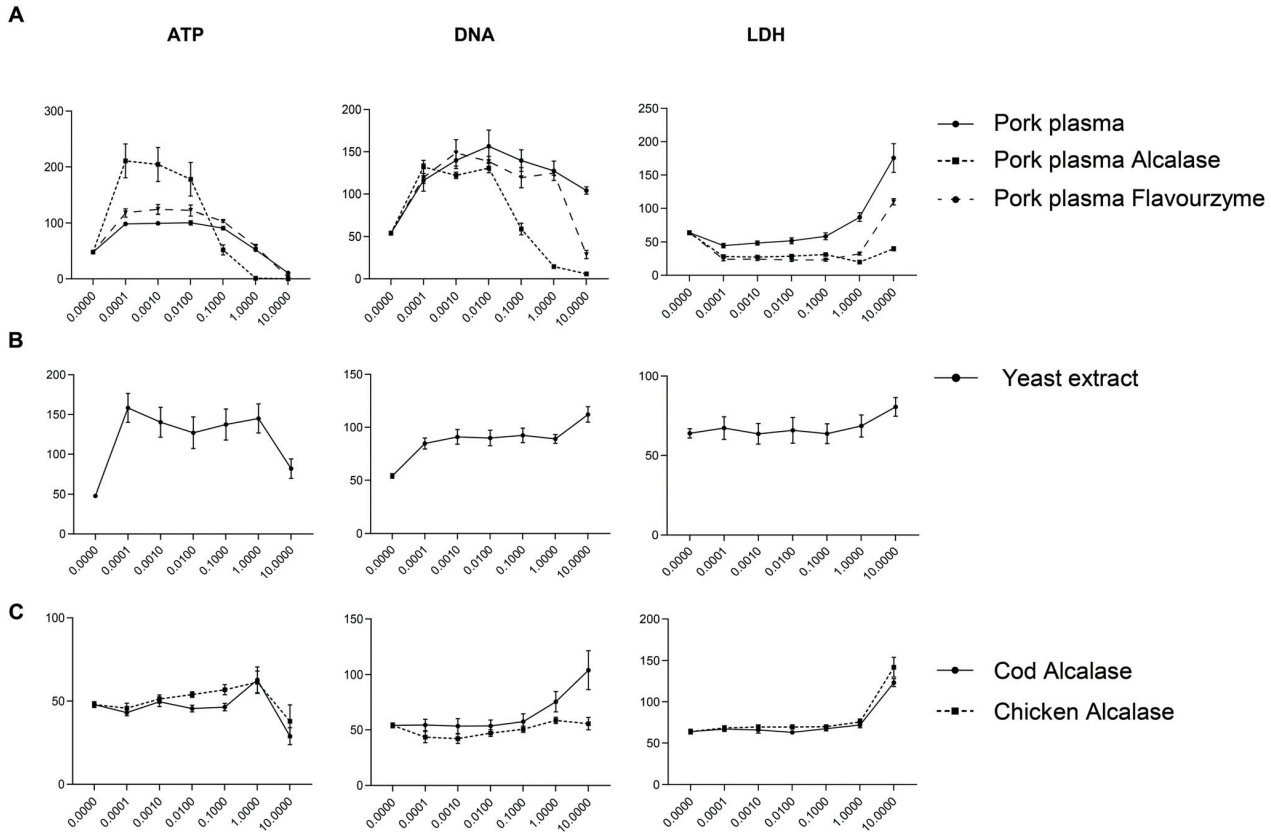

Fig. 7 Alcalase digested pork plasma (A), yeast extract (B) and chicken and cod by-products (C) recover and boost cell growth in serum-free conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with serum-free conditions (0% FBS and % Ultroser G) supplemented with protein hydrolysates, lyophilized pork plasma or yeast extract in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

Plasma is the liquid cell-free part of blood that has been treated with anticoagulants. Blood from six-month-old pigs were collected during slaughter by open draining and added sodium citrate (0.6% w/v) to prevent blood clotting. The blood was fractionated by 30 min centrifugation (10 000*g*), resulting in a liquid phase supernatant of plasma and a solid phase of cells. The cell fraction was discarded, and the liquid phase was lyophilized by freeze drying. The pork plasma and egg white dry powder were hydrolyzed with Alcalase and Flavourzyme using the same protocol previously described for enzymatically hydrolyzed ESM power. The hydrolysates and materials used in this study are listed in Table 1.

Total nitrogen, carbon and sulfur determination

Nitrogen, carbon and sulfur were determined using elemental combustion analysis (varioEL CER 2019, CHNS system, Elementar, Langenselbold, Germany) from solid dry powder samples that are converted to N_2, CO_2 and SO_2, the concentrations were measured by gas chromatography. The calibration curve was generated using 3 aliquots of pure sulfanilamide standards. The Kjeldhal method was used to estimate total nitrogen content with a conversion factor of 6.25.[37]

Degree of hydrolysis (DH)

The DH was measured using the trinitrobenzene sulfonate (TNBS) method described by Kristoffersen *et al.*[38] The buffer (0.21 M sodium phosphate buffer; pH 8.2) was prepared and stirred for 60 minutes at room temperature. Calibration solutions were prepared by a dilution series containing 0, 0.075, 0.15, 0.3, 0.6, 0.9, 1.2 and 1.5 mM Leucine in 1% SDS solution. The samples were prepared by dissolving 10 mg mL^{-1} hydrolysate powder in 0.1 M Tris-HCl pH 8.0 buffer followed by a dilution in 1% SDS-solution to 0.5 mg mL^{-1}. All samples and calibration solutions were measured in triplicate in Pierce™ 96-Well Polystyrene Plates, Corner Notch (Thermo Fisher Scientific, Waltham, MA, USA). 15 μL sample (reference or calibration solution) was added per well followed by the addition of 45 μL 0.21 M sodium phosphate buffer (pH 8.2) and 45 μL

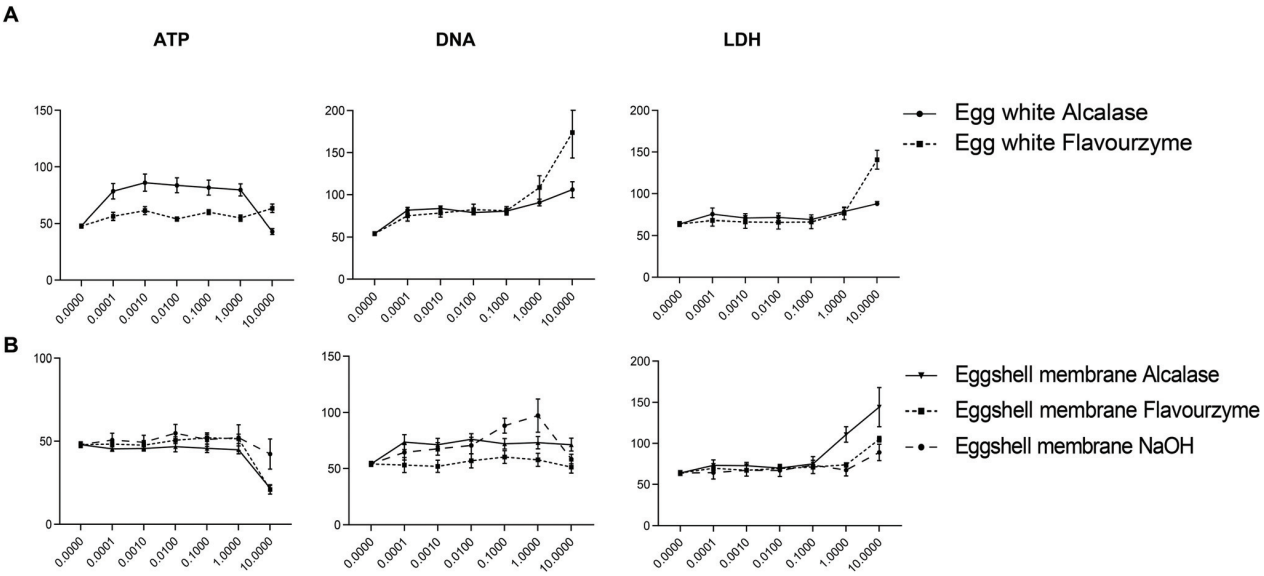

Fig. 8 By-product hydrolysates from egg white by-products (A), and eggshell membrane by-products (B) recover muscle cell metabolic activity and proliferation in serum-free conditions. The graphs show the relative cell metabolic activity (ATP), proliferation (DNA) or cytotoxicity (LDH) (*y*-axis) in skeletal bovine muscle cells after 48 hours incubation with the different materials compared to control cells, *i.e.*, untreated cells grown in normal serum conditions (DMEM media with 2% FBS and 2% Ultroser G). Cells were seeded 3000 per well in 96-well plates in triplets cultivated in media with serum-free conditions (0% FBS and % Ultroser G) supplemented with protein hydrolysates in dilution series with concentrations ranging from 0.0001–10 mg ml^{-1} (*x*-axis). Luminescence, fluorescence or absorbance were measured using CellTiter-Glo ®Luminescent assay (Promega), CyQuant™ Assay (Invitrogen), and Cytotoxicity Detection Kit (Roche Applied Science). Data represent mean ± SEM (n = 3–4 independent cell experiments seeded out in triplicates).

