WPI

Leef Jerky™: A Sustainable Meat Product for a Better Future

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From left to right: Brian, Fatin, Alex, Daniel

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Abstract

The current state of meat production is resulting in numerous global environmental issues. Raising animal livestock for slaughter involves significant land and water usage, and is a leading contributor of global greenhouse gas emissions. Lab-grown meat has been investigated as an alternative to conventional meat production. Despite recent advancements in cellular agriculture, perfusable scaffolding remains a prominent issue when trying to develop thick and structured meat. This project aimed to develop an environmentally conscious, lean, structured meat product using decellularized plant leaf scaffold technology. In addition, dried meats were identified as an ideal entry market for lab-grown meat due to their lean nature and high profit margins. Experimental results showed that isolated bovine muscle cells successfully adhered to each of the chosen decellularized plant leaf scaffolds. The adhered cells exhibited alignment, proliferation, confluence, viability, and differentiation into myocytes without the use of adherent protein coatings. The results of this project demonstrate that decellularized plant leaf technology is promising in the future production of dried meat products.

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1 Introduction

Approximately 97% of all U.S. adults consume meat on a regular basis, with the average American estimated to have consumed a record 222 pounds of red meat and poultry in the year 2018 alone [1,2]. Agriculture uses 51% of all land in the United States, 80% of which is used to raise animal livestock [3]. Because it is projected that both global population and meat production will continue to rise, there is a real risk that there will be insufficient land to keep up with the growing demand for meat [4].

Agriculture contributes to 24% of global greenhouse gas emissions [5]. Experts suggest that increasing greenhouse gas levels within the Earth's atmosphere are leading to global warming [6]. Some of the consequences of this climate change include rising ocean levels, stronger and potentially catastrophic weather events, and global drought [6]. In addition, agriculture is responsible for consuming 70% of all freshwater globally [7]. It is estimated that, by 2050, over half of the global population will be living in moderately water scarce areas [7].

It is clear that the current state of meat production and agriculture is causing large-scale environmental harm. There is a need for an alternative meat source that satisfies the growing demands of consumers, while significantly reducing land usage, greenhouse gas emissions, and water consumption. Two viable alternatives to conventional meat products include plant-based protein and cellular agriculture.

Because only 3% of U.S. citizens follow a vegan or vegetarian lifestyle, the market and environmental impact for plant-based products is relatively small [1]. Cellular agriculture, also commonly referred to as cultured meat, is an emerging industry which utilizes tissue engineering technology to grow authentic meat products [8]. Cellular agriculture presents a unique opportunity because it caters to the larger audience of meat eaters, and can have a significant environmental impact. One of the biggest challenges in the field of cellular agriculture is the development of perfusable scaffolds that can produce structured meat [8]. The goal of this project was to utilize decellularized plant leaf scaffolds to produce an environmentally conscious structured meat product.

Dried meat products, such as beef jerky, were chosen as the focus of this project. Dried meat snacks are a $2.8 billion industry in the United States, and are currently an untapped market within cellular agriculture [9]. The nature of dried meat snacks is that they are primarily made from the leanest cuts of meat. Choosing to grow a lean food product simplifies the cell culture process because only muscle cells would be required. Dried meat products also rely less on the taste of the meat itself, as they are heavily flavored during processing. An additional

consideration is a suitable scaffolding material that would support the alignment of cells to produce a structured product. Beef was selected as the meat of choice because it is widely consumed by Americans and is the least sustainable in terms of environmental impact [2].

The preliminary design objectives for a suitable scaffold included low cost, familiarity to consumers, edibility, and scalability to industrial standards. Based on these objectives, and inspired by the decellularized plant leaf technology developed by the Gaudette lab, leek, iceberg lettuce, and spinach were selected as possible scaffolding materials for growing meat [10]. The procedure for decellularizing plant leaves included a needle cannulation and washing step that was time consuming, user intensive, and non-scalable [10]. A bulk decellularization system was developed to simplify and scale this process. The project team identified the use of food-grade materials during the decellularization process as an important design consideration as well. The bulk decellularization process utilized polysorbate (tween) 20, sodium dodecyl sulfate (SDS), sodium hypochlorite (bleach), and TRIS buffer, all of which are FDA classified as common food safe additives [11].

Several proof of principle seeding experiments were performed to determine if adhesion, viability, differentiation, confluency, and contraction of bovine skeletal muscle cells was possible. The experiments were completed in culture and on the decellularized leaf scaffolds without the use of adherent protein coatings. The first step in this process was the isolation of bovine skeletal muscle cells from a meat sample. The isolated cells were cultured to determine if they could proliferate, differentiate, and contract. The isolated cells were then seeded onto decellularized plant leaf scaffolds at a density of 250k. After four days of incubation, the leaves were stained using both Hoechst 33342 and Phalloidin, and were imaged using a fluorescence microscope. The same seeding protocol was performed using Hoechst 33342 and MF20 as markers for the detection of myocytes.

2 Literature Review

This chapter examines some of the contemporary, ethical, and health issues associated with meat consumption in the United States. We also investigate the current state of the meat industry and lab-grown meat as an alternative. Next, considerations are made into some of the common materials and techniques employed in tissue engineering and animal meat growing applications.

2.1 Contemporary Issues with Meat Consumption

From 2000 to 2015, the number of Americans adopting a vegan or vegetarian diet has seen a staggering increase from approximately 3-500,000 to 2.5-6 million consumers [12]. Moreover, the number of sustainable or "organic" producers was 2.7 million in the year 2016, with a corresponding market of over 100 billion dollars [13]. These dramatic increases are due in large part to the many issues arising in contemporary animal agriculture, most notably environmental sustainability, animal ethics, and health.

2.1.1 Environmental Sustainability

Environmental damage can be defined as all associated costs caused by humans, ecosystems, and natural resources [14]. Currently, animal agriculture as an industry is estimated to contribute between 4.6 and 7.1 billion tons of greenhouse gases, which accounts for 19-23% of actual warming caused in the atmosphere [15]. Of these total emissions, 74% is attributed to beef and dairy cattle, which represents a carbon footprint larger than that of human energy usage [16]. Animal agriculture has a significant effect on natural resources such as soil nutrients and water. Approximately 908 trillion liters (240 trillion gallons) of water is used to sustain current livestock production levels globally. Consequently, animal agriculture is the largest source of global water pollution in the world [17]. The manure lagoons used to contain the liquid animal waste produced through animal agriculture have been correlated to high concentrations of nitrogen and chloride contamination into surrounding groundwater reserves [18]. In addition, animal agriculture for meat consumption is also responsible for over half of the total erosion of major waterways, causing 40 billions tons of soil sedimentation loss per year [17]. Furthermore, the need for land to raise livestock has cleared a staggering 70% of all grasslands, 50% of savannahs, 45% of temperate rainforests, and 27% of tropical forests worldwide in order to keep up with the growing demand for animal products [17]. The resultant land encroachment of animal agriculture activities is responsible for driving over one thousand animal species to extinction per year [17].

2.1.2 Animal Ethics

As global meat consumption continues to grow, the trend of animal slaughter has increased dramatically. In the United States, land animal slaughter has increased from 1.8 billion in 1960 to 9.1 billion total animals in 2013, with the majority of these being avian species [19]. Avian species include ducks, geese, chickens, and turkeys, which constitute approximately 59 billion animal deaths worldwide in the year 2014 [19]. Industrial scale agriculture of avian species includes selective breeding, genetic modification, confinement, and antibiotic treatment [19]. Most avian species in these operations grow so large so quickly that they crush underdeveloped bones from the weight of their own muscle mass and are unable to move [19]. In further evaluating the impact of animal agriculture, the use of land mammals must also be considered. In 2011 alone, the global number of cattle slaughtered for meat was 296 million. The conditions in which the cattle live have also become increasingly inhumane. Similar to the avian industry, the majority of large-scale beef production involves selective breeding, genetic modification, as well as the pharmaceutical (primarily antibiotic) enhancement of animals [19]. Due to space limitations in agriculture, feeding operations called CAFOs (concentrated animal feed operations) are routinely used where cattle movement is minimized in combination with increased food intake and growth hormone treatment to develop cattle as fast as possible, resulting in a growth period of 12-16 months before slaughter [19]. There is also the concern over the slaughtering process itself. Cattle are first shipped on a vessel where many die under the weight of others. During processing, cattle are hanged by their feet and bloodlet mechanically through an assembly line process without the use of anesthetic, where they can suffer for an extended period of time before death [19].

2.1.3 Health Concerns

Currently, there are a variety of serious public health threats due to increasing meat consumption globally. Meats harvested through animal agriculture are attributed to higher a risk of cardiovascular disease such as atherosclerosis, various cancers, and diabetes [20]. In a cohort study of the National Institutes of Health-AARP (NIH-AARP), there was found to be a significant correlation between overall mortality and the consumption of red meat (Figure 1). Increased consumption showed a 50% increased chance of mortality for men, and a 35% increased chance of mortality for women [21].

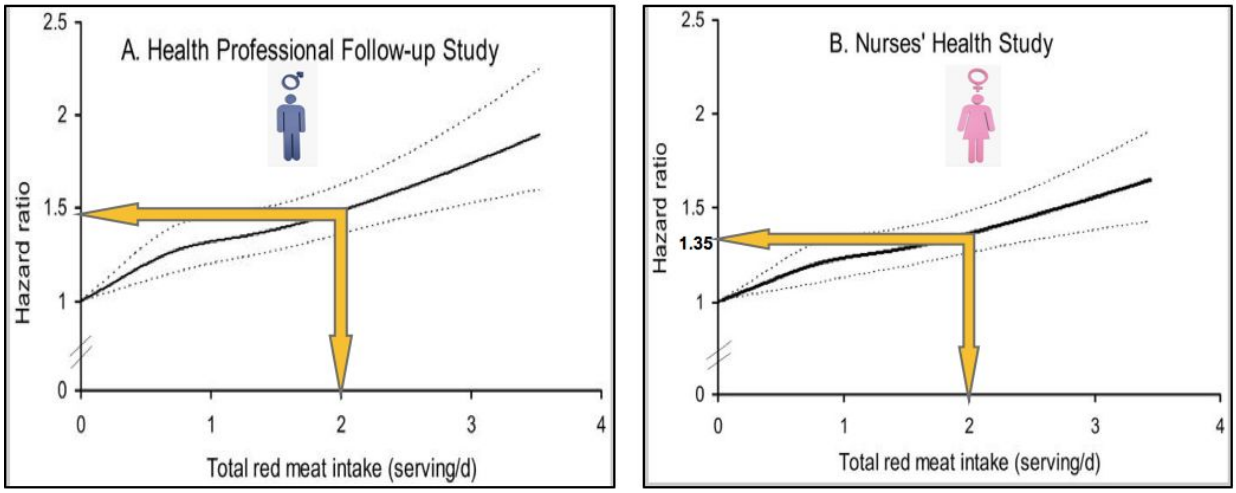

Figure 1: Mortality Cohort Study]22]

An even more pressing concern is the antibiotic resistance which bacteria are developing in response to the uncontrolled use of antibiotics in the animal agriculture industry, as well as the widespread bacterial contamination of livestock that these antibiotics are being used to treat. An estimated 90% of all chickens grown in the United States and 50-75% in the United Kingdom are infected with Campylobacter species, which is now the most common cause of bacterial gastroenteritis in developed nations [20]. Furthermore, disease outbreaks such as bovine spongiform encephalopathy, swine flu, and foot and mouth disease have been directly correlated to the animal agriculture industry, where increasing antibiotic resistance is posing a major concerns globally for new drug development [20].

2.2 State of the Meat Industry

In this section, we analyze the expanding meat industry in the United States and some current trends. We mention some potential global problems that the meat industry poses, and discuss lab-grown meat as a developing market and alternative to slaughtered meat. Lastly, we explain how lab-grown meat is a potential solution to some of the problems the current meat industry creates.

2.2.1 Expanding Market

Despite the problems resulting from the meat industry, meat has a larger market today than ever before. According to the United States Department of Agriculture, meat consumption increases every year, with 2018 expected to have record-high amounts of meat consumption and production [23]. It is estimated that the United States alone will produce about 140 billion

pounds of red meat and poultry by the end of 2018, four billion more pounds than the amount produced in 2017 [23]. This steady yearly increase creates a significant market for meat production. This trend continues year to year, despite the consequences and problems that result from the meat industry. Considering the future course of meat consumption, it is important to develop and implement a method to tackle these problems while satisfying the current market for meat.

2.2.2 Lab Grown Meat as a Solution

Cellular agriculture is an alternative method for growing clean or cultured meat, and is defined as the process of creating edible animal muscle-skeletal tissue in vitro using tissue engineering techniques [24]. With increasing public awareness of the ethical, environmental, and sustainability concerns surrounding the animal agriculture industry, cultured meat is an ecological alternative to satisfying consumers' taste for meat [24]. With respect to sustainability, cellular agriculture can minimize the environmental impact dramatically, where equivalent land and water usage to produce animal tissue is 99% less than that of traditional animal agriculture methods [20]. Additionally, cellular agriculture uses less total energy and produces less pollution than all conventional animal agriculture areas except poultry [20]. For animal welfare, cellular agriculture has been recognized by PETA and other organizations as a means to eliminate the need for animal slaughter and dramatically minimize the amount of animal harm involved in meat production [20]. In order to obtain initial cell samples, only small, harmless biopsies would be required to produce thousands of pounds of meat. Public health can also be improved using cellular agriculture because it is done in a sterile environment without the inherent risks of factory farming, and the meat produced can contain nutritionally beneficial compounds [20].

Although lab grown meat may sound unnatural or unusual to some, it has been demonstrated that many people would be willing to try it. A study was administered by Matti Wilks and Clive J.C. Phillips, researchers for the Early Cognitive Development Centre and for The Centre for Animal Welfare and Ethics, respectively, at The University of Queensland in Brisbane, Australia. During the study, they utilized Amazon Mechanical Turk (mTurk) to run an online survey [25]. Of the 673 survey participants, two-thirds stated that they would probably or definitely try lab grown meat. A third of these participants stated that they were willing to eat lab grown meat regularly and use it as a replacement for meat in their diet. However, 34% stated that their choices would be valid only if the price was comparable to that of traditional meat. Only 16% of participants were willing to pay more for lab grown meat. The study surveyed 328 males, 340 females, and 5 others, with an age range between 18 to 70. The average age was 32.58 ± 10.79. The demographic was about five years younger on average, had a slightly lower income, and were more likely to have an undergraduate degree than the average person.

