BME SENIOR CAPSTONE PROJECT:

3D Printed Whole-Cut Meats with Nutraceuticals

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**Abstract:** Concerns surrounding the meat industry, including its climate impact, unethical treatment of animals, and unsustainability for future populations illustrate the growing desire for alternatives to animal products. While some ground meat alternatives and cultured-cell fish products are available on the market, 3D bioprinting of cultured cells provides the opportunity to incorporate nutritional additives during the printing process of a whole-cut meat product. This project will involve the synthesis of whole-cut meat through the use of FRESH, a thermoreversible support bath that melts away from complex 3D printed structures when heated to body temperature (37ºC). Nutritional additives have the potential to address numerous health concerns, such as heart disease and antibiotic resistance, which increase strain on the healthcare system. In the past, limited efforts have been made to infuse cultured meat with nutrients, but there are no nutritionally-enhanced cultured meat products that are currently on the market. Thus, this project aims to develop a 3D printed whole-cut meat product of multiple cell types and with nutritional additives of melatonin and caffeine as a proof of concept for future meat alternatives.

**Keywords:** 3D bioprinting; thermoreversible support; nutraceuticals;

1. **Elements of Engineering Design**

Our plan of action involves the use of the FRESH reservoir system as a method to accomplish our primary objective of 3D printing cell-based, whole-cut meats by printing with undifferentiated bovine muscle, fat, and endothelial cells onto a biocompatible and edible scaffold. After removal of the FRESH system, the co-culture of muscle cells and adipose cells seeded during printing will then be proliferated and differentiated while on the scaffolds. The scaffold will remain as part of the final product to provide support and structure. In addition to using multiple cell types and a scaffold material as our bioinks, we will also be enhancing the product with nutraceuticals with the chosen nutraceuticals being melatonin and caffeine.

There are a number of engineering principles we will be exploring in the project. Our overarching project consists of combining the field of 3D bioprinting and cellular agriculture, and the project will mainly focus on using cell culture techniques, bioprinting methods, characterization assays, and statistical analysis to create a final product and measure success. Additionally, since our project involves printing a scaffold, the science of biomaterials and its considerations will be an integral part of our project.

 Our project will proceed in three phases. Phase 1 of this project consists of adding nutraceuticals to the cells and measuring potential induced differentiation and toxicity. Induced differentiation and toxicity will be tested through adding the melatonin and caffeine to the cells and then performing ELISA assays to test for undifferentiated, differentiated, and apoptotic markers at various time points after the addition of the nutraceuticals. For Phase 1, we will need to take into account the differentiation process of the murine myoblasts and how both of our nutraceuticals affect differentiation. We will be looking at the levels of toxicity both melatonin and caffeine induce, if those levels of toxicity are tolerable, and if the nutraceuticals induce or prevent differentiation. These studies are important for us to not only be able to keep the cells alive and growing, but to also have full control of differentiation throughout the entire printing process. The objectives for Phase 2 consist of ensuring that we can induce cell differentiation while the cells remain viable and deciding on what biomaterial to use for the scaffold. To determine success, we will need to perform the ELISA for the differentiation markers and measure the concentrations of our nutraceuticals to ensure they remain relatively constant and are not being metabolized by the cells. Finally, moving onto Phase 3, the final objective is to create a finished whole-cut meat steak that closely mimics a real cut of meat, and we will be quantifying that success through cell viability and differentiation capability post-print and shear stress and strain measurements for texture evaluation.

