Serum Reduced Culture of Tuna Fish Cells

A thesis submitted by

Kourosh (Cyrus) F. Karimy

In partial fulfillment of the requirements for the degree of

Master of Science

In

Biomedical Engineering

Tufts University

May 2024

Advisor: Dr. David Kaplan

Thesis Committee: Dr. David Kaplan, Dr. Reza Ovissipour, Dr. Qiaobing Xu

ABSTRACT

Overfishing is a major global issue, leading to declining fish populations, habitat damage, and poorer water quality, which threatens our seafood supply. Aquaculture has been proposed to decrease wild fish capture and encourage sustainable fish growth. Yet, the Blue-Fin Tuna still faces critically low numbers, and ocean health continues to decline. Cultivated meat or cellular agriculture from animal cells emerges as an innovative solution to reduce reliance on wild fish stocks and improve oceanic health. This approach could offer sustainable food sources without harming animals, while also improving quality. However, cultivated meat production currently faces hurdles like the costly use of fetal bovine serum (FBS) in cell cultures, raising both economic and ethical concerns. My thesis aims to minimize the use of Fetal Bovine Serum (FBS) in Bluefin Tuna cell cultures, thereby pioneering a method to improve the feasibility of cultivated meat. This result is achieved through the development of serum-reduced media that sustains cell growth, laying the foundation for sustainable and healthier seafood production as well as contributing to a healthier ocean environment.

ACKNOWLEDGEMENTS

I would like to thank my close friends, family, and mentors for supporting my academic goals. Without them, I would not be where I am today. I would like to especially thank Dr. David Kaplan for providing me with guidance and tools to strive for a profound education and being a great mentor throughout my graduate studies.

Table of Contents

ABSTRACT ii
ACKNOWLEDGEMENTS iii
LIST OF TABLES v
LIST OF FIGURES vi

1. INTRODUCTION
    a. Overfishing & Biomass Sustainability 1
    b. Overfishing and Environmental Impact 3
    c. Overfishing Implications on Economic Sustainability & Food Security 4
    d. Dangers of Consumption (Pesticides, Heavy Metals, and Plastics) 5
    e. Fish Freshness, Stability, Spoilage, and Waste of Resources 7
    f. Cell Agriculture and Cell-Based Seafood 7
    g. Cell Culture Media and FBS 9
    h. Aims of The Study 13
2. Materials & Methods
    a. Tuna Isolation & Cell Culture 14
    b. Basal Medium Proliferation Screening Materials 15
    c. Media Supplement Screening Materials 16
    d. Short-Term Combination Materials 18
    e. Short-Term Proliferation Assay Procedure 19
    f. Statistical Analysis 22
3. RESULTS
    a. Basal Medium Combination Short-Term Proliferative Effects 24
    b. Media Supplement Short-Term Proliferative Effects 24
    c. Short-Term Combination Proliferative Effects 28
4. DISCUSSION
    a. Basal Medium 30
    b. Supplements 35
    c. Combination Testing 36
5. FUTURE WORK
    a. Cell Line Characterization 38
    b. Further Identification of Serum Replacement Components 38
    c. Media Optimization Through DOE 39
    d. Spent Media Analysis 40
    e. Long-term SRM Combination Culture Studies 40
6. REFERENCES 42

LIST OF TABLES

Table 1: FBS contents (Andreassen et al.) 11

Table 2: Components added to SFM and their respective purpose (Andreassen 2020) 12

Table 3: Impacts of conventional meat production vs. cultivated meat 12

Table 4: The combinations of the basal medium that were tested. 16

Table 5: Table of supplements tested in SRM 18

Table 6: Different media combinations that were tested on their proliferative effects in short-term 19

Table 7: DMEM Glutamax components and respective Concentrations that are not found in L-15 Medium. Adapted from Gibco™ (n.d.) 30

Table 8: F-12K Components and Respective Concentrations that Are Not Found in L-15 Medium. Adapted from Gibco™ (n.d.) 31

Table 9: F-12 Components and Respective Concentrations that Are Not Found in L-15 Medium. Adapted from Gibco™ (n.d.) 32

LIST OF FIGURES

Figure 1: Sustainability standing of various species (Costello 2012)____________________ 2

Figure 2: Climate change impacts on our oceans and economy (Sumaila 2020)___________ 4

Figure 3: Overall process of cellular agriculture___________________________________ 8

Figure 4: Serial dilution process of each supplement tested__________________________ 20

Figure 5: Example 96 Well Plate set up of supplement testing________________________ 21

Figure 6: Overall process of short-term proliferation analysis________________________ 22

Figure 7: Proliferative effects of different basal medium conditions in SRM_____________ 24

Figure 8: Proliferative effects of different CPI and RPI conditions in SRM______________ 25

Figure 9: The proliferative effects of different growth factors in SRM_________________ 26

Figure 10: Proliferative effects of different ITS conditions in SRM____________________ 27

Figure 11: 2.5% Control (left image) vs SRM with 62.5 ng/mL Oleic Acid (right image)___ 28

Figure 12: Proliferative effects of different Fatty Acid conditions in SRM_______________ 28

Figure 13: Proliferative effects of different combinations in SRM_____________________ 29

Figure 14: Comparison of combination #2 to the controls___________________________ 37

Chapter 1: Introduction

The demand for fish, specifically blue-fin tuna is on the rise, and has been for some time. According to the World Wildlife Fund, more than 3 billion people in the world rely on farmed and wild-caught seafood as a significant source of protein for their diet. This equates to roughly 40% of the world's population relying on seafood for their diets. This increasing demand for resources and diminishing stock pushes a need to find an alternative or supplementary source of fish. One way to supplement this deficiency is through cellular agriculture. Here, I review the current knowledge on fishing practices and the negative effects on fish stocks, the environment, and human health. In addition, I explore cellular agriculture as a potential solution to these issues. Simultaneously, issues of Fetal Bovine Serum (FBS), a crucial component in the process of cell growth, are discussed, as are the possibilities to reduce and remove it from cell-based seafood cultivation. With the potential success of cell-based seafood, we can cultivate the same tuna while simultaneously removing the negative implications of typical fish farming.

1.1 Overfishing and Biomass Sustainability

Overfishing is the process of the removal of certain fish species from water bodies at a rate that the species cannot replenish. This decrease in combination with poor recovery, results in the loss of an entire species from certain areas (Du, 2021). The Food and Agriculture Organization (FAO) estimates that 53% of Bluefin Tuna stock is fully depleted (Boon 2013). The loss of different species can impact the overall health of the ocean's environment (Hanafiah, 2011), thus negatively affecting our environment and food security (Sumaila, 2020).

Fishing practices have lowered ocean biomass by an estimated 80%, a problem made worse by global warming (Rubio, 2019). Aquaculture, the practice of farming fish and aquatic organisms (Martinez-Porchas, 2012), is capable of reducing wild capture. Unfortunately, outside of Asia, 67% of all farmed fish are carnivorous, which equates to over 800,000 tons of aquaculture requiring wild aquatic animals for their nutrient source (New, 1995). As aquaculture increases, wild-fish capture simultaneously increases (Rubio, 2019). A sustainability analysis was performed by Costello et al. in 2012 about the sustainability status of specific fish species. A multivariate regression approach was used to identify predictors of stock status. Many species were below the line of sustainability, including Tuna. For a species to be safe, it must be well above this line (Costello, 2012) as seen in the figure below.

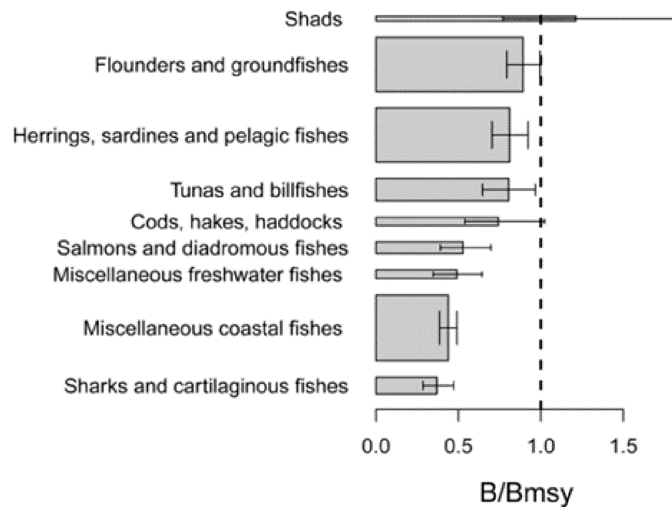

Figure 1: Sustainability standing of various species (Costello, 2012)

There are risks beyond ocean biomass sustainability that arise with overfishing. Empirical and modeling studies have shown that the loss of these top predators can cause population decline in dependent predators such as seabirds, larger fishers, and marine mammals. Fishing at maximum

sustainable yield levels (MSY) for fish has been shown to lead to population declines of 54% for seabirds and 27% for all predators combined (Pikitch, 2012).

1.2 Overfishing and Environmental Impact

The depletion of fish stocks, as seen in the critical reduction of Bluefin Tuna, not only threatens biodiversity but also precipitates broader environmental consequences. Habitat degradation, a direct fallout of overfishing practices, further exacerbates the vulnerability of our marine ecosystems. Habitat degradation is embedded in overfishing through destructive fishing equipment and plastic pollution. (Sumaila, 2020). Fishing strategies such as dredging, bashing, spearfishing, and blast fishing negatively impact the habitats by disrupting, flattening and exploding the seabed, which in turn alters the physical structure of the habitat while simultaneously destroying fragile ecosystems like coral reefs and seagrass meadows (Carneiro, 2021). Ghost fishing, also refers to the common scenario where plastic from overfishing techniques remains in the ocean, which leads to continued marine debris and plastic pollution, which negatively impacts the health of the ecosystem (Carneiro, 2021).

Overfishing has resulted in habitat loss leading further to the undermining of coastal protection and carbon storage (Sumaila, 2020), increasing our susceptibility to global warming. The depletion of fish from an ecosystem can disrupt the food chain, which in turn affects the survival of other marine life forms. Every link in the food web of the ocean is crucial to maintaining ocean abundance. The interactions between different species are complex and interwoven. A reduction in fish can impact life forms from phytoplankton to predators, which can increase the ocean ecosystems' carbon dioxide production (Du, 2021).

The current state of tuna fishing produces 7.63 kg of carbon dioxide per kg of tuna produced (Gephart, 2021) producing heavily more carbon per kg than fish. Maintaining healthier fish stocks is important for essential planetary such as carbon storage, and coastal protection/erosion. The role of the oceans in the regulation of the global carbon cycle is well known. It is estimated that the ocean contains about 38,000 Gigatons (Gt) of carbon, and this is by far the largest reservoir of carbon on Earth. The ocean captures and stores carbon that would otherwise enter the atmosphere and contribute to climate change. Healthy fish stocks and marine ecosystems regulate ocean health, which can help to mitigate global warming. Climate-related impacts have impacted species, populations, and ecosystems already as the figure below demonstrates (Sumaila, 2020).