Table 1 Raw materials and hydrolysates used

Material	Hydrolysis/enzyme	Abbreviations
Pork plasma	—	PBP
	Alcalase	PBP-A
	Flavourzyme	PBP-F
Chicken carcass	Alcalase	K1-A
Cod backbone	Alcalase	T1-A
Egg white powder	Alcalase	EW-A
	Flavourzyme	EW-F
Eggshell membrane	Alcalase	ESM-A
	Flavourzyme	ESM-F
	NaOH	ESM-NaOH
Yeast extract	—	YE

TNBS solution (0.05% w/v in water). The plate was sealed with a sticker and wrapped in aluminum foil to avoid UV degradation during the one-hour incubation time at 50 °C. After incubation, 90 μL 0.1 M HCl was added to all wells before absorbance was measured at 340 nm using a BioTek Synergy™ H1 spectrophotometer (BioTek Instruments, Winooski, VT, USA). The DH% values were then calculated according to eqn (1), using h_{tot} estimated from total nitrogen estimations from elemental combustion analysis (h is the number of cleaved peptide bonds).[39] Protein content analysis data is presented in Fig. 1A.

$$\mathrm{DH} = \frac{h}{h_{tot}} \times 100\% \quad (1)$$

Size exclusion chromatography

The SEC was preformed according to Wubshet *et al.*[36] 2 mg mL^{-1} injection solutions of standards and rehydrated hydrolysates (1% w/v), filtrated using Millex-HV PVDF 0.45 μm 33 mm filter (Millipore Sigma, Burlington, MA, USA) of the supernatants were directly used as injection solution without further modifications. Chromatographic separation of standards and samples was performed with a Thermo Scientific Dionex UltiMate 3000 Standard System (Thermo Fisher Scientific, Waltham, MA, USA). The injection volume was 10 μL for the standards and 15 μL for samples. Separation was performed at 25 °C using a BioSep-SEC-s2000 column (300 × 7.8 mm, Phenomenex, Torrence, CA, USA). The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion 30 : 70 (v/v), containing 0.05% trifluoroacetic acid. Isocratic elution was carried out using a flow rate of 0.9 mL min^{-1} for 20.0 minutes. Between 20.0 and 20.1 minutes the mobile phase was changed to NaH_2PO_4 (0.10 M) and maintained until 23.0 minute for column cleaning. Elution conditions were restored between minute 23.0 and 23.1 and the column was equilibrated for an additional 27 minutes. Chromatographic runs were controlled from Chromeleon™ Chromatography Data System (CDS) software (Thermo Fisher Scientific, Waltham, MA, USA). From chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was used in the M_w calculations. The retention times of analytical standards were obtained from manual peak picking

Table 2 Content of maintenance and experimental media

Material	Maintenance	Experimental		
		Normal serum (S)	Reduced serum (RS)	Serum free (SF)
DMEM	500 mL	50 mL	50 mL	50 mL
FBS	2%	2%	1%	0%
Ultroser G	2%	2%	1%	0%
P/S	10 000 units per mL	0 units per mL	0 units per mL	0 units per mL
Fungizone	250 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}

Chromeleon (CDS). The retention times of the standards were used to construct a third polynomial (r^2 = 0.97) fitted calibration curve.[40] The retention times for the standards are presented in ESI Table S-2.† Finally, M_w were calculated using PSS winGPC UniChrom V 8.00 (Polymer Standards Service, Mainz, Germany) for each chromatogram. The calculation from the software was based on a slicing method, similar to those previously used for analysis of protein hydrolysates.[41]

Bovine primary skeletal muscle cell isolation

The muscle cells were extracted from *Longissimus thoracis* (beef sirloin, Nortura AS, Rudshøgda, Norway) as previously described.[42,43] In short, muscle biopsy samples (1–2 g) were digested with 0.72 mg mL^{-1} collagenase in 10 mL DMEM containing 10 000 units per mL P/S and 250 μg mL^{-1} amphotericin B for 1 hour at 37 °C with 70 rpm shaking. The tissue was further digested for 25 minutes with 0.05% trypsin/EDTA and added 10% FBS for enzyme inactivation, this step was repeated three times before the cells were pooled. To purify the myogenic cells (*i.e.*, fibroblast removal), the cells were incubated in uncoated 25 cm^2 cell culture flasks for one hour at 37 °C and 5% CO_2 in maintenance culture media containing DMEM medium supplemented with 2% fetal bovine serum (FBS), 2% Ultroser G, 250 μg mL^{-1} fungizone, and 10 000 units per mL penicillin/streptomycin (P/S. Fibroblasts adhere to the plastic and the non-adhering primary muscle cells were collected and seeded into cell flasks coated with 1 mg mL^{-1} Entactin-Collagen-Laminin (ECL). When the cells reached 70–80% confluence they were harvested in freezing media (8% dimethyl sulfoxide in DMEM media) and stored in a liquid nitrogen tank.[42,43]

Cell culture and treatment

Primary skeletal muscle cells were kept at 37 °C and 5% CO_2 in maintenance culture media containing DMEM medium supplemented with 2% fetal bovine serum (FBS), 2% Ultroser G, 250 μg mL^{-1} fungizone, and 10 000 units per mL penicillin/streptomycin (P/S). Ultroser G is a commercially available serum substitute with semi-defined composition and is considered to have a concentration five times higher than fetal calf serum. The experimental culture media had three different serum conditions (serum free, reduced serum and normal serum) and did not contain P/S or amphotericin B. The constituents of the different culture media used in this study are listed in Table 2. All experiments were performed in the 2nd or 3rd cell culture passage.

Determination of cell proliferation, viability and cytotoxicity

The cells were seeded 3000 cells per well grown in ECL coated 96-well plates (BD Falcon, Franklin Lakes, NJ, USA) in triplicates and kept at 37 °C and 5% CO_2 in maintenance culture medium. 24 hours after seeding, the cells were treated with increasing concentrations (0.0001–10 mg mL^{-1}) of hydrolysates in experimental media with normal serum, reduced serum or serum free conditions (Table 2). 48 hours after treatment cell viability and proliferation was analyzed using CellTiter-Glo ®Luminescent assay (Promega, Madison, WI, USA) and CyQuant™ Assay (Invitrogen, Carlsbad, CA, USA), respectively. Muscle cell cytotoxicity was measured as lactate dehydrogenase (LDH) leakage into the media using Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). Luminescent, fluorescent and absorbance signals, respectively, were measured using Synergy H1 hybrid multi-mode microplate reader (Biotek, Winooski, VT, USA).

Data treatment

Each cell culture experiment was seeded out in triplicates and repeated at least three individual biological replicates. The experiments (cell metabolic activity, proliferation and cytotoxicity) were performed in triplicates and repeated three to four times. Data are presented as mean ± SEM. Significant variance by treatments in comparison to the control sample (cells grown in normal serum conditions) was determined by one-way ANOVA using Dunnett's multiple comparison test. Differences were considered significant at $p < 0.05$. All statistical analysis was performed in Graph Pad Prism version 7.04 (GraphPadSoftware, La Jolla, CA, USA), and presented in detail in Tables S-3 to S-13.†

Conclusion

In this study we show that choice of by-product material and enzyme have impact on protein degradation during hydrolysis. The results show that none of the hydrolysates were harmful to the skeletal muscle cells. In fact, hydrolysates rich in peptides with approximately 2–15 amino acids in length enhanced cell growth. Most interestingly, this response was dependent on both material and choice of enzyme used. Of all the by-pro-

ducts tested pork plasma hydrolysates and yeast extract were the most promising materials. We suggest that these materials have the potential to replace serum during cultivation and as such be included in a tailor-made serum-free media.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Ingrid Måge is acknowledged for excellent statistical assistance. Rune Rødbotten is greatly acknowledged for generously providing pork slaughter blood and Norilia for providing avian by-products. Internal funding from Nofima through the project "PepTek" (no. 11889) is acknowledged. The Research Council of Norway is greatly acknowledged for the financial support through the project "GrowPro" (no. 280381). Funding from Norwegian Fund for Research Fees for Agricultural Products (no. 262300) is also acknowledged.

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Electronic Supplementary Material (ESI) for Food & Function.

Supporting Information

Screening of by-products from the food industry as growth promoting agents in serum-free media for skeletal muscle cell culture

R. Christel Andreassen,[ab] Mona E. Pedersen,[a] Kenneth A. Kristoffersen,[a] Sissel B. Rønning [a]*

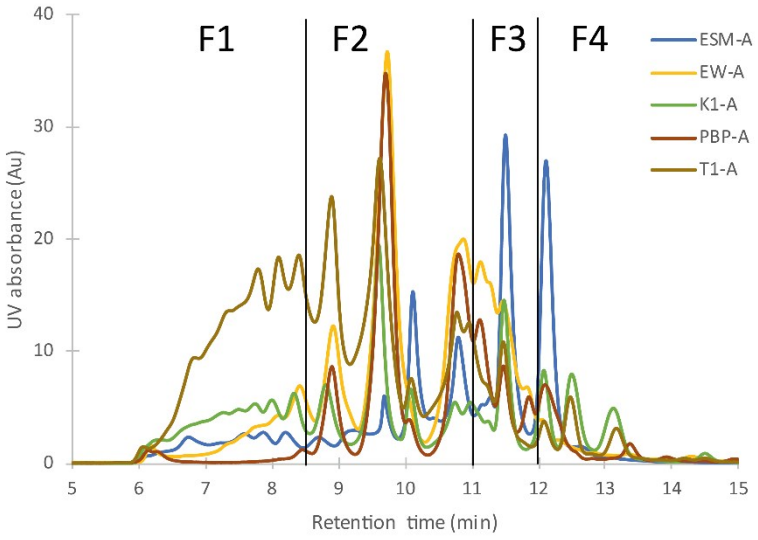
a) Nofima - Norwegian Institute of Food, Fisheries and Aquaculture Research, P.O. Box 210, N-1431 Ås, Norway. b) Institute of basic medical sciences, Faculty of medicine, Norwegian University of Oslo, sognsvannsveien 9, 0372 Oslo, Norway.

