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Mammalian-Algal Co-Culture For Macroscale Cultured Fat Purposes

Abstract

Algae is a type of photosynthetic aquatic plant with a long history of medicinal, nutritional, and agricultural applications. In cellular agriculture, the use of algae is already being explored with hopes of increasing environmental sustainability, plant-based scaffolding, and fatty acid customization. However, the full potential of algae has yet to be harnessed. This paper explores the use of *Characium* algae co-culture to improve not only the texture, but the flavor profile of cultured meat. In certain mutualistic conditions, this algae has the advantageous ability to accumulate lipids, which holds potential to address some of the current issues with culturing fat.

Background

Animal husbandry, the act of domesticating animals for human benefit, has faced much controversy in recent years. Concerns regarding the environment, animal welfare, cardiovascular health, and antibiotic resistance have sparked an interest in developing alternatives to conventional meat products. While some have shifted their diets to include plant-based sources of protein, the overwhelming majority still rely on slaughtered-meat. Cellular agriculture is an emerging multidisciplinary field that seeks to combat these concerns by instead producing meat through cell culture. To create such a product, a harmless biopsy is first taken from an animal of choice. Relevant cells, namely satellite (muscle) and adipocyte (fat), are isolated and allowed to expand in a bioreactor. These cells are then allowed to differentiate and placed on scaffolds to mature into the final meat-like product (Post et. al, 2020). By *growing* meat in such a way, as opposed to merely *mimicking* meat using plant-based ingredients, a theoretically identical textural, flavor, and nutritional profile to conventional meat can be obtained.

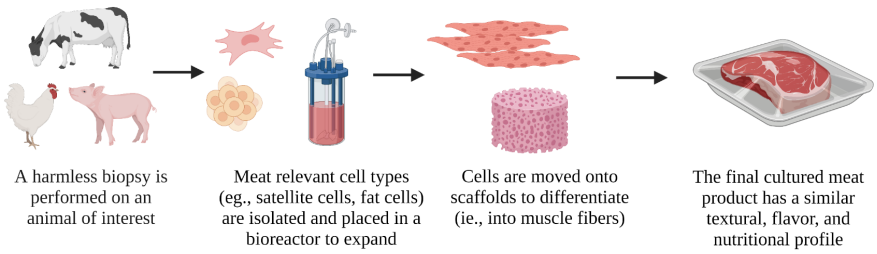

Figure 1. A generic overview of the process behind creating a cultured meat product. (Created with BioRender.com)

Algae, a type of aquatic photosynthetic organism, has a versatile nutrient composition that could prove beneficial in addressing certain nutritional and production needs within the food and agricultural industry. The main classifications include *macro*algae, which are large in size, and *micro*algae, referring to microscopic single cells (Khan et. al., 2018). The latter have been found to be rich in a broad range of compounds such as amino acids, vitamins, and essential fatty acids (Haraguchi and Shimizu, 2021). Aside from their nutritional properties, microalgae are an attractive resource to explore within cellular agriculture due to their growth efficiency and ability to tolerate a variety of environmental conditions. Additionally, autotrophic microalgae have been found to be capable of consuming toxic compounds, namely ammonia and carbon dioxide, while releasing beneficial byproducts, such as oxygen and other unique bioproducts. (Diaz et. al., 2023). In terms of its physico-chemical surface properties, most species have cell walls made of cellulose fibrils and polysaccharides. In the presence of ethyl and methyl groups, these cell walls become hydrophobic, while in the presence of carbonyl and carboxylic groups, the surfaces become more hydrophilic. This knowledge is important for the industry in order to assess and evaluate the most viable harvesting technology that increases production that is economically feasible (Ozkan and Berberoglu, 2013). While there is growing interest in researching the potential uses of microalgae, much of what is currently being explored is related to the production of biofuels and wastewater treatments. As a food source, it is mainly used as a food additive or a dietary supplement and is also used in animal feed, some drugs and cosmetics. Nonetheless, there is very limited knowledge and resource on the use of microalgae, and most data is constrained to only a select few species: Spirulina, Chlorella, and Dunaliella (Araujo et. al, 2021).

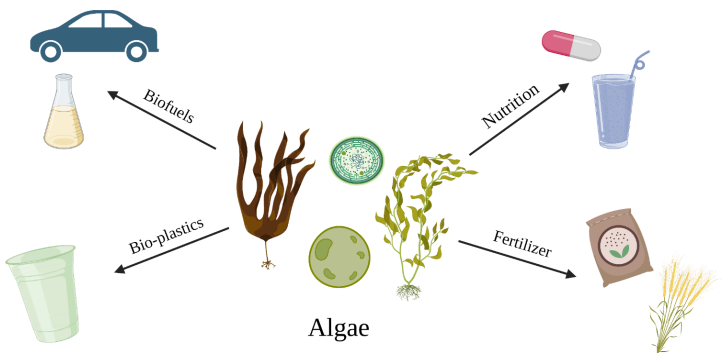

Figure 2. Key examples of the current commercial uses of algae. (Created with BioRender.com)

For many of the reasons listed above, algae holds promise in improving cultured meat and is already being explored to some extent. Kappa-carrageenan, derived from seaweed, has very distinct properties that make it a hypothetically ideal bio-ink for 3-D printed scaffolds to grow mammalian cells (Marques et .al, 2022). The ability of certain species of algae to metabolize undesired compounds produced in cell culture, such as ammonia, could prove beneficial in improving media recycling by reducing these harmful byproducts (Marko et. al). Marine algae could be used to supplement essential omega-3 fatty acids eicosapentaenoic acid (DHA) and docosahexaenoic acid (DHA) in cultured seafood to better replicate the health benefits of conventional fish products (Rubio et. al, 2019), (Chauton et. al, 2015). However, the use of algae to improve the flavor of cultured meat remains understudied. Thus, this hypothetical technique will serve as the focus of our proposal.