 The transition from murine to bovine cells, potential toxicity of melatonin and/or caffeine, the difficulty of printing with multiple bioinks, and being able to control the differentiation of the cells at the ideal time points are a few constraints that we need to keep in mind as we move forward. The first hurdle will be moving from murine to bovine cells as there is always the possibility that the effects of the chosen nutraceutical on the murine cells will not be the same as the effect they will have on bovine cells. Since murine cells are cheaper and are generally easier to work with, we will be starting with murine cells for proof of concept and to optimize the characterization assays before moving onto bovine cells. The characterization assays will still be done on the bovine cells, but those studies will ideally be streamlined due to the work we will have already done on the murine cell lines. There is also the possibility that melatonin or caffeine will prove to be too toxic for cell growth and differentiation especially since some studies in cancer and endothelial cells have shown that caffeine especially has toxic and apoptotic effects on some cell types [37]. To mitigate this potential problem, we would choose different nutraceutical enhancements or perhaps move from nutraceuticals to nutritional enhancements like higher protein content or lower cholesterol. Finally, the biggest hurdle this project will likely face is the same issue that has plagued all of cellular agriculture the past few decades: printing live cells into a 3D structure that mimics real whole-cuts of meat. For the 3D printing phase, we will start with only printing the scaffold with muscle cells. We will also be deciding whether to print the scaffold on its own and then seed the scaffold with cells post-print or if printing the cells onto the scaffold during print would lead to better results. The end goal for this project would be to print the scaffold and all undifferentiated cell types simultaneously since real meat has vascularization and marbling that would be difficult to introduce post-print. A potential concern is keeping the cells healthy, alive, and undifferentiated during print, but proof of concept studies performed by FluidForm have shown that cells printed in a media solution remain viable for the duration of longer prints [24]. Ideally, if the proper mitigations for these considerations are implemented, we will be able to overcome all future challenges to successfully printing whole-cut cell-based meat.



**Figure 1: Project design flowchart.** Having identified a gap in the non-traditional meat products market, the goal of our project is to develop a whole-cut meat product derived from multiple cell types to mimic natural animal products. The “whole-cut” structure is accomplished by bioprinting into the FRESH thermoreversible support which, when subjected to high stress and temperature, leaves only the printed material and allows for complex printed structures. Our 3D printed meat product will incorporate melatonin and caffeine as nutraceuticals. Phase I is the 2D culturing of murine myoblasts in the presence of melatonin and caffeine. If characterization assays indicate appropriate viability, differentiation and nutrient uptake, the project moves onto Phase II. In this phase, murine myoblasts are replaced with bovine which are 3D printed with FRESH suspension and CAD-designed scaffold. Phase III is the assessment of the 3D printed product, including nutrient uptake assays and shear stress-strain assessment of texture. If at any point in the project, the product fails to meet expectations, new cell types, scaffold materials or designs, and nutraceuticals are to be considered and the 3D printing restarted.

1. **Introduction and Background**

One of the most pressing issues facing the development of cellular agriculture as a viable, large-scale alternative to traditional meat sources is the inability to produce whole-cut, cell-based meat such as steaks or chicken breasts. This inability is detrimental to cellular agriculture as a long-term replacement to traditional meat products since different types of whole-cut meat compromise over 50% of the meat market by sales annually. For example, Americans consume over 97 lbs of chicken each year, yet only 1.5% of that chicken is ground with the other 98.5% coming almost entirely from different forms of whole-cut meat [1,2]. Likewise, even the most popular type of ground meat, ground beef, only covers 60% of the beef market, meaning that 40% of an 800 billion dollar market is based on whole-cut meat [3].

One of the biggest pulls of cell-based meat products is the idea that it gives society the ability to move away from traditional animal agriculture and all the problems associated with naturally made meat and dairy products. Traditional agriculture takes up roughly 50% of habitable land on Earth in which almost 80% of agricultural land is used for livestock [4]. This massive amount of space the meat, poultry, and dairy industry consumes makes up 15% of global carbon emissions annually and it takes almost 2,000 gallons of water to produce a single pound of beef [5, 6]. In addition to the sustainability concerns the meat industry proposes, the meat industry is also the main target for animal rights activism as livestock, especially poultry and animals marked for slaughter, are treated very inhumanely [5]. However, with the global meat industry being valued at around 800 billion U.S. dollars in 2020 and being estimated to hit 1 trillion dollars by 2025, it is apparent that animal activism, climate change, and global sustainability efforts are not enough to reduce or mitigate meat consumption worldwide [7].