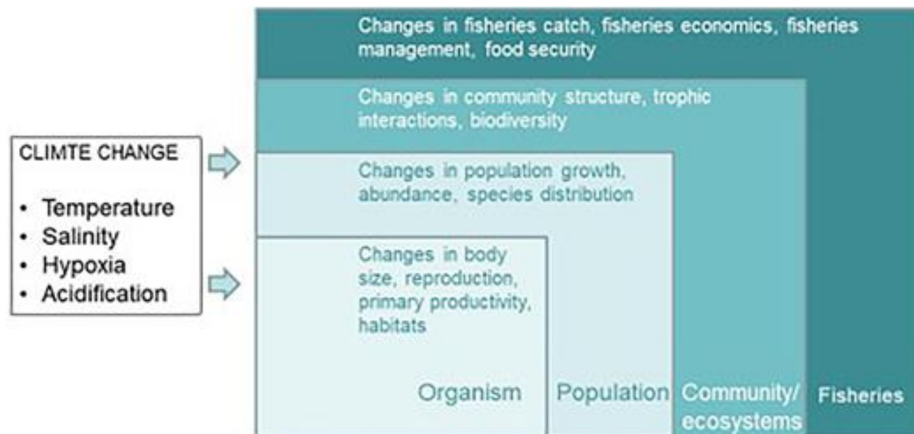

Figure 2: Climate change impacts on our oceans and economy (Sumaila, 2020)

1.3 Overfishing Implications on Economic Sustainability and Food Security

The environmental toll of overfishing, through habitat destruction and ecosystem imbalance, has profound repercussions beyond marine life. It directly impacts economic sustainability and food security, sectors deeply intertwined with healthy marine environments. Overfishing leads to a decline in fish stocks, which can reduce the productivity of fisheries. This

decline can negatively impact the economic visibility of fishing industries, leading to a loss of income for fishermen and associated businesses. Fisheries represent around 1% of the total U.S. GDP, sales have been valued higher than $212 billion, and contribute to around 1.7 million jobs. Marine ecosystems in the U.S. and globally and their associated marine capture fisheries have a significant societal and economic impact (Link, 2021). Thus, this system's decline can negatively affect our quality of life beyond the ocean. Fisheries produce over 4.5 million metric tons of fish a year in the U.S, comparable statistics are found globally (Link, 2021), indicating the vital role of fisheries on food security, which can be compromised due to overfishing practices.

1.4 Dangers of Consumption (Pesticides, Heavy Metals, and Plastics)

The ripple effects of overfishing extend into the realm of human health, where the economic and food security implications of dwindling fish stocks are compounded by the dangers posed by seafood consumption. As fish populations are over-exploited and ecosystems disrupted, the risk of consuming contaminated seafood, carrying heavy metals, plastics, and other pollutants, escalates, highlighting a pressing need for safer, sustainable alternatives. Pesticides used in agriculture and other applications have the potential to reach waterways through rain or the atmosphere. These pollutants are absorbed by plankton and small fish at the bottom of the food chain. Bluefin tuna are an apex predator, which consumes large quantities of contaminated prey. Tuna has been found to be contaminated with persistent organic pollutants (POPs) and Polychlorinated biphenyls (PCBs) (Klinčić, 2020), which if consumed in high volumes can negatively impact neurodevelopmental problems in children (Ribas-Fito, 2001), increase cancer risk (Rokni, 2023) suppress the immune system (Gascon, 2013) and lead to reproductive issues (Guillotin, 2022).

Industrial activities and natural disastrous effects such as volcano eruptions can release heavy metals such as mercury, lead, and cadmium into the ocean (Lozano-Bilbao, 2023). Heavy metals introduced into the rivers and coastal waters are primarily absorbed by plankton. Tuna, belonging the upper stage of the food chain consumes organisms that consume the plankton, allowing for toxicity of these metals to transfer through their diet (Bae, 2023). Studies have shown high amounts of Iron (Pb), Cadmium (Cd), and Lead (Pb) (Lozano-Bilbao, 2023). Concentrations of Cd and Pb were found to be over the legislative limit in Tuna samples, thus posing a risk to human health (Lozano-Bilbao, 2023). Mercury poisoning is also relevant in tuna consumption. The FDA has a limit of 0.1 ppm of mercury (Hg); however, studies have shown that mercury in tuna is up to 1.17 ppm (Rasmussen, 2005). Hg is highly toxic and severely damaging to tissue and cellular function. Hg can accumulate in the kidneys, liver, and brain. It can also block intestinal nutrient transport and can impose various immunotoxin effects. Cadmium can cause acute and chronic poisoning by primarily targeting the kidneys. Over time, Cadmium can also contribute to osteoporosis by inhibiting calcium absorption. Lead also has severe effects on the nervous, gastrointestinal, and renal systems. Gastrointestinal colic and death are results of acute lead poisoning, while chronic poisoning leads to anemia and damage to organs (Lichtfouse, 2012). Excessive iron (Fe) can also cause organ damage, specifically to the liver, heart and endocrine organs (McDowell, 2024).

Plastics have also contaminated our seafood, specifically our bluefin tuna. Studies have shown that around 31% of bluefin tuna are contaminated with microplastics, mesoplastics, and macroplastics (Romeo, 2015). Ingesting plastics is associated with cancer, birth defects, impaired

immunity endocrine disruption, and negative developmental and reproductive effects (Rustagi, 2011).

1.5 Fish Freshness, Stability, Spoilage, and Waste of Resources

Beyond the risks posed by contaminants in seafood, the industry faces significant challenges in ensuring the freshness and stability of fish, a concern directly tied to the health and safety of consumers. Fish are particularly prone to spoiling, mainly because they contain a lot of water—around 70-80% (Sabu & Sasidharan, 2020). The decline in fish quality involves complex processes such as bacterial growth, chemical alterations, and changes in appearance and taste. These issues not only raise the risk of foodborne diseases but also lead to financial losses due to the reduced market appeal of spoiled fish. Prompt and proper cooling after catching, along with careful handling and transportation, is key to maintaining fish quality and prolonging its shelf life. Neglecting these steps can significantly shorten the time fish remain fresh, emphasizing the need for immediate action post-catch. Additionally, it's estimated that improper handling results in 10 to 12 million tons of fish wasted each year because of spoilage (Sabu & Sasidharan, 2020). The effort and resources invested in fish capture, which includes labor, fuel, and the costs associated with fishing gear and vessels, become unsustainable when faced with the loss of the catch due to spoilage or degradation in quality. This not only directly impacts the availability of fish as a food source but also represents a waste of both environmental and economic resources involved in the capture process (Sabu & Sasidharan, 2020).

1.6 Cell Agriculture and Cell-Based Seafood

Given the significant dangers associated with conventional seafood consumption, the search for sustainable alternatives is imperative. Cellular agriculture presents a groundbreaking

solution, offering the potential to produce seafood without the environmental and health risks inherent in traditional fishing and aquaculture practices. By cultivating fish cells in a controlled environment, we can envision a future where seafood is both safe and sustainable. An alternative to meat from farmed animals is Cellular Agriculture. Cellular agriculture is the production of agricultural products from cell cultures rather than from animals (Rubio, 2019). Cellular agriculture employs cells isolated from animals, which are grown in a lab. Removing the animal from the equation limits the need for high-scale conventional meat production, thus creating a potential avenue for a more human and environmentally friendly process (Tuomisto, 2011). The main goal of cellular agriculture is to manufacture agricultural products that are similar to those made in traditional agricultural methods on a molecular level. Cultures of microorganisms such as plants, animals, and tissue cultures can be used (Eibl, 2021). Cellular agriculture can address the issues within public health, environmental, and animal welfare challenges (Rubio, 2019). The world population is estimated to reach 9-11 billion people by 2050. The pressure of limited arable land and the threat of climate change can compromise the ability to supply this population with food and other agricultural products by the current standard of food production. Implementing cellular agriculture is a potential solution to this problem (Eibl, 2021).

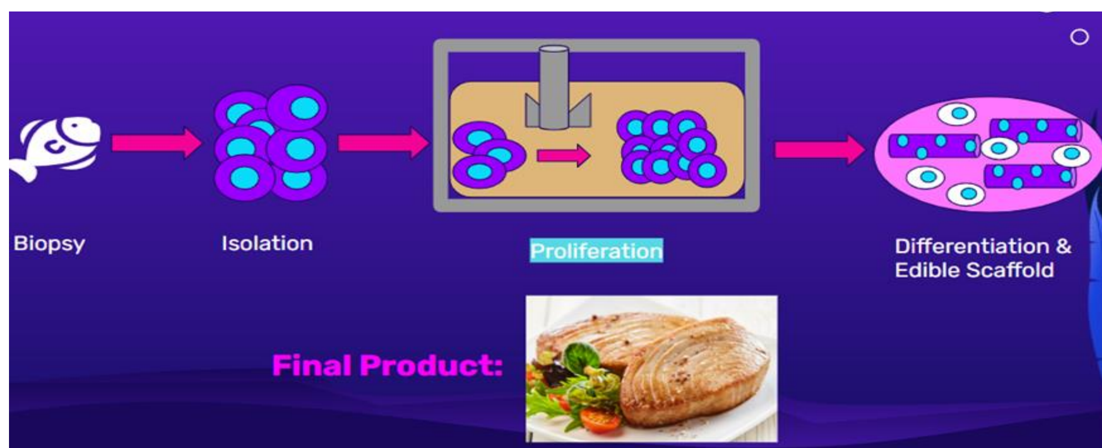

Figure 3: Overall process of cellular agriculture

Cellular agriculture can extend beyond mammalian species, it can include fish and seafood products. This subtopic has been labeled as cultivated meat, and specifically for sea creatures it has been labeled cell-based seafood (Rubio, 2019). Cell-based seafood offers a sustainable alternative to traditional fishing. Cell-based seafood has the potential to mitigate the environmental impacts of overfishing and marine pollution. By cultivating seafood from cells it removes the harmful impacts from microplastics, radioactivity, and heavy metals consumed by sea creatures. Simultaneously, it reduces the strain on marine ecosystems and contributes to the conservation of biodiversity. Cell-based seafood provides a means to meet the growing demand for food without further depleting natural fish stocks, thus supporting food sustainability and environmental conservation (Jung, 2022).

1.7 Cell Culture Media and FBS

The current challenges that face cultivated meat are cost, scalability, food-safe, and free of animal-derived products. In terms of cost, the biggest hurdle is that cell culture media typically consists of at least 99% of the cost (Stout, 2022). A growth medium or culture medium is a liquid designed to support the growth of microorganisms, cells, or small plants (Arora, 2013). Culture medium supplies essential components for cell growth such as carbohydrates, vitamins, amino acids, minerals, growth factors, hormones, and components that control physicochemical properties such as the culture's pH and cellular osmotic pressure (Segeritz, 2017). Cell culture media has always relied on fetal bovine serum (FBS) to proliferate and differentiate cells.

FBS is the most expensive component of the media. It is also uncharacterized, inconsistent, animal-derived, and unsustainable (Stout, 2022). There is yet to be a serum-free or serum-reduced media formulation that allows tuna cells to proliferate at a comparable rate to traditional serum-

containing media. It is estimated that about 500,000 liters of serum from 1,000,000 fetuses are produced annually, and the use of FBS is expected to increase in the coming years with the continuous boom of *in vitro* biological research (Van Der Valk, 2004). FBS use has increased within diagnostics, research, and the pharmaceutical industry. The serum supply is lower than the demand, which makes it very expensive. It is not uncommon for the cost of serum to be up to 95% of the total medium cost. FBS is loosely regulated, allowing for discrepancy and deception in selling FBS within scientific communities (Andreassen, 2020). FBS is commonly harvested from fetal cows using a cardiac puncture without any form of anesthesia, causing undue harm to the fetus and parent (Jochems, 2004). More specifically, FBS is the supernatant of clotted blood from a 3-9 month-old bovine fetus. The reproductive tract is also removed from the slaughtered pregnant cow before the fetus' heart is punctured (Andreassen, 2020). Furthermore, FBS composition varies drastically between batches, with risks of contamination with viruses, mycoplasma, and prions. This may interfere with the reproducibility of experiments and the safety of products (Van Der Valk, 2004), especially in cultured meat. Although FBS has never been fully characterized, the typical components are shown in Table 1 (Andreassen, 2020).