** Corresponding author: Sissel Beate Rønning, sissel.ronning@nofima.no*

This document contains:

One figure containing all size exclusion chromatograms (SEC). 13 tables; the first contains M_w and molecular weight distribution (MWD) values, the second table contains retention times for all standards used to calculate M_w, The remaining tables contains statistical data from one-way ANOVA using Dunnett`s multiple comparison test for all treatments.

A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F4
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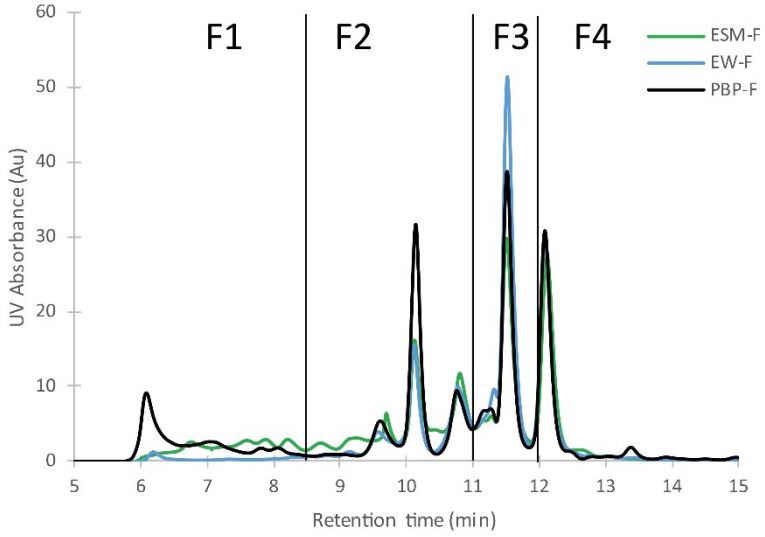
EW-A
K1-A
PBP-A
T1-A
UV absorbance (Au)
Retention time (min)

B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F3
F4
ESM-F
EW-F
PBP-F
UV Absorbance (Au)
Retention time (min)

C

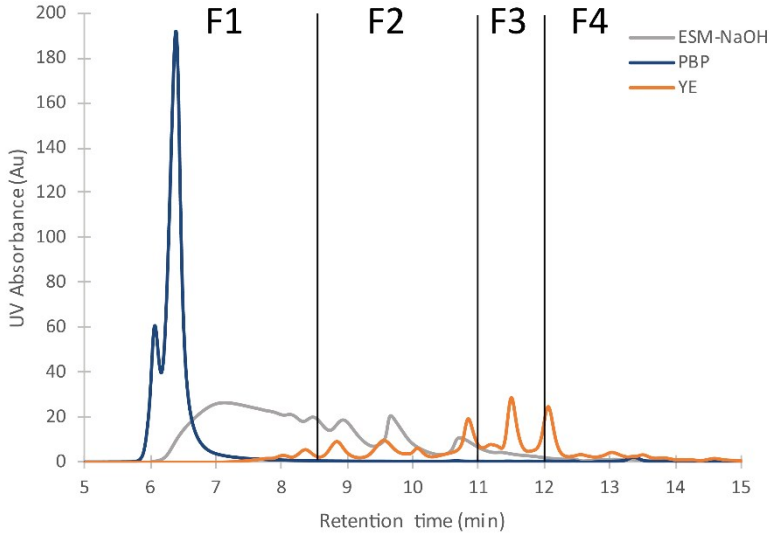
F1
F2
F3
F4
ESM-NaOH
PBP
YE
UV Absorbance (Au)
Retention time (min)

Fig S-1: All SEC chromatograms from 5-15 min of the 11 different materials studied. A: Eggshell membrane (ESM), egg whites (EW), cod (T1), Chicken (K1), and pork blood plasma (PBP) hydrolyzed with Alcalase (A). B: ESM, EW, and PBP hydrolyzed with Flavourzyme (F). C: ESM chemically hydrolyzed with NaOH, and lyophilized PBP and yeast extract (YE) powder.

Table S-1: M_w (g/mol) and molecular weight distribution (MWD) values for all samples.

Sample	M_w	Area	F1 (%)	F2 (%)	F3 (%)	F4 (%)
ESM-A	1137,6	46,37	7,1	14,3	68,1	10,5
ESM-F	1054,6	31,31	7,9	9,8	62,0	20,3
ESM-NaOH	4128,0	82,82	50,9	21,7	22,6	4,9
EW-A	1222,2	46,43	8,0	14,7	71,0	6,3
EW-F	707,8	28,91	2,4	2,8	69,7	25,1
K1-A	2467,6	31,59	25,7	13,6	41,5	19,2
PBP	16831,0	61,45	95,0	0,9	1,3	2,9
PBP-A	1005,3	30,54	2,1	8,9	75,7	13,3
PBP-F	2923,8	34,79	17,3	2,7	56,6	23,5
T1-A	2791,6	66,07	33,5	24,9	36,0	5,6
YE	723,7	38,35	4,4	12,7	51,2	31,7

All samples were measured once

Table S-2: The molecular weights (Mw) and the retention times (RT) for the analytical standards used for the BioSep-SEC-s2000 column calibration.

Compound name	Mw (g/mol)	RT (min)
Albumin from chicken egg white	44287	6.071
Carbonic anhydrase	29000	6.003
Lysozyme	14300	6.626
Aprotinin from bovine lung	6511	6.865
Insulin chain B oxidized from bovine pancreas	3496	8.763
Renin substrate tetradecapeptide porcine	1759	8.133
Angiotensin II human	1046	8.724
Bradykinin Fragment 1-7	757	9.208
[D-Ala^2]-leucine enkephalin	570	11.377
Val-Tyr-Val	379	10.925
Tryptophan	204	11.950

The column was calibrated using the mean of three replicates of each standers.

Table S-3 to S-13: Statistics of all treatments, significant variance by treatments in comparison to the control sample (cells grown in normal serum conditions), analysed by one-way ANOVA using Dunnett`s multiple comparison test of all samples. Asterix indicate significant differences, *<0.05, **<0.001, *** <0.001, ****p<0.0001. Abbreviations: ESM (eggshell membrane), ns (no significant difference).

TABLE S-3: COD ALCALASE

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-2,645	-20,13 to 14,84	No	ns	0,9976
		Control vs. 1	-18,97	-36,45 to -1,487	Yes	*	0,0277
		Control vs. 10	-7,463	-24,94 to 10,02	No	ns	0,7490
		Control vs. 100	-8,533	-26,01 to 8,949	No	ns	0,6345
		Control vs. 1000	-2,939	-20,42 to 14,54	No	ns	0,9954
		Control vs. 10000	42,08	24,6 to 59,56	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	17,86	2,416 to 33,3	Yes	*	0,0163
		Control vs. 1	20,94	5,499 to 36,39	Yes	**	0,0032
		Control vs. 10	4,649	-10,79 to 20,09	No	ns	0,9302
		Control vs. 100	20,59	5,145 to 36,03	Yes	**	0,0039
		Control vs. 1000	6,31	-9,135 to 21,75	No	ns	0,7813
		Control vs. 10000	44,59	29,14 to 60,03	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	56,81	45,02 to 68,6	Yes	****	<0,0001
		Control vs. 1	50,2	38,41 to 61,99	Yes	****	<0,0001
		Control vs. 10	54,36	42,57 to 66,15	Yes	****	<0,0001
		Control vs. 100	53,49	41,7 to 65,28	Yes	****	<0,0001
		Control vs. 1000	37,05	25,26 to 48,84	Yes	****	<0,0001
		Control vs. 10000	71,03	59,24 to 82,82	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	0,3265	-15,92 to 16,58	No	ns	>0,9999
		Control vs. 1	-11,9	-28,15 to 4,345	No	ns	0,2360
		Control vs. 10	-16,02	-32,27 to 0,2337	No	ns	0,0549
		Control vs. 100	-11,73	-27,98 to 4,52	No	ns	0,2488
		Control vs. 1000	-1,146	-17,4 to 15,1	No	ns	0,9997
		Control vs. 10000	10,11	-9,394 to 29,62	No	ns	0,5747
	Reduced-serum conditions	Control vs. 0.1	3,038	-17,92 to 24	No	ns	0,9978
		Control vs. 1	3,069	-17,89 to 24,03	No	ns	0,9977
		Control vs. 10	2,575	-18,39 to 23,53	No	ns	0,9995
		Control vs. 100	2,381	-18,58 to 23,34	No	ns	0,9996
		Control vs. 1000	-2,402	-23,36 to 18,56	No	ns	0,9996
		Control vs. 10000	12,9	-8,062 to 33,86	No	ns	0,4013
	Serum-free conditions	Control vs. 0.1	44,61	23,15 to 66,08	Yes	****	<0,0001
		Control vs. 1	45,5	24,03 to 66,96	Yes	****	<0,0001
		Control vs. 10	45,32	23,86 to 66,78	Yes	****	<0,0001
		Control vs. 100	41,53	20,07 to 63	Yes	****	<0,0001
		Control vs. 1000	23,49	2,026 to 44,95	Yes	*	0,0260
		Control vs. 10000	-4,988	-26,45 to 16,48	No	ns	0,9782
LDH	Normal serum conditions	Control vs. 0.1	6,264	-3,668 to 16,2	No	ns	0,3784
		Control vs. 1	6,511	-3,421 to 16,44	No	ns	0,3400
		Control vs. 10	7,828	-2,104 to 17,76	No	ns	0,1785
		Control vs. 100	7,105	-2,827 to 17,04	No	ns	0,2581
		Control vs. 1000	8,281	-1,651 to 18,21	No	ns	0,1391
		Control vs. 10000	-28,29	-38,22 to -18,36	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	20,09	11,93 to 28,26	Yes	****	<0,0001
		Control vs. 1	24,58	16,41 to 32,75	Yes	****	<0,0001
		Control vs. 10	25,41	17,24 to 33,57	Yes	****	<0,0001
		Control vs. 100	23,02	14,86 to 31,19	Yes	****	<0,0001
		Control vs. 1000	20,5	12,34 to 28,67	Yes	****	<0,0001
		Control vs. 10000	-18,21	-26,37 to -10,04	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	32,74	25,23 to 40,24	Yes	****	<0,0001
		Control vs. 1	33,8	26,3 to 41,31	Yes	****	<0,0001
		Control vs. 10	36,67	29,16 to 44,17	Yes	****	<0,0001
		Control vs. 100	32,19	24,68 to 39,7	Yes	****	<0,0001
		Control vs. 1000	27,71	20,2 to 35,21	Yes	****	<0,0001
		Control vs. 10000	-23,65	-31,16 to -16,15	Yes	****	<0,0001