In addition to the sustainability concerns within the modern-day meat industry, the issue of antibiotic resistance in both humans and livestock arising in the last few decades also has heavy societal implications in relation to cellular agriculture. Antibiotic resistance is a growing issue that has already wreaked havoc on some species of plants and livestock due to the unnecessary and excessive usage of antibiotics in animal agriculture [8]. The use of antibiotics is a practice used in order to prevent bacterial infections in livestock and can slightly improve growth rates and product yield [8]. Unfortunately, overuse can cause resistance in livestock that produce unsafe meat and dairy products that could cause antibiotic-resistant strains of disease in humans that might lead to global pandemics [8, 9]. Since most people will not move to a completely plant-based diet, a solution to the antibiotic resistance crisis is critical to prevent future antibiotic resistant food-borne illness. Luckily, current cellular agricultural practices are sterile, and the use of antibiotics is entirely unnecessary and often undesirable in certain cell types [10]. Cell culturing practices that would produce food-grade products will be highly regulated by the FDA and USDA and fall under the jurisdiction of both organizations, so cell-based products will have many restrictions to ensure that the process is completely sterile. The sterility of these processes will also reduce the rates of current food-borne illness as sterile cell culture protects against not only antibiotic-resistant disease strains, but all microbial disease strains.

 Another significant health concern inherent to traditional meat is cardiovascular disease. Every thirty-six seconds, an American dies from heart disease [11]. In fact, one of every four deaths in America are linked to heart disease for a total of 659,000 deaths each year; as the leading cause of death worldwide, cardiovascular disease accounts for 17.9 million deaths every year [12]. The American Heart Association estimated the total cost of cardiovascular disease treatment to be $363 billion in 2016 and 2017 [13]. Research indicates a higher risk of heart disease — a 3-7% increase — from consuming only two servings of red meat, processed meat or poultry per week [13]. With the prevalence of these products in diets around the world and their link to the most significant cause of death, innovation in the meat industry is clearly warranted.

 The current market for traditional meat alternatives is quite limited in scope. The brands Wild Type and Finless Foods have developed cultured fish, salmon and tuna respectively [14,15]. Their products seek to address concerns of overfishing and the presence of microplastics and mercury in natural fish products. Meatable is a Dutch startup developing cultivated beef and pork products [16]. However, there is no mention of whole-cut meats nor nutraceuticals and products are not expected to be available until 2025. In terms of nutraceutical-enhanced meats, Beyond Meat produces a reduced saturated fat plant-based meat alternative but all Beyond Meat products are ground or shredded, which limits their usability to consumers compared to whole-cut meats [17].

Nutraceuticals are not a new concept, but their application is currently limited in meat products. Some previous research in the cellular agriculture space has focused on nutritionally enhancing cultured meat; Stout et al. generated carotenoids in bovine stem cells by using bacterial enzymes [18]. They accomplished this by inducing myogenic differentiation in cultured mouse skeletal myoblasts and isolated primary bovine satellite cell; then, Gibson assembly facilitated the insertion of the genes of interest into the plasmid, and the Sleeping Beauty transposon helped transfer genes from the plasmids to the genome. Subsequently, the cells were able to endogenously produce beta carotene after plasmid transfection and the addition of a lipid reagent. In addition, an unpublished paper by Dave et al. proposes a similar method to nutritionally enhance meat; namely, the researchers sought to reduce the risk of developing cardiovascular disease by both reducing the amounts of unsaturated fats and LDL cholesterol and increasing vitamin C and fiber levels [19]. Our selection of a melatonin or caffeine-enhanced meat is an opportunity to show the applications of nutraceuticals.

Methods to create whole-cut, cell-based meat are actively being researched, but companies still have a long way to go before achieving a viable solution. For instance, in the reproduction of steaks, the industry leader can currently produce slabs with a thickness of up to 10 mm via 3D bioprinting [20]. However, a typical steak will have a thickness of around 1.5 inches, which is equivalent to approximately 40 mm, meaning that current state-of-the-art processes are still only able to achieve ¼ of the desired thickness. Additionally, the aforementioned 10 mm steak only consisted of bovine fat cells and thus did not adequately resemble the structural, chemical, or nutritional make-up of a traditional steak.