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

Table 1: FBS contents (Andreassen, 2020)

Since different cell lines have specific individual requirements, developing a universal serum-free media (SFM) is not probable. SFMs are currently commercially available for many cell types within industrial and medical applications. Since these SFMs are commercially available, the composition has not been published for commercial reasons. This makes it difficult to transfer and update knowledge between large-scale cell biotechnology companies and cellular agriculture research. Commercially available SFMs are currently not food-grade. Cost and performance are still issues. Typically, developing an SFM includes a basal medium that provides a suitable environment for the cells, containing macronutrients and micronutrients. Macronutrients and micronutrients consist of carbon and nitrogen sources and vitamins and minerals, respectively. Carbon sources consist of carbohydrates and lipids. Amino acids are the most effective nitrogen source. Insulin, recombinantly produced or purified growth factors, transferrin, selenium, and serum proteins (such as albumin) are also essential for cell culture. Some genetically modified cells can be maintained with these media elements, but most cells need many additional

supplements for efficient cell adhesion, growth, survival, and proliferation. The most common components added to SFM and their purpose are shown in Table 2.

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.

Table 2: Components added to SFM and their respective purpose (Andreassen, 2020)

Meat Production Methods	**Food Security**	**Food Safety**	**Environmental Impact**
Conventional Seafood Production Methods	Issues with stock depletion (Boon, 2013) Below line of sustainability (Costello, 2012)	Toxic Pesticides in fish (Klinčić, 2020) Heavy metals in fish (Lozano-Bilbao, 2023) Plastics in fish (Romeo, 2015)	Destroys ocean environment (Carneiro, 2021) Damaging the ocean keeps more Carbon dioxide in the air (Sumaila, 2020)
Cultivated Meat	Tissue isolations and cell line immortalizations can remove the need to continuously kill animals for meat (Reiss, 2021)	Potentially less susceptible to contamination (Ong, 2021)	Potentially less land, less water, and less greenhouse gas emissions (Stout, 2022)

Table 3: Impacts of conventional meat production vs. cultivated meat

1.8 Aims of the Study

The goal of this study was to contribute a foundation of supplements to reduce serum in the field of cell-based seafood cultivation, thus further developing the avenue for reducing serum in cultivated meat. As we develop serum-reduced and serum-free media, we simultaneously become closer to our goal of developing a more sustainable food resource for all. Through the development of cultivated meat, we possess the capability to substantially enhance the quality of life on our planet, benefiting not only its diverse ecosystems but also all of its inhabitants.

This study aimed to [1] Identify the combination of basal medium that significantly outperformed the conventional Fish growth media basal medium [2] Identify and quantify individual supplements and respective optimal concentrations that aided in proliferation within the serum-reduced culture (2.5% FBS) of Tuna fish cells (Thunnus Thynus) [3] Quantify short-term proliferative efficacy of these optimal supplements in combination with each other in serum-reduced culture.

2. Materials & Methods

2.1 Tuna Isolation & Cell Culture

Tuna tissue was provided and isolated from Gulf of Maine Research Institute (Portland, ME, USA). To isolate tuna cells, the work area was cleaned with 70% ethanol, and all dissection tools were sterilized. A piece of tissue was cut using sterile scissors, then disinfected with 70% ethanol before being placed in a hood on a Petri dish. Next, the tissue was cut into 4-8 sections, each about 2x2cm. These sections were treated with 5-10 mL of PBS supplemented with antibiotics and allowed to incubate for a minute to prevent bacterial contamination. The antibiotics solution was then aspirated, and each section was transferred to a sterile Petri dish. For cell isolation, the tissue sections were minced into 1mm fragments. Using the explant method, the fragments, about 2 g, were transferred directly into a cell culture T25 flask, kept at 27°C, and 3 ml of DMEM with 10% FBS was added. The flask was monitored daily for migrating cells, which were collected, and the explant tissue was removed with a filter. Alternatively, the enzymatic method involved transferring the fragments into sterile 50ml tubes with 5 mL of collagenase II solution. The tubes, labeled "tissue," were gently shaken to dissociate the tissue and then placed on a rotary shaker for 10 minutes at room temperature. After centrifugation at 1600 RCF for 7 minutes, the supernatant was aspirated. The cell tissue pellet was treated with 5 mL of trypsin solution, gently pipetted, and shaken again for 10 minutes at room temperature. Following centrifugation, the supernatant was collected in a tube labeled "cells," and FBS was added to inactivate the enzyme. This process was repeated three times. Finally, the collected cells were centrifuged, the supernatant was aspirated, and the cells were resuspended in appropriate media for culture, either DMEM 20% FBS or L-15 20% FBS, and then seeded in cell culture T25 flasks.

The cell cultures were incubated at 27°C in an incubator without CO2. After 48 hours, the cell suspension was moved to a laminin-coated T25 flask, and the original flask was fed. This isolation procedure was performed at the Science and Technology Center at Tufts University (Medford, MA, USA) on October 19, 2022.

First, fish growth media (Fish GM) was prepared with Leibovitz L-15 Medium (L-15) [ThermoFisher (Waltham, MA, USA) #11415064], 20% Fetal Bovine Serum (FBS) [ThermoFisher #A5256801], 1% Gentamicin [ThermoFisher #15750060], 1% Antibiotic/Antimycotic (Anti/Anti) [ThermoFisher #15240062], 1 ng/ml Fibroblast Growth Factor-2 (FGF-2) [ThermoFisher #SRP4037] and 2% Hepes buffer [ThermoFisher #15630106]. Media was 0.22 um sterile filtered [ThermoFisher #1680045] before use. The tuna satellite cells (TSCs) were grown at 37°C with three to four days between passaging, aiming for around 80-90% confluency.

The TSCs were passaged using a standard protocol. Briefly, the media was removed, and the cells were washed in phosphate-buffered saline [ThermoFisher #J67670-AP] (PBS). 0.05 Trypsin [ThermoFisher #25200056] was added and incubated at room temperature (RT) for 10 minutes to allow for detachment. The trypsin was neutralized with FBS by adding Fish GM and spun down at 300 rpm at RT for 5 minutes. The cell pellet was resuspended in Fish GM media and counted using Countess 3 at each passage. Cells were seeded from 4-8k/cm^2 into a new flask.

2.2 Basal Medium Proliferation Screening Materials

Basal media screening was performed by analyzing the proliferative effects of various media combinations with the TSC's. Mediums chosen to test in combinations were Dulbeccos's Modified Eagle Medium Glutamax (DMEM) [ThermoFisher #10569010], Ham's F12 Nutrient

Mix (F12) [ThermoFisher #1765054], Ham's F12K (Kaighn's) Medium (F12K) [ThermoFisher #21127022], Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) [ThermoFisher #11320033], CO_2 Independent Medium (CO_2 ind.) [ThermoFisher 18045088], Medium 199 (M199) [ThermoFisher #11150067]. Each basal medium combination was combined with 10% Fish GM in a 1:1 ratio to create 5% FBS media for each combination. 5% FBS for each condition was chosen based on previous basal media screenings done by the Kaplan lab for fish cell culture. TSCs were grown in each media combination to observe the short-term proliferation effects of the media formulations. Each condition was tested in triplicate. The combinations tested are listed in the table below. Each combination was chosen due to the nutrients that it added.

Basal Media Formula	Component 1	Component 2	Component 3
L-15/DMEM	L-15 10% FBS Fish GM	DMEM Glutamax	-
L-15 + DMEM/F12	L-15 10% FBS Fish GM	DMEM/F12 Glutamax	-
L-15 + DMEM + F12K	L-15 10% FBS Fish GM	DMEM Glutamax	F-12K
L-15 + F-12	L-15 10% FBS Fish GM	F-12	-
L-15 + F12K	L-15 10% FBS Fish GM	F12K	-
L-15 + CO2 Ind.	L-15 10% FBS Fish GM	CO2 Independent	-
L-15 + M199	L-15 10% FBS Fish GM	M199	-

Table 4: The combinations of the basal medium that were tested

2.3 Media Supplement Screening Materials

Media supplements were chosen based on their previous positive effects on fish cell proliferation in the Kaplan Lab. Each condition's proliferation efficacy was tested in 2.5% serum-reduced media (SRM) Fish GM. (Insulin-Transferrin-Selenite) ITS was a premade supplement and did not require any further dilution. All growth factors and fatty acids were diluted in PBS and ready for use by previous members of the Kaplan Lab. Chlorella (*Chlorella vulgaris*) and Rapeseed (*Brassica napus*) Protein isolates were extracted in-house and provided by members of the Kaplan lab. Rapeseed was bought in cake form, while Chlorella was bought in powder form. The proteins were isolated through an albumin extraction process (Stout 2023) with protein content calculated with a Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit [ThermoFisher #23225]. The Bicinchoninic Acid (BCA) assay is a technique used for quantifying total protein concentration in a sample. The assay involves the biuret reaction, where proteins under alkaline conditions reduce Cu^{2+} ions to Cu^{+}. This reduction is facilitated by the peptide bonds in the protein structure, making the reaction directly proportional to the amount of protein present. The reduced copper ion (Cu^{+}) then reacts with bicinchoninic acid to form a purple-colored complex. The intensity of this color is directly proportional to the protein concentration in the sample.

Supplement Classification	**Component**	**Range Tested in SRM**	**Vendors & Catalog Number**
Hormone/Protein/Trace Element	Insulin-Transferrin-Selenium (ITS)	0.25% - 4%	Millipore Sigma (Danvers, MA, USA) #I3146
Albumin	Chlorella Protein Isolate (CPI)	0.1 - 1.6 mg/mL	Anthony's Goods (Glendale, California, USA) #B0788ZFQWQ
Albumin	Rapeseed Protein Isolate (RPI)	0.1 - 1.6 mg/mL	Joshua Roth Limited (Albany, OR, USA) #6046

Growth Factor	FGF-2	1.25 - 20 ng/mL	ThermoFisher #SRP4037
Growth Factor	Human Insulin-like Growth Factor (IGF-1)	1 - 16 ng/mL	ThermoFisher #PGH0071
Growth Factor	Human Transforming growth factor-β (TGF-β)	1 - 16 ng/mL	ThermoFisher #PGH9214
Growth Factor	Platelet-derived Growth Factor (PDGF BB)	1.25 - 20 ng/mL	Millipore Sigma #P3201
Growth Factor	Human Interleukin-6 (IL-6)	0.1 - 1.6 ng/mL	ThermoFisher #AF-200-06-1MG
Fatty Acid	Linoleic Acid	37.5 - 600 ng/mL	ThermoFisher #215041000
Fatty Acid	Oleic Acid	62.5 - 1000 ng/mL	ThermoFisher #031997.06

Table 5: Table of supplements tested in SRM

2.4 Short-Term Combination Materials

After the optimal concentration for each component was identified as mentioned above, each was added in combination with each other to the 3 different basal mediums that were deemed significantly more effective than L-15 Fish GM on its own. These optimal Fish GM basal mediums were L-15 & DMEM/F-12, L-15 & F-12, and L-15 & F-12K. These optimal components & their respective concentrations were ITS at 1%, CPI 0.4 mg/mL, and Oleic Acid at 62.5 ng/mL, all added to the 3 Fish GM basal mediums. The 3 combinations were tested in a short-term proliferation analysis.