A similar alternative on the market is grass fed meat. Consumers choose to buy this meat because it is more sustainable and thought to be healthier than non-grass fed meat. Retail sales of grass fed beef grew 15 times in the four years since June 2012, reaching $272 million by June 2016. In addition, The grass fed beef industry generated an estimated $4 billion in retail and food services sales in 2015. Although this only represents about 4% of the United States' total meat market, these numbers have been rising rapidly over the years [26]. This shows that consumers are becoming more conscious of the unsustainable and damaging consequences of inorganic and non-grass fed meats. It can be estimated that lab grown meat would follow the same trend as grass fed meat. It is likely that the people who already purchase grass fed and organic meats, the "conscious consumers", would likely try lab grown meat.

There are several companies that are currently growing animal meat, including Memphis Meats and Mosa Meat. These companies are making significant headway into the industry and have several important figures and companies backing them. For example, Memphis Meats has Bill Gates, the founder of Microsoft Corporation, and Tyson, the largest meat producer in the United States, as partners [27]. Tyson believes that "today's consumers want more protein. Sixty percent of us are actively trying to add more protein to our diets, and when we think about the attributes we want in our food, protein tops the list...At the same time, our global population continues to grow. That's why Tyson Foods is investing in alternative proteins… [to give] our growing population more ways to feel good about the protein they're eating" [28]. Although these companies have significant financial backing, none of them have lab grown meat that is available for purchase commercially as of yet. One of the reasons for this is the cost of the meat. In 2013, Memphis Meat's first hamburger cost about $330,000. They claim that they would like to release it to the public at a much more reasonable price of about $11 per hamburger. The company plans to release chicken nuggets, sausage, and foie gras along with hamburgers before the end of 2018 [29]. Mosa Meat estimates that by the next three to four years, they will be selling hamburgers at about $12 each when they scale to industrial size [30].

According to Mosa Meat, the greatest scientific challenge these companies face is the development of a replacement for fetal bovine serum (FBS) (2018). FBS is the most commonly used serum in tissue engineering applications and needs to be replaced because it is harvested from bovine fetuses during slaughter [31]. Utilizing FBS for growing animal meat would be going against its main purpose. Serum-free media does exist, but it is more expensive than FBS. Mosa Meat has been able to formulate their own serum-free medium that works, but it still needs to be optimized. This is a major technological advancement, since media accounts for about 80% of the cost of cultured meat. However, the price of meat is a significant problem for these companies because they have a difficult time scaling up production and bringing the price down to a competitive level. Mosa Meat hopes to address these problems in the coming years [30]. They hope to further reduce their costs by increasing the volume of meat production using

different scaffolds, further decreasing the cost of media, or utilizing methods of recycling media, to name a few.

2.3 Tissue Engineering Materials

In this section, we examine some of the commonalities that shown in Figure 2 which involved in tissue engineering and meat growing applications such as: scaffolding, cells types, media, and growing conditions. The goal of this section is to outline these current techniques and materials that used to produce lab grown meat.

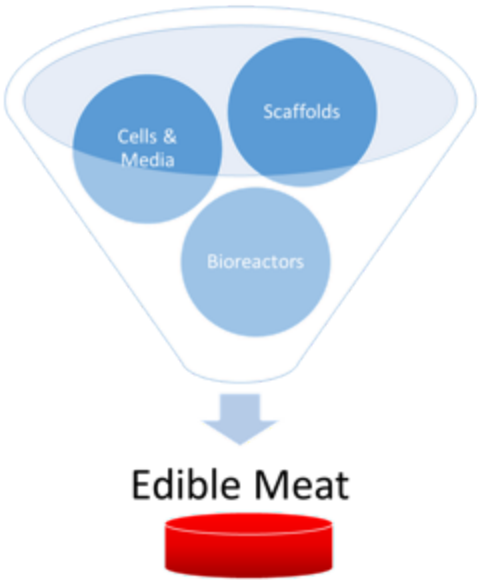

Figure 2: Current Challenges in Lab-Grown Meat

2.3.1 Scaffolding

An important material which acts as a base for and stretches cells is a scaffold. There are many types of scaffolding mechanisms available depending on shape, composition, and characteristics to optimize muscle cell and tissue morphology. The perfect scaffold has large surface area for cell attachment and growth. Also, effective scaffolds can maximize medium diffusion before the separation of cultured cells. Different natural polymers successfully utilized as a scaffold include collagen meshworks, edible beads, cellulose, alginate, or chitosan. These safe materials can add textural quality to cultured meat and can stretch regularly with changes to temperature or pH and support the growth of myoblast cell layers [32]. These scaffolds have diffusional limitations with processed meat products; most notably the maximum thickness of the myocyte layer is about 100–200 μm [33]. Synthetic or inedible polymers allow for good quality tissue formation. Micropatterned surfaces and thermoresponsive coatings are useful techniques that deal with separating cultured cells from scaffolds [33]. One of the technical challenges of growing meat

with scaffolds is removing the scaffolding system without damaging the cells and the extracellular matrix that is produced [33].

Currently, lab-grown meat companies can only create amorphous, or unstructured, meat products. These are products such as ground chicken, beef, etc. In order to address this, many of the companies are looking into using scaffolds to align the cells and to promote a structure. The difficulty lies in finding a scaffold that fits this need while also being edible and nontoxic. If a scaffold is inedible, the creation of the product would require additional steps. The scaffold would have to be removed, most likely through the use of chemicals. It is possible that the material or chemicals would then leave behind inedible or toxic byproducts or remnants. In addition, growing a 3D structured meat product requires a scaffold that is able to direct nutrients to the cells through the thickness of the product. Otherwise, the cells would not proliferate and would die.

Decellularization techniques used on plant tissues aim to create a sustainable scaffold that allows cultured cells to attach and proliferate in a proper manner. The plant scaffold can transport the nutrients in the media to the distal end. During decellularization, a leaf is made colorless (its tissue has lost chloroplasts) but its vascular network can provide human umbilical vein endothelial cells (HUVEC) with the proper environment to stay alive when coated with fibronectin and acetylated low-density lipoprotein (Dil-Ac-LDL) as shown in Figure 3 [10].

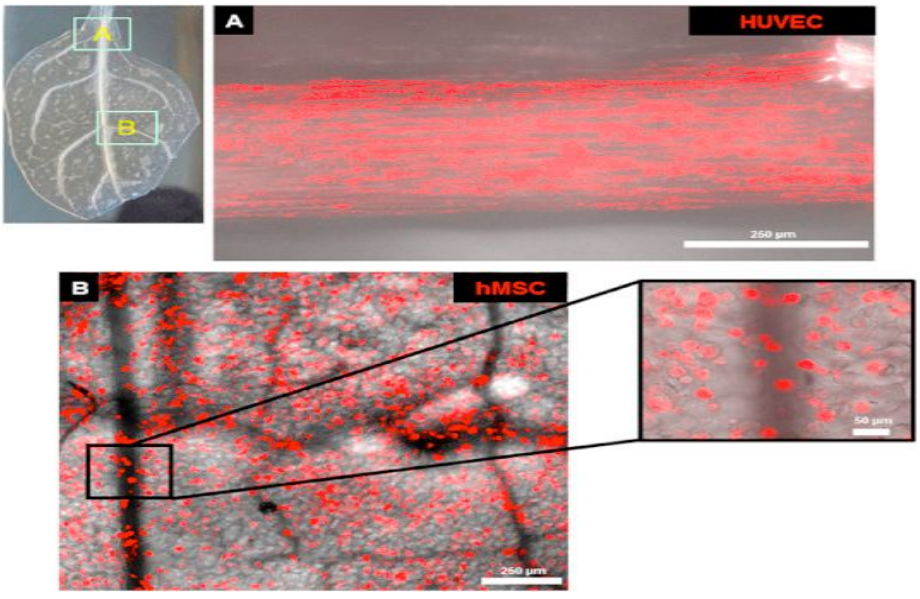

Figure 3: Decellularized Spinach Leaves used to Culture HUVEC and hMSC Cells [10]

In addition to spinach, decellularized apple tissue has been used as a scaffold for mammalian cell growth. Researchers are working to create a meat stick rather than ground meat by using the structure of the scaffold as the basis to build muscle. Mushroom, chitosan, and micropatterned cellulose surfaces are examples of scaffolds that researchers are currently using in clean meat [34].

Another method for growing meat is by volume expansion of muscle tissue. To produce fish meat, Benjamin et al expanded the volume of a fish explant using a medium that held a crude cell mixture of the fish. In spite of the good quality of the product, there are diffusional limitations that prevent large-scale production [33].

2.3.2 Cell Types

For a good source of viable cells, a living animal is chosen for muscle biopsy. The cells will then proliferate *in vitro* and be grown into muscle fibers [35]. Stem cells are a specific type of cell that are selected and modified to develop *in vitro* meat because of their abilities to retain themselves in an undifferentiated form and proliferate quickly. Stem cells (also known as satellite cells) are differentiated into muscle cells via growth factors in the cell culture media [32]. Adult stem cells (ADSCs) are isolated from adipose tissue which is highly susceptible to differentiate into myogenic cell breeds. These cells, which can be obtained from the subcutaneous fat layer, have properties to greatly expand but have short-term culturing (4–5 months) [33]. Memphis Meats is the first company that expanded skeletal myosatellite stem cells using a serum-free method [35]. Although the myosatellite cells have limited regenerative potential, they can be isolated from different muscles and regulated by growth and differentiation adapters [33]. Myoblasts/muscle cells are anchorage-dependent cells that have the ability to normally contract [33].

Many cell divisions are necessary to create cultured muscle tissue for *in vitro* meat production systems (IMPSs). Hayflick's Limit is the theoretical limit of cell doublings that can occur before cells reach senescence. This number can be improved by replenishing the cell culture periodically, using a cell line, or inducing immortalization. Immortalization of cells requires genetic manipulation but is a high specialized practice [33]. Another factor known as the diffusion limit represents the rate of growing cells along the scaffold surface as an extracellular matrix. The diffusion limit for good cell growth must be within 200 microns between the diffuse cell and its nutrient supply [36].

2.3.3 Media

Media refers to the food source that helps cells grow and divide. Media is typically expensive because it relies on an animal-based serum to obtain desirable results. Because of this, animal-free media formulations have been created to make cells grow [37]. One source of culture media is cyanobacteria. They contain up to 70% protein and they can grow fast as photosynthetic bacteria. In addition, they provide specific nutrients including vitamins, lipids, salts, and amino acids that cells need to grow and survive. Serum and plasma are used to deliver these factors to the cells [38]. Fetal bovine serum (FBS) contains most of these factors and hormones that help the cells. FBS is added at concentrations of 5-20% to the final medium formulation. In this way, a cell culture can have delayed growth by manipulating the amount of growth factors in the media. Otherwise, in serum-free conditions, serum replacements that consist of insulin, transferrin, selenium, putrescine, and progesterone are added to the cell medium. Also, vegetal serum or peptones can be used [35].

2.3.4 Bioreactors and Growing Conditions

Bioreactors are used to provide cells with optimal conditions during growth including oxygen concentration, pH, and temperature. The oxygen supply rate for both culture medium and cells must be in high concentration to allow for high cell viability. Bioreactors are designed to keep a lower shear force and optimized perfusion for a high quantity of cultured meat [38]. Figure 4 shows a typical cultured meat production system for myoblasts or myosatellites seeded on a scaffold and grown in a bioreactor. For achieving a good taste and quality of meat, the bioreactor must provide a favorable environment for the growth of cells on the scaffold [32].

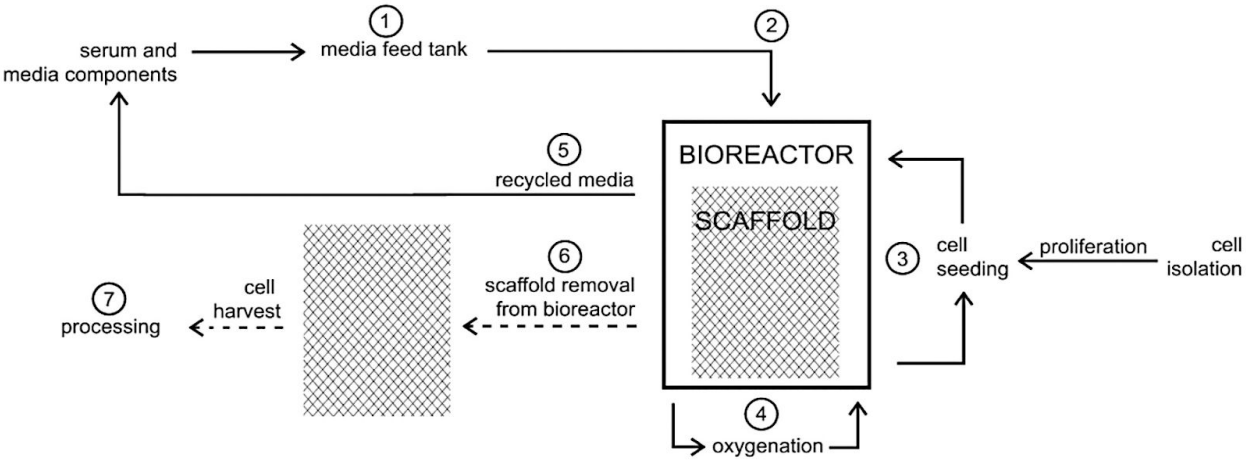

Figure 4: Cultured Meat Production System [38]

Finally, the cultured meat is harvested from the bioreactor. In order to give the final product the same taste of conventional meat, the grown tissue should contain muscle fibers and connective tissue which comprises collagen, elastin, and fat cells which provide meat with its flavor [32]. Sometimes a mix of saffron and red beet juice is used to provide color of cultured meat [33].

2.4 Cell Seeding

Cell seeding is the process of spreading a certain number of cells upon an area such as a petri dish, flask, or 3D tissue engineering scaffold. For almost all tissue engineering applications, it is crucial that a cost-effective, replicable, and effective seeding technique is employed [39]. Furthermore, efficient seeding techniques are easier to mass produce [39]. Successful cell seeding is defined as having high efficiency (or number of cells), fast cell attachment, high cell viability, and uniform cell distribution [40]. To maximize the volume of meat produced in this project, it is paramount that successful cell seeding is obtained. This section discusses cell seeding techniques commonly employed in tissue engineering, factors affecting cell seeding, and ways to measure cell seeding.