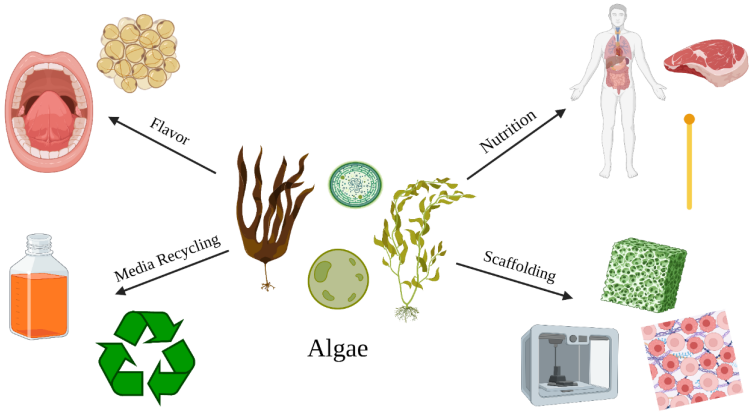

Figure 3. Examples of potential uses of algae within cellular agriculture. (Created with BioRender.com)

The Problem

If cultured meat products are to be brought to market, it is important that they closely mimic conventional agriculture products. In other words, consumer acceptance is of the utmost importance for the long term success of a cultured meat product. Therefore, achieving a similar flavor profile is very important. Previous studies have discussed the importance of *in vitro* fat, as it is a strong contributor to meat flavor (Yuen et. al, 2022.) Not only does fat contribute to flavor, but it is highly responsible for *variability* between different kinds of meat. While fat makes up a significantly smaller amount of a meat product as opposed to muscle (about 1-10% for fat compared to 20-25% muscle), fat content is the characteristic that changes most between various species, cuts, and preparation (Fish et. al, 2020). Despite fat being crucial to the signature flavors of various meats, most current cellular agriculture research focuses on growing skeletal muscle. Consumer acceptance is imperative in ensuring a longstanding future for cultivated meat, so it is just as important to develop innovative approaches to cultivating fat (separately from or in conjunction with muscle), as well. The numerous benefits of cellular agriculture cannot be reaped if these products do not appeal to the general public as a true alternative to conventional meat.

Previous work by Yuen et. al, 2022, explored the use of aggregated mammalian adipocytes to produce fat for food applications. Further, Letcher et. al, 2022, explored the use of mycelium scaffolds along with *Manduca Sexta* embryonic cells treated with free fatty acids to produce *in vitro* fat. While these are two ways to produce *in vitro* fat for cellular agriculture applications, both of these studies rely on lipid accumulation via free fatty acid treatment to produce fat. This may create issues when scaling up, as this adds a new component that needs to be purchased. Further, for free fatty acid treatment to be viable, cell types capable of lipid uptake are necessary. This is an issue as mammalian muscle cells are *not capable of lipid accumulation*. “Marbling” is also an important aspect of conventional agriculture products, which may be difficult to replicate if fat and muscle are grown separately. Thus, exploration into muscle-fat co-culture or fatty acid synthesis are necessary for further improvement within the field.

While cultivating fat for cellular agriculture purposes or using algae in mammalian cell culture is not a novel concept, the combination of the two is unexplored thus far. However, it is crucial to go into some

background on the current techniques and limitations of both algal co-culture and culturing mammalian fat for human consumption.

Scaffolds within cell culture are necessary not only to help create 3-D structures, but are important for cell growth. This is because cells are essentially programmed to die when they become detached from the extracellular matrix (ECM) in a process coined *anoikis* (McKee and Chaudhry, 2017). In suspension bioreactors, microcarriers (small objects for cells to adhere to) or scaffolding is typically required to maintain cell health and growth. Some FDA-approved and edible biomaterials (Valle et. al, 2017) include: pectin, chitosan, cellulose, gelatin, gellan gum, starch, gluten, alginate, textured soy protein (TVP), and poly-ethyl glycol. Typically, gelatin is considered one of the best choices as it is made of collagen, same as native ECM. However, gelatin is sourced from animals, which goes against the higher purpose of cellular agriculture to eliminate any dependence on animals.

Aside from its sourcing, scaffolding has many difficulties in itself. The two main types of scaffolds are ‘top-down’ and ‘bottom-up’. In the top-down approach, the scaffold is fully fabricated before cells are seeded. The main drawback of this approach is that it’s difficult to recreate tissue microstructure, which is very important when creating a cultured meat product. Alternatively, in the bottom-up approach, cells are seeded while the scaffold is generated. The main drawback in this process is that it is far more complex. When trying to meet such high demands for meat, ease of scalability is an important factor to consider when looking at scaffolding (Bomkamp et. al, 2022). Yet another aspect of complexity is added when trying to make a food-safe product. All scaffolding involved thus must be fit for human consumption. Alginate has risen as an ideal scaffold for cellular agriculture due to its biocompatibility and prior use in the food industry (Mehta et. al, 2018).