Combination #	Media Base (2.5% FBS)	ITS %	CPI mg/mL	Oleic Acid ng/mL
1	Fish GM + DMEM/F12K	1	0.4	62.5
2	Fish GM + F12	1	0.4	62.5
3	Fish GM + DMEM/F12K	1	0.4	62.5

Table 6: Different media combinations that were tested on their proliferative effects in short-term

2.5 Short-Term Proliferation Assay Procedure

During a TSC passage, after the cells were spun down and counted, enough cells were pipetted in 20% FBS Fish GM so that triplicates of each condition and control could then be seeded onto a 96-well plate. Cells were incubated at 37 degrees C for 3 hours to allow cells to adhere.

During the 3-hour incubation time, the testing media was created. Serial dilution was used to achieve the concentration range of each supplement tested. This process allowed each concentration to have 5 separate concentrations tested (not including the 0% concentration or other controls). The maximum concentration of the desired supplement was added to the right-most well in 1500 ul of our SRM and mixed with a pipette tip multiple times. Then to serially dilute, 750 ul of the rightmost well was pipetted into the next well to the left and mixed thoroughly. This process was repeated with the following 3 wells, thus, developing 5 different concentrations to test in triplicate. Serially diluted test media conditions were then seeded onto a 96-well plate after the cell attachment step. All concentration ranges were tested in triplicate on 2-3 separate runs.

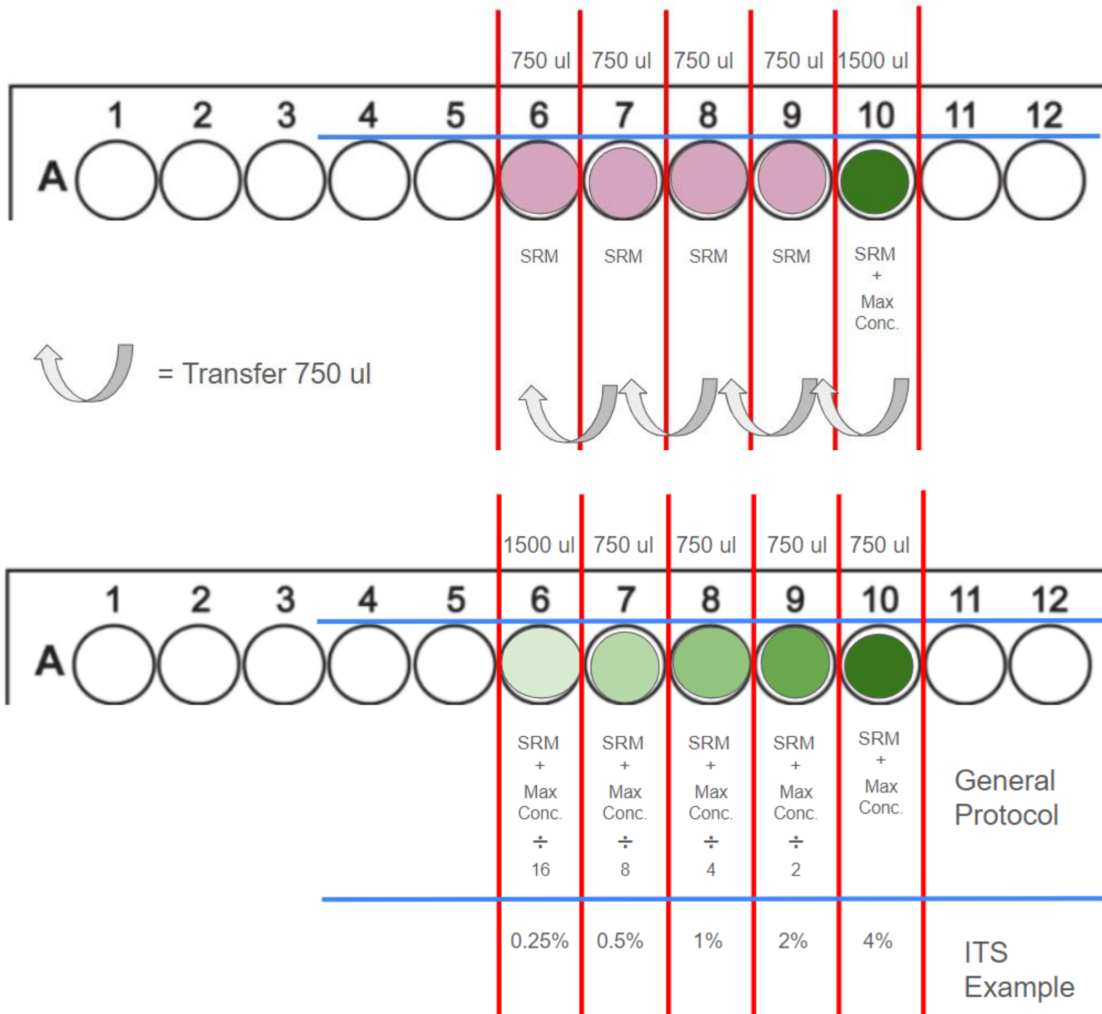

Figure 4: Serial dilution process of each supplement tested

After the cells adhered to the well plate, the 20% Fish GM was aspirated from each well. The controls and supplements (each condition in triplicate) were added with a multichannel pipette at 200 ul to each well respectively. The wells surrounding the perimeter of the pipetted media were filled with 200 ul of PBS to mitigate evaporation of the testing media. The controls and supplements were then incubated at 37 degrees C for 72 hours to allow for short-term proliferation to occur. After 72 hours, the well plate was then taken out of the incubator and imaged on the

microscope one row for each supplement was captured to analyze and cross-reference with the proliferation assay. Post imaging, the media was aspirated, and each well was washed with 200 ul of PBS, which was once again aspirated. Then, the well plate was stored in a -80 freeze for at least 24 hours. The freezing step is important for efficient cell lysis in the CyQUANT® proliferation assay.

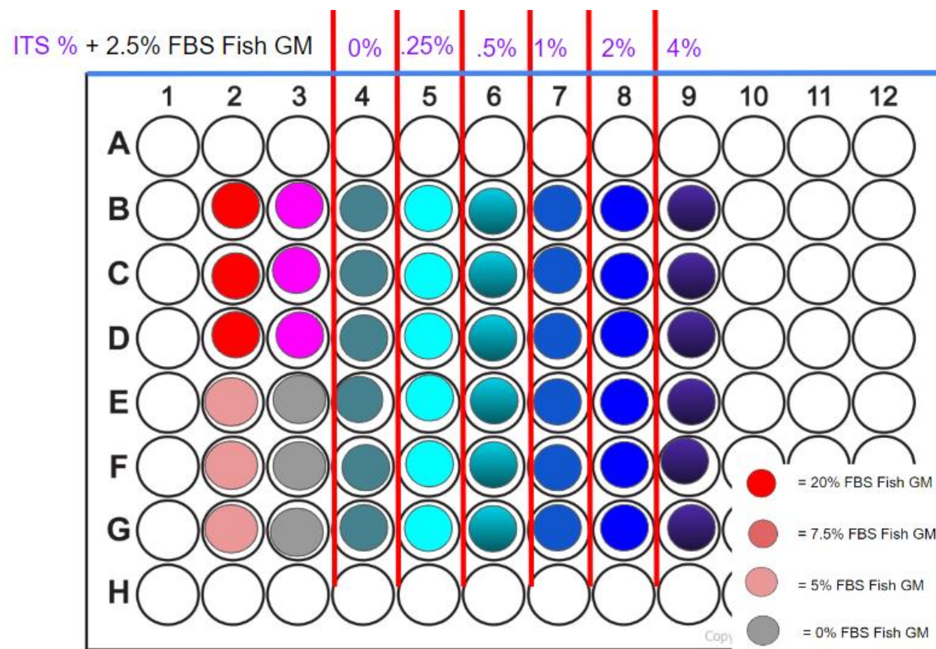

Figure 5: Example 96 Well Plate set up of supplement testing

After at least 24 hours, the well plates were removed from the freezer and the proliferation assay was performed. The proliferation assay working solution is created with 3 components, RNase-free distilled water, along with the Cell lysis buffer and CyQUANT® GR-dye which were both provided by the manufacturer. On the day of the experiment, the concentrated cell-lys-is--buffer stock solution was diluted 20-fold in distilled water. For each assay, 200 µL was required. Before running the experiment, CyQUANT® GR stock solution was diluted 400-fold into the 1X cell-lysis buffer. Typically, to prepare 20 mL of CyQUANT® GR working solution (enough for

~100 assays-), 1 mL of the 20X stock cell lysis buffer would be mixed with 19 mL of nuclease-free distilled water; next 50 µL of the CyQUANT® GR stock solution would be added and mixed thoroughly. The working solution is added to each well using a multichannel pipette, at 200 ul for each well. The well plate was surrounded in aluminum foil for 5 minutes for incubation of the solution in darkness due to the light sensitivity of the fluorescence dye. After 5 minutes of incubation, the well plate was inserted into a plate reader where the fluorescence was read at 480 nm excitation and 520 nm emission. This assay stains the nuclei of each cell, indicating that the higher the fluorescence the higher the cell count.

The proliferative effects of all basal mediums, supplements, and short-term combinations were tested using this procedure. The conditions from Aim 1 and 2 with the highest nuclei count displayed by the plate reader, and deemed as significant through statistical analysis were selected for the Aim 3 study, where the same assay was performed to analyze the proliferative efficacy once again.

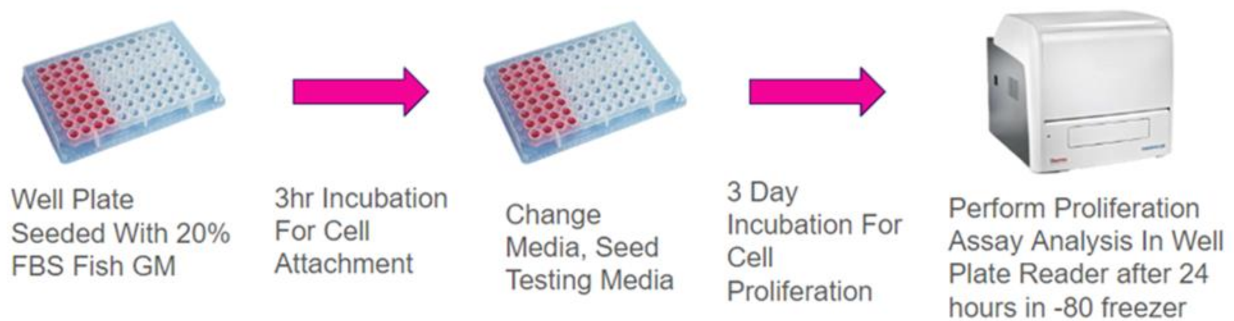

Figure 6: Overall process of short-term proliferation analysis

2.6 Statistical Analysis

The fluorescence numbers, indicating cell growth from the plate reader were then normalized to 2.5% FBS SRM to observe the significance of growth to the base control. Statistical analysis was performed with GraphPad Prism 10 software. All data was analyzed via one-way ANOVA. Multiple comparisons of all analyses were performed with Tukey's HSD post-hoc test. P values ≤ 0.05 were treated as significant. **** $p \leq 00001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, not significant (ns) $p > 0.05$.