2.4.1 Cell Seeding Techniques

In most tissue engineering applications, static/passive seeding is a commonly employed seeding technique [39]. Static seeding involves pipetting a suspension of cells onto a scaffold or desired location. Upon addition of the cells, the scaffold is placed in an incubator for a few minutes, then incubated in a petri dish containing cell media for a few hours to a few days in order to maximize cell attachment. Although static seeding is regarded as one of the simplest seeding techniques, it has relatively low efficiency (10-25%) and has low scaffold penetration by cells. To overcome these disadvantages, alternative seeding techniques have been developed [39].

Dynamic seeding utilizes rotational force and/or pressure to increase cell seeding efficiency and scaffold penetration [39]. In rotational dynamic seeding, a scaffold is attached to a needle and is placed in the center of a container with a rotator at the bottom. The needle is submerged in the cell suspension, and cells attach to the scaffold via centrifugal force. Rotation speeds have been used up to 2500 rpm, and incubation times have varied from twelve hours to three days. Efficiency for rotational seeding was reported at 38 to 90%. In vacuum dynamic seeding, an internal or external pressure is applied to a cell suspension to encourage seeding on a scaffold. Although this technique can be employed relatively rapidly and has high efficiency (60-90%), it can only be applied to porous scaffolds [39].

Perfusion seeding is another technique which involves the continuous flow of a cell suspension through a 3D porous scaffold. Wendt et al [41] developed an automated perfusion bioreactor which provided continuous oscillatory flow of cell suspension through a porous polymeric scaffold. When compared to static and dynamic seeding techniques, Wendt et al reported that their perfusion technique seeded approximately 18-25% more cells with up to 3.8-times more uniformity [41].

Haiyan et al [42] developed a cell seeding method using surface acoustic wave (SAW) technology. SAWs were created using an interdigital electrode fabricated on lithium niobate. The seeding process was reported to last ten seconds when compared to static technique which relies on diffusion and can take up to 30 minutes. The use of SAWs was linked to increased scaffold penetration, along with cell seeding uniformity and distribution [41]. There are many more types of cell seeding techniques, but those briefly discussed above are some of the most common ones. While each technique has different effects on cell seeding, there are other outside factors which must be considered.

2.4.2 Factors Affecting Cell Seeding

Chen et al [43] performed a study on different factors affecting cell seeding. They used the following experimental design:

- Input factors
    - 2 cell types
        - Human periosteum-derived cells (hPDCs)
        - Human osteosarcoma cell line (SaOS-2)
    - 2 scaffold types
        - Foamed titanium
        - 3D fiber-deposited titanium
- Continuous process factors
    - 2 seeding densities
        - 60,000 and 1,200,000 cells
    - 2 seeding volumes
        - 50% and 150% of total scaffold volume
    - 2 seeding times
        - 30 minutes and 4 hours
- Response variables
    - Cell seeding efficiency (CSE): percentage of initially seeded cells that successfully remained attached to the scaffold
    - Specific cell viability (CSV): metabolic activity of the cells
    - Cell special distribution (CSD): uniformity of the cells

The authors noticed a strong dependance of cell type on CSE, with the hPDCs performing better than SaOS-2 cell line in all three response variables [43]. The authors proposed that different cells have different cell adhesion and overall cell behavior. The authors decided to use titanium scaffolds in their experiment due to its orthopedic applications, and found no significant difference between either scaffold type. For the three continuous process factors manipulated in the study--seeding density, volume, and time--the authors found results similar to that of other studies. Seeding density did not have a significant effect on CSE. They found that increased seeding volume had a negative effect on CSE, while increasing seeding time had a positive effect. For CSV, increased seeding volume had a positive effect while increased seeding density had a negative effect. Seeding time had no observable change for CSV. Lastly, the authors found that increased cell volume had increased CSD [43].

Another study by Bueno et al [40] looked into some hydrodynamic factors and their effect on cell seeding. Hydrodynamics is the study of how fluid forces affect the environment. The authors obtained primary chondrocytes from the cartilage of calf knee joints. Two different bioreactors with different hydrodynamic properties were used: a wavy-walled bioreactor (WWB) and spinner flasks. A WWB has periodic and turbulent flow when compared to spinner flasks. The cells contained within the spinner flask would experience higher and more uniform shear forces than WWB. The WWB has outer lobes, which experience less and more uniform shear than the center. PGA scaffolds were used in each bioreactor, and seeding density from 2.5 to $10x10^{-6}$ cells and seeding volume of 6.4 to $25.5x10^{7}$ cells/cm^3 were used. Cell viability, seeding efficiency, and spatial distribution were measured over the period of three days. Bueno et al found no observable differences in cell viability from either bioreactor. Regarding seeding efficiency, the authors found that $2.5x10^{6}$ seeding density had significantly more attached cells in both the lobes and center of the WWB. For $10x10^{6}$ seeding density, there was 1.7-2.1 times attachment in the lobes of the WWB. Furthermore, at $10x10^{6}$ seeding density, the scaffolds in the lobes of the WWB contained 100% of the cells at all seeding densities. For spatial distribution, the center of the WWB had the most uniform distribution of cells. In this study, the WMB (turbulent flow environment) had more uniform and efficient cell seeding than the spinner flask [40].

2.4.3 Quantifying Cell Seeding Efficiency, Viability, and Uniformity

It is clear that many factors affect cell seeding efficiency, viability, and uniformity, but how are these parameters quantified? Cell seeding efficiency can be measured as the total number of cells that have attached or have embedded into a scaffold compared to the total number of cells seeded initially [39]. There are a variety of different techniques available to measure cell seeding efficiency. Villalona et al [39] provide a helpful table (Figure 5) which organizes the principal techniques. All seeding efficiency techniques can be divided into two groups: observation and counting, and indirect quantification via assays. Observation and counting can be cumbersome

and time consuming, but are a clear measure of efficiency. Indirect assays are faster and ensure that dead cells are not counted, but are generally less accurate [39].

Methods	*Advantages*	*Disadvantages*
Hemocytometer	Simple, fast	Not accurate, overestimates cell seeding
Histology	Real counting, best overall	Tedious, time consuming, good different cell lines if labeled
SEM	Scaffold surface analysis	Only good for scaffold surfaces
DNA	Accurate, reliable	Changes when comparing different cell lines or different donor
MTS, MTT, WST-1	Quantitative and qualitative	Changes when comparing different cell lines or different donor

SEM, scanning electron microscopy; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide); WST-1, (4-(3-4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzenedisulfonate).

Figure 5: Quantification Methods Used in Cell Seeding [39]

Cell viability is measured to ensure that cells attached to a scaffold are able to grow and contribute to the forming tissue. Cell viability can be measured using an MTT or live-dead cell assay. Cell uniformity can be measured using histology, live-dead staining, or image analysis [40,41,42].

3 Project Strategy

This chapter examines our initial client statement, and the design constraints, objectives, and standards that were considered over the course of the project. These were used to influence the design process in the following chapter.

3.1 Initial Client Statement

Because the current state of meat production is resulting in environmental harm and is not sustainable, the project team sought to create an alternative meat product. This meat product should be able to satisfy consumer demand for meat, but also be more environmentally responsible. The project team came up with the following client statement which served as a guide throughout the entire project.

There is a need for an alternative meat source that meets the growing demand of consumers. The meat source should be created using significantly reduced land usage, greenhouse gas emissions, and water consumption. The final meat product should be slaughter and hormone-free and comparable to conventional meat in taste, texture, and structure.

3.2 Engineering Criteria

The project team identified an untapped market within the lab-grown meat industry: dried meat products, specifically beef jerky. Dried meat products are a $2.8 billion market in the United States, and may be a viable entry point for lab-grown meat [9]. Beef jerky products have a few important design considerations: 1) they are mostly lean, so only muscle cells are required; 2) there is the need for a proper scaffold that can give the meat product proper structure and texture; 3) dried meat products have higher margins than other meat products such as ground beef or chicken.

The team decided that the alternative meat product would be called Leef Jerky™. The engineering criteria for Leef Jerky™ were separated into two groups based on the initial client statement: design constraints and design objectives.

3.2.1 Design Constraints

The design constraints are presented in Table 1, and are defined as critical design components necessary for the final product to be successful and acceptable to meat eaters. The design constraints have been separated into biological, mechanical, and industrial constraints.

Table 1: Design Constraints

Biological Constraints
● Completely edible ● Slaughter-free ● Safe to eat and no contamination risk
Mechanical Constraints
● Scaffold must be sturdy or rigid enough to support its own weight without falling apart ● Comparable to beef jerky in taste, texture, and aesthetic
Industrial Constraints
● FDA and USDA compliant ● Any detergents used must be food-grade

3.2.2 Design Objectives

The design objectives are presented in Table 2, and are defined as design components that would be beneficial if met, but are not required for the design to be successful. The design objectives have been separated into biological, mechanical, and industrial objectives.

Table 2: Design Objectives

Biological Objectives
• Cell differentiation and viability • Completely uniform distribution of cells • No other animal products used • Hormone-free
Mechanical Objectives
• Thickness of regular beef jerky (¼ inch)
Industrial Objectives
• Potential to scale and automate all processes involved in creating Leef Jerky™

3.3 Engineering Standards

Design standards are guidelines created by regulatory bodies to ensure that a product meets a minimum performance, is safe to use/consume, and can be reproduced consistently. As of March 7th, 2019, the FDA and USDA are both responsible for regulating lab-grown meat used for human consumption. The FDA is responsible for overseeing cell collection, cell banks, and cell growth and differentiation. FSIS, a branch of the USDA, is responsible for the product once the cells are harvested. FSIS will also oversee the production and labeling of the product. However, FSIS' regulations are currently unclear and are not fully publicized as of yet [44]. Therefore, the standards that need to be considered for the project are in progress, and an adaptation of the procedure will have to be completed upon their publication. Meanwhile, the project will adhere to the following general ISO regulations:

- Sterility (ISO/FDIS 11737-2, sterilization of medical devices)
- Food Safety Management (ISO 22000:2018 Food safety management systems -- Requirements for any organization in the food chain)

3.4 Revised Client Statement

The project team came up with a revised client statement based on the constraints and objectives for the product. The revised client statement reflects the practice of using completely food-grade materials and scalable processes (changes are bolded).

There is a need for an alternative meat source that meets the growing demand of consumers. The meat source should be created using significantly reduced land usage, greenhouse gas emissions, and water consumption. The final meat product should be slaughter and hormone-free and comparable to conventional meat in taste, texture, and structure. ***The meat source should be created using completely food-grade materials and processes that can be scaled to an industrial size.***

3.5 Management Approach

A-Term was spent primarily determining the scope of the project and the type of meat product the project team wanted to make. During A-Term, all four team members came up with the name of the project, Leef Jerky™, and decided to focus on dried meat products. The team came up with the design objectives and constraints for Leef Jerky™, and created multiple conceptual and preliminary designs. Chapters 2, 3, and 4 of the final paper were started at the end of A-Term. In B-Term the project team started with experimentation. After significant discussion, the bulk decellularization apparatus was fabricated and all of the plant leaf scaffolds were decellularized for the first time. Seeding experiments 1 and 2 were completed during B-Term. Three bovine skeletal muscle cell isolations were conducted in B-Term as well. At the end of B-Term, chapters 3 and 4 were added to, while chapter 5 was started. The WPI Campus Survey was conducted at the end of B-Term as well. During C-Term, seeding experiments 3 and 4 were conducted, along with the rest of the bulk decellularization experiments. An isolation was attempted in C-Term as well. As part of the NSF I-Corps program, the project team conducted almost 30 interviews with potential customers and stakeholders involved with the project. Sections 3, 4, and 5 were completed at the end of C-Term. During D-Term, another isolation was attempted and a final fully-processed Leef Jerky™ strip was fabricated. The final paper and presentation were also completed at the end of D-Term.

4 Design Process

This chapter examines the needs involved in the creation of Leef Jerky™. It then examines several alternative and conceptual designs that were created to address these needs. The designs are then analyzed using Pugh Analyses in order to determine which options were best for the project.

4.1 Needs Analysis

In this section, the needs most important for the development of Leef Jerky™ were considered and were separated into three groups: scaffolding needs, decellularization needs, and seeding and growing needs. Scaffolding refers to the needs that must be met by the scaffold used to grow the meat. Decellularization refers to the process of decellularizing plant leaf scaffolds that were selected. Seeding and growing refers to the needs that must be met by the process of seeding and growing cells to form muscle.

4.1.1 Scaffolding Needs

Inspired by the decellularized plant leaf technology pioneered by the Gaudette lab, the project team decided to use decellularized plant leaf scaffolds to grow Leef Jerky™. In evaluation of the needs most important for the development of Leef Jerky™, several objective criteria for a plant leaf scaffold were identified and weighted on a scale from 1 (least important) to 5 (most important) as shown in Table 3.

Table 3: Weighting of Design Objectives for Scaffold

Objective	Weight
Surface Area	5
Thickness	3
Availability	5
Commonly Eaten	4
Familiarity	4
Price	5
Shape	3
Edibility	5

There are several factors that are required for a successful plant leaf scaffold. The most important factors are surface area, availability, price, and edibility. Surface area is important because it deems how much meat can be grown, dictating the volume of production. Availability is important because the scaffold should be easily obtained and readily available, making the operation simpler. Price is a major factor because current clean meat costs are very high. Lastly, the leaf should be edible so it does not need to be removed during processing.

The next tier of important factors is whether the material is commonly eaten. Commonly eaten materials play an important role in the potential marketability of the product, as consumers are less likely to eat something that is not recognized or commonly eaten. For example, grass is edible and also readily available, but is not commonly eaten.

The final tier of important factors includes the shape and thickness of the material. The shape is important because some shapes may be too large or difficult to fabricate. The thickness is also important, because thicker scaffolds may require more media and nutrients to sustain seeded cells. Conversely, a thicker scaffold would be helpful in meeting a desired thickness of the final product.