There are also several current approaches to culturing fat cells. Similar to scaffolding, each of these approaches comes with a variety of challenges and complications. There are many different cell sources that can be used for cultivated fat: pluripotent stem cells, dedifferentiated fat cells, mesenchymal stem cells, adipose derived stem cells, preadipocytes, etc.. When choosing which cell type is ideal, it is important to consider their proliferative ability over long periods of time, their ability to form fat cells, and scalability

(Yuen et. al, 2020). Some techniques show much promise in generating authentic fat, such as fat vascularization achieved by endothelial cell co-culture. However, several issues become apparent when operating at even a slightly larger scale, such as inadequate capillaries to support larger size tissue (Yuen et. al, 2022). Thus, it still remains imperative to develop novel approaches to cultivating fat that have the ability to be replicated on far larger scales.

The predominant method of animal fat production is *in vitro* cultivation of fat tissue from adipogenic cell lines of relevant species. The cell lines must have sufficient proliferation capacity to scale from primary isolation to commercial production. One key issue with proliferation is that after 30-50 cell divisions, senescence is reached and the cells stop dividing (the Hayflick limit) (Rodriguez-Brenes et. al). One way to improve long term proliferation is through genetically induced cell immortalization. The controversy with this method is that it would require the product to be labeled as genetically modified, which may negatively impact consumer acceptance. One of the main challenges in culturing fat cells is that adipose tissue is highly vascularized and has been hard to mimic *in vitro* (Gu et. al, 2013). However, the significance of cultured mad is immeasurable. Aside from improving the sensory qualities of cultured meat, cultured fat could be incorporated into plant-based foods, as well. Ideally, the creation of a 'hybrid' plant/cell based product would improve flavor and mouthfeel (Joshi et. al 2015).

Research Plan

There are two noteworthy studies that have inspired the development of this resource proposal. Within the algal biofuel sector, increasing efficiency of biomass and lipid production is essential. *Characium* freshwater algae were grown alongside several microorganisms native to their original habitat. While the exact mechanism behind this is unknown, the mutualistic relationship between algae and certain microorganisms is believed to be the underlying cause (Berthold et. al, 2019). In an unrelated study, *Chlorococcum littorale* algae were found to improve the thickness of rat cardiac cell tissue when co-cultured. Similarly to the previous paper, a symbiotic relationship was observed between the two, in which the algae significantly reduced the amount of ammonia and lactate present in the tissue and media (Haraguchi et. al,

2016). Combining these two observations, this proposal involves the co-culture of lipid-producing *Characium* freshwater algae alongside meat-relevant muscle cells. Hypothetically, similar symbiotic relationships as seen in Haraguchi et. al and Berthold et. al will be observed, leading to the formation of thicker muscle with increased lipid production. Therefore, co-culture of lipid-producing *Characium* freshwater algae with mammalian cells could lead to an improved texture, taste, and nutritional value for cellular agriculture applications.

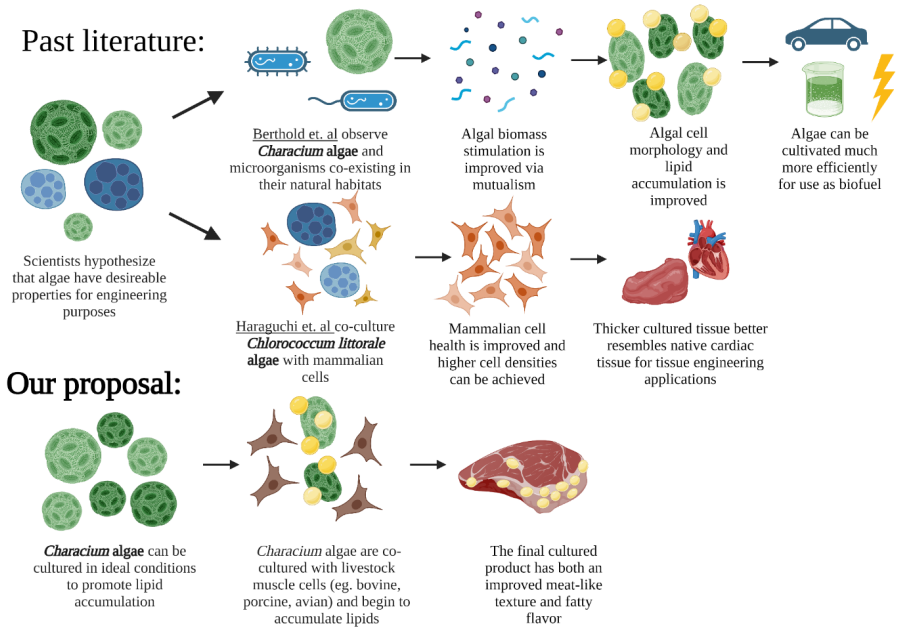

Figure 4. An overview of the behavior of algal co-culture in past literature that led to the development of the proposal outlined in this paper. (Created with BioRender.com)

To validate this proposal of co-culturing algae with mammalian cells, we propose several experimental aims. The first involves assessing the growth and viability of the algal-mammalian co-culture. The next step confirms lipid production and characterizes the lipids that were produced. Next, textural analysis via double compression testing will be performed. Finally, to gain a better idea of the taste, lipid profiles will be compared to conventional meat lipid profiles. Further, sensory evaluation will be done. (Figure 5.)