3. Results

3.1 Basal Medium Combination Short-Term Proliferative Effects

The proliferation assay showed that there is a significant statistical difference between different basal medium combinations in comparison to the typical Fish GM on its own. Specifically, cell growth significantly increased when DMEM + F12K, F-12, and F-12K were combined with the typical Fish GM across a 3 day-incubation period. This increase makes sense because many of these other basal mediums added to the culture have nutrients that aren't included with the L-15 basal medium.

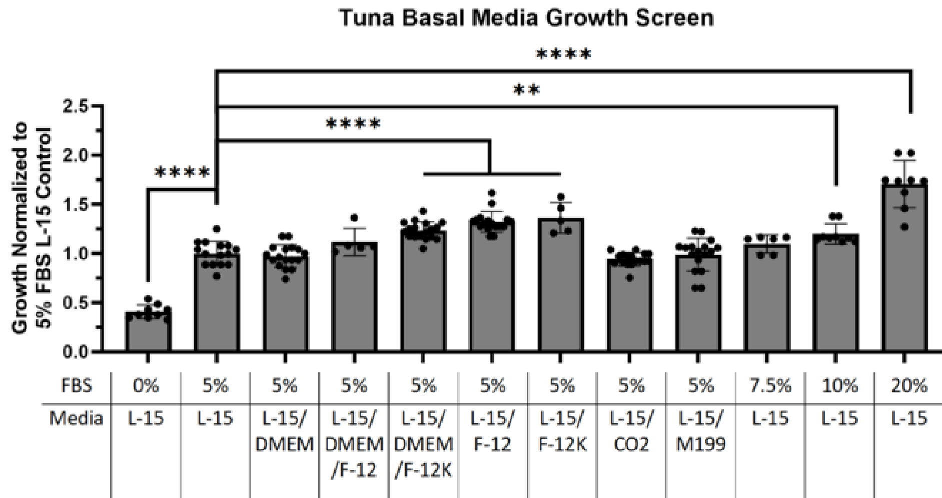

Figure 7: Proliferative effects of different basal medium conditions in SRM

3.2 Media Supplement Short-Term Proliferative Effects

For the proteins tested, the proliferation assay showed there were significant proliferative effects when CPI was added to the cell culture, but not when RPI was added. This finding was surprising considering how effective RPI has been for previous cell lines in terms of cell growth.

RPI was cytotoxic at specific concentrations. However, because algae are consumed in the diets of fish, it makes sense that it has more synergistic effects with fish cells.

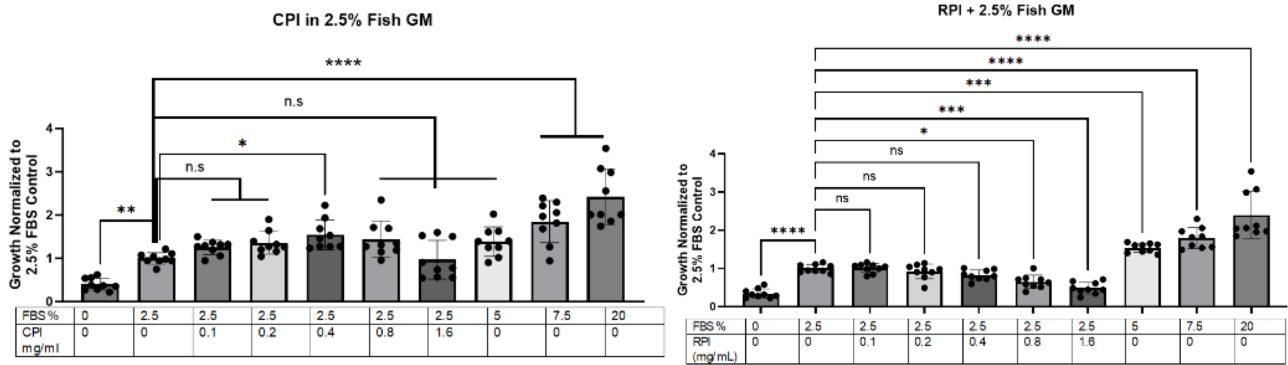

Figure 8: Proliferative effects of different CPI and RPI conditions in SRM

There were no significant enhanced proliferative effects from the supplementation of all the growth factors tested. Throughout the growth factor studies, the 5% and 7.5% FBS containing Fish GM controls were not deemed significant in comparison to the 2.5% Fish GM control. This was slightly abnormal because the protein extract studies showed significant difference between 5% and 7.5% FBS controls in comparison to the 2.5% FBS control. Regardless of this limitation, there was no positive significant effect from any growth factor. With this result, no growth factors tested were included in the combination stage of this study. Growth factors with proliferative promise were tested on 3 different assays. Growth factors that immediately showed no positive effect or even immediate negative effects were not experimented on after 2 assays.

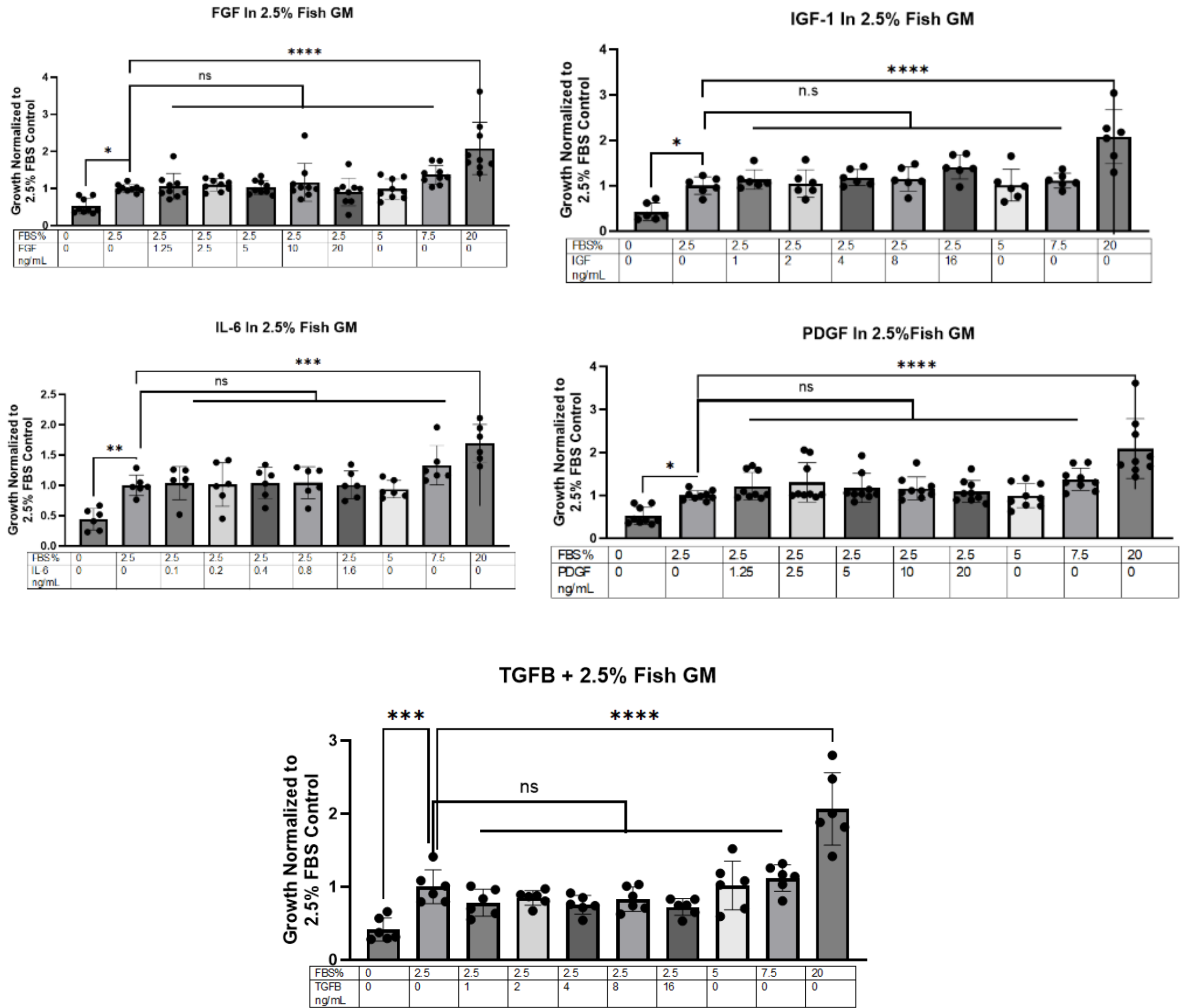

Figure 9: The proliferative effects of different growth factors in SRM

ITS proved to enhance proliferation significantly. ITS was tested 3 times and had 6 replicates for each condition. Although the procedure was slightly different than the other tests, the accuracy only increased with more data points. All concentrations of ITS proved to be significantly effective. 1%, 2%, and 4% had the highest averages as shown in the graph below and were all around the same fluorescence level. 1% ITS was chosen to be implemented into the

combination step because it had one of the highest averages while simultaneously using the least amount of material to get the same result.

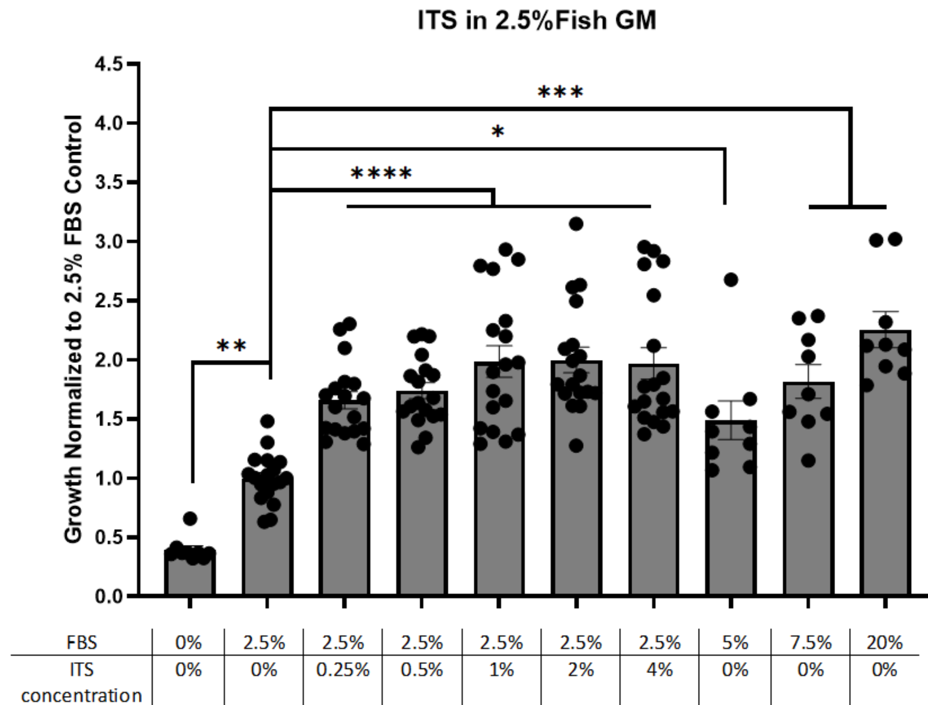

Figure 10: Proliferative effects of different ITS conditions in SRM

According to the statistical analysis, fatty acids were not significantly enhancing proliferation. Both Linoleic acid and Oleic acid proved cytotoxic at higher concentrations. However, Oleic acid had a higher fluorescent average value than the 2.5% control at 62.5 ng/mL. Microscope images also showed that there seemed to qualitatively be more cells for oleic Acid at 62.5 ng/mL. Due to these two points, along with its known synergy with albumin in cell culture (van der Vusse, 2009), oleic acid at 62.5 ng/mL was deemed effective and added into the combination study. For the image below, the microscope image on the right visibly shows more

cells taking up the space of the 96 well, indicating higher confluency, which we can infer is higher cell growth.