4.1.2 Decellularization Needs

In evaluation of the needs most important for the development of Leef Jerky™, several objective criteria for a decellularization technique were identified and weighted on a scale from 1 (least important) to 5 (most important) as shown in Table 4.

Table 4: Weighting of Design Objectives for Decellularization

Objective	**Weight**
Chemical Recycling	3
Ability to Automate	5
Quickness	4
Easy to Use	4
Amount of Leaves	5
Chemical Usage	3

The first tier of design objectives is ability to automate and the number of leaves capable of being decellularized at a time. In order for the production of the dried meat products to ever be scalable, these two factors must be kept in mind. Automating processes will reduce cost, increase production time, and remove human error. Increasing the number of leaves that can be decellularized at one time will also reduce cost and make the process more scalable.

The second tier of design objectives is quickness of the process. Although we saw ability to automate and the number of leaves decellularized at a time as the most important design objectives, the speed of the entire process is also important. The faster leaves are decellularized the faster the dried meat product can be produced.

The final tier of factors, although not most important but still important nonetheless, are chemical recycling and usage. While the chemicals and detergents required for decellularization are quite cheap and easily accessible, lowering chemical usage and recycling chemicals helps reduce waste and lower the cost of the entire process.

4.1.3 Seeding and Growing Needs

In evaluation of the needs most important for the development of Leef Jerky™, several objective criteria for a cell seeding and growing technique were identified and weighted on a scale from 1 (least important) to 5 (most important) as shown in Table 5.

Table 5: Weighting of Design Objectives for Cell Seeding and Growing

Objective	Weight
Media Recycling	3
Ability to Automate	5
Confluency	4
Cell Viability	5
Cell Seeding Efficiency	5
Media Usage	3
Form Factor	4
Easy to Use	4

4.2 Conceptual and Alternative Designs

Many alternative designs were considered as a possible final design of the project. Designs were grouped into two different categories: 1) decellularization and 2) seeding and growing devices. The goal of these preliminary designs is to try to optimize cell growth and meat volume, decrease the amount of space and time required to get the final meat product, and conserve resources such as detergents and cell media.

4.2.1 Decellularization Designs

A design choice for the apparatus used in bulk decellularization for this project was a constant or intermittent flow system designed to perfuse several stages of detergents through the vasculature of plant leaves. The need for a bulk decellularizing system became evident due to the intensive user interfacing and non-scalability of the current leaf cannulation process. The standard

cannulation process involved suturing surgical needles into the stems of all the leaves, and washing them rigorously with hexanes (Figure 6).

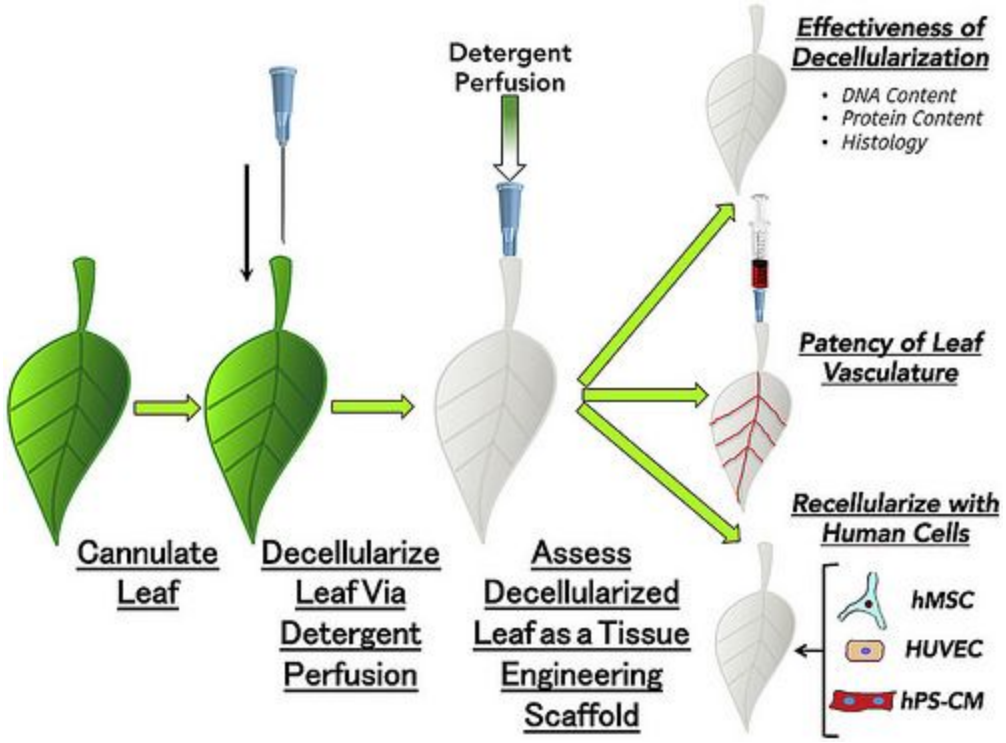

Figure 6: Original Cannulation and Decellularization of Plant Leaves [10]

Our original prototype for decellularization was designed to continuously stir leaves in a beaker filled with different chemicals in 24 hour increments (SDS → Tween 20 + Bleach → DI Water → Tris Buffer). Although the design was successful in decellularizing several leaves at once, it was observed that many of the leaves were settling to the bottom and making contact with the stirring mechanism which damaged the leaves. Revisions were made to the system, which included a prototype aluminum protective grate. The grate would not disrupt the stir bar and still allowed flow in the system, but protected the leaves from being damaged (Figure 7).

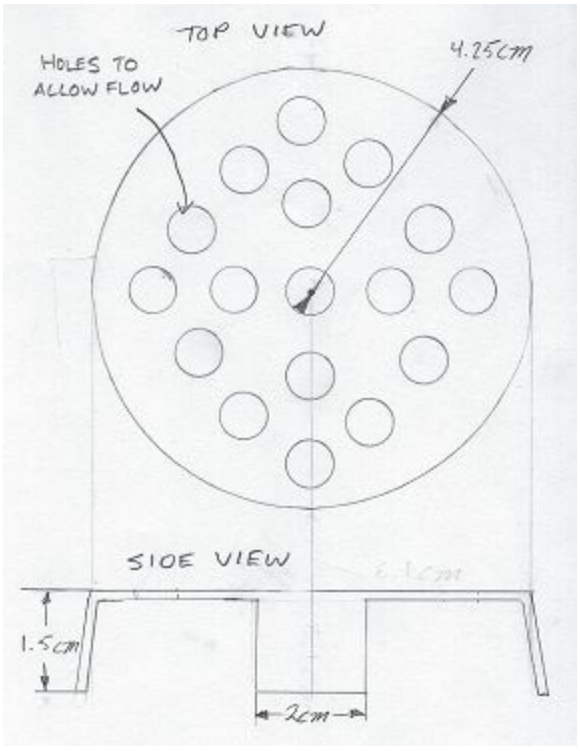

Figure 7: Prototype Protective Grate for Bulk Decellularization

After the second revision, it was observed that the force and direction of the flow were causing the leaves to stick to the protective grate, and highly overlapped sections were not perfused enough for desired levels of decellularization. The final system was designed to prevent the leaves from not only becoming damaged but clumping together on top of the grate. We came up with a final tiered grate system for the leaves (Figure 8).

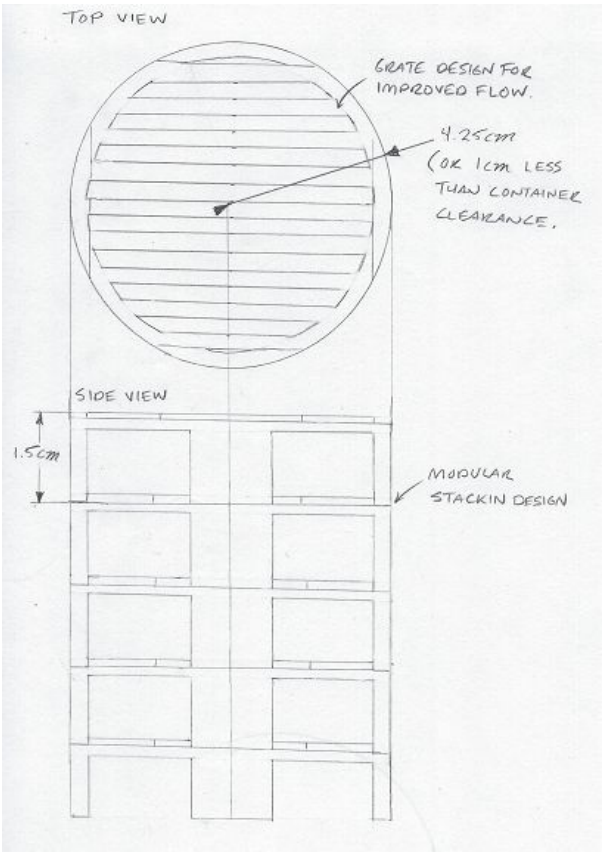

Figure 8: Improved Modular Grate Design for Bulk Decellularization

The final bulk decellularization design is scalable to industrial levels and consistently decellularizes both lettuce and spinach leaves. Its potential modularized design also allows for easy setup and removal of the plant leaves.

4.2.2 Seeding and Growing Designs

The first design incorporates the use of a carboy and hanging the leaves. The top of the carboy is removed and the leaves are hung along a vertical rod across the box. The leaves will be seeded with cells, and instead of putting them in cell media directly they are spritzed with media. This technique is very similar to that of fruits and vegetables in a grocery store. The leaves would be sprayed twice an hour. In Figure 9, there is a rough sketch of what this design would entail.

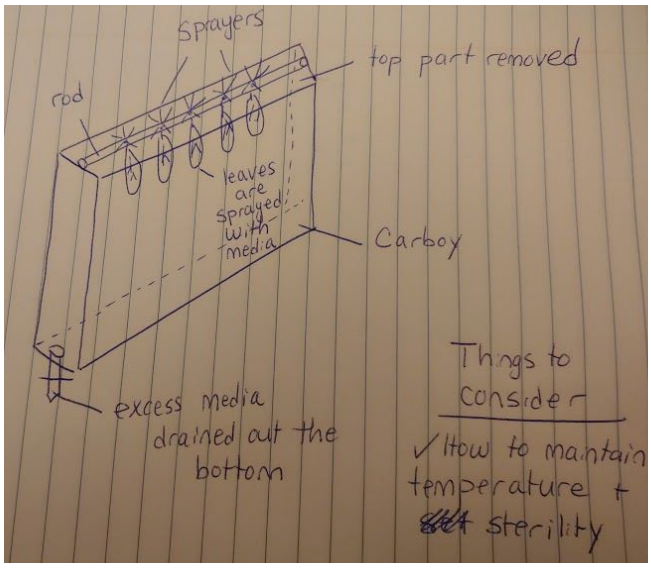

Figure 9: Carboy Watering Design

The second design choice involves the use of a ThermoFisher Scientific Nunc™ EasyFill™ Cell Factory™ System or equivalent. This type of system would help maximize the amount of laboratory space when trying to grow meat on leaves. Each layer would contain one, or possibly more, seeded leaves bathed in cell media. Media is added and removed from the top of the system and can equally distribute cell media between all layers. Figure 10 is an image of such a system.

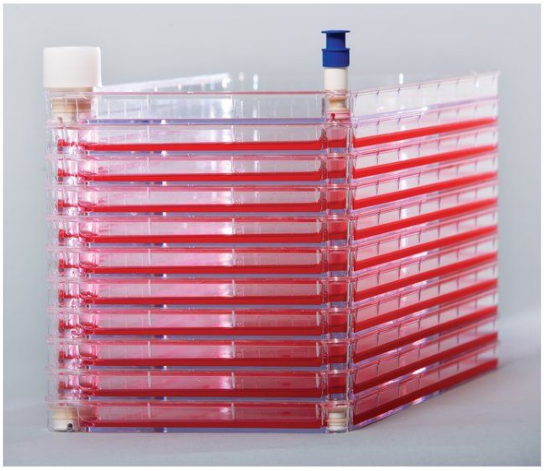

Figure 10: Thermo-Fisher Cell Factory

The third design involves the placement of seeded leaves in a media bath that is agitated by a magnetic stir bar. The agitation could provide a few possible benefits to the cells: 1) additional oxygenation, 2) increased perfusion of cell media throughout the leaf, 3) increased shear forces

that could stimulate the leaf and drive the growth and differentiation of cells. Figure 11 is a general idea of the system.

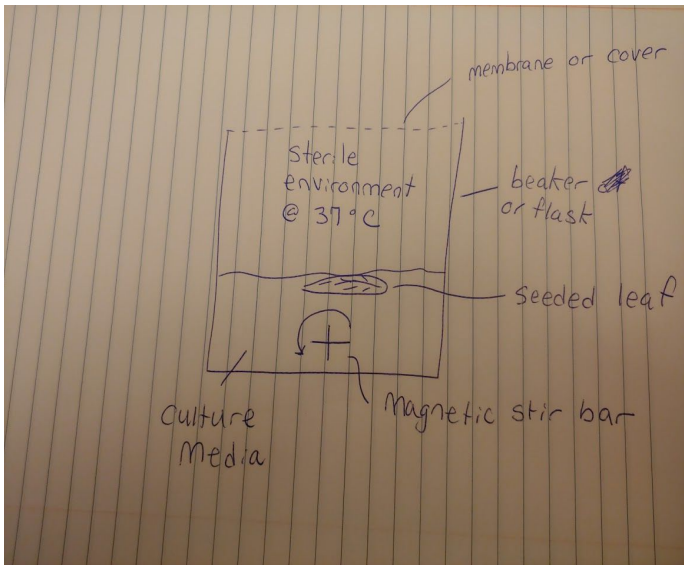

Figure 11: Agitation Design

The fourth design as shown in Figure 11 involves the use of a carboy similar to that of Figure 10, but instead of spraying the cells with culture media, the media is perfused throughout the leaf's vasculature. The goal of this design would be to maximize cell viability and growth by providing nutrients in a more efficient manner. A potential consideration for this design might be the type of leaf venation used (Figure 12). The design is shown in Figure 13.