Although FRESH is a new technology, it has already been used successfully on several occasions, which indicates its viability as a method for our proposed project. For example, as a proof-of-concept experiment, a scaled-down version of a human femur was printed and tested to confirm that individual layers of the print had fused together properly within the secondary substance [21]. While the mock-femur was solid and used only to show the ability of layers to adhere, a bifurcated tube was later printed to ensure that hollow substances were also able to be printed using FRESH [21]. Additionally, further experiments validated the ability of FRESH to print common bioinks such as Collagen Type 1 or Fibrin, Matrigel, Alginate, and Decellularized ECMs [21]. While the previously listed materials have all been printed using the FRESH system, Collagen Type I and Matrigel are the most commonly used bioinks when using FRESH technology.

Other bioinks being considered for our process include Cellulose, Textured Soy Protein (TSP) and Soy Protein Isolate (SPI). These inks have not been printed using FRESH technology yet. However, this lack of data is not indicative of a belief that these options would fail as bioinks, but rather, that FRESH-supported bioprinting efforts and research has currently and almost exclusively revolved around replicating tissues and organs that could be found within a human body. Cellulose, TSP, and SPI are currently much more closely associated with cellular agriculture than with human tissue engineering and thus choosing one of these bioinks would likely be the first noteworthy change in the transition from using FRESH solely for human organs to using FRESH for cellular agriculture.

Outside of printing biomedical objects and bioink selection, FRESH’s system has also been confirmed to be capable of running for extended periods of time (4 hours) while maintaining the thermal settings necessary for success [21]. A final proof-of-concept experiment demonstrated that FRESH was able to 3D print C2C12 and MC3T3 fibroblasts suspended within various bioinks (fibrinogen, collagen type 1, or Matrigel) and have these cells survive and proliferate for at least 7 days post-printing [21].

More complex and intricate geometries have also been created using FRESH, namely a mimicry of the right coronary artery vascular tree [21]. This structure was confirmed to have well-formed details and in places to have achieved a wall thinness of < 1 mm. Ink pumped into this vascular tree was shown to move throughout the system [21]. This successfully printed vascular tree bodes well for the hope that using FRESH technology will allow us to create a scaffolding system complex and detailed enough to mimic *in vivo* vasculature and to allow for the perfusion of oxygen within the system.

While FRESH has not yet been used for printing products for cellular agriculture, it has seen success as a way to 3D print structurally functional and biocompatible coronary arteries that will ideally be seeded with cells and eventually used as viable implants [21]. We are confident that this technology is highly transferable to the field of cellular agriculture, and that we will be able to 3D print a cell-based cut of meat which adequately mimics a real cut of meat in taste, texture, and nutritional value.

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For a clearer image, please see: <https://docs.google.com/presentation/d/1WsHi2HM78fqIXjtVuRsv02EdlScltYReLs_G7nYItr0/edit?usp=sharing>

1. **Specific Aims, Methods, and Results**

*3.1 Specific Aims*

In search for a method to vascularize these tissues and provide ample oxygen diffusion, scaffolds seem to be one of the most promising options. Scaffolds are a particularly good option because they are able to provide mechanical support and integrity while also possibly serving as part of the vasculature [22]. Therefore, scaffolds can aid in both creating a structure capable of growing cells to mimic whole-cut meat, and in ensuring all cells in this structure are able to receive the oxygen and nutrients needed for survival. However, scaffolds come with their own set of issues - issues which are often exacerbated by the specific requirements of cellular agriculture. These problems include cost, lack of nutritional value, lack of resemblance to the taste and texture of real meat, edibility, and the varying ability to scale-up [23]. At the very minimum, any and all scaffolds considered for use in cellular agriculture must either degrade before consumption or else be able to be consumed as part of the final product [23].