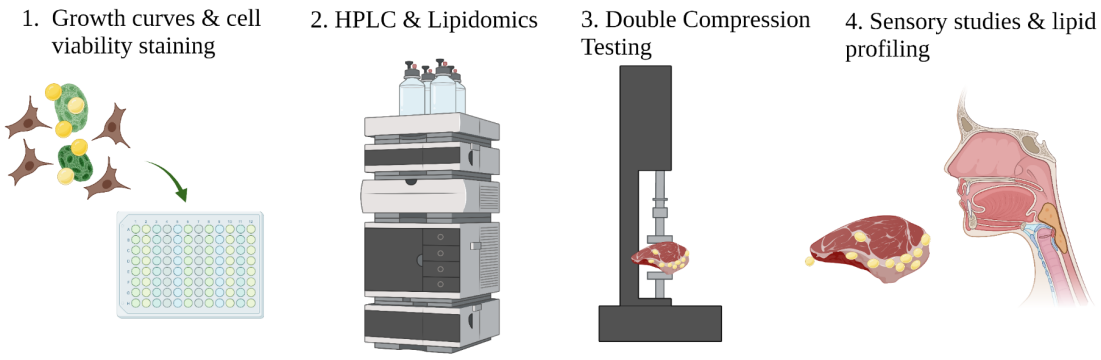

Figure 5. An overview of the 4 key components of the research plan to validate the use of algal co-culture to improve the flavor of cultured meat. (Created with BioRender.com)

Aim #1: Assess whether co-culture impacts viability, growth, and health of mammalian cells in culture

First, it is important to establish the effect that co-culture has on viability, growth, and overall health of mammalian cells in culture. This will be accomplished in a variety of methods. To assess overall cell viability, live/dead staining will be used. Widely available from ThermoFisher (#R37601), this dye utilizes Calcein-AM and Bobo-3 Iodide and assesses cell viability based on intracellular esterase activity and membrane permeability. With this dye, live cells will fluoresce green and dead cells will fluoresce red. That said, image analysis can be used to assess cell viability. Alternatively, this dye can also be used to assess cell viability by flow cytometry. Flow cytometry would also be beneficial as the cells could be identified based on size. That said, one would be able to obtain data for the viability of both algal cells and mammalian cells in culture. Using this one dye and two different methods, we can assess overall cell viability. It is also important to assess growth of cells. This can be accomplished using double stranded DNA (dsDNA) quantification. DsDNA quantification can be used to measure growth as the relative amount of DNA will correlate with the relative amount of cells present over time. This entails the use of a lysis buffer, which will lyse cells of interest, a fluorescent dye (such as FluoroReporter or CyQUANT) that will bind to dsDNA, and a plate reader, to measure relative fluorescence. Finally, using commercially available assays, lactate and ammonia (harmful metabolic byproducts) levels in media can be measured to determine the effect of co-culture. Previously, Haraguchi et. al, 2016 showed that co-culture improved growth of C2C12 cells due to

the symbiotic relationship created with algae (see Appendix #1). Thus, we would expect improved viability and growth, along with a decrease in lactate and ammonia (Figure 6).

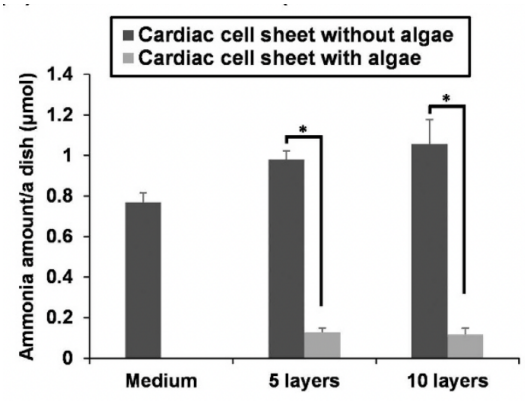

Figure 6. Figure taken from **Haraguchi et. al, 2016** showing that co-culture significantly decreased ammonia concentrations.

Aim #2: Assess lipid accumulation

Next, it is important to assess lipid production within the co-culture. This is important for several reasons. Primarily, it will allow us to confirm that the co-culture is producing lipids. Further, lipid characterization will allow for future nutritional tuning and sensory analysis. To first determine whether lipids will be produced, BODIPY (a stain for neutral lipids) will be used. Thus, one can obtain visual confirmation of lipid accumulation through fluorescence. Further, one can use image analysis to quantify lipid content. Oil Red O can also be used as a means of staining lipids and quantifying via fluorescence. To further characterize the lipid content, lipidomics can be used to obtain lipid profiles. First, lipids are extracted via phase separation. Then, lipid samples are sent to a centralized facility with a mass spectrometer to perform lipidomics analysis, which will give a list of all lipids present. This has been performed several times in the past for cellular agriculture applications, as described in Yuen et. al, 2022, Letcher et. al, 2022, and Saad et. al, 2022. Further, in order to determine omega-3 and omega-6 content, HPLC can be used. While lipidomics will show which fatty acids are present, HPLC will confirm omega-3 and omega-6 content, which will be an important measure of nutritional value.