TABLE S-4: CHICKEN ALCALASE

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	1,667	-17,61 to 20,95	No	ns	0,9997
		Control vs. 1	-16,55	-35,83 to 2,726	No	ns	0,1207
		Control vs. 10	-17,46	-36,74 to 1,817	No	ns	0,0912
		Control vs. 100	-18,88	-38,15 to 0,4023	No	ns	0,0574
		Control vs. 1000	-14,24	-33,52 to 5,035	No	ns	0,2298
		Control vs. 10000	87,81	68,54 to 107,1	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	9,842	-4,122 to 23,81	No	ns	0,2712
		Control vs. 1	7,941	-6,023 to 21,9	No	ns	0,4834
		Control vs. 10	2,574	-11,39 to 16,54	No	ns	0,9933
		Control vs. 100	-4,269	-18,23 to 9,695	No	ns	0,9256
		Control vs. 1000	6,632	-7,332 to 20,6	No	ns	0,6596
		Control vs. 10000	84,43	70,47 to 98,39	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	54,19	40,40 to 67,99	Yes	****	<0,0001
		Control vs. 1	48,62	34,83 to 62,42	Yes	****	<0,0001
		Control vs. 10	45,93	32,13 to 59,73	Yes	****	<0,0001
		Control vs. 100	42,98	29,19 to 56,78	Yes	****	<0,0001
		Control vs. 1000	38,44	24,64 to 52,23	Yes	****	<0,0001
		Control vs. 10000	62,05	48,25 to 75,85	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	9,18	-8,264 to 26,62	No	ns	0,5599
		Control vs. 1	2,856	-14,59 to 20,3	No	ns	0,9957
		Control vs. 10	-3,168	-20,61 to 14,28	No	ns	0,9937
		Control vs. 100	-13,86	-31,3 to 3,584	No	ns	0,1717
		Control vs. 1000	-9,402	-26,85 to 8,042	No	ns	0,5360
		Control vs. 10000	39,83	20,31 to 59,34	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	28,78	12,27 to 45,3	Yes	****	<0,0001
		Control vs. 1	19,79	3,277 to 36,31	Yes	*	0,0119
		Control vs. 10	11,37	-5,142 to 27,89	No	ns	0,2913
		Control vs. 100	4,035	-12,48 to 20,55	No	ns	0,9723
		Control vs. 1000	11,08	-5,439 to 27,59	No	ns	0,3164
		Control vs. 10000	35,72	19,2 to 52,23	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	55,51	40,69 to 70,34	Yes	****	<0,0001
		Control vs. 1	56,96	42,13 to 71,78	Yes	****	<0,0001
		Control vs. 10	51,82	36,99 to 66,64	Yes	****	<0,0001
		Control vs. 100	48,34	33,52 to 63,17	Yes	****	<0,0001
		Control vs. 1000	40,39	25,57 to 55,22	Yes	****	<0,0001
		Control vs. 10000	43,36	28,54 to 58,19	Yes	****	<0,0001
LDH	Normal serum conditions	Control vs. 0.1	2,515	-7,035 to 12,06	No	ns	0,9614
		Control vs. 1	6,583	-2,967 to 16,13	No	ns	0,2920
		Control vs. 10	4,849	-4,701 to 14,4	No	ns	0,5976
		Control vs. 100	7,671	-1,879 to 17,22	No	ns	0,1646
		Control vs. 1000	1,98	-7,57 to 11,53	No	ns	0,9882
		Control vs. 10000	-63,09	-72,64 to -53,54	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	16,45	7,239 to 25,66	Yes	****	<0,0001
		Control vs. 1	16,68	7,471 to 25,89	Yes	****	<0,0001
		Control vs. 10	18,66	9,444 to 27,87	Yes	****	<0,0001
		Control vs. 100	20,91	11,7 to 30,12	Yes	****	<0,0001
		Control vs. 1000	13,47	4,261 to 22,68	Yes	**	0,0012
		Control vs. 10000	-44,79	-54 to -35,57	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	31,43	20,27 to 42,6	Yes	****	<0,0001
		Control vs. 1	30,27	19,1 to 41,43	Yes	****	<0,0001
		Control vs. 10	30,14	18,97 to 41,3	Yes	****	<0,0001
		Control vs. 100	29,75	18,58 to 40,91	Yes	****	<0,0001
		Control vs. 1000	23,99	12,83 to 35,16	Yes	****	<0,0001
		Control vs. 10000	-42,3	-53,46 to -31,13	Yes	****	<0,0001

TABLE S-5: EGGSHELL MEMBRANE ALCALASE

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-2,811	-13,73 to 8,109	No	ns	0,9648
		Control vs. 1	-13,04	-23,96 to -2,116	Yes	*	0,0123
		Control vs. 10	-10,8	-21,72 to 0,1177	No	ns	0,0536
		Control vs. 100	-12,54	-23,46 to -1,616	Yes	*	0,0175
		Control vs. 1000	-4,719	-15,64 to 6,2	No	ns	0,7380
		Control vs. 10000	45,32	34,4 to 56,24	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	11,97	1,332 to 22,61	Yes	*	0,0208
		Control vs. 1	14,8	4,161 to 25,44	Yes	**	0,0024
		Control vs. 10	9,328	-1,31 to 19,97	No	ns	0,1082
		Control vs. 100	12,69	2,055 to 23,33	Yes	*	0,0124
		Control vs. 1000	19,53	8,89 to 30,17	Yes	****	<0,0001
		Control vs. 10000	50,31	39,67 to 60,94	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	54,48	43,64 to 65,32	Yes	****	<0,0001
		Control vs. 1	54,3	43,46 to 65,14	Yes	****	<0,0001
		Control vs. 10	53,15	42,31 to 63,99	Yes	****	<0,0001
		Control vs. 100	54,14	43,3 to 64,98	Yes	****	<0,0001
		Control vs. 1000	55,06	44,22 to 65,9	Yes	****	<0,0001
		Control vs. 10000	78,22	67,38 to 89,06	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	-10,16	-28,77 to 8,451	No	ns	0,5234
		Control vs. 1	-11,44	-30,05 to 7,172	No	ns	0,4026
		Control vs. 10	-15,2	-33,81 to 3,412	No	ns	0,1523
		Control vs. 100	-25,04	-43,65 to -6,431	Yes	**	0,0036
		Control vs. 1000	-14,14	-32,75 to 4,465	No	ns	0,2057
		Control vs. 10000	14,37	-4,237 to 32,98	No	ns	0,1931
	Reduced-serum conditions	Control vs. 0.1	-27,99	-63,86 to 7,885	No	ns	0,1860
		Control vs. 1	-30,12	-65,99 to 5,755	No	ns	0,1345
		Control vs. 10	-37,01	-72,88 to -1,139	Yes	*	0,0403
		Control vs. 100	-37,57	-73,44 to -1,696	Yes	*	0,0362
		Control vs. 1000	-25,43	-61,3 to 10,44	No	ns	0,2659
		Control vs. 10000	-4.314	-40.19 to 31.56	No	ns	0.9995
	Serum-free conditions	Control vs. 0.1	25,38	10,08 to 40,67	Yes	***	0,0002
		Control vs. 1	27,68	10,99 to 44,37	Yes	***	0,0002
		Control vs. 10	22,88	8,138 to 37,61	Yes	***	0,0006
		Control vs. 100	26,9	10,96 to 42,84	Yes	***	0,0001
		Control vs. 1000	25,87	9,928 to 41,81	Yes	***	0,0003
		Control vs. 10000	27,7	11,01 to 44,39	Yes	***	0,0002
LDH	Normal serum conditions	Control vs. 0.1	6,508	-14,91 to 27,92	No	ns	0,9270
		Control vs. 1	1,606	-19,81 to 23,02	No	ns	0,9997
		Control vs. 10	15,05	-6,369 to 36,46	No	ns	0,2735
		Control vs. 100	6,32	-15,09 to 27,73	No	ns	0,9355
		Control vs. 1000	18,88	-2,533 to 40,3	No	ns	0,1053
		Control vs. 10000	-4,228	-26,64 to 17,19	No	ns	0,9955
	Reduced-serum conditions	Control vs. 0.1	12,73	-13,71 to 39,17	No	ns	0,6462
		Control vs. 1	15,85	-10,59 to 42,3	No	ns	0,4285
		Control vs. 10	5,663	-20,78 to 32,11	No	ns	0,9856
		Control vs. 100	-0,9636	-27,41 to 25,48	No	ns	0,9999
		Control vs. 1000	4,37	-22,07 to 30,81	No	ns	0,9956
		Control vs. 10000	-12,12	-38,56 to 14,32	No	ns	0,6900
	Serum-free conditions	Control vs. 0.1	35,3	20,06 to 50,53	Yes	****	<0,0001
		Control vs. 1	32,36	17,13 to 47,59	Yes	****	<0,0001
		Control vs. 10	32,71	17,48 to 47,94	Yes	****	<0,0001
		Control vs. 100	26,18	10,95 to 41,41	Yes	***	0,0001
		Control vs. 1000	32,21	16,98 to 47,44	Yes	****	<0,0001
		Control vs. 10000	10,71	-4,525 to 25,94	No	ns	0,2731