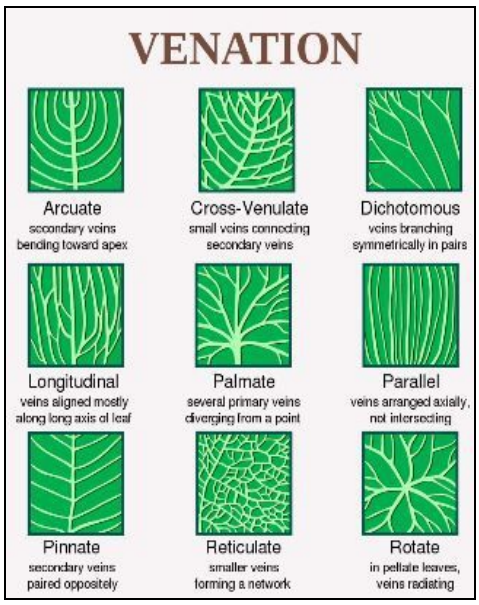

Figure 12: Different Types of Leaf Venation [45]

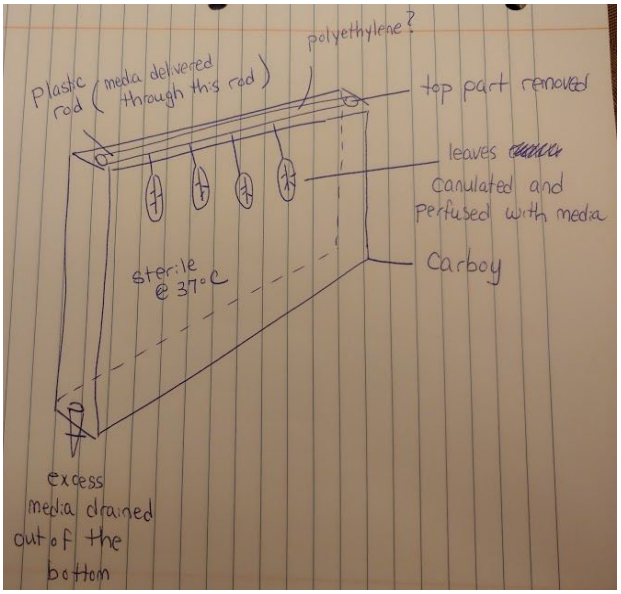

Figure 13: Carboy Perfusion Design

The fifth design being considered is the use of a centrifuge to deliver cells. The leaves would be placed in a centrifuge along with a cell suspension. The leaves would be lined along the outer edge of the centrifuge. Once it spins, the cells will be driven along the centrifuge to guide their attachment to the cells. An alternative design can be used where cells are shot out of the center of the centrifuge and are guided to attach to the leaves lining the outer wall. Figure 14 depicts this design.

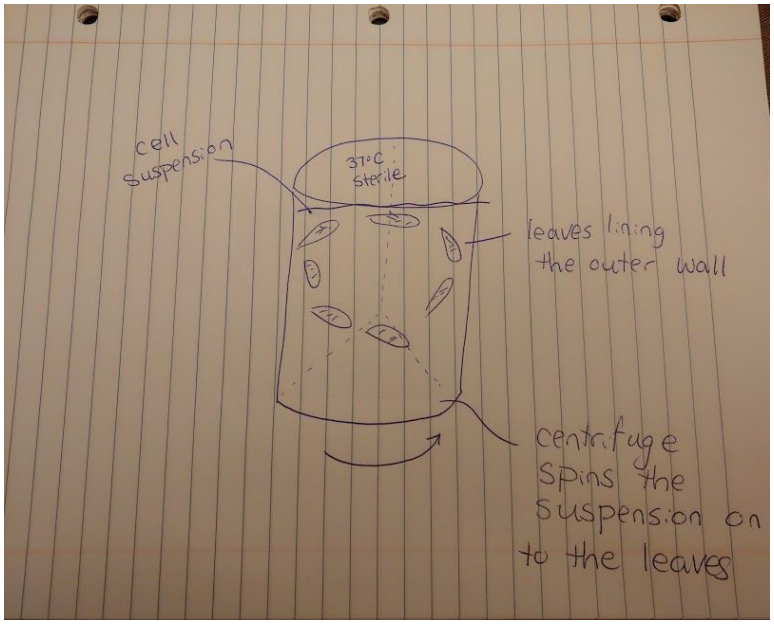

Figure 14: Centrifuge Design

The final design was considered to be used for growing and seeding the beef muscle-skeletal cells is an incubator “tackle box” design. The incubation “tackle box” design is made from polystyrene so that it is gamma irradiation, autoclaving, and ethylene oxide (EtO) sterilizable [46]. The base of the design is a compartmented container with dimensions desirable for the form factor of the dried meat product (Figure 15).

Figure 15: Base of the Incubation Box Design

Leaves will be placed into each of the compartments for initial seeding and proliferation. The dividing sections are perforated with small holes to allow for the equal exchange and leveling of media between compartments. A reserverior is attached to the lengthwise portion of the design, where media can be aspirated and added by tilting the box and allowing gravity to pool into the reservoir (Figure 16).

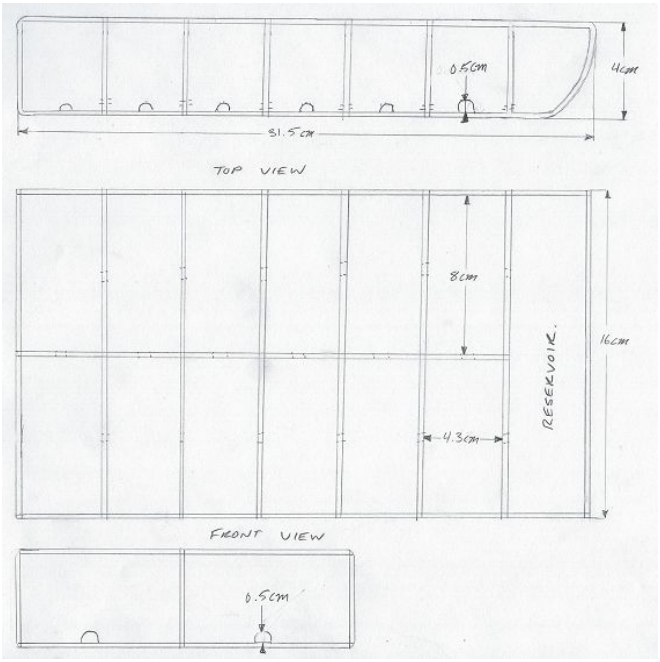

Figure 16: Diagram with Dimensions of Tackle Box design with Perforated Sections

The incubation "tackle box" design eliminates the potential of damaging the leaves during media exchange and can be used for seeding and reseeding on scaffolding materials. The portable design and form of the box allow for it to be placed from a biosafety cabinet into an incubator. The design is fabricated to not allow airflow exchange into the compartments of the box to prevent contamination during transfer to and from biosafety cabinets and during incubation.

4.3 Final Design Selection

In this section, we discuss how we chose our final designs and parameters for each of the following: leaf type, decellularization design, and seeding and growing design.

4.3.1 Leaf Selection

Shown below in Tables 6 and 7 are Pugh Analyses for several prospective plant leaves as decellularized scaffolds. The evaluation criteria were based off of the factors identified in the Needs Analysis and weighted according their importance. No inedible materials were considered, as this was the most important design consideration.

Table 6: Pugh Analysis for Leaves Part I

Evaluation Criteria	Weight Factor	Baseline Spinach	Grass	Onion	Scallion	Celery	Iceberg Lettuce
Surface Area	5	0	-1	+1	+1	+1	+1
Thickness	3	0	0	-1	0	-1	0
Availability	5	0	+1	+1	-1	+1	+1
Commonly Eaten	4	0	-1	+1	+1	+1	+1
Price	5	0	+1	-1	-1	-1	+1
Shape	3	0	-1	-1	-1	-1	0
Score		**0**	**-2**	**-7**	**-4**	**+3**	**+19**

Table 7: Pugh Analysis for Leaves Part II

Evaluation Criteria	Weight Factor	Baseline spinach	Leek Leaves	Broccoli	Swiss Chard	Romaine Lettuce
Surface Area	5	0	+1	+1	+1	+1
Thickness	3	0	0	-1	0	0
Availability	5	0	0	0	0	+1
Commonly Eaten	4	0	0	+1	+1	+1
Price	5	0	+1	-1	0	+1
Shape	3	0	0	-1	0	0
Score		**0**	**+10**	**-2**	**+9**	**+19**

Based on Tables 6 and 7, lettuce and leek will be considered as possible scaffolds to be used in Leef Jerky™. Spinach will also be tested as well because it has been extensively used in prior research for this application.

4.3.2 Decellularization Technique Selection

Shown below in Table 8 is a Pugh Analysis for several prospective decellularization procedures. The evaluation criteria were based on the factors identified in the Needs Analysis and weighted according to their importance.

Table 8: Pugh Analysis for Decellularization

Evaluation Criteria	**Weight Factor**	**Perfusion (Current Standard)**	**Beaker Design**
Chemical Recycling	3	0	0
Ability to Automate	5	0	+1
Quickness	4	0	0
Easy to Use	4	0	+1
Amount of Leaves	5	0	+1
Chemical Usage	3	0	+1
Score		**0**	**+17**

Based on Table 8, the beaker decellularization design was chosen as the final design.

4.3.3 Growing and Seeding Technique Selection

Shown below in Table 9 is a Pugh Analysis for several prospective seeding and growing procedures. The evaluation criteria were based off of the factors identified in the Needs Analysis and weighted according their importance.

Table 9: Pugh Analysis for Seeding and Growing

Evaluation Criteria	**Weight Factor**	**Well Plate (Current Standard)**	**Carboy Watering**	**Cell Factory**	**Agitation**	**Carboy Perfusion**	**Centrifuge**	**Tackle Box**
Media Recycling	3	0	+1	0	0	+1	N/A	0
Scalable/ Automatable	5	0	+1	+1	+1	0	+1	+1
Confluency	4	0	0	0	0	0	N/A	0
Cell Viability	5	0	0	0	+1	0	N/A	+1
Cell Seeding Efficiency	5	0	N/A	N/A	N/A	N/A	+1	0
Media Usage	3	0	+1	0	0	0	N/A	0
Form Factor	4	0	-1	0	0	0	0	+1
Less User interfacing	4	0	0	0	+1	0	0	+1
Score		**0**	**+7**	**+5**	**+14**	**+3**	**+10**	**+18**

* N/A denotes a category where that device does not apply. For example, the carboy watering design is for growing cells, and cells are seeded onto the leaves prior to its use.

The tackle box was chosen as the final design because it combines seeding and growing cells and scored the highest out of the seven possible designs.

5 Final Design Verification and Testing

The development of Leef Jerky™ can be broken down into four steps: isolation of cells, decellularization of plant leaf scaffolds, seeding the isolated cells onto the scaffold, and post processing which includes flavoring, dehydration, and packaging (Figure 17).

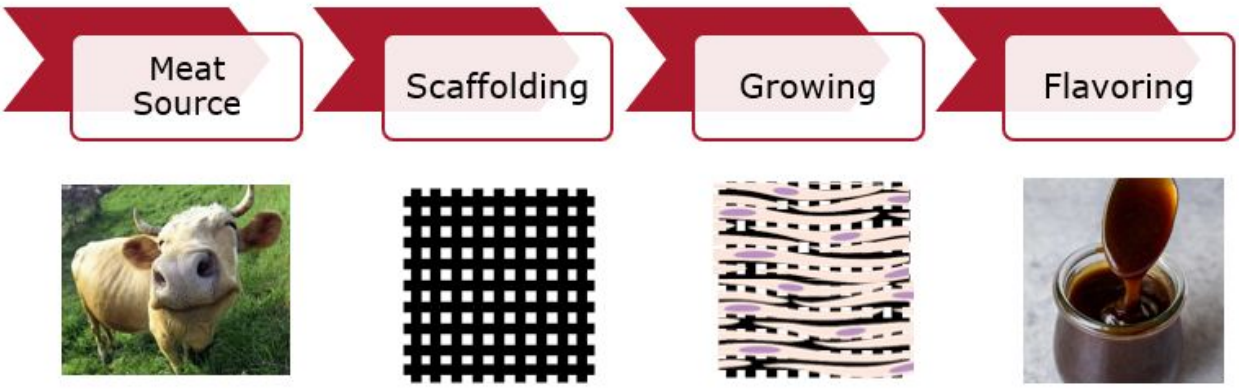

Figure 17: The Process of Developing Leef Jerky™

In this chapter, we discuss the experiments involved to develop Leef Jerky™and evaluate the final design selections. The design verification chapter was divided into four sections, each related to a step of the meat growing process: isolation of primary cells and contraction (meat source), bulk decellularization experiments (scaffolding), seeding experiments (growing), and post-processing experiments (flavoring). The tacklebox design that was chosen as a final design for growing and seeding cells was not tested due to time constraints.

5.1 Isolation of Primary Cells and Contraction

During the project, our team conducted five primary cell isolations from a bovine muscle sample from a local butcher (Appendix A). Three isolations were conducted in B-Term, one was conducted in C-Term, and one was conducted in D-Term. All isolations were conducted with the assistance of PhD student Jordan Jones. One isolation was successful in B-Term, one in C-Term, and one in D-Term. For the purpose of this chapter and subsequent chapters, the cells isolated in B-Term will be denoted **Isolation # 1**, the cells isolated in C-Term will be denoted **Isolation # 2**, and the cells isolated in D-Term will be denoted **Isolation # 3**. A representative image of the cells from **Isolation # 1** is shown below (Figure 18). The cells are at P8 and are imaged at 20x magnification. Any isolated cells were grown and maintained according to the protocols in Appendix B and Appendix C (feeding and passaging muscle cells). Isolated cells were frozen in

liquid nitrogen and thawed when needed according to the protocols in Appendix D (Freezing muscle cells) and Appendix E (Thawing muscle cells).