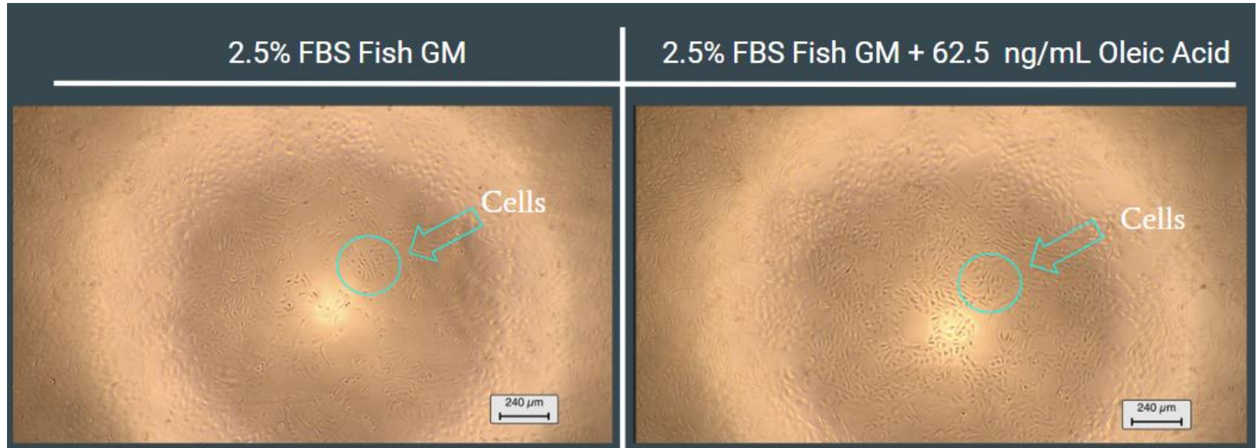

Figure 11: 2.5% Control (left image) vs SRM with 62.5 ng/mL Oleic Acid (right image).

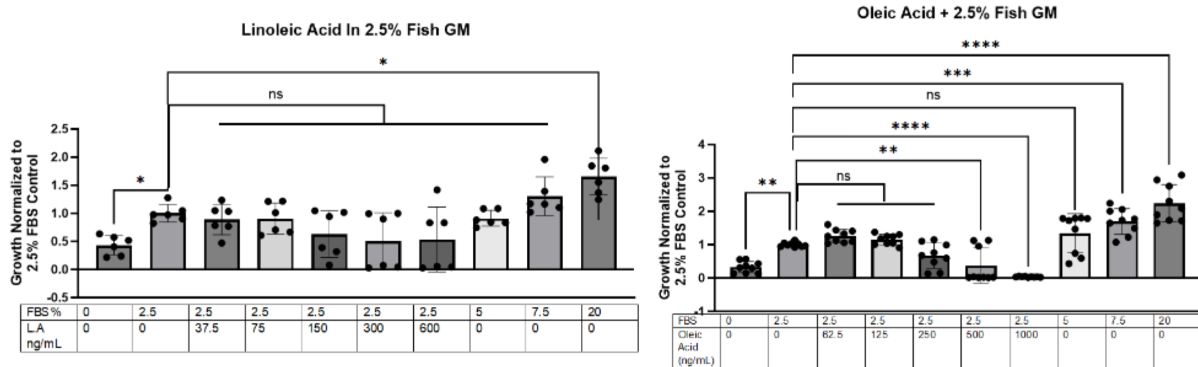

Figure 12: Proliferative effects of different Fatty Acid conditions in SRM

3.3 Short-Term Combination Proliferative Effects

The results from Aim 1 and Aim 2 allowed us to develop 3 different combinations with the potential to increase cell growth. Each of the 3 basal mediums that were selected as effective in Aim 1 were used as the base for each combination. Next, ITS, CPI, and Oleic Acid, the three

supplements with enhanced proliferation capabilities, were added at 1%, 0.4 mg/mL, and 62.5 ng/mL, respectively, to each basal medium base to create 3 different combinations to be tested in short-term proliferation. Out of the 3 combinations tested, only combination 2 showed significant growth in comparison to just 2.5% FBS SRM on its own, indicating that F-12 was the best addition to SRM Fish GM, along with the three effective supplements. This result is the first effective SRM formulation produced for the short-term culture of TSCs.

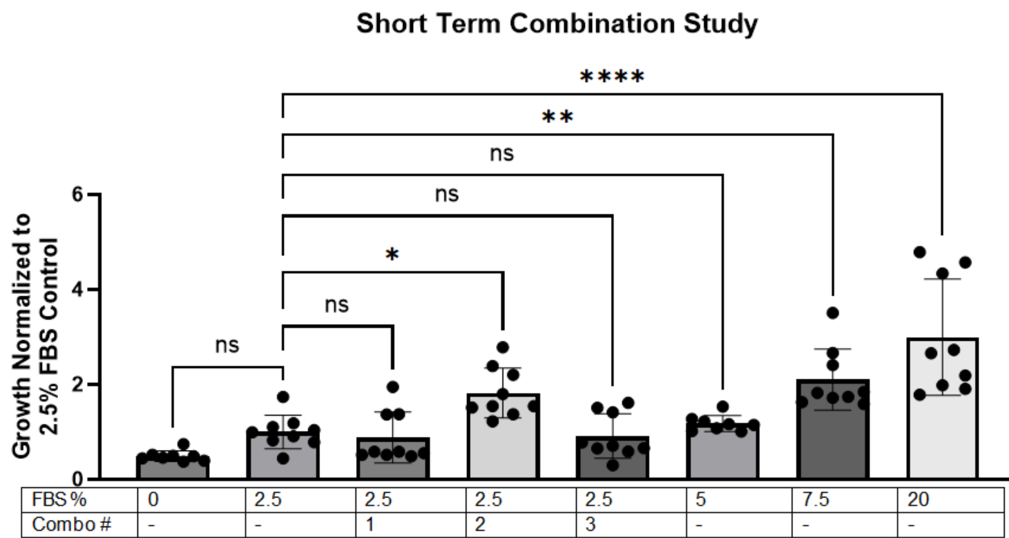

Figure 13: Proliferative effects of different combinations in SRM

4. Discussion

The purpose of this study was to analyze different basal medium and medium supplements and implement them together to create a new SRM for TSCs.

4.1 Basal Medium

The findings suggest that the addition of DMEM Glutamax, F-12, and F-12K boosts the cell growth of TSCs in SRM. This inference is most likely true because these three basal mediums include different components that are not typically found in L-15 medium. These additional components can contribute to increasing cell growth. Below is a list of tables for each of the three basal mediums. The tables consist of components that are not found in L-15 but are included in the respective medium.

DMEM Glutamax Components	***Concentration (mg/L)***
Amino Acids	
L-Alanyl-Glutamine	862
L-Cystine 2HCl	63
L-Arginine hydrochloride	84
L-Histidine hydrochloride-H2O	42
L-Lysine hydrochloride	146
L-Tyrosine disodium salt dihydrate	104
Vitamins	
Pyridoxal hydrochloride	4
Riboflavin	0.4
Inorganic Salts	
Ferric Nitrate	0.1

Sodium Bicarbonate	3700
Sodium Phosphate monobasic	125
Other Components	
D-Glucose (Dextrose)	4500

Table 7: DMEM Glutamax components and respective Concentrations that are not found in L-15 Medium. Adapted from Gibco™ (n.d.)

F-12K Components	***Concentration mg/L***
Amino Acids	
L-Alanine	18
L-Arginine hydrochloride	422
L-Asparagine-H2O	30
L-Aspartic acid	26.6
L-Cysteine hydrochloride - H2O	70
L-Glutamic Acid	29
L-Histidine hydrochloride - H2O	45.8
L-Isoleucine	7.88
L-Leucine	26.2
L-Leucine hydrochloride	73
L-Methionine	8.96
L-Phenylalanine	9.92
L-Proline	69
L-Serine	21
L-Threonine	23
L-Tryptophan	4.1

L-Tyrosine disodium salt dihydrate	13.5
L-Valine	23
Vitamins	
Biotin	0.07
Choline chloride	14
D-Calcium pantothenate	0.5
Folic Acid	1.3
Niacinamide	0.037
Pyridoxine hydrochloride	0.06
Riboflavin	0.04
Thiamine hydrochloride	0.3
Vitamin B12	1.4
i-Inositol	18
Inorganic Salts	
Calcium Chloride	102
Culpric sulfate	0.002
Ferric sulfate	0.8
Magnesium Chloride	49.7
Magnesium Sulfate	192
Potassium Chloride	285
Sodium Bicarbonate	2500
Sodium Chloride	7530
Sodium Phosphate	115.5
Sodium Phosphate monobasic	59

Table 8: F-12K Components and Respective Concentrations that Are Not Found in L-15 Medium. Adapted from Gibco™ (n.d.)

F-12 Components	*Concentration mg/L*
Amino Acids	
L-Alanine	18
L-Asparagine-H2O	422
L-Aspartic acid	30
L-Cysteine hydrochloride - H2O	26.6
L-Glutamic Acid	70
L-Proline	34.5
Vitamins	
Biotin	.0073
Vitamin B12	1.4
Inorganic Salts	
Culpric sulfate	0.0025
Ferric sulfate	0.834
Zinc sulfate	0.863
Other Components	
Hypoxanthine Na	4.77
Linoleic Acid	0.084
Lipoic Acid	0.21
Putrescine 2HCl	0.161
Thymidine	0.7

Table 9: F-12 Components and Respective Concentrations that Are Not Found in L-15 Medium. Adapted from Gibco™ (n.d.)

The tables show a variety of components that are found in the effective basal mediums that are not found in L-15. The only commonality between the basal mediums that is not included in L-15 already is L-glutamine, which is an amino acid often added in various forms (e.g., L-Alanyl-Glutamine in DMEM Glutamax). L-glutamine has been stated as vital to fish cell growth because it is utilized as a main energy source (Bols, 1994). Because all of the significantly effective basal mediums include glutamine, we can infer that the cell growth of TSCs can increase with the addition of L-glutamine.

It's not entirely clear which specific elements from the three selected basal mediums—DMEM Glutamax, Ham's F-12, and Ham's F-12K—are responsible for the noticeable boost in cell growth. However, the significant improvement observed in the proliferation of TSCs when using these mediums suggests that they contain critical components lacking in the L-15 Fish GM. This discovery points to the specialized nutritional needs of TSCs and indicates that the composition of these mediums closely aligns with those needs, resulting in a mix of amino acids, vitamins, and minerals that precisely cater to their growth requirements. The considerable enhancement in cell growth not only underscores the importance of refining culture conditions to meet the specific demands of tuna cells but also opens up new avenues for advancing cell-based seafood techniques and the broader field of cellular agriculture.