TABLE S-6: EGGSHELL MEMBRANE FLAVOURZYME

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-14,38	-27,09 to -1,673	Yes	*	0,0198
		Control vs. 1	-27,55	-40,26 to -14,84	Yes	****	<0,0001
		Control vs. 10	-29,36	-42,07 to -16,65	Yes	****	<0,0001
		Control vs. 100	-14,77	-27,48 to -2,057	Yes	*	0,0158
		Control vs. 1000	-11,73	-24,44 to 0,9848	No	ns	0,0822
		Control vs. 10000	56,3	43,59 to 69,01	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	4,429	-6,472 to 15,33	No	ns	0,7838
		Control vs. 1	5,317	-5,585 to 16,22	No	ns	0,6336
		Control vs. 10	-2,331	-13,23 to 8,57	No	ns	0,9856
		Control vs. 100	-1,189	-12,09 to 9,712	No	ns	0,9996
		Control vs. 1000	5,307	-5,594 to 16,21	No	ns	0,6352
		Control vs. 10000	64,85	53,95 to 75,75	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	51,54	40,54 to 62,53	Yes	****	<0,0001
		Control vs. 1	52,27	41,27 to 63,26	Yes	****	<0,0001
		Control vs. 10	49,2	38,2 to 60,19	Yes	****	<0,0001
		Control vs. 100	47,84	36,84 to 58,83	Yes	****	<0,0001
		Control vs. 1000	48,4	37,41 to 59,4	Yes	****	<0,0001
		Control vs. 10000	79	68 to 89,99	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	0,4341	-16,82 to 17,69	No	ns	>0,9999
		Control vs. 1	-5,571	-22,82 to 11,68	No	ns	0,943
		Control vs. 10	-12,87	-30,13 to 4,378	No	ns	0,251
		Control vs. 100	-16,49	-33,74 to 0,7638	No	ns	0,0686
		Control vs. 1000	-1,273	-18,53 to 15,98	No	ns	0,9998
		Control vs. 10000	15,3	-1,949 to 32,56	No	ns	0,1088
	Reduced-serum conditions	Control vs. 0.1	14,35	-0,795 to 29,49	No	ns	0,0704
		Control vs. 1	9,56	-5,583 to 24,7	No	ns	0,3770
		Control vs. 10	6,579	-8,564 to 21,72	No	ns	0,7353
		Control vs. 100	7,036	-8,107 to 22,18	No	ns	0,6792
		Control vs. 1000	12,64	-2,506 to 27,78	No	ns	0,1384
		Control vs. 10000	14,52	-0,6225 to 29,66	No	ns	0,0655
	Serum-free conditions	Control vs. 0.1	45,91	30,18 to 61,65	Yes	****	<0,0001
		Control vs. 1	47,03	31,3 to 62,76	Yes	****	<0,0001
		Control vs. 10	42,09	26,36 to 57,82	Yes	****	<0,0001
		Control vs. 100	38,76	23,03 to 54,49	Yes	****	<0,0001
		Control vs. 1000	41,22	25,49 to 56,95	Yes	****	<0,0001
		Control vs. 10000	47,66	30,65 to 64,68	Yes	****	<0,0001
LDH	Normal serum conditions	Control vs. 0.1	-1,458	-10,07 to 7,157	No	ns	0,9952
		Control vs. 1	-2,553	-11,17 to 6,062	No	ns	0,9343
		Control vs. 10	-2,638	-11,25 to 5,977	No	ns	0,9247
		Control vs. 100	-3,468	-12,08 to 5,147	No	ns	0,7908
		Control vs. 1000	-6,843	-15,46 to 1,772	No	ns	0,1723
		Control vs. 10000	-28,95	-37,56 to -20,33	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	15,73	9,326 to 22,14	Yes	****	<0,0001
		Control vs. 1	17,31	10,9 to 23,71	Yes	****	<0,0001
		Control vs. 10	16,89	10,49 to 23,3	Yes	****	<0,0001
		Control vs. 100	15,68	9,277 to 22,09	Yes	****	<0,0001
		Control vs. 1000	13,07	6,664 to 19,48	Yes	****	<0,0001
		Control vs. 10000	-15,18	-21,58 to -8,774	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	30,09	23,48 to 36,7	Yes	****	<0,0001
		Control vs. 1	32,38	25,77 to 38,98	Yes	****	<0,0001
		Control vs. 10	30,2	23,59 to 36,81	Yes	****	<0,0001
		Control vs. 100	28,47	21,87 to 35,08	Yes	****	<0,0001
		Control vs. 1000	25,86	19,25 to 32,47	Yes	****	<0,0001
		Control vs. 10000	-5,418	-12,03 to 1,19	No	ns	0,1500

TABLE S-7: EGGSHELL MEMBRANE NAOH

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-3,654	-14,38 to 7,068	No	ns	0,8841
		Control vs. 1	-4,377	-16,52 to 7,77	No	ns	0,8572
		Control vs. 10	-4,83	-16,98 to 7,317	No	ns	0,7988
		Control vs. 100	-4,947	-17,09 to 7,2	No	ns	0,7823
		Control vs. 1000	7,81	-4,336 to 19,96	No	ns	0,3577
		Control vs. 10000	22,26	10,11 to 34,4	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	12,84	2,448 to 23,23	Yes	**	0,0089
		Control vs. 1	12,31	0,5389 to 24,08	Yes	*	0,0367
		Control vs. 10	9,734	-2,037 to 21,5	No	ns	0,1440
		Control vs. 100	3,095	-8,676 to 14,87	No	ns	0,9612
		Control vs. 1000	19,68	7,911 to 31,45	Yes	***	0,0002
		Control vs. 10000	27,9	16,13 to 39,67	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	49,18	36 to 62,35	Yes	****	<0,0001
		Control vs. 1	50,45	35,52 to 65,37	Yes	****	<0,0001
		Control vs. 10	45,06	30,13 to 59,98	Yes	****	<0,0001
		Control vs. 100	48,84	33,91 to 63,77	Yes	****	<0,0001
		Control vs. 1000	47,74	32,81 to 62,67	Yes	****	<0,0001
		Control vs. 10000	57,61	42,68 to 72,54	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	5,104	-20,4 to 30,61	No	ns	0,9955
		Control vs. 1	-6,702	-32,21 to 18,8	No	ns	0,9811
		Control vs. 10	-4,492	-30 to 21,01	No	ns	0,9978
		Control vs. 100	-14,51	-40,02 to 10,99	No	ns	0,5691
		Control vs. 1000	-19,13	-44,64 to 6,371	No	ns	0,2515
		Control vs. 10000	-8,235	-33,74 to 17,27	No	ns	0,9485
	Reduced-serum conditions	Control vs. 0.1	4,228	-15,55 to 24,01	No	ns	0,9859
		Control vs. 1	-4,213	-25,92 to 17,5	No	ns	0,9916
		Control vs. 10	-10,72	-32,43 to 10,99	No	ns	0,6238
		Control vs. 100	-14,69	-36,4 to 7,016	No	ns	0,3089
		Control vs. 1000	-22,3	-44,01 to -0,5931	Yes	*	0,0416
		Control vs. 10000	-6,798	-28,51 to 14,91	No	ns	0,9179
	Serum-free conditions	Control vs. 0.1	34,5	17,52 to 51,49	Yes	****	<0,0001
		Control vs. 1	31,47	13,68 to 49,26	Yes	****	<0,0001
		Control vs. 10	28,46	10,67 to 46,24	Yes	***	0,0004
		Control vs. 100	10,7	-7,088 to 28,49	No	ns	0,4234
		Control vs. 1000	1,736	-24,05 to 27,53	No	ns	0,9997
		Control vs. 10000	40,48	10,91 to 70,05	Yes	**	0,0029
LDH	Normal serum conditions	Control vs. 0.1	3,27	-6,446 to 12,99	No	ns	0,8898
		Control vs. 1	4,775	-6,251 to 15,8	No	ns	0,7370
		Control vs. 10	1,884	-9,142 to 12,91	No	ns	0,9950
		Control vs. 100	0,9095	-10,12 to 11,94	No	ns	0,9997
		Control vs. 1000	0,8482	-10,18 to 11,87	No	ns	0,9997
		Control vs. 10000	-5,734	-19 to 7,53	No	ns	0,7385
	Reduced-serum conditions	Control vs. 0.1	12,84	1,714 to 23,97	Yes	*	0,0167
		Control vs. 1	9,875	-2,751 to 22,5	No	ns	0,1839
		Control vs. 10	1,873	-10,75 to 14,5	No	ns	0,9977
		Control vs. 100	3,63	-8,995 to 16,26	No	ns	0,9424
		Control vs. 1000	5,01	-7,616 to 17,64	No	ns	0,8007
		Control vs. 10000	10,71	-1,92 to 23,33	No	ns	0,1281
	Serum-free conditions	Control vs. 0.1	26,42	12,6 to 40,23	Yes	****	<0,0001
		Control vs. 1	26,98	11,3 to 42,66	Yes	***	0,0001
		Control vs. 10	29,7	14,02 to 45,38	Yes	****	<0,0001
		Control vs. 100	24,56	8,883 to 40,24	Yes	***	0,0005
		Control vs. 1000	-11,21	-26,89 to 4,468	No	ns	0,2577
		Control vs. 10000	-44,61	-63,47 to -25,74	Yes	****	<0,0001