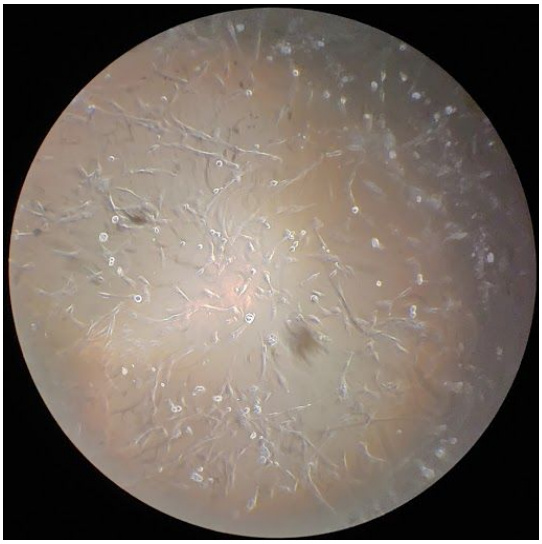

Figure 18: Isolated Cells in a Flask

250k cells from **Isolation # 1** were seeded onto a treated six well plate. After three days of incubation in growth factor media and 7 days in non-growth factor media, the plate was viewed using an inverted microscope. An electrical current of 15V was applied across the well plate using a C-PACE machine at a frequency of 0.5 Hz for 10 ms. Several contracting myocytes were observed. These contractions were recorded using a high speed video camera. A representative image of one of these myocytes can be seen below (Figure 19).

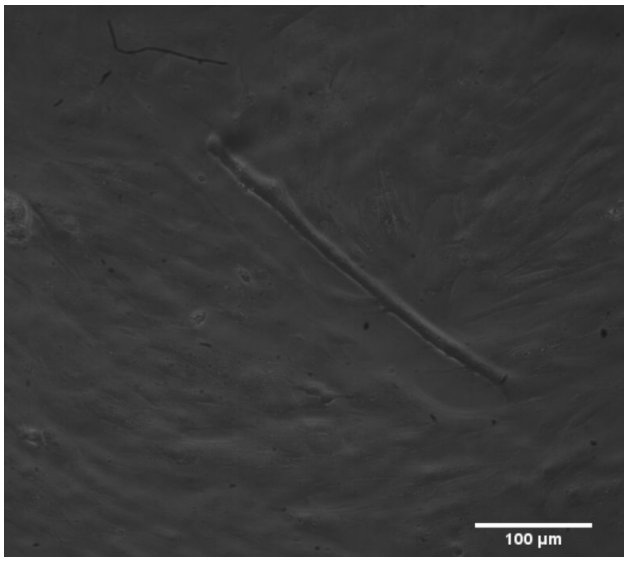

Figure 19: Myocyte contacted using C-PACE

5.2 Bulk Decellularization Experiments

All bulk decellularization experiments were carried out using the beaker and stir bar design discussed in Chapter 4. The purpose of this section was to evaluate the effectiveness of the design and add to it as experiments were performed. All decellularization procedures were conducted according to the protocol in Appendix G. Information on preparing necessary decellularization reagents is present at the start of Appendix F. Iceberg lettuce, spinach, and leek were chosen as final leaf scaffolds to be decellularized according to Chapter 4.

5.2.1 Decellularization of Iceberg Lettuce

A bulk decellularization experiment was performed with iceberg lettuce. This is the first decellularization experiment conducted, and did not feature the stir bar protector plate. The leaves were exposed to four solutions (SDS → Triton-X100 + Bleach → D.I. H2O → Tris Buffer) for 24 hours each. The stir plate was set to 60 rpm.

The goal of the SDS step is to wash away oils or contaminants on the surface of the leaf. The Triton-X100 + Bleach step washes away all of the cells and chloroplasts, leaving behind a clear cellulose backbone. The D.I. H2O and Tris Buffer steps are used to wash out the excess SDS, Bleach, and Triton-X100 before the leaves are lyophilized, rehydrated, and seeded with muscle cells. The leaves become completely translucent after the bleaching step.

The images in Figure 20 below show the progress of the leaves throughout the decellularization process. Figure 20 (A) shows the iceberg lettuce leaves immersed in SDS at 0h. Figure 20 (B) shows the leaves after 24h in SDS.

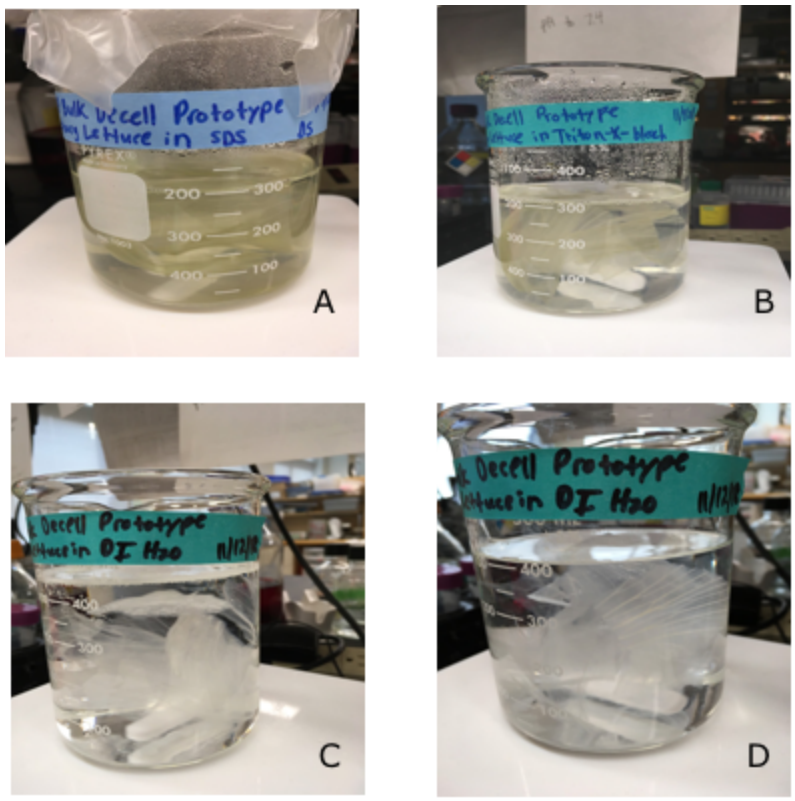

Figure 20: Iceberg Lettuce Decellularization Days 0 and 1

Figure 20 (C) shows the leaves after 24h in Triton-X100 + Bleach. Figure 20 (D) right shows the leaves after 24h in D.I. H2O. The leaves were not shown after the Tris Buffer step because their physical appearance does not change.

5.2.2 Decellularization of Spinach

This experiment utilized the second iteration of the bulk decellularization design with the addition of the aluminum protective plate cut with circular holes. The plate is used to protect the leaves from the stirring mechanism. Tween 20 was substituted for Triton-X100 because it is commonly used as a food additive [47]. The stir plate was set to 60 rpm. Some of the leaves didn't come out as desired, which has to do with the rpm and amount of time in SDS. Figure 21 left shows the leaves after 24 hours in SDS and Figure 20 right shows the leaves after 24h in Tween 20 + Bleach. For future experiments with spinach, the rpm and the amount of time in SDS was increased.

Figure 21: Results of Spinach Decellularization # 1

When the rpm was increased to 100 and the amount of time in SDS was increased to 48 hours, a new batch of spinach leaves decellularized significantly better (Figure 22).

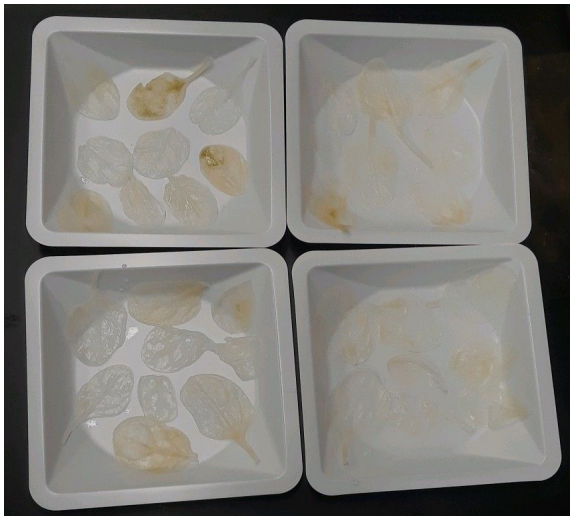

Figure 22: Results of Spinach Decellularization # 2

5.2.3 Decellularization of Leek

A batch of leek was decellularized using the bulk decellularization apparatus including the stir plate protector. The leek was cut into small square pieces and was torn in half, exposing an aligned thread-like network within. Tween 20 was used instead of Triton-X100. The stir plate was set to 100 rpm. Figure 23 left shows the leek after soaking in SDS for 24 hours, and Figure 23 right shows the leek after soaking in (Tween 20 + Bleach) for 24 hours.

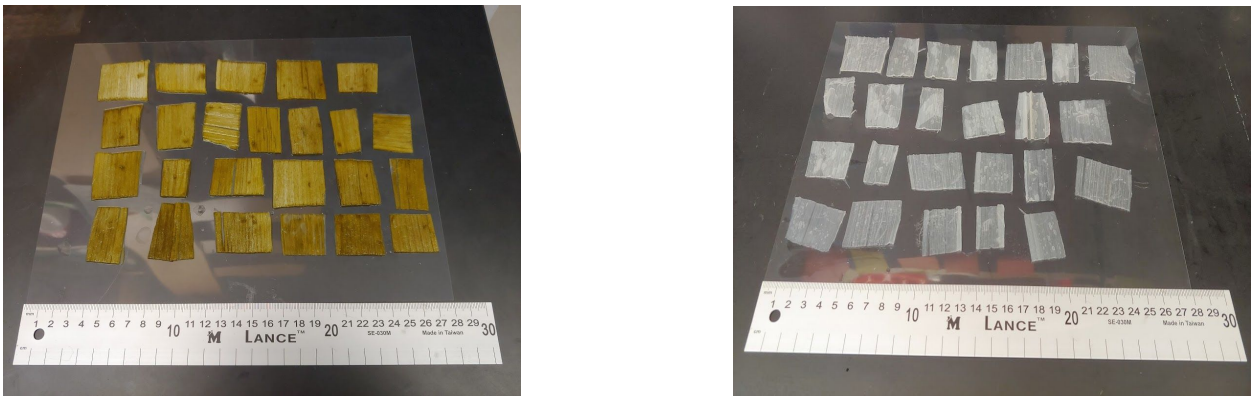

Figure 23: Leek Decellularization after 24 hours SDS (left) and 24 hours tween 20 (right)

5.2.4 Maximum Volume Decellularization of Iceberg Lettuce # 1

The project team wanted to determine the maximum amount of iceberg lettuce that could be decellularized at one time, so an experiment was performed in which 45 segments of lettuce (7.5cm x 4cm - the approximate size of a piece of Jerky) were added to the bulk decellularization apparatus. After 24h in SDS, the solution was extremely saturated with green material from the leaves. In addition, almost all of the leaves were shredded from the stir bar. Because most of the leaves had ripped, they were unusable. This was most likely attributed to the apparatus being overcrowded with leaves, forcing the leaves under the protective plate and into the stirring bar. In addition, the stirring speed was set to over 100 rpm, which was too high. Figure 24 left shows the 45 leaf segments at 0 hours, and Figure 24 right shows the 45 leaf segments after 24h in SDS.

Figure 24: Maximum Volume Decellularization of Iceberg Lettuce # 1

5.2.5 Maximum Volume Decellularization of Iceberg Lettuce # 2

After the first maximum volume decellularization experiment failed, it was repeated with half of the amount of iceberg lettuce segments. 22 segments sized 7.5cm x 4cm were added to the bulk decellularization apparatus. At the end of the four days, only four of the 22 leaves were still intact. The leaves were destroyed by the stirring bar because the protective cover is inadequate. As more leaves are added to the beaker, some of the leaves are forced under the protector plate and directly into the stirring bar. Future design changes need to be made to ensure that a large number of leaves can be decellularized, but remain intact. Figure 25 left shows the leaves at 0 h, and Figure 25 right shows the four remaining leaves after four days of decellularization.

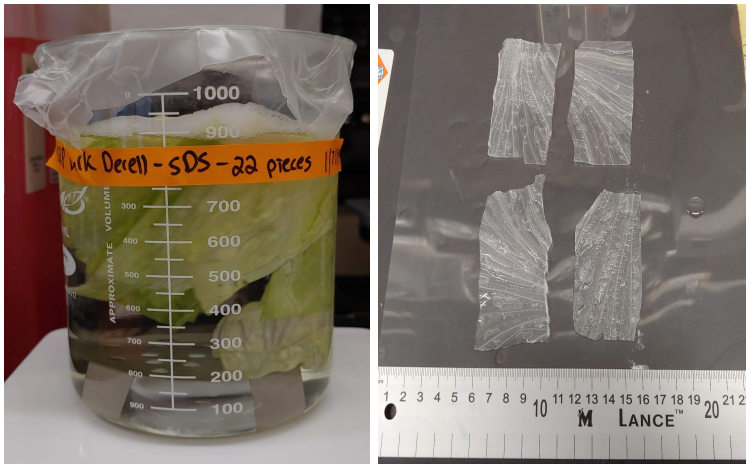

Figure 25: Maximum Volume Decellularization of Iceberg Lettuce # 2

5.3 Seeding Experiments

Once the project team was able to successfully decellularize lettuce, spinach, and leek, the next step in creating Leef Jerky™ involved seeding the isolated muscle cells on each decellularized plant leaf scaffold. Various different seeding experiments were performed determining whether the muscle cells could proliferate, align, and survive on each of the leaves without the use of adherent protein coatings. In addition, the team wanted to determine if the cells could differentiate and form confluent layers on the leaf scaffold.

5.3.1 Seeding Experiment 1: Phalloidin/Hoechst Staining

An experiment was performed in which P7 cells from **Isolation # 1** were seeded onto a 24 well plate containing 12 wells of decellularized spinach and 12 wells of decellularized iceberg lettuce leaves. Each of the leaves was lipholized, and then rehydrated and seeded according to the

protocol in Appendix H. Leaves were seeded at a density of 200k cells per well using pyrex cloning wells. The cells were left to incubate for four days in growth factor media. The cells were then fixed and stained according to the Phalloidin 488 and Hoechst 33342 protocol (Appendix I). Cells were then imaged under a fluorescent microscope

The spinach showed a confluent monolayer of cells spread along the top of leaves, with the green coloring represented actin and the blue representing nuclei (Figure 26).