Aim #3: Conduct a textural profile analysis using double compression focusing on meat-relevant properties

To evaluate whether *Characium* algae co-culture improves the textural profile of cultured meat, a double mechanical compression test could be utilized. This type of mechanical testing aims to mimic chewing of the sample. Specific food-relevant properties will be assessed, namely springiness, cohesiveness, chewiness, and resilience. A similar study has been conducted in which the properties of a cultured meat product were compared to conventional sausage, turkey, and chicken to determine texturally which the cultured product mimics most closely (Paredes et. al, 2022). As described in this paper, material stiffness is typically assessed using the Young's Modulus, which is determined by the *slope* of the linear portion of a stress-strain (force-displacement) graph. In other words, this is the ratio of stress to strain of a material (σ/ε), or the slope of the linear portion of the curve. Hardness, which is equated to the "first bite" of meat, can be assessed by looking at the first compression cycle at the point of maximum load. Cohesiveness is determined by looking at the *area* under a force vs. time curve and determining the ratio between the second to first cycle. Cohesiveness provides insight to how consistent the texture of a material remains with time, with ratios of ≈ 1 indicating no disintegration and values < 1 indicating high disintegration (ie. falling apart during chewing). Furthermore, 'springiness' is defined as the ratio between the *time* needed to reach the maximum load for the second cycle compared to the first cycle. An indicator for chewiness can be obtained by multiplying together hardness, cohesiveness, and springiness for a parameter detailing how easy it is to *bite* a material. Finally, resilience is determined by assessing the first cycle and comparing the area under the force vs. time curve before and after the maximum load is reached. This gives insight on the amount of plastic deformation faced by a material, with resilience values of 1 indicating no plastic deformation, and values > 1 indicating a lack of shape recovery (deformation of a product while chewing).

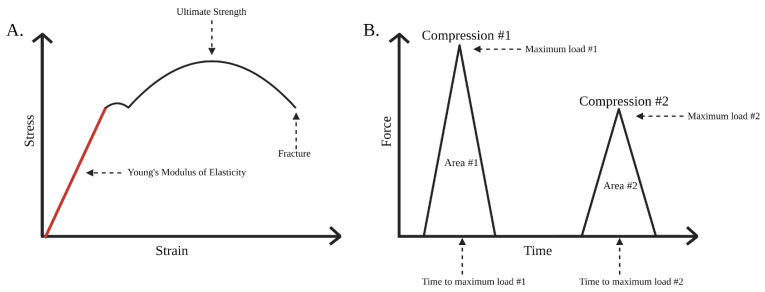

Figure 7. Examples of stress-strain (force-displacement) curve and a force-time curve data for textural profile analysis purposes. (Created with BioRender.com)

To determine the effect of algal co-culture (and a resulting increase in lipid accumulation) on the textural profile of a cultured meat product, a double compression test can be used as a means of comparison to mono-cultured meat and various traditional meat products. Ideally, the presence of algae will improve the thickness of the cultured tissue and also add variation in texture to better resemble fat.

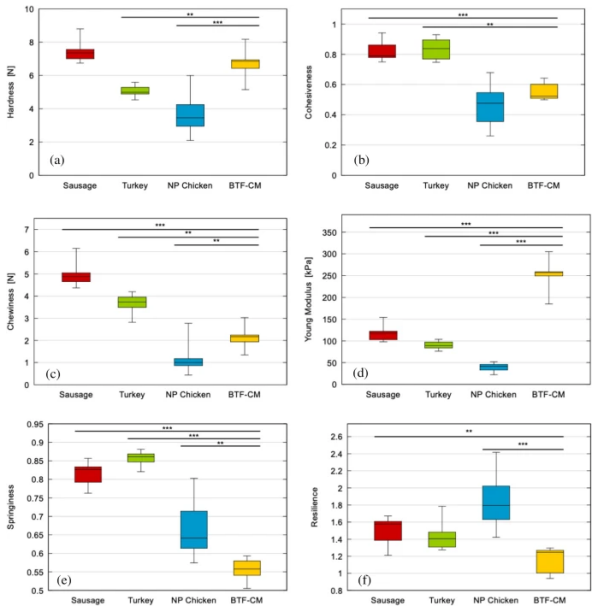

Figure 8. This image is from **Paredes et. al**, and compares the hardness (a), cohesiveness (b), chewiness (c), Young's Modulus (d), springiness (e), and resilience (f) of a cultured tissue compared to sausage, turkey, and chicken. Similar results will be assessed for co-culture tissue, mono-culture tissue (mammalian only), and conventional meat.

Aim #4: Determine whether algal co-culture effectively alters the taste of cultured meat

Finally, with regards to the overall goal of this proposal, it is important to determine whether this co-culture system will improve the taste of cultured meat. One way to test this would be to simply taste it. However, through the development process it is important to have a more analytical method of assessing the taste. As was already touched on, fat is a significant contributor to meat taste. One way to predict the taste could be to compare lipidomics data to previously obtained lipidomics data that is associated with a taste. For example, fatty acid profiles of co-cultured cells can be compared to fatty acid profiles of other conventional meat as a way to show what the cells may taste similar to. Comparison to other species has been done before, such as in Letcher et. al, 2022 (Figure 9).

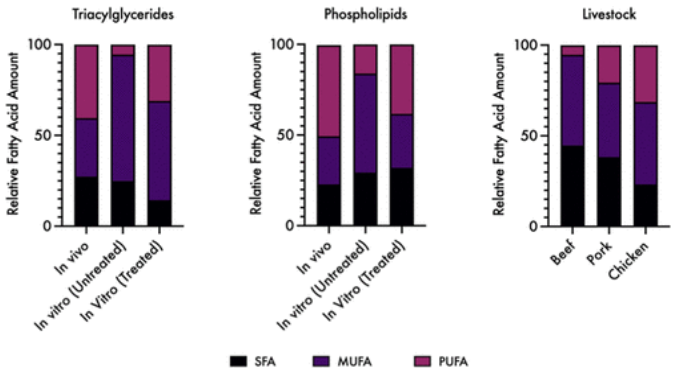

Figure 9. Comparison of lipid profiles of treated cells in **Letcher et. al, 2022**'s study compared to traditional meat sources.