TABLE S-8: EGG WHITE ALCALASE

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-32,51	-56,77 to -8,244	Yes	**	0,0037
		Control vs. 1	-33,39	-57,65 to -9,128	Yes	**	0,0027
		Control vs. 10	-34,66	-58,92 to -10,4	Yes	**	0,0017
		Control vs. 100	-33,33	-57,59 to -9,072	Yes	**	0,0027
		Control vs. 1000	-28,38	-52,65 to -4,124	Yes	*	0,0147
		Control vs. 10000	29,19	4,929 to 53,45	Yes	*	0,0114
	Reduced-serum conditions	Control vs. 0.1	-13,92	-36,74 to 8,895	No	ns	0,4114
		Control vs. 1	-17,61	-40,43 to 5,203	No	ns	0,1942
		Control vs. 10	-13,78	-36,6 to 9,032	No	ns	0,4214
		Control vs. 100	-10,94	-33,76 to 11,87	No	ns	0,6508
		Control vs. 1000	-10,84	-33,65 to 11,98	No	ns	0,6595
		Control vs. 10000	32,84	10,03 to 55,66	Yes	**	0,0015
	Serum-free conditions	Control vs. 0.1	21,49	6,615 to 36,37	Yes	**	0,0015
		Control vs. 1	13,86	-1,017 to 28,74	No	ns	0,0777
		Control vs. 10	16,2	1,32 to 31,08	Yes	*	0,0271
		Control vs. 100	18,27	3,392 to 33,15	Yes	**	0,0094
		Control vs. 1000	20,33	5,451 to 35,21	Yes	**	0,0030
		Control vs. 10000	56,88	43,75 to 70,02	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	-18,87	-36,01 to -1,731	Yes	*	0,0247
		Control vs. 1	-20,62	-37,76 to -3,477	Yes	*	0,0114
		Control vs. 10	-18,47	-35,61 to -1,324	Yes	*	0,0293
		Control vs. 100	-16,27	-33,41 to 0,8697	No	ns	0,0696
		Control vs. 1000	-27,98	-45,12 to -10,84	Yes	***	0,0002
		Control vs. 10000	-23,68	-43,1 to -4,267	Yes	**	0,0100
	Reduced-serum conditions	Control vs. 0.1	-9,027	-24,92 to 6,864	No	ns	0,4844
		Control vs. 1	-6,701	-22,59 to 9,19	No	ns	0,7582
		Control vs. 10	-0,7502	-16,64 to 15,14	No	ns	0,9998
		Control vs. 100	3,451	-12,44 to 19,34	No	ns	0,9846
		Control vs. 1000	-12,17	-28,06 to 3,724	No	ns	0,2006
		Control vs. 10000	-25,9	-41,8 to -10,01	Yes	***	0,0003
	Serum-free conditions	Control vs. 0.1	17,28	3,076 to 31,48	Yes	*	0,0102
		Control vs. 1	15,22	1,019 to 29,42	Yes	*	0,0305
		Control vs. 10	20,27	6,073 to 34,47	Yes	**	0,0017
		Control vs. 100	18,32	4,119 to 32,52	Yes	**	0,0056
		Control vs. 1000	8,19	-6,009 to 22,39	No	ns	0,4688
		Control vs. 10000	-7,071	-21,27 to 7,129	No	ns	0,6156
LDH	Normal serum conditions	Control vs. 0.1	8,317	-0,6519 to 17,29	No	ns	0,0799
		Control vs. 1	10,58	1,614 to 19,55	Yes	*	0,0137
		Control vs. 10	11,15	2,183 to 20,12	Yes	**	0,0083
		Control vs. 100	12,08	3,11 to 21,05	Yes	**	0,0035
		Control vs. 1000	8,193	-0,7759 to 17,16	No	ns	0,0869
		Control vs. 10000	-9,373	-18,34 to -0,4039	Yes	*	0,0368
	Reduced-serum conditions	Control vs. 0.1	26,2	20 to 32,4	Yes	****	<0,0001
		Control vs. 1	28,05	21,84 to 34,25	Yes	****	<0,0001
		Control vs. 10	26,73	20,52 to 32,93	Yes	****	<0,0001
		Control vs. 100	25,66	19,45 to 31,86	Yes	****	<0,0001
		Control vs. 1000	19,29	13,09 to 25,5	Yes	****	<0,0001
		Control vs. 10000	2,495	-3,708 to 8,698	No	ns	0,7914
	Serum-free conditions	Control vs. 0.1	24,14	14,57 to 33,72	Yes	****	<0,0001
		Control vs. 1	28,84	19,27 to 38,41	Yes	****	<0,0001
		Control vs. 10	28,19	18,62 to 37,77	Yes	****	<0,0001
		Control vs. 100	30,63	21,06 to 40,2	Yes	****	<0,0001
		Control vs. 1000	21,08	11,51 to 30,66	Yes	****	<0,0001
		Control vs. 10000	11,67	2,1 to 21,24	Yes	*	0,0100

TABLE S-9: EGG WHITE FLAVOURZYME

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-2,847	-14,39 to 8,694	No	ns	0,9713
		Control vs. 1	-7,497	-19,04 to 4,043	No	ns	0,3483
		Control vs. 10	-8,009	-19,55 to 3,532	No	ns	0,2852
		Control vs. 100	-17,02	-28,56 to -5,482	Yes	**	0,0011
		Control vs. 1000	-23,65	-35,19 to -12,1	Yes	****	<0,0001
		Control vs. 10000	10,05	-3,021 to 23,13	No	ns	0,1971
	Reduced-serum conditions	Control vs. 0.1	11,09	-0,7085 to 22,9	No	ns	0,0738
		Control vs. 1	8,357	-3,445 to 20,16	No	ns	0,2669
		Control vs. 10	5,973	-5,828 to 17,78	No	ns	0,6001
		Control vs. 100	4,728	-7,074 to 16,53	No	ns	0,7949
		Control vs. 1000	-0,339	-12,14 to 11,46	No	ns	>0,9999
		Control vs. 10000	7,718	-4,084 to 19,52	No	ns	0,3421
	Serum-free conditions	Control vs. 0.1	43,62	32,11 to 55,13	Yes	****	<0,0001
		Control vs. 1	38,57	27,05 to 50,08	Yes	****	<0,0001
		Control vs. 10	45,98	34,47 to 57,49	Yes	****	<0,0001
		Control vs. 100	39,91	27,03 to 52,79	Yes	****	<0,0001
		Control vs. 1000	45,13	33,62 to 56,65	Yes	****	<0,0001
		Control vs. 10000	36,61	25,62 to 47,6	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	-0,9177	-17,17 to 15,33	No	ns	0,9998
		Control vs. 1	-10,7	-26,95 to 5,551	No	ns	0,3340
		Control vs. 10	-17,99	-34,24 to -1,747	Yes	*	0,0237
		Control vs. 100	-18,06	-34,31 to -1,816	Yes	*	0,0229
		Control vs. 1000	-21,17	-37,42 to -4,919	Yes	**	0,0051
		Control vs. 10000	-10,46	-28,64 to 7,721	No	ns	0,4697
	Reduced-serum conditions	Control vs. 0.1	4,752	-10,04 to 19,54	No	ns	0,9079
		Control vs. 1	5,031	-9,762 to 19,82	No	ns	0,8847
		Control vs. 10	3,434	-11,36 to 18,23	No	ns	0,9783
		Control vs. 100	-1,254	-16,05 to 13,54	No	ns	0,9997
		Control vs. 1000	-5,382	-20,17 to 9,411	No	ns	0,8519
		Control vs. 10000	-10,48	-25,28 to 4,31	No	ns	0,2653
	Serum-free conditions	Control vs. 0.1	23,96	0,3077 to 47,61	Yes	*	0,0459
		Control vs. 1	20,66	-2,986 to 44,31	No	ns	0,1101
		Control vs. 10	16,57	-7,078 to 40,22	No	ns	0,2752
		Control vs. 100	18,07	-5,576 to 41,72	No	ns	0,2012
		Control vs. 1000	-9,614	-33,26 to 14,04	No	ns	0,7830
		Control vs. 10000	-74,87	-103,3 to -46,48	Yes	****	<0,0001
LDH	Normal serum conditions	Control vs. 0.1	16,94	9,279 to 24,59	Yes	****	<0,0001
		Control vs. 1	15,26	7,601 to 22,91	Yes	****	<0,0001
		Control vs. 10	14,99	7,333 to 22,65	Yes	****	<0,0001
		Control vs. 100	15,81	8,152 to 23,46	Yes	****	<0,0001
		Control vs. 1000	14,95	7,291 to 22,6	Yes	****	<0,0001
		Control vs. 10000	16,57	8,915 to 24,23	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	23,72	13,29 to 34,15	Yes	****	<0,0001
		Control vs. 1	24,52	14,09 to 34,95	Yes	****	<0,0001
		Control vs. 10	25,33	14,9 to 35,76	Yes	****	<0,0001
		Control vs. 100	24,63	14,2 to 35,06	Yes	****	<0,0001
		Control vs. 1000	20,7	10,27 to 31,13	Yes	****	<0,0001
		Control vs. 10000	22,1	11,67 to 32,53	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	31,52	16,45 to 46,59	Yes	****	<0,0001
		Control vs. 1	33,42	18,34 to 48,49	Yes	****	<0,0001
		Control vs. 10	33,84	18,77 to 48,91	Yes	****	<0,0001
		Control vs. 100	33,23	18,16 to 48,3	Yes	****	<0,0001
		Control vs. 1000	23,14	8,07 to 38,21	Yes	***	0,0006
		Control vs. 10000	-41,04	-56,11 to -25,97	Yes	****	<0,0001