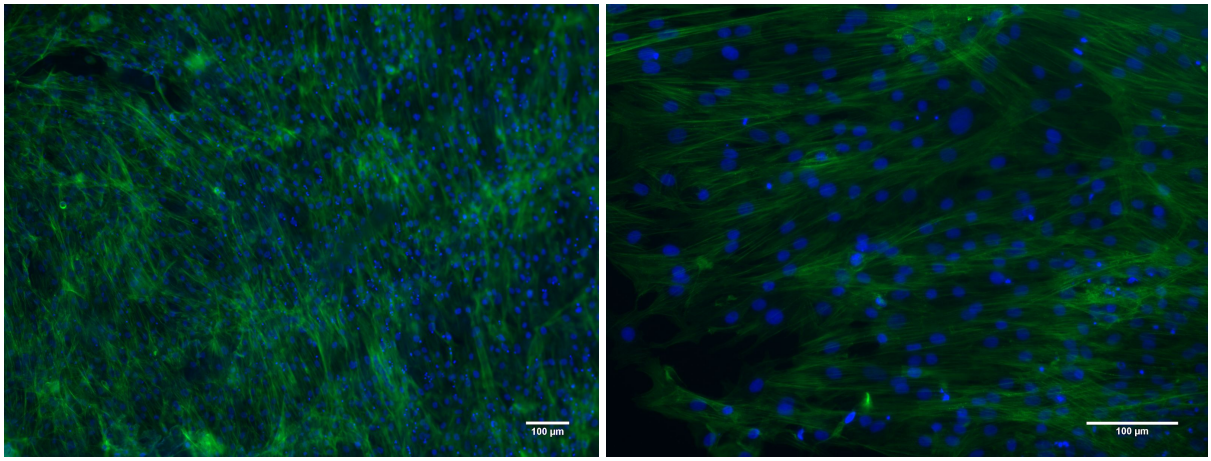

Figure 26: Confluent Monolayer of Cells on Spinach Leaves
(Actin - Green, Nuclei - Blue)

In addition to the confluency observed on the leaves, there was evidence of multinucleation and myocyte development on the leaves as well (Figure 27). There was also evidence of cellular alignment.

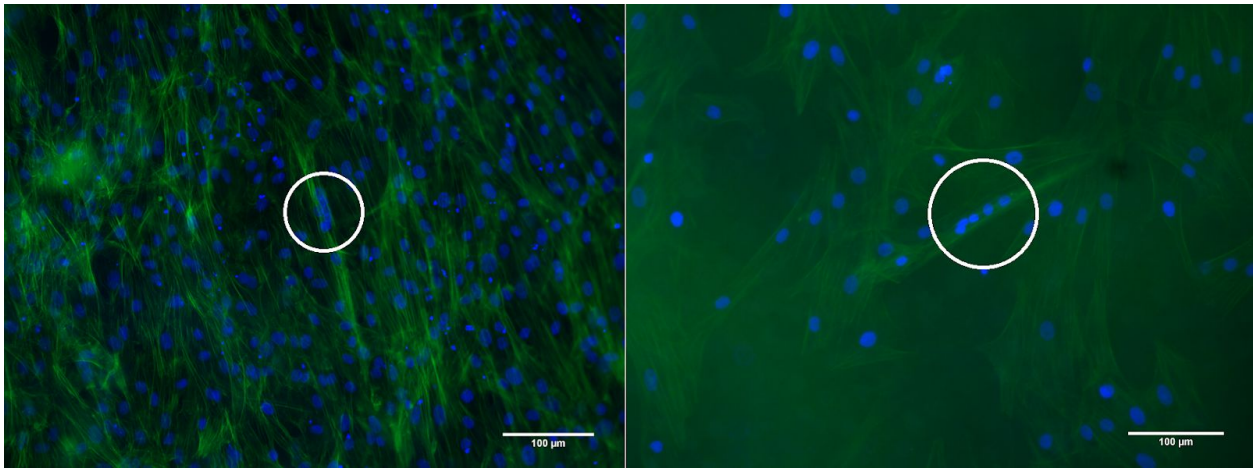

Figure 27: Aligned Cells and Myocytes on Spinach Leaves
(Actin - Green, Nuclei - Blue)

Iceberg lettuce also displayed a confluent monolayer of aligned muscle cells (Figure 28).

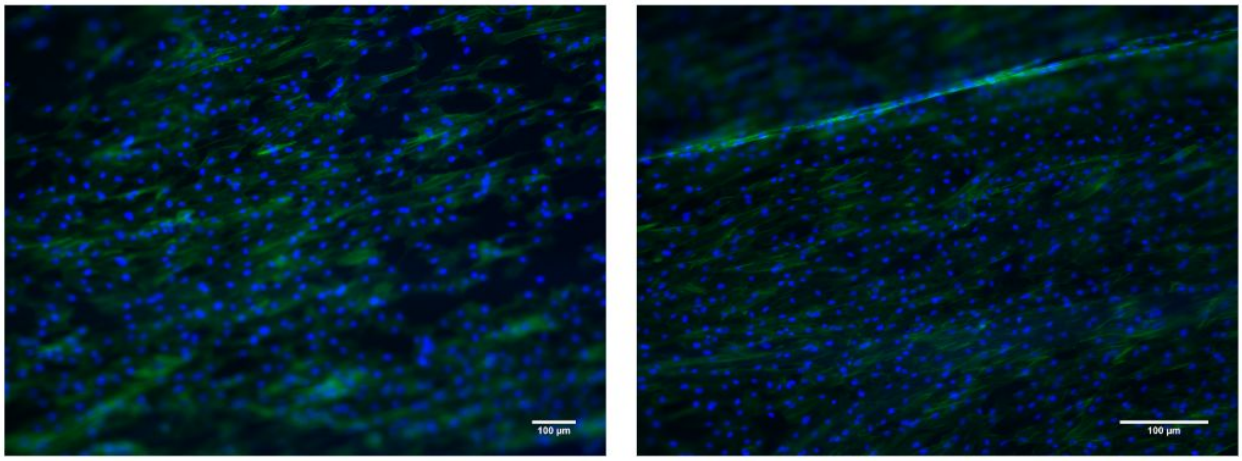

Figure 28: Confluent Monolayer of Aligned Cells on Iceberg Lettuce Leaves (Actin - Green, Nuclei - Blue)

There was some evidence of muscle differentiation from these images. However, since these samples were not stained for MF20 (myosin heavy chain, a component of differentiated muscle) the actual extent of differentiation was only speculative. These results were conclusive with a triplicate control well that was seeded in parallel without the use of leaves (Figure 29).

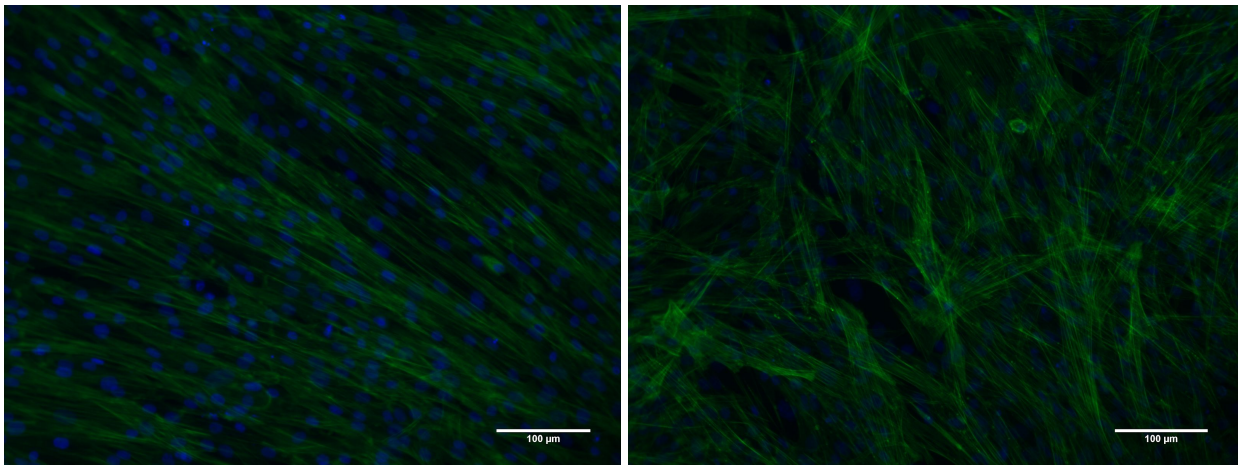

Figure 29: TCP Control Wells (Actin - Green, Nuclei - Blue)

5.3.2 Seeding Experiment 2: MF20 Staining of Well Plates

P8 cells from **Isolation #1** were used in seeding experiment 2. In this experiment, approximately 400k cells were seeded per well on a 24 well plate. The cells were allowed to grow for four days

with growth factor media. The cells were then fixed and stained according to the MF20/Hoechst staining protocol (Appendix J). No decellularized plant leaves were used.
There was evidence that some myoblasts were differentiating due to contact with other myoblasts in the wells. The green myosin heavy chain stain shows multiple areas in which there are multinucleated myocytes (Figure 30). The next step of this experiment would be to induce differentiation of myoblasts on an actual plant leaf. This is explored in seeding experiment 3.

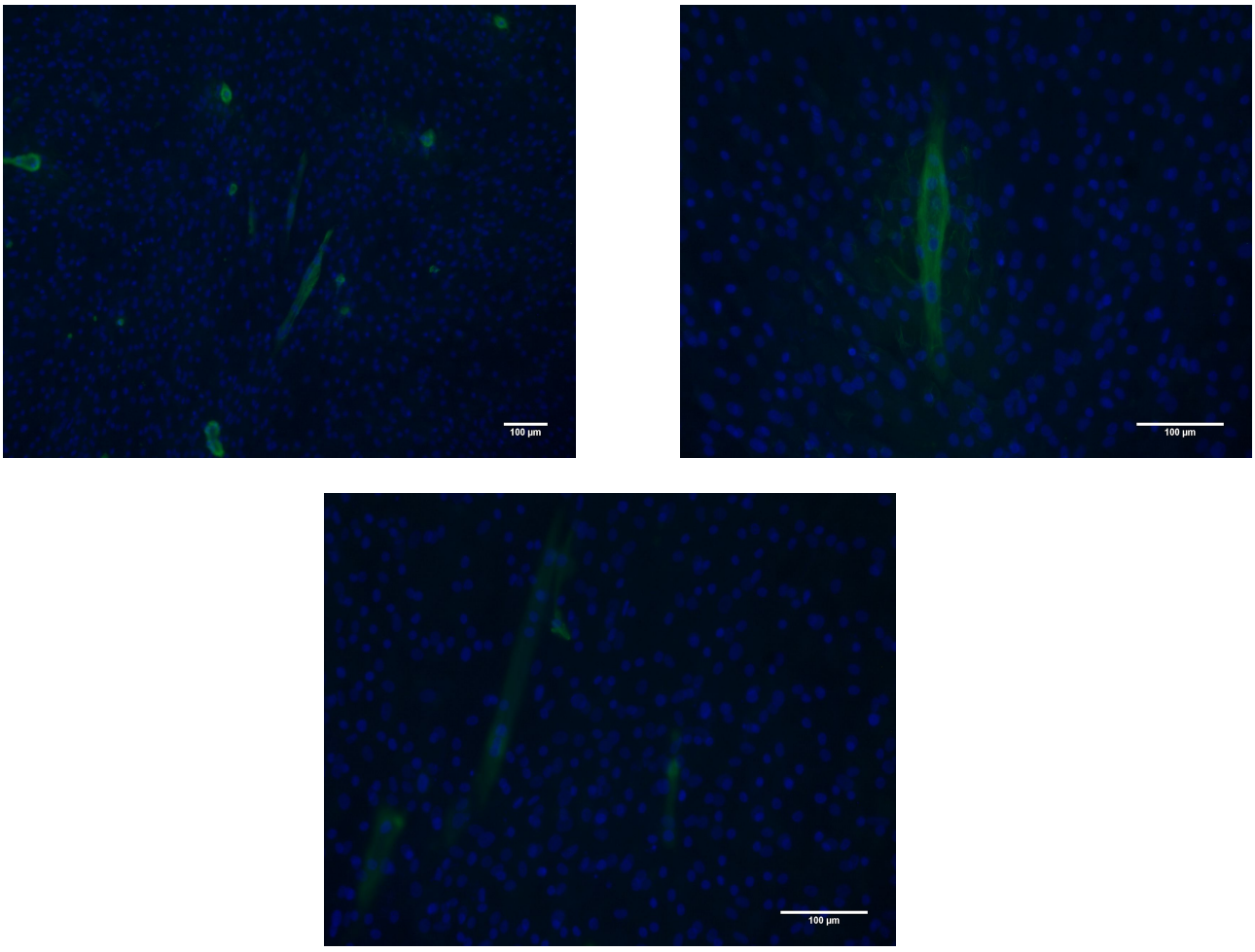

Figure 30: Myocytes Imaged on TCP Well Plate
(Myosin - Green, Nuclei - Blue)

5.3.3 Seeding Experiment 3: MF20 Staining of Leaves

For this series of experiments, three different differentiation-focused experiments were conducted on decellularized plant leaves seeded with P9 cells from **Isolation # 1**. The first of the three experiments involved the use of growth factor media exclusively for a nine day period, replacing the media every 2 days. After nine days, the cells were stained with MF20 and Hoechst

to observe the presence of differentiated myoblasts (Figure 31). 200k cells were seeded per leaf. Iceberg lettuce leaves were used exclusively for this experiment.

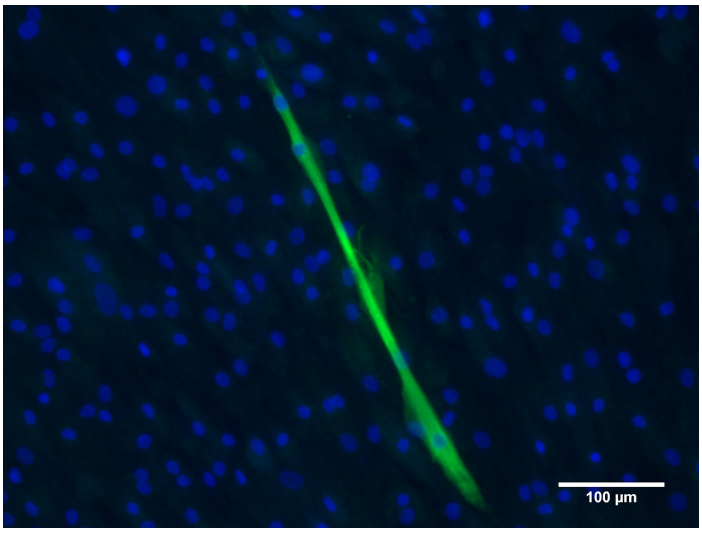

Figure 31: Myocyte on Iceberg Lettuce Leaf # 1

The second experiment featured the same cells cultured for nine days total, four days in growth factor media and then five days in non-growth factor media. The removal of growth factors is supposed to drive the differentiation of the myoblasts. Media was changed every two days. 200k cells were seeded per leaf. After nine days the wells were stained with MF20 and Hoechst to examine the areas of differentiation (Figure 32). The green markers show multiple areas of differentiation beginning on the surface of the leaf.