It is also noteworthy to mention that these accumulated lipid profiles can also be "fine tuned" via free fatty acid treatment. However, it was previously mentioned that this technique may not be realistic to be brought to scale, as it could require copious amounts of fatty acids to supplement into the culture media. Nonetheless, it remains a viable option for consideration as a secondary modification, not the sole source of fatty acids. Previously, in Letcher et. al 2022's study, free fatty acids were able to be taken up by *Manduca Sexta* embryonic cells, which then altered the lipid profile. That said, algae can also do the same. Thus, exogenous media supplementation of free fatty acids could provide a way to alter the lipid profile of co-cultured cells to make them resemble species-specific lipid profiles that are already widely accepted.

Finally, one possible sensory test that may be simpler but also effective could be sensory-smell tests of cultured and cooked cells. This would give consumer input, along with sensory data without having people taste a product, as this would require significantly more logistical considerations.

Evaluate Scalability

The integration of an algal-mammalian cell co-culture that incorporates mammalian muscle cells and *Characium* microalgae offers the opportunity to propose a sustainable system that can address the environmental problems often associated with traditional cell culture. This underutilized resource will leverage the existing cell-based meat culturing processes. The difference will be that during the cell culturing phase of production, the bioreactors will be inoculated with both algae and mammalian cells. The ratio of algae to mammalian cells of 200:1 (Marko et. al) is a good starting point. One possible configuration is the

use of a hollow fiber bioreactor. The benefits of this setup is that the cells could adhere and have better growth. The setup of the hollow fiber bioreactor is shown in Figure 9 with a light source for the algae to undergo photosynthesis for growth. The drawback is that hollow fiber bioreactors are more expensive and have small capacities. A suppression bioreactor with microcarriers would be a preferred method for scalability because of larger volumes and cheaper cost.

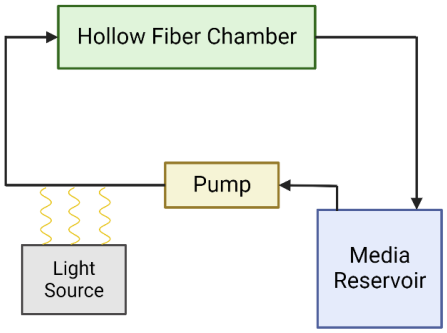

Figure 9. Conceptual Diagram of Hollow Fiber Bioreactor from **Marko et. al.**

Microalgae have the potential to provide the necessary nutrients required for animal cell cultivation, which can serve as a possible substitute for certain expensive or unethical culture media components. At an industrial level, mammalian cells have a significant demand for culture media formulated with grain-derived nutrients. The production of such nutrients requires the use of agrochemicals that emit large amounts of greenhouse gasses and demand increased energy and resources. Furthermore, waste treatment of these culture media can potentially lead to negative environmental impacts on water bodies (Haraguchi et. al, 2022). On the contrary, microalgae are capable of synthesizing many of the required nutrients through photosynthesis and can also utilize nitrogen or ammonia to produce vital amino acids without generating significant waste. Additionally, it has been found that a co-culture strategy can reduce the ammonia present in cell culture mediums that contain FBS (Haraguchi et. al, 2022), promoting a sustainable system since algae can also benefit from the waste medium released by animal cells while simultaneously thriving through the absorption of carbon dioxide (Haraguchi and Shimizu, 2021).

One of the greatest advantages of this unique organism is its high productivity, as it requires less space compared to other agricultural crops that need arable lands (Ullmann and Grimm, 2021). Microalgae can be produced in open pond systems or photobioreactors, allowing for the utilization of lands with no

industrial use and providing the opportunity for unconstrained growth. Although space requirements can be minimized by utilizing closed systems, open ponds have been found to be the most sustainable alternative for its cultivation (Resurrection et al. 2012).

The overall production can be limited if the process is intensified and the species utilized require freshwater. This leads to an extensive demand for water resources and fertilizers, resulting in increased energy consumption and greenhouse gas emissions (Yin et. al, 2020). A variety of environmental impacts studied in these types of cultures come from the production of biofuels, which demand the use of treated wastewaters. However, the possible presence of pathogens and pollutants undermines its effectiveness as a solution to reduce the need for freshwater. Its applicability is dependent on the process, and while it can be recycled, it will require a proper treatment, resulting in higher costs and energy consumption (Guieysse and Plouviez, 2021).

In terms of greenhouse gas emissions, algae has the capacity to reduce levels of carbon dioxide in the atmosphere, while its biomass serves to reduce carbon emissions and avoid the biofixation of residual carbon. It is also deemed carbon-neutral, with research indicating that roughly 1 kg of biomass only requires 1.8 kg of CO2 (Ahmad et. al, 2011). It is important to note that these characteristics are unique to algae and do not necessarily represent possible emissions for the entire cultivation process. One of the gasses produced during algae cultivation is nitrous oxide (N_2O), which is commonly associated with the use of fertilizers. Certain species of cultured algae can release some of this gas as part of the nitrogen supply or indirectly through ammonia volatilization or nitrification at higher pH levels if the culture conditions are not regulated. While there may be other gasses that could be emitted, there is currently no research or evidence to suggest that high emissions to the atmosphere from algae cultivation could have a detrimental impact on the environment (Guieysse and Plouviez, 2021).