TABLE S-10: YEAST EXTRACT

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-71,56	-104,6 to -38,47	Yes	****	<0,0001
		Control vs. 1	-77,38	-110,5 to -44,29	Yes	****	<0,0001
		Control vs. 10	-100,4	-136,7 to -64,06	Yes	****	<0,0001
		Control vs. 100	-108,8	-145,2 to -72,52	Yes	****	<0,0001
		Control vs. 1000	-101,6	-137,9 to -65,3	Yes	****	<0,0001
		Control vs. 10000	77,88	44,79 to 111	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	-42,36	-70,48 to -14,24	Yes	***	0,0008
		Control vs. 1	-40,34	-68,46 to -12,22	Yes	**	0,0016
		Control vs. 10	-46,11	-74,23 to -17,98	Yes	***	0,0002
		Control vs. 100	-45,82	-73,94 to -17,7	Yes	***	0,0002
		Control vs. 1000	-50,97	-79,09 to -22,85	Yes	****	<0,0001
		Control vs. 10000	68,16	40,03 to 96,28	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	-58,48	-102,5 to -14,46	Yes	**	0,0040
		Control vs. 1	-40,56	-75,97 to -5,161	Yes	*	0,0177
		Control vs. 10	-27,21	-66,07 to 11,65	No	ns	0,2774
		Control vs. 100	-37,64	-76,5 to 1,219	No	ns	0,0615
		Control vs. 1000	-45,2	-80,6 to -9,797	Yes	**	0,0062
		Control vs. 10000	18,08	-17,32 to 53,48	No	ns	0,5918
DNA	Normal serum conditions	Control vs. 0.1	-6,546	-23,75 to 10,66	No	ns	0,8285
		Control vs. 1	-8,479	-25,69 to 8,729	No	ns	0,6264
		Control vs. 10	-14,36	-31,56 to 2,853	No	ns	0,1389
		Control vs. 100	-39,71	-56,91 to -22,5	Yes	****	<0,0001
		Control vs. 1000	-19,2	-38,08 to -0,3133	Yes	*	0,0447
		Control vs. 10000	27,83	8,945 to 46,71	Yes	**	0,0011
	Reduced-serum conditions	Control vs. 0.1	8,657	-14,81 to 32,12	No	ns	0,8964
		Control vs. 1	5,551	-17,91 to 29,02	No	ns	0,9874
		Control vs. 10	5,716	-17,75 to 29,18	No	ns	0,9852
		Control vs. 100	-5,412	-28,88 to 18,05	No	ns	0,9891
		Control vs. 1000	-23,53	-46,99 to -0,05937	Yes	*	0,0491
		Control vs. 10000	-10,66	-34,12 to 12,81	No	ns	0,7713
	Serum-free conditions	Control vs. 0.1	14,21	-2,147 to 30,57	No	ns	0,1137
		Control vs. 1	9,832	-6,529 to 26,19	No	ns	0,4268
		Control vs. 10	11,15	-5,21 to 27,51	No	ns	0,3022
		Control vs. 100	6,507	-9,854 to 22,87	No	ns	0,7996
		Control vs. 1000	9,908	-6,453 to 26,27	No	ns	0,4190
		Control vs. 10000	-13,19	-29,55 to 3,17	No	ns	0,1617
LDH	Normal serum conditions	Control vs. 0.1	4,461	-8,524 to 17,45	No	ns	0,8814
		Control vs. 1	6,021	-6,964 to 19,01	No	ns	0,6811
		Control vs. 10	5,9	-7,085 to 18,89	No	ns	0,6987
		Control vs. 100	5,998	-6,988 to 18,98	No	ns	0,6845
		Control vs. 1000	8,365	-4,62 to 21,35	No	ns	0,3570
		Control vs. 10000	-45,48	-58,47 to -32,49	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	11,15	-5,898 to 28,2	No	ns	0,3450
		Control vs. 1	11,91	-5,138 to 28,96	No	ns	0,2818
		Control vs. 10	-3,237	-20,28 to 13,81	No	ns	0,9927
		Control vs. 100	15,26	-1,789 to 32,31	No	ns	0,0979
		Control vs. 1000	9,705	-7,342 to 26,75	No	ns	0,4861
		Control vs. 10000	-51,09	-68,13 to -34,04	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	32,42	17,94 to 46,89	Yes	****	<0,0001
		Control vs. 1	36,03	21,56 to 50,51	Yes	****	<0,0001
		Control vs. 10	33,86	19,39 to 48,33	Yes	****	<0,0001
		Control vs. 100	35,94	21,47 to 50,41	Yes	****	<0,0001
		Control vs. 1000	31,09	16,61 to 45,56	Yes	****	<0,0001
		Control vs. 10000	19,03	4,56 to 33,51	Yes	**	0,0045

TABLE S-11: PORSK PLASMA

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-13,04	-25,52 to -0,5571	Yes	*	0,0370
		Control vs. 1	-14,65	-27,81 to -1,501	Yes	*	0,0226
		Control vs. 10	-11,8	-24,95 to 1,359	No	ns	0,0962
		Control vs. 100	1,939	-11,21 to 15,09	No	ns	0,9977
		Control vs. 1000	48,41	35,93 to 60,89	Yes	****	<0,0001
		Control vs. 10000	87,62	75,13 to 100,1	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	-17,08	-28,28 to -5,883	Yes	***	0,0007
		Control vs. 1	-16,29	-27,49 to -5,092	Yes	**	0,0014
		Control vs. 10	-13,37	-24,57 to -2,173	Yes	*	0,0123
		Control vs. 100	-2,501	-13,7 to 8,698	No	ns	0,9821
		Control vs. 1000	42,58	31,38 to 53,77	Yes	****	<0,0001
		Control vs. 10000	88,22	77,02 to 99,42	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	1,506	-9,473 to 12,49	No	ns	0,9980
		Control vs. 1	0,3312	-10,65 to 11,31	No	ns	0,9999
		Control vs. 10	-0,5427	-11,52 to 10,44	No	ns	0,9998
		Control vs. 100	8,952	-2,028 to 19,93	No	ns	0,1535
		Control vs. 1000	47,67	36,69 to 58,65	Yes	****	<0,0001
		Control vs. 10000	89,47	78,49 to 100,5	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	16,45	-3,081 to 35,98	No	ns	0,1320
		Control vs. 1	-3,735	-24,31 to 16,84	No	ns	0,9937
		Control vs. 10	-0,4781	-20,01 to 19,05	No	ns	>0,9999
		Control vs. 100	-27,42	-47,99 to -6,84	Yes	**	0,0040
		Control vs. 1000	-19,58	-40,15 to 1,002	No	ns	0,0686
		Control vs. 10000	17,81	-1,72 to 37,34	No	ns	0,0876
	Reduced-serum conditions	Control vs. 0.1	-15,17	-39,92 to 9,582	No	ns	0,4053
		Control vs. 1	-14,76	-39,52 to 9,989	No	ns	0,4329
		Control vs. 10	-34,64	-59,39 to -9,887	Yes	**	0,0023
		Control vs. 100	-39,12	-63,88 to -14,37	Yes	***	0,0004
		Control vs. 1000	-20,09	-46,17 to 5,988	No	ns	0,1950
		Control vs. 10000	-3,459	-28,21 to 21,29	No	ns	0,9979
	Serum-free conditions	Control vs. 0.1	-17,05	-41,12 to 7,008	No	ns	0,2651
		Control vs. 1	-40,92	-64,99 to -16,86	Yes	***	0,0001
		Control vs. 10	-57,63	-81,69 to -33,57	Yes	****	<0,0001
		Control vs. 100	-40,69	-64,76 to -16,63	Yes	***	0,0001
		Control vs. 1000	-28,58	-53,93 to -3,224	Yes	*	0,0205
		Control vs. 10000	-5,168	-29,23 to 18,89	No	ns	0,9852
LDH	Normal serum conditions	Control vs. 0.1	-15,83	-34,63 to 2,975	No	ns	0,1471
		Control vs. 1	-15,81	-34,61 to 2,992	No	ns	0,1479
		Control vs. 10	-17,93	-36,74 to 0,8699	No	ns	0,0698
		Control vs. 100	-25,15	-43,96 to -6,349	Yes	**	0,0030
		Control vs. 1000	-53,79	-72,59 to -34,99	Yes	****	<0,0001
		Control vs. 10000	-134	-152,8 to -115,2	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	27,58	8,528 to 46,63	Yes	**	0,0014
		Control vs. 1	23,8	4,744 to 42,85	Yes	**	0,0079
		Control vs. 10	19,47	0,4188 to 38,53	Yes	*	0,0431
		Control vs. 100	13,26	-5,798 to 32,31	No	ns	0,2825
		Control vs. 1000	-30,09	-49,14 to -11,03	Yes	***	0,0004
		Control vs. 10000	-115,6	-134,7 to -96,57	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	55,12	37,72 to 72,51	Yes	****	<0,0001
		Control vs. 1	51,22	33,83 to 68,62	Yes	****	<0,0001
		Control vs. 10	47,83	30,44 to 65,23	Yes	****	<0,0001
		Control vs. 100	41,11	23,72 to 58,51	Yes	****	<0,0001
		Control vs. 1000	12,44	-4,954 to 29,84	No	ns	0,2576
		Control vs. 10000	-75,91	-93,31 to -58,52	Yes	****	<0,0001