The limitations of this Aim stem from inconsistent testing as each formulation was not tested the same number of times. Certain formulations were tested later on in the process due to delay of material. However, this limitation does not invalidate the results completely because each formulation was tested in triplicate, and statistical analysis was repeatedly used. Formulations were not tested in different combination ratios. The formula ratios consisted of one ratio per

combination, rather than different ratios for each basal media combination. Nevertheless, it does not take away from the significant proliferative effects shown by the data.

4.2 Supplements

As previously mentioned, developing an SFM includes more than a basal medium, it contains macronutrients and micronutrients. Carbohydrates, lipids, GFs (such as FGF2 and TGF-Beta), transferrin, selenium, and serum proteins (such as albumin) are some of the most common components added to SFM for efficient cell growth, survival, and proliferation (Andreassen, 2020). Current cell-based seafood literature lacks a foundation for SRM or SFM cell culture. Channel catfish ovary cell lines have been adapted to serum-free conditions over multiple passages (Radošević, 2016). A member of the Kaplan lab has tried to adapt the TSCs to low serum conditions, but the cells went through apoptosis when they reached 5% FBS SFM.

In this study, the supplements that statistically significantly enhanced proliferation were ITS and CPI. There are no current studies that implement these specific components into serum-reduced fish cell culture. However, members in the Kaplan lab are currently developing SRM for mackerel cells and have found these components to be effective. Insect protein hydrolysates have been effectively implemented into SRM for zebrafish proliferation and cell culture (Batish, 2022). This finding aligns with the positive effects of our CPI, proving that proteins are important for serum reduction in TSCs and seafood cell culture. The use of GFs has been shown to prove generally ineffective when attempting to reduce serum (Nikkhah, 2023), aligning with the insignificant proliferative results of the GFs implemented in this study.

Limitations in Aim 2 consist of errors in some of the controls. Within the studies of the GFs, the 5% and 7.5% FBS SRM controls were not significantly different than 2.5% FBS SRM.

This result is inconsistent with the other studies in this thesis, as there is ITS, CPI, and Oleic Acid typically some significant difference in proliferation between these controls. It is possible that this impacted the results. However, the TSCs are adapted to growing in 1 ng/mL FGF-2, and it is possible that, due to this adaptation, the cells do not require extra GF supplementation. This possibility would make the error in controls less significant, as additional GF supplementation does not enhance proliferative ability. Another limitation involves the ranges used for each component. Each range was recommended based on the best proliferative effects after supplementing to SRM for mackerel cell proliferation in the Kaplan Lab. Other concentrations within or outside of the range could be tested and have a stronger effect on cell growth than what was tested in this study. A third limitation is that there is a potential for other components that have not been utilized or considered to have a profound proliferative effect in the culture of TSCs. Further screening and investigation are required to find potential components.

4.3 Combination Testing

Out of the three combinations tested, only one showed significant proliferative effects in comparison, which was combination #2, which consists of 2.5% FBS Fish GM, F-12, 1% ITS, 0.4 mg/mL CPI, and 62.5 ng/mL Oleic Acid. Simultaneously, through statistical analysis, we see that combination #2 is statistically insignificant in comparison to 5% and 7.5% FBS SRM controls. Thus, this finding indicates that this formulation was successful in producing a SRM formulation that was comparable to Fish GM, which contained 3x of the serum. This result suggests that F-12 not only has components that are missing in TSC culture that are beneficial to proliferation, but also has the best synergistic effect with typical SRM or SFM additives.

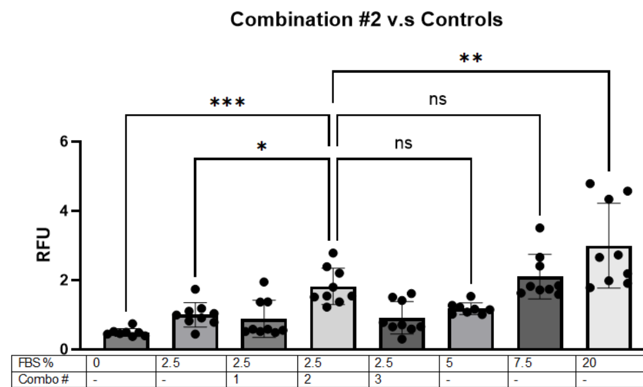

Figure 14: Comparison of combination #2 to the controls

The largest limitation of this aim is that DOE was not implemented. DOE allows for every combination to be tested, including all of the significantly effective concentrations for each supplement tested in Aim 2, respectively. Utilizing a DOE would have also tested combinations without some of the effectively proven components. It is possible that better combinations were missed through the approach used. A DOE was unable to be performed due to the limited amount of CPI in stock at the time this study was performed. A second limitation is that a spent media analysis was not performed. A spent media analysis would allow us to further understand what components are in excess, desired amount, or deficit of the requirements for optimized cell growth. Nevertheless, the results from this study can be implemented in the field of cell-based seafood. This study sets a foundation for components and respective concentrations that can be implemented to reduce or potentially eliminate serum from fish cell culture, furthering the practicality of the application.

5. Future Directions

Future examination into cell line characterization, more components for serum replacement, DOE, spent media analysis, and long-term combination study would increase the impact of this study.

5.1 Cell Line Characterization of Tuna Cells

This foundational step is critical for identifying the unique requirements of tuna cells. Characterization is essential for formulating strategies to reduce serum dependence without compromising cell health. These strategies will aid in the development of more sustainable and cost-effective cell culture practices.

Aim: Establish a comprehensive profile of tuna cell lines.

Objectives: 1) Utilize qPCR to assess gene expression profiles characteristic of muscle or adipose (fat) tissue. 2) Implement immunocytochemistry to visualize protein expression and cellular morphology related to muscle and fat cell phenotypes. 3) Employ flow cytometry to quantify the proportion of cell populations expressing muscle or fat-specific surface markers.

5.2 Further Identification of Serum Replacements for Tuna Cell Culture

Identifying serum replacements specific to the nutritional requirements of tuna cells can significantly reduce the reliance on animal-derived serum, which is not only ethically desirable but also reduces the risk of contamination and variability, leading to more reproducible and scalable cultures.

Aim: Explore and identify potential components and supplements that can serve as effective replacements for serum in tuna cell cultures.

Objectives: 1) Conduct a review of peer-reviewed literature and databases to compile a list of non-serum supplements with demonstrated efficacy in supporting cell-based seafood cultures. 2) Implement factorial design experiments to assess the individual impact of selected non-serum supplements on cell viability and growth. 3) Analyze the individual proliferative ability of each supplement using one-way ANOVA, monitoring which supplements are effective by identifying which components showed significant difference in comparison to multiple controls.

5.3 Media Optimization Through DOE

Utilizing DOE for media optimization enables a more efficient and thorough exploration of the media formulation space, potentially uncovering non-intuitive interactions between components. This approach streamlines the development of a tailored, serum-reduced media that supports the specific needs of tuna cells.

Aim: Systematically optimize the culture medium for tuna cells using DOE, focusing on reducing serum content while maintaining cell culture performance.

Objectives: 1) Identify critical media components and their interaction effects on tuna cell culture performance. 2) Execute a series of DOE, including central composite designs, to model the multidimensional response of cell growth and metabolism to variations in media composition. 3) Validate the optimized media formulation against standard benchmarks through a series of confirmatory runs, employing rigorous statistical analysis to ensure reproducibility and robustness of the results.

5.4. Spent Media Analysis

Spent media analysis offers direct insights into the metabolic state of the cells, revealing whether the cells are experiencing stress or nutritional deficiencies. This information is vital for iteratively refining serum-reduced media formulations, ensuring that the cells are supported optimally throughout their growth, which is crucial for both research applications and industrial-scale cell culture operations.

Aim: Investigate the metabolic byproducts in spent media of tuna cell cultures grown under serum-reduced conditions to further refine media formulations.

Objectives: 1) Characterize nutrient depletion and metabolic byproduct accumulation in spent media from serum-reduced tuna cell cultures using mass spectrometry and nuclear magnetic resonance spectroscopy. 2) Correlate metabolic profiles with phenotypic markers of cell stress and viability, applying multivariate statistical methods to elucidate relationships between metabolic changes and cell health. 3) Iteratively adjust media formulations based on metabolomic data, utilizing algorithm-driven optimization to enhance media supportiveness for cell growth and metabolic efficiency.

5.5 Long-term SRM Combination Culture Studies

Conducting long-term culture studies is essential for assessing the real-world applicability of serum-reduced media formulations. It ensures that the cells remain healthy, productive, and genetically stable over time, which is crucial for any application requiring prolonged cell culture. This step validates the effectiveness of the serum reduction strategy and provides confidence in

the scalability and reliability of the cell culture process for both academic research and industrial biotechnology applications.

Aim: Evaluate the long-term viability and performance of tuna cell cultures using the optimized serum-reduced media.

Objectives: 1) Maintain tuna cell lines in optimized SRM conditions over two weeks, monitoring cumulative population doublings. 2) Analyze long-term cellular productivity by comparing doubling rates and morphology of the SRM to the controls over successive passages.

References

Andreassen, R. C., Pedersen, M. E., Kristoffersen, K. A., & Rønning, S. B. (2020). Screening of by-products from the food industry as growth promoting agents in serum-free media for skeletal muscle cell culture. *Food & Function, 11*(3), 2477-2488. https://doi.org/10.1039/C9FO02690H

Arora, M. (2023). Cell Culture Media: A Review. *Materials and Methods, 3*(175). https://dx.doi.org/10.13070/mm.en.3.175

Bae, S.-J., Shin, K.-S., Park, C., Baek, K., Son, S.-Y., & Sakong, J. (2023). Risk assessment of heavy metals in tuna from Japanese restaurants in the Republic of Korea. *Annals of Occupational and Environmental Medicine, 35*, e3. https://doi.org/10.35371/aoem.2023.35.e3

Batish, I., Zarei, M., Nitin, N., & Ovissipour, R. (2022). Evaluating the potential of marine invertebrate and insect protein hydrolysates to reduce fetal bovine serum in cell culture media for cultivated fish production. *Biomolecules*, *12*(11), 1697. https://doi.org/10.3390/biom12111697

Bols, N. C., Ganassin, R. C., Tom, D. J., Lee, L. E. J., & Stevenson, R. M. W. (1994). Growth of fish cell lines in glutamine-free media. *Cytotechnology, 16*(3), 159–166. https://doi.org/10.1007/BF00749903

Boon, K. E. (2013). Overfishing of Bluefin Tuna: Incentivizing inclusive solutions. *University of Louisville Law Review, 52*(1). Retrieved from https://ssrn.com/abstract=2356566 or http://dx.doi.org/10.2139/ssrn.2356566

Carneiro, M., & Martins, R. (2021). Destructive fishing practices and their impact on the marine ecosystem. In W. Leal Filho, A. M. Azul, L. Brandli, P. G. Özuyar, & T. Wall (Eds.), *Life Below Water: Encyclopedia of the UN Sustainable Development Goals*. Springer, Cham. https://doi.org/10.1007/978-3-319-71064-8_10-1