Conventional agriculture practices (i.e., animal husbandry) is expected to become less popular in the next few years; Some analysts venture to estimate a displacement of around 60-70%. Further, the consumption of meat is calculated to double by 2050, thus there is a necessity to create a scalable model for the production of cell-based meat, considering financial productivity that can reach the demand of the

market. The scalability to industry is best advised to include aspects that are estimated for pharmaceutical cultured cells such as capital expenditures, operating costs, ingredients and raw material, utility related expenses and labor related expenses (Risner et. al, 2020).

This techno-economic assessment (TEA) is only analyzing the cost of producing lipids on top of the cost of culturing cells. First, algae serves as a source of CO_2; In many cases, CO_2 is one of the most expensive components of culturing mammalian cells. This reduces the capital expenditure (CapEx) to only the cost of lights for autotrophic growth, with an estimated cost of $3000 (Eco-Industrial Supplies). The increase in operating expenditure (OpEx) would be electricity for the light source and additional media costs if supplementation is needed to accommodate for necessary algal cell nutrients. A similar TEA involving the use of microorganisms to produce palm oil states that co-products could reduce cost to effectively $0 (Karamerou et. al). Using the same TEA, the estimate of electricity was 0.08 $/kg and for sterility 0.01 $/kg. With minimal CapEx, the cost of producing lipids on top of culturing would be ~ 0.09 $/kg. The cell density of algae is (0.57 ± 0.04) g cell/cm^3 (Hu, W) and the lipid content of algae is 20-50% and up to 80% w/w (Chisti, Y et. al, 2017). So with an engineered strain, a lipid content of 50% should be achievable. The cell density for muscle cells is estimated to be 1060 kg/ m^3, though it's important to take into account that this density reduces if an external fat source is used. This gives a yield of 285 kg lipid/m^3 reactor.

$$0.57 \frac{g\ cell}{cm^3\ reactor}\ 0.5 \frac{g\ lipid}{g\ cell} 0.001 \frac{kg}{g} 10^6 \frac{cm^3}{m^3} = 285 \frac{kg\ lipid}{m^3\ reactor}$$

Concluding Remarks

The presented proposal involving the co-culture of lipid-producing *Characium* freshwater algae alongside meat-relevant muscle cells presents a novel method to produce cell cultured fat and improve the textural properties of cell cultured meat. Further, it reduces the costs needed for culture of mammalian cells, improves the taste, and nutritional value by creating a customizable fatty acid profile. Due to the symbiosis between algae and muscle cells, the low-cost culture found for algae in the industry, and the potential market for this novelty product, the scalability of the project increases and makes it more accessible and possible to commercialize meat more environmentally friendly and cost-effective.

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Appendix 1: Algae as a Good Feedstock Source for a Circular Cellular Agriculture

Algae, as a potential input feedstock material, for the use in cellular agriculture, comes as a natural drive to reduce the reliance of the in-vitro meat industry on bovine serum and other animal products that would require the harvesting of animals, which goes against the main tenets of cellular agriculture and mission to find more efficient, more ethical and more environmentally friendly meat options. As per Michael Selden, the CEO Finless Foods, a startup company focused on cultivating seafood items in the lab, Fetal Bovine Serum (FBS) is the primary growth media for cultivating meat in a lab due to the variety of proteins, nutrients, amino acids and chemicals needed to sustain multicellular growth (Reynolds, 2018). Aside from the ethical and sustainability issues of FBS, cost is a major factor to consider as Mark Post, Co-Founder of “Mosa Meat”, has illustrated that one burger would require at least 50 liters of FBS, making the initial cost of the first burger they produced go up to 220,000 GBP and after more years, the industry managed to get the costs down to 4,400 GBP, nowhere close to any commercial status. The company, Mosa Meat, then went on

to begin a line of products that is free from FBS as per their patented research findings published in January 2022 (Reynolds, 2018).

The efforts towards utilizing plant-based serum replacements vary in content, efficacy and potential and some aim to develop mixtures, like Galileo & Timothy study published in 2023 where extract of algae (Chlorella Vulgaris), combined with insulin and two growth factors, seem to show promise as growth factors could be produced from genetically modified bacteria and insulin, as a hormone, is also produced without harvesting complex multicellular organisms. Their study uses tissue culture media of Dulbecco's Modified Eagle's Medium (DMEM), that is free from animal products and while the findings support utilization of algae to significantly reduce reliance on FBS, the mammalian cells thrived at a 10% FBS content, meaning that the reliance on FBS was reduced by 90% due to supplementing the solution with algae extracts and other products produced by single-celled organisms. A similar study, also utilizing *Chlorella Vulgaris* extract, published in 2020 by a team from Singapore, also supported the finding that the algae extract helped with cellular differentiation and simulating growth, both on 2D and 3D culture basis and helped preserve the cells at reduced / FBS serum at starvation levels reaching 5% (Messmer, T, 2022). The study categorized Chlorella Vulgaris extract components as follows:

Moisture (%)	Crude protein† (%)	Crude carbohydrate‡ (%)	Crude ash (%)	Trace metals§ (mg/kg)	Microbial content¶ (CFU/g)
Chlorella growth factor (CGF)					
5.5	67.1	27.4	5.7	Arsenic < 0.20 Mercury < 0.02 Cadmium < 0.10 Lead < 0.20	Total aerobic microbe < 110 *E. Coli* undetectable Salmonella species undetectable