TABLE S-12: PORK PLASMA ALCALASE

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-169,2	-233 to -105,5	Yes	****	<0,0001
		Control vs. 1	-170,4	-234,2 to -106,7	Yes	****	<0,0001
		Control vs. 10	-154,7	-218,4 to -90,91	Yes	****	<0,0001
		Control vs. 100	-95,74	-159,5 to -31,98	Yes	***	0,0009
		Control vs. 1000	39,68	-27,51 to 106,9	No	ns	0,4434
		Control vs. 10000	98,53	34,77 to 162,3	Yes	***	0,0006
	Reduced-serum conditions	Control vs. 0.1	-155,8	-206,3 to -105,3	Yes	****	<0,0001
		Control vs. 1	-141,6	-192,1 to -91,14	Yes	****	<0,0001
		Control vs. 10	-126,7	-177,2 to -76,25	Yes	****	<0,0001
		Control vs. 100	-54,06	-104,5 to -3,571	Yes	*	0,0308
		Control vs. 1000	95,37	44,88 to 145,8	Yes	****	<0,0001
		Control vs. 10000	99,58	49,09 to 150,1	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	-110,9	-151,1 to -70,72	Yes	****	<0,0001
		Control vs. 1	-104,6	-144,8 to -64,35	Yes	****	<0,0001
		Control vs. 10	-77,86	-118,1 to -37,65	Yes	****	<0,0001
		Control vs. 100	48,15	7,939 to 88,35	Yes	*	0,0120
		Control vs. 1000	98,74	58,54 to 139	Yes	****	<0,0001
		Control vs. 10000	99,79	59,58 to 140	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	-46,5	-66,68 to -26,32	Yes	****	<0,0001
		Control vs. 1	-57,79	-76,95 to -38,64	Yes	****	<0,0001
		Control vs. 10	-62,04	-82,22 to -41,86	Yes	****	<0,0001
		Control vs. 100	-41,55	-60,7 to -22,4	Yes	****	<0,0001
		Control vs. 1000	-2,408	-22,59 to 17,77	No	ns	0,9995
		Control vs. 10000	94,58	75,42 to 113,7	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	-27	-69,68 to 15,67	No	ns	0,3733
		Control vs. 1	-38,57	-81,24 to 4,106	No	ns	0,0921
		Control vs. 10	-47,28	-89,95 to -4,606	Yes	*	0,0236
		Control vs. 100	-36,14	-78,81 to 6,534	No	ns	0,1286
		Control vs. 1000	25,84	-16,84 to 68,51	No	ns	0,4177
		Control vs. 10000	95,02	52,35 to 137,7	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	-33,21	-49,07 to -17,35	Yes	****	<0,0001
		Control vs. 1	-23,1	-38,96 to -7,241	Yes	**	0,0014
		Control vs. 10	-31,73	-47,59 to -15,87	Yes	****	<0,0001
		Control vs. 100	39,97	24,11 to 55,83	Yes	****	<0,0001
		Control vs. 1000	84,32	68,47 to 100,2	Yes	****	<0,0001
		Control vs. 10000	93,05	77,19 to 108,9	Yes	****	<0,0001
LDH	Normal serum conditions	Control vs. 0.1	-10,5	-17,89 to -3,103	Yes	**	0,0019
		Control vs. 1	-6,412	-13,8 to 0,9807	No	ns	0,1146
		Control vs. 10	-8,39	-15,78 to -0,9979	Yes	*	0,0193
		Control vs. 100	-5,865	-13,26 to 1,528	No	ns	0,1732
		Control vs. 1000	-4,907	-12,3 to 2,486	No	ns	0,3269
		Control vs. 10000	64,95	57,56 to 72,34	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	30,37	23,68 to 37,07	Yes	****	<0,0001
		Control vs. 1	31,38	24,68 to 38,08	Yes	****	<0,0001
		Control vs. 10	29,09	22,4 to 35,79	Yes	****	<0,0001
		Control vs. 100	25,17	18,48 to 31,87	Yes	****	<0,0001
		Control vs. 1000	64,57	57,88 to 71,27	Yes	****	<0,0001
		Control vs. 10000	66,65	59,96 to 73,35	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	71,46	65,75 to 77,16	Yes	****	<0,0001
		Control vs. 1	72,25	66,54 to 77,95	Yes	****	<0,0001
		Control vs. 10	71,01	65,31 to 76,72	Yes	****	<0,0001
		Control vs. 100	68,35	62,65 to 74,06	Yes	****	<0,0001
		Control vs. 1000	79,92	74,22 to 85,62	Yes	****	<0,0001
		Control vs. 10000	59,85	54,14 to 65,55	Yes	****	<0,0001

TABLE S-13: PORK PLASMA FLAVOURZYME

		DUNNETT'S MULTIPLE COMPARISONS TEST	MEAN DIFF	95,00% CI OF DIFF	SIGNIFICANT?	SUMMARY	ADJUSTED P VALUE
ATP	Normal serum conditions	Control vs. 0.1	-47,57	-60,29 to -34,86	Yes	****	<0,0001
		Control vs. 1	-46,94	-59,65 to -34,22	Yes	****	<0,0001
		Control vs. 10	-43,53	-56,25 to -30,81	Yes	****	<0,0001
		Control vs. 100	-21,27	-33,98 to -8,551	Yes	***	0,0002
		Control vs. 1000	43,72	31,01 to 56,44	Yes	****	<0,0001
		Control vs. 10000	96,33	83,61 to 109	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	-26,52	-40,96 to -12,08	Yes	****	<0,0001
		Control vs. 1	-15,75	-30,19 to -1,315	Yes	*	0,0266
		Control vs. 10	-5,232	-19,67 to 9,205	No	ns	0,8541
		Control vs. 100	11,41	-3,028 to 25,85	No	ns	0,1757
		Control vs. 1000	66,02	51,58 to 80,46	Yes	****	<0,0001
		Control vs. 10000	97,35	82,91 to 111,8	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	-17,84	-32,78 to -2,907	Yes	*	0,0123
		Control vs. 1	-24,16	-39,1 to -9,227	Yes	***	0,0003
		Control vs. 10	-22,28	-37,21 to -7,339	Yes	***	0,0010
		Control vs. 100	-3,321	-18,26 to 11,62	No	ns	0,9824
		Control vs. 1000	41,39	26,45 to 56,33	Yes	****	<0,0001
		Control vs. 10000	97,44	82,51 to 112,4	Yes	****	<0,0001
DNA	Normal serum conditions	Control vs. 0.1	-21,05	-40,37 to -1,733	Yes	*	0,0269
		Control vs. 1	-25,72	-45,04 to -6,404	Yes	**	0,0041
		Control vs. 10	-33,49	-52,8 to -14,17	Yes	****	<0,0001
		Control vs. 100	-40,29	-60,65 to -19,94	Yes	****	<0,0001
		Control vs. 1000	-60,32	-88,32 to -32,31	Yes	****	<0,0001
		Control vs. 10000	45,9	26,58 to 65,22	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	-15,21	-46,23 to 15,81	No	ns	0,6280
		Control vs. 1	-21,15	-52,17 to 9,869	No	ns	0,3006
		Control vs. 10	-20,47	-53,16 to 12,21	No	ns	0,3833
		Control vs. 100	-64,23	-95,25 to -33,21	Yes	****	<0,0001
		Control vs. 1000	-136,6	-169,3 to -103,9	Yes	****	<0,0001
		Control vs. 10000	1,35	-29,67 to 32,37	No	ns	0,9999
	Serum-free conditions	Control vs. 0.1	-20,59	-43,45 to 2,281	No	ns	0,0940
		Control vs. 1	-49,74	-75,33 to -24,16	Yes	****	<0,0001
		Control vs. 10	-39,59	-67,04 to -12,14	Yes	**	0,0016
		Control vs. 100	-20,42	-43,28 to 2,449	No	ns	0,0982
		Control vs. 1000	-25,82	-55,68 to 4,039	No	ns	0,1159
		Control vs. 10000	70,15	47,28 to 93,02	Yes	****	<0,0001
LDH	Normal serum conditions	Control vs. 0.1	8,957	-1,532 to 19,45	No	ns	0,1237
		Control vs. 1	8,737	-1,752 to 19,23	No	ns	0,1394
		Control vs. 10	7,318	-3,171 to 17,81	No	ns	0,2798
		Control vs. 100	8,223	-2,266 to 18,71	No	ns	0,1819
		Control vs. 1000	0,1011	-10,39 to 10,59	No	ns	>0,9999
		Control vs. 10000	-38,14	-48,63 to -27,65	Yes	****	<0,0001
	Reduced-serum conditions	Control vs. 0.1	44,97	37,21 to 52,72	Yes	****	<0,0001
		Control vs. 1	43,43	35,68 to 51,19	Yes	****	<0,0001
		Control vs. 10	43,14	35,38 to 50,89	Yes	****	<0,0001
		Control vs. 100	40,9	33,15 to 48,66	Yes	****	<0,0001
		Control vs. 1000	26,66	18,91 to 34,41	Yes	****	<0,0001
		Control vs. 10000	-27,04	-34,79 to -19,28	Yes	****	<0,0001
	Serum-free conditions	Control vs. 0.1	75,63	69,09 to 82,17	Yes	****	<0,0001
		Control vs. 1	75,42	68,88 to 81,96	Yes	****	<0,0001
		Control vs. 10	76,88	70,34 to 83,42	Yes	****	<0,0001
		Control vs. 100	76,6	70,05 to 83,14	Yes	****	<0,0001
		Control vs. 1000	67,26	60,72 to 73,8	Yes	****	<0,0001
		Control vs. 10000	-10,25	-16,79 to -3,711	Yes	***	0,0005