As seen by the 3D printed, 10 mm steak, traditional 3D bioprinting is also a promising potential way to create whole-cut meat. But, there are severe limitations in current 3D printing methods that have stunted the progress of printing soft, vascularized tissues. Due to the nature of most tissues, the majority of bioinks do not have the inherent structural integrity required immediately after being extruded from the nozzle head to maintain shape [24]. Therefore, 3D printed tissues are often oversimplified and entirely nonfunctional. Many bioinks can obtain structural integrity post-print through processes like crosslinking, however, since crosslinking must occur post-print or after each layer, 3D bioprinting is still limited to very simple geometries and can take many hours or days to complete a simple print [24]. Therefore, innovation is needed if cell-based meat will one day be able to replace traditional meat on a global scale.

In 2050, there will be about 9.6 billion humans living on Earth. As a result, there will be a large demand for food, especially animal products. Overall, livestock consumption will not prove unsustainable for the global population. due to a lack of caloric efficiency; only about 13% of calories consumed by animals are turned into meat [27]. As such, there is a critical need to alter the nutritional content of meat during production. Studies have indicated that perceived healthiness is positively associated with purchase intention. If the nutraceuticals added to this product are branded effectively, our product would not only commercially succeed, but perhaps the 38% of Americans unwilling to try cultured meat to give it a chance [28].

Our solution to the problem of producing whole-cut cell-based meat is based on mitigation of the current limitations of 3D bioprinting through the use of Freeform Reversible Embedding of Suspended Hydrogels (FRESH™) from FluidForm [25]. The technology behind FRESH is a thermoreversible yield-stress material, which acts as a hard body under low shear stress and a viscous fluid under high shear stress. These properties allow for the chosen bioink to be printed directly into a supportive, yet removable, reservoir [24]. The specific design of FRESH is crucial as it allows for a 3D printed material to rest upon a supporting structure as soon as the biomaterial has been deposited from the nozzle, whereas in traditional methods the biomaterial is deposited directly into open air. This thermoreversible support bath solves the current primary issue with the 3D printing of biomaterials, as the tissues that are ultimately too soft to be used as bioink in traditional 3D printing are now able to maintain the desired geometry until the print has finished and solidification or crosslinking can occur. After stabilization has been completed, the support bath holding the finished print can be heated to 37ºC, which will fully melt the support bath and allow for easy print removal from the reservoir [26].

 The *long-term goal* of this project is to fill a market gap in the meat alternative industry with an ethical animal-based whole-cut product. Our *specific objective* is to develop a 3D printed, 3-cell type whole-cut meat mimic with melatonin and caffeine nutraceuticals. Our *central hypothesis* is that our cultured cells will take up significant concentrations of melatonin and caffeine while remaining viable and differentiated. The *rationale* for this project is that future research could develop a 3D printed meat mimic with a variety of nutraceuticals. Our *team* will work with the Kaplan lab which specializes in biomaterials for tissue engineering and regenerative medicine. Our advisor, Vincent Fitzpatrick, and his team are already successfully bioprinting with murine myoblasts. We will have access to the materials and equipment needed for this project.

*Specific Aim 1: Culture C2C12 murine myoblasts with nutraceuticals*

 We hypothesize that the C2C12 murine myoblast cells will continue to be viable after differentiation and will have taken up nutraceuticals at concentrations comparable to currently available melatonin and caffeine supplements. These factors will be assessed through the use of ELISA tests. The expected outcome is the verification of melatonin and caffeine as usable nutraceuticals and the most effective time during cell culturing to introduce them.

*Specific Aim 2: 3D print bovine myoblasts with nutraceuticals in FRESH suspension*

Bovine myoblasts will be cultured using the same methods as the C2C12 murine myoblasts before them. Characterization assays will quantify viability, differentiation and nutraceutical concentrations. After 3D printing the cells into the FRESH suspension, we will assess texture by comparing the shear stress and strain mechanics of our product with a similar natural meat. We recognize that we may need to redesign our scaffold and reprint for our product to exhibit a comparable texture. The expected outcome is a methodology for 3D printing myoblasts with melatonin and caffeine nutraceuticals.

*Specific Aim 3: 3D print bovine whole-cut meat mimic with three cell types*

 The same culturing, 3D printing and assessment techniques will be used to incorporate additional cell types. The expected outcome here is a 3D printed meat mimic with nutraceuticals and three cell types of myoblasts, lipoblasts and angioblasts for muscle, adipose and vasculature.