Costello, C., Ovando, D., Hilborn, R., Gaines, S. D., Deschenes, O., & Lester, S. E. (2012). Status and solutions for the world's unassessed fisheries. *Science*, *October 2012*. https://doi.org/10.1126/science.1223389

Du, Y., Sun, J., & Zhang, G. (2021). The impact of overfishing on environmental resources and the evaluation of current policies and future guideline. In *Proceedings of the 2021 International Conference on Public Relations and Social Sciences (ICPRSS 2021)* (Advances in Social Science Education and Humanities Research, vol. 586). Atlantis Press. https://doi.org/10.2991/assehr.k.211020.316

Eibl, R., Senn, Y., Gubser, G., Jossen, V., van den Bos, C., & Eibl, D. (2021). Cellular Agriculture: Opportunities and challenges. *Annual Review of Food Science and Technology, 12*, 51–73. https://doi.org/10.1146/annurev-food-063020-123940

Gascon, M., Morales, E., Sunyer, J., & Vrijheid, M. (2013). Effects of persistent organic pollutants on the developing respiratory and immune systems: A systematic review. *Environmental International, 52*, 51-65. https://doi.org/10.1016/j.envint.2012.11.005

Gephart, J. A., Henriksson, P. J. G., Parker, R. W. R., Shepon, A., Gorospe, K. D., Bergman, K., Eshel, G., Golden, C. D., Halpern, B. S., Hornborg, S., Jonell, M., Metian, M., Mifflin, K., Newton, R., Tyedmers, P., Zhang, W., Ziegler, F., & Troell, M. (2021). Environmental performance of blue foods. *Nature, 597*(7876), 360–365. https://doi.org/10.1038/s41586-021-03889-2

Gibco™. (n.d.). DMEM, high glucose, GlutaMAX™ Supplement. Thermo Fisher Scientific. Retrieved March 26, 2024, from https://www.thermofisher.com/order/catalog/product/10566016

Gibco™. (n.d.). Ham's F-12 Nutrient Mix. Thermo Fisher Scientific. Retrieved March 26, 2024, from https://www.thermofisher.com/order/catalog/product/11765054

Gibco™. (n.d.). Ham's F-12K (Kaighn's) Medium. Thermo Fisher Scientific. Retrieved March 26, 2024, from https://www.thermofisher.com/order/catalog/product/21127022

Gibco™. (n.d.). Leibovitz's L-15 Medium. Thermo Fisher Scientific. Retrieved March 26, 2024, from https://www.thermofisher.com/order/catalog/product/11415064

Guillotin, S., & Delcourt, N. (2022). Studying the impact of persistent organic pollutants exposure on human health by proteomic analysis: A systematic review. *International Journal of Molecular Sciences, 23*(22), 14271. https://doi.org/10.3390/ijms232214271

Hanafiah, M. M., Xenopoulos, M. A., Pfister, S., Leuven, R. S. E. W., & Huijbregts, M. A. J. (2011). Characterization factors for water consumption and greenhouse gas emissions based on freshwater fish species extinction. *Environmental Science & Technology, 45*(4), 5272–5278. https://doi.org/10.1021/es1039634

Jochems, C. E. A., van der Valk, J. B. F., Stafleu, F. R., & Baumans, V. (2002). The use of fetal bovine serum: Ethical or scientific problem? *ATLA, 30*, 219–227. https://doi.org/10.1177/026119290203000208

Jung, J.-K., Park, Y.-M., Kim, S.-G., & Lee, S.-Y. (2022). Research trends and development direction in cell-based seafood. *Food Science and Industry, 55*(1), 95–104. https://doi.org/10.23093/FSI.2022.55.1.95

Klinčić, D., Herceg Romanić, S., Katalinić, M., Zandona, A., Čadež, T., Matek Sarić, M., Šarić, T., & Aćimov, D. (2020). Persistent organic pollutants in tissues of farmed tuna from the Adriatic Sea. *Marine Pollution Bulletin, 158*, 111413. https://doi.org/10.1016/j.marpolbul.2020.111413

Lichtfouse, E., Schwarzbauer, J., & Robert, D. (Eds.). (2012). *Environmental Chemistry for a Sustainable World Volume 1: Nanotechnology and Health Risk*. Springer Dordrecht Heidelberg London New York. https://doi.org/10.1007/978-94-007-2442-6

Link, J. S. (2021). Evidence of ecosystem overfishing in U.S. large marine ecosystems. *ICES Journal of Marine Science, 78*(9), 3176–3201. https://doi.org/10.1093/icesjms/fsab185

Lozano-Bilbao, E., Delgado-Suárez, I., Paz-Montelongo, S., Hardisson, A., Pascual-Fernández, J. J., Rubio, C., González Weller, D., & Gutiérrez, Á. J. (2023). Risk assessment and characterization in tuna species of the Canary Islands according to their metal content. *Foods*, 12(7), 1438. https://doi.org/10.3390/foods12071438

Martinez-Porchas, M., & Martinez-Cordova, L. R. (2012). World Aquaculture: Environmental impacts and troubleshooting alternatives. *The Scientific World Journal, 2012*, Article ID 389623. https://doi.org/10.1100/2012/389623

McDowell, L. A., Kudaravalli, P., Chen, R. J., & Sticco, K. L. (2024). Iron Overload. In *StatPearls [Internet]*. StatPearls Publishing. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK526131/

Montano, L., Pironti, C., Pinto, G., Ricciardi, M., Buono, A., Brogna, C., Venier, M., Piscopo, M., Amoresano, A., & Motta, O. (2022). Polychlorinated Biphenyls (PCBs) in the environment: Occupational and exposure events, effects on human health and fertility. *Toxics, 10*(7), 365. https://doi.org/10.3390/toxics10070365

New, M. (1995). The use of marine resources in aquafeeds. In H. Reinersten & H. Haaland (Eds.), *Sustainable Fish Farming* (pp. 45-46). A.A Balkema Publishers. https://books.google.com/books/about/Sustainable_Fish_Farming.html?id=9eOOKo9mQhgC

Nikkhah, A., Rohani, A., Zarei, M., Kulkarni, A., Batarseh, F. A., Blackstone, N. T., & Ovissipour, R. (2023). Toward sustainable culture media: Using artificial intelligence to optimize reduced-serum formulations for cultivated meat. *Science of the Total Environment*, *894*, 164988. https://doi.org/10.1016/j.scitotenv.2023.164988

Ong, K. J., Johnston, J., Datar, I., Sewalt, V., Holmes, D., & Shatkin, J. A. (2021). Food safety considerations and research priorities for the cultured meat and seafood industry. *Comprehensive Reviews in Food Science and Food Safety, 20*(6), 5421-5448. https://doi.org/10.1111/1541-4337.12853

Pikitch, E. K. (2012). The risks of overfishing. *Science*, 338(6107), 474-475. https://doi.org/10.1126/science.1229965

Radošević, K., Dukić, B., Andlar, M., Slivac, I., & Gaurina Srček, V. (2016). Adaptation and cultivation of permanent fish cell line CCO in serum-free medium and influence of protein hydrolysates on growth performance. *Cytotechnology, 68*(1), 115–121. https://doi.org/10.1007/s10616-014-9760-x

Rasmussen, R. S., Nettleton, J. DSc, RD, & Morrissey, M. T. PhD. (2005). A review of mercury in seafood: Special focus on tuna. *Journal of Aquatic Food Product Technology, 14*(4), 71-100. https://doi.org/10.1300/J030v14n04_06

Reiss, J., Robertson, S., & Suzuki, M. (2021). Cell sources for Cultivated Meat: Applications and considerations throughout the production workflow. *International Journal of Molecular Sciences*, 22(14), 7513. https://doi.org/10.3390/ijms22147513

Ribas-Fito, N., Sala, M., Kogevinas, M., & Sunyer, J. (2001). Polychlorinated biphenyls (PCBs) and neurological development in children: A systematic review. *Journal of Epidemiology and Community Health, 55*(8), 537–546. https://doi.org/10.1136/jech.55.8.537

Rokni, L., Rezaei, M., Rafieizonooz, M., Khankhajeh, E., Mohammadi, A. A., & Rezania, S. (2023). Effect of persistent organic pollutants on human health in South Korea: A review of the reported diseases. *Sustainability*, 15(14), 10851. https://doi.org/10.3390/su151410851

Romeo, T., Pietro, B., Pedà, C., Consoli, P., Andaloro, F., & Fossi, M. C. (2015). First evidence of presence of plastic debris in stomach of large pelagic fish in the Mediterranean Sea. *Marine Pollution Bulletin*, 95(1), 358-361. https://doi.org/10.1016/j.marpolbul.2015.04.048

Rubio, N., Datar, I., Stachura, D., Kaplan, D., & Krueger, K. (2019). Cell-Based Fish: A novel approach to seafood production and an opportunity for Cellular Agriculture. *Frontiers in Sustainable Food Systems, 3*, 43. https://doi.org/10.3389/fsufs.2019.00043

Rustagi, N., Pradhan, S. K., & Singh, R. (2011). Public health impact of plastics: An overview. *Indian Journal of Occupational and Environmental Medicine, 15*(3), 100–103. https://doi.org/10.4103/0019-5278.93198

Sabu, S., & Sasidharan, A. (2020). Impact of fishing on freshness and quality of seafood: A review. *International Journal of Fisheries and Aquatic Studies, 8*(2), 193-198. https://dx.doi.org/10.22271/fish

Segeritz, C.-P., & Vallier, L. (2017). Cell Culture: Growing cells as model systems in vitro. In *Basic Science Methods for Clinical Researchers* (pp. 151-172). Elsevier. http://dx.doi.org/10.1016/B978-0-12-803077-6.00009-6

Stout, A. J., Mirliani, A. B., Rittenberg, M. L., Shub, M., White, E. C., Yuen Jr., J. S. K., & Kaplan, D. L. (2022). Simple and effective serum-free medium for sustained expansion of bovine satellite cells for cell cultured meat. *Communications Biology, 5*(466). https://doi.org/10.1038/s42003-022-03423-8

Stout, A. J., Rittenberg, M. L., Shub, M., Saad, M. K., Mirliani, A. B., & Kaplan, D. L. (2023). A Beefy-R culture medium: Replacing albumin with rapeseed protein isolates. *Biomaterials*, *122092*. https://doi.org/10.1016/j.biomaterials.2023.122092

Sumaila, U. R., & Tai, T. C. (2020). End overfishing and increase the resilience of the ocean to climate change. *Frontiers in Marine Science, 7*, 523. https://doi.org/10.3389/fmars.2020.00523

Tuomisto, H. L., & Teixeira de Mattos, M. J. (2011). Environmental impacts of cultured meat production. *Environmental Science & Technology, 45*(14), 6117–6123. https://doi.org/10.1021/es200130u

van der Valk, J., Mellor, D., Brands, R., Fischer, R., Gruber, F., Gstraunthaler, G., Hellebrekers, L., Hyllner, J., Jonker, F. H., Prieto, P., Thalen, M., & Baumans, V. (2004). The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicology in Vitro, 18*(1), 1-12. https://doi.org/10.1016/j.tiv.2003.08.009

van der Vusse, G. J. (2009). Albumin as fatty acid transporter. *Drug Metabolism and Pharmacokinetics, 24*(4), 300–307. https://doi.org/10.2133/dmpk.24.300

World Wildlife Fund. (n.d.). Sustainable seafood. World Wildlife Fund. Retrieved March 23, 2024, from https://www.worldwildlife.org/industries/sustainable-seafood