*†Crude protein composition = expressed as percentage of dry base. ‡% carbohydrate = 100% - (
C. vulgaris). §Trace element content complied with Singapore Medicines Act, under Medicines (
medicinal products containing excessive levels of toxic heavy metals -p3 (1) (c). ¶Microbial conte
of Sale and Supply) Order for prohibition of import, sale or supply of Chinese proprietary medicir*

Amino acids	[a]	[b]	[c]	[d]	WHO/FAO
Isoleucine	4.44	4.82	3.8	0.09	4.0
Leucine	9.38	10.78	8.8	6.91	7.0
Methionine	1.24	1.55	2.2	0.60	N/A
Phenylalanine	5.51	6.02	5.0	5.36	N/A
Tyrosine	3.14	3.02	3.4	7.78	6.0
Threonine	5.15	5.60	4.8	5.62	4.0
Valine	6.61	7.86	5.5	2.85	5.0
Lysine	6.68	7.70	8.4	6.30	5.5
Arginine	6.22	7.97	6.4	6.81	N/A
Histidine	1.97	2.40	2.0	1.16	N/A
Alanine	8.33	7.18	7.9	10.05	N/A
Aspartic acid	9.80	10.39	9.0	10.09	N/A
Glutamic acid	12.66	11.6	11.6	8.37	N/A
Glycine	6.07	5.05	5.8	7.93	N/A
Proline	4.90	3.88	4.8	2.74	N/A
Serine	4.32	7.17	4.1	3.50	N/A
Cysteine	1.28	0.18	1.4	0.50	3.5
Tryptophan	2.30	2.04	2.1	1.1	1.0
Ornithine	n.d	0.12	n.d	n.d	N/A

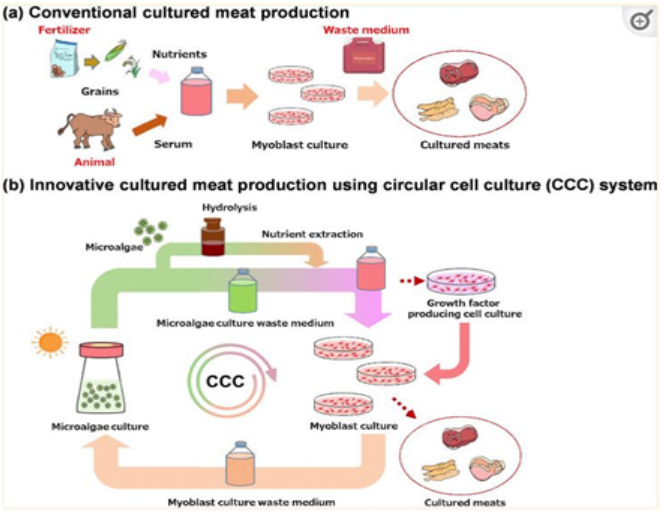
*n.d: not detected; N/A: not available; a = (Maruyama et al., 1997); b = (Safi et al., 2013); c = Becker (2007); d = (Morris Quevedo et al., 1999).

Algae as a growth media feedstock source, has the added advantage of avoiding zoonotic contamination that is possible when handling animal-derived input materials like FBS (Messmer, 2022). Algae also provides an impressive amino acid profile as cited in different studies in comparison with the World Health Organization (WHO) and the Food & Agriculture Organization (FAO) (Kim et. al, 2021) as seen in this table listing amino acids in *Chlorella Vulgaris* extract:

The table below shows a comparison between the amino acid profile in algae extract and FBS:

Compound	Chlorella Vulgaris Extract (gram per 100 gram protein) (Shalaby, Samah., 2013)	Fetal Bovine Serum (FBS) (gram per 100 gram protein, experimental figures) (Pacheco, et. al, 2008)
Aspartic Acid	10.5	11.9
Threonine	5.24	4.9
Serine	5.08	4.22
Glutamic Acid	10.74	17.4
Glycine	5.1	1.84
Alanine	8.44	5.11
Valine	6.44	5.4
Isoleucine	5.01	2.86
Leucine	6.84	9.61
Tyrosine	5.2	5.03
Phenylalanine	4.2	6.07
Histidine	5.02	3.86
Lysine	5.6	10.4
Arginine	8.2	5.56
Proline	6.4	4.45

Since algae is efficient at harvesting nutrients and metabolites from waste streams (hence the proposed utilization of algae to recover nutrients from excess agricultural fertilizers), a joint study was carried out by Tokyo Women's Medical University and Waseda University to propose and highlight the circular applications of algae utilization in



cellular agriculture as illustrated in the diagram below illustrated within the study (Ng et. al, 2020):

The system utilizes saltwater algae strain "*Chlorococcum littorale*" is highly tolerant to CO2 (Ng et. al, 2020) which means that the production system could be supplemented with CO2 injection for fixation and could be cultivated successfully without having to rely on precious and scarce freshwater resources. The utilized algae strain has a high capacity for lipid accumulation that could reach up to 56% of its dry weight. The accumulated lipids could help with the biosynthesis of fatty animal cells; greatly improving texture and flavor. The process of harvesting the nutrient solution for supplementing following patches of production is simple as it involves the usage of HCL then acidity neutralization using NaOH then centrifuging the solution to acquire the supernatant that could then be added to the growth medium of next round of production.