*3.2 Methods*

For Phase 1, we will be culturing C2C12 cells which are murine myoblasts and adding the nutraceuticals into the culture media. The starting concentrations of our nutraceuticals will be around 80 mg of caffeine and 8 mg of melatonin per 3-4 oz of meat serving, but we will be testing a range of concentrations of the chosen nutraceuticals [29,30,31]. We will be able to conclude if melatonin and caffeine were successfully added if after addition only notable concentrations of undifferentiated markers are found in the cells. If differentiated markers or apoptotic markers are found, we will need to evaluate if our additives cause early differentiation or cell death respectively. The markers that have associated ELISA kits are mouse CAF-1 for undifferentiated cells, mouse MYOD1 for differentiated cells, and mouse BAX for apoptotic cells [32,33,34]. Once the murine ELISA assays are finished, we will be re-running the same characterization assays on the bovine myoblasts. After the ELISA experiments are complete, we will move onto Phase 2.

The measurements and methods for completing Phase 2 will be done with additional ELISA kits that detect melatonin and caffeine to ensure the nutraceuticals have not been metabolized, and cell viability and proper differentiation will be determined based on cell counts and the use of the differentiation marker ELISA kit [35,36]. Phase 2 will also consist of evaluating different types of scaffolds to see how well they print and how well the cells adhere to them. After all characterization assays and differentiation studies have been finished, we will move onto the last phase which is Phase 3.

Measuring success in Phase 3 is fairly straightforward as the final product will ideally be an edible whole-cut piece of cell-based meat that mimics real meat in taste and texture, although Phase 3 will have the biggest barriers to success. This step is far down the line, but success will be determined through 3D printing of the scaffold biomaterial and the three types of bovine cells into a meat-mimicking 3D structure. The three cell types we will be printing with are myoblasts, lipoblasts, and angioblasts which are the undifferentiated precursors of muscle, fat, and the endothelial cells that make-up vascular walls in tissues. Once these precursors are seeded into the printed scaffold, they will be allowed to proliferate until the desired confluence is achieved. At that point, we will use the differentiation study results from Phase 2 to differentiate the cells to create a finished product. The product will then undergo shear stress and strain tests; even though this product will not be taste-tested, research may also be performed to determine additives that may enhance the product’s flavor.

*3.3 Results*

 There are no results to report at this time. We will begin cell culturing next week.

1. **Discussion and Future Work**

*4.1 Discussion*

 Currently, we have no results to discuss.

*4.2 Future work*

 There are several aspects of this project we will not have time to explore further. First, we are not assessing taste in this project so, if the process could be completed according to proper food safety regulations, this is a future area for research. Human taste tests could also determine the exogenous effects of nutraceuticals to verify the dosage is appropriate. Secondly, cooked meats are required for proper human testing. But from this research alone, we cannot assess the effect of cooking on nutraceutical concentration nor texture of the meat mimic. Future work may find additional adjustments are required for a cookable product.

Additional forays into the cultured meat could investigate alternate types of meat cuts - perhaps efforts could be made to marble the meat and create a sort of highly striated steak. Besides murine and bovine cell lines, other types of stem cells could be utilized, including porcine or ovine cell lines.

Finally, if our project proves successful, it opens up possibilities for other nutraceuticals in cultured meat products. Other interesting options for future experimentation for whole-cut meat mimics include reduced fat and cholesterol, increased fiber, added vitamins or compounds addressing specific nutrient deficiencies. The most significant limitation, assuming the cell culture is viable, is in the marketability of the selected nutraceutical-meat combination. Neural analyses could also be done to investigate alternate ways to maximize the impact of nutraceuticals.

1. **Conclusions**
* A 3D printed nutritionally-enhanced meat mimic may fill an existing need within consumer markets for an ethical whole-cut alternative.
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**Individual Contributions**

* Abstract- Hanna, Soham
* Engineering design- Emma
* Design flowchart & description- Emma, Hanna
* Introduction & background- All
* Unifying figure- Hanna
* Specific Aims- All
* Methods- Emma
* Future work- Hanna, Soham