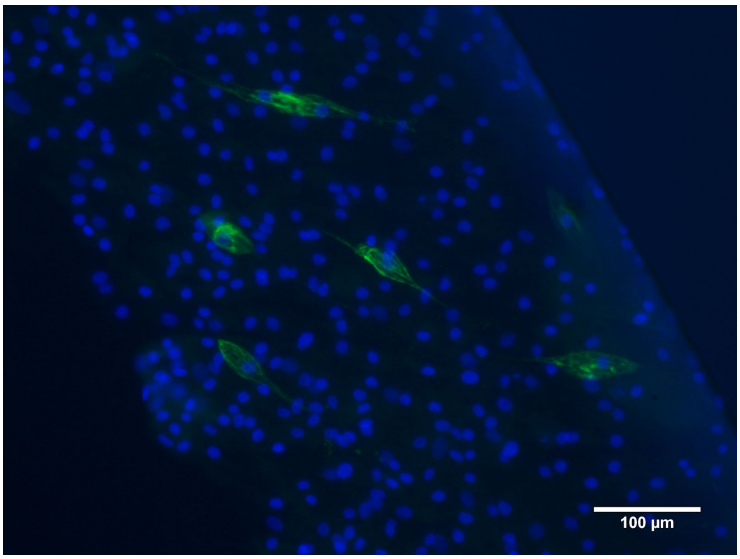

Figure 32: Myocyte on Iceberg Lettuce Leaf # 2

The third and final experiment used the same cells incubated for a period of 18 days and growth factor media for four days. Then, non-growth factor media for 14 days was used and media was changed every 2 days. Approximately 200k cells were seeded onto each leaf. The MF20 markers were still observed on the lettuce, but no significant difference was observed when compared to the other two experiments (Figure 33).

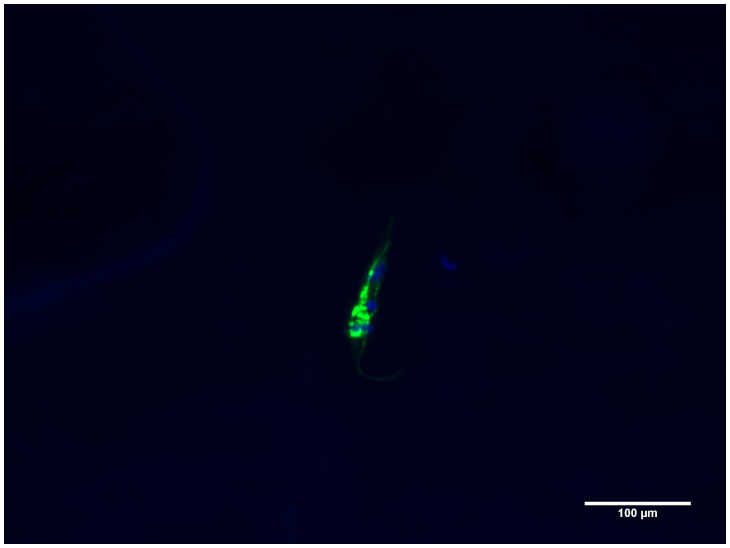

Figure 33: Myocyte on Iceberg Lettuce Leaf # 3

5.3.4 Seeding Experiment 4: Multiple Seedings

In this set of experiments, two different tests for multiple cell seedings on decellularized leaves were conducted. P10 cells from **Isolation # 1** were seeded at a density of approximately 200k per well onto a 24 well plate. The first experiment used growth factor media exclusively for 18 days, reseeding with 200k cells on every fifth day. Media was changed every 2 days. After the 18 days, the cells were stained with MF20 and Hoechst (Figure 34). The nuclei overlay was omitted in this picture due to the high autofluorescence caused by multiple layers of nuclei present.

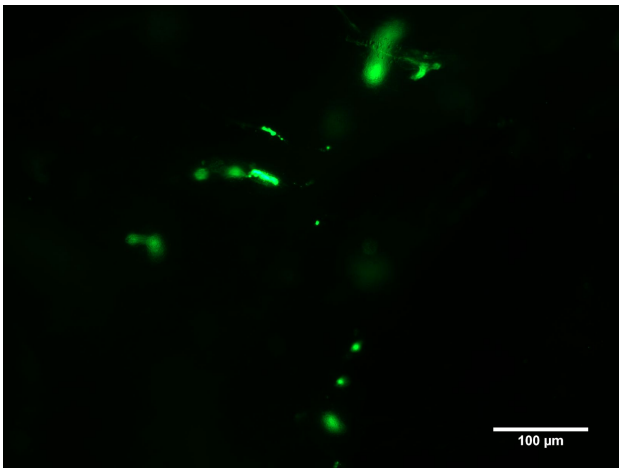

Figure 34: Multiple Layers of Myocytes on Iceberg Lettuce

In the second experiment, the same cells were seeded with 200k cells and incubated for 4 days with growth factor media, and then changed to non growth factor media for 5 days. The decellularized leaves were reseeded with 200k cells on day 9. On day 9 the media was replaced with growth factor media for 4 days, and then replaced with non growth factor media for 5 days. After 18 days, or two cycles, the cells were fixed and stained using MF20 and Hoechst. During imaging, it was observed the most of the cell layers had sheared off, likely due to the shearing of cells during seeding and media replacement. As a result, there are no images of myocytes to show.

5.4 Post-Processing Experiments

For a dried meat product, the main post-processing steps include flavoring and dehydration. The project team decided to make a Leef Jerky™ sample for MQP Project Presentation Day. The (7.5x4) cm strip of iceberg lettuce was decellularized and seeded with approximately 37 million cells. A high cell number was used because the team had never attempted to seed so many cells at once and there were cells leftover. The leaf was incubated in growth factor media for 5 days without the change of media (to reduce the shearing of cells). After 5 days, the leaf was flavored in teriyaki sauce for 3 hours. In the future, the team plans to dehydrate Leef Jerky™ samples. Figure 35 shows a sample of a processed strip of Leef Jerky™.

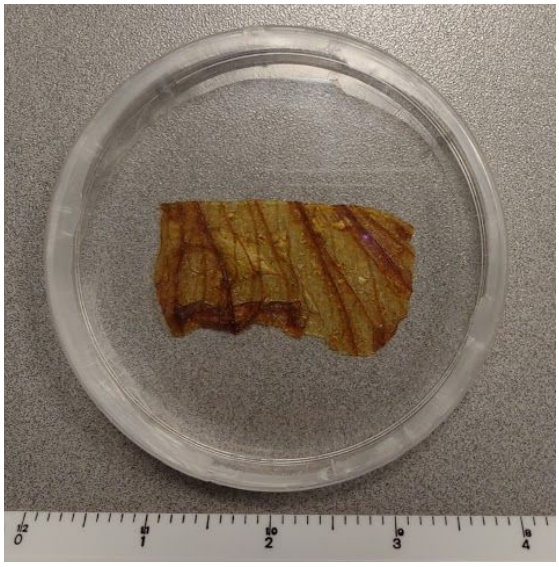

Figure 35: Processed Strip of Leef Jerky™

6 Final Design Validation

This chapter is used to validate the final design. This validation includes an examination of the design considerations, economics, environmental impact, societal impact, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability of the final design. The chapter also includes the analysis of a survey completed to examine the the WPI community's opinion on Leef Jerky™. In addition, the chapter includes an analysis of interviews that were conducted with several executives and scientists in order to examine the market for Leef Jerky™.

6.1 Evaluation of Design Criteria

This section examines our final design and whether it addresses the design considerations that we mentioned earlier. These considerations include the design constraints, objectives and standards that were used to influence the various designs that were developed.

6.1.1 Evaluation of Constraints

A. Completely edible

The components of the product are the decellularized leaf and bovine muscle cells. In order to create the decellularized leaf, the components are the leaf, sodium dodecyl sulfate (SDS), Polysorbate 20 (Tween-20), Sodium Hypochlorite (Bleach), Tris Buffer, and deionized water. In order to grow the cells, growth factor cell media that contained penicillin and streptomycin was utilized. According to the FDA, all of these constituents except for Tris are considered food safe additives [47]. The individual components of cell media are edible since media is composed of vitamins, amino acids, lipids, nucleosides, and salts, which are all edible [48]. Tris is not mentioned by the FDA. However, it has shown to not pose any safety or health risks when in concentrations under 5mg/kg in food [49]. Since all of the constituents of the product are considered safe and edible, the product itself is completely edible and safe for consumers to eat.

B. Slaughter-free

The sample of cow muscle that was used to isolate cells was obtained from a butcher. However, the process can be adapted to use isolation from a muscle biopsy taken from a living cow. It is estimated that the minimum amount of muscle needed can be obtained from a sesame seed sized biopsy [50]. Therefore, the process can be adapted to be completely slaughter-free.

C. No contamination

The lab utilizes the aseptic technique, as well as the use of penicillin and streptomycin as antibiotics. In addition, any tools used during the process are sterilized using either an autoclave or ethylene oxide gas. As a result, the process does not provide the opportunity for contamination.

D. Scaffold integrity

No testing was performed on the integrity of the scaffold. However, it always maintained its shape and structure upon handling during our various experiments. Occasionally, the scaffold ripped if the handling was excessively rough. This was accounted for and more care was taken afterwards.

E. Comparable to beef jerky in taste, texture, and aesthetic

The final product was not a complete meat product. The team was able to achieve one layer of cells on the leaves. Therefore, the taste, texture, and aesthetic were not comparable. Future considerations address this, as more research and experimentation is required.

6.1.2 Evaluation of Objectives

A. Cell differentiation and viability

Cells were successfully isolated from the meat sample. They were then successfully cultured repeatedly and showed no signs of decreased proliferation or differentiation abilities throughout the project. Through seeding and staining experiments, it was demonstrated that cells were able to adhere to the non-coated leaves and differentiate.

B. Uniform distribution of cells

Upon staining of the seeded leaves, it was demonstrated that confluent monolayers of cells adhered. Since the monolayer was confluent, the distribution is uniform.

C. Hormone-free

The process did not satisfy this objective. The media that was used was combined with growth factors for proliferation. These growth factors are considered hormones. Future work recommends experiments without these factors.

D. No use of animal products other than cells

The process did not satisfy this objective. The media that was used contained fetal bovine serum (FBS), a component isolated from the blood of fetal cows. However, other lab-grown meat companies are focusing on developing and bringing the price down of serum-free media. Some companies, such as Mosa Meats, have already completed this. Due to a low budget, these alternatives were not considered, and the project was more focused on proof of principle using the serum.

E. Thickness comparable to beef jerky

The final product contained only a single layer of cells. Therefore, the thickness was not comparable to that of conventional beef jerky. Future experiments address this objective.

F. Scalable to industrial size

A maximum quantity bulk decellularization demonstrated that 20 segments of iceberg lettuce (7.5x4cm each) could be decellularized using a volume of 1L. A 500 gallon beer vat contains 1892.71 L. If this process were scaled to a vat of this size, the process would be able to decellularize 37854.2 segments at a time. This would result in 113.5626 m^2 of processed scaffold per batch.

6.1.3 Evaluation of Standards

A. Sterility (ISO/FDIS 11737-2, sterilization of medical devices)

In compliance with ISO/FDIS 11737-2, the International Standard on Sterility of Medical Devices, all cell culture practices, scaffold seeding, and material handling was done in a sterile biosafety cabinet and/or sterile incubator. All the tools that were used for these procedures were either autoclaved or sterilized with ethylene oxide gas prior to use. In addition, all objects or materials coming into the biosafety cabinet were sterilized with rinses of ethanol [51].

B. Food Safety Management (ISO 22000:2018 Food safety management systems -- Requirements for any organization in the food chain)

ISO 22000:2018 dictates that all relevant food safety hazards are identified and adequately controlled at each step within the food chain. The product of the project is produced using completely food grade and food safe materials, so there are no food safety hazards with regards to any of the materials. Therefore, the product is compliant with ISO 22000:2018 [52].

C. FDA and USDA Standards and Regulations

The implementation and publicization of these standards and regulations are still in progress. The procedure and product will have to be altered or adapted once they become public and official. Until then, the first two standards will be considered.

6.2 Additional Considerations

This section examines the economics, environmental impact, societal impact, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability of the final design.

6.2.1 Economics

Until the process of growing lab-grown meat becomes significantly cheaper, or at least comparable to conventional meat, and is scaled to industrial size, it will never realize its potential in the marketplace. Mosa Meat states that their lab-grown hamburger quarter-pound costs approximately $10 to produce. This is significant progress from over $250,000 just three years ago. The likely entry market for these products is high-end restaurants while costs are being reduced and processes are being scaled. Lab-grown meat is likely 5 or more years away from being is a common grocery store. Meat is a $1 trillion market in the U.S., so there is tremendous potential for alternative meat products in the future. The main costs from creating Leef Jerky™ and other types of lab-grown meat is the cost of media and cells. Trying to eliminate animal-based products from the entire process as well is also extremely costly. If lab-grown meat products become the new societal norm for meat, there will be significantly fewer farmers, cattle ranchers, and slaughterhouses and factories.

6.2.2 Environmental Impact

Leef Jerky™ and other lab-grown meat products can have a major positive impact on the global environment. It is calculated that the lab-grown meat process uses 99.5% less land and 99% less water [53]. In addition, it is expected that lab-grown meat will have 88% reduced greenhouse gas emissions when compared to conventional meat [54]. The process of developing Leff Jerky ™can also be made entirely slaughter-free. Without the requirement for livestock, there will be reduced land required for animals to graze and live. Methane emissions from cows will also be significantly reduced, as this is a major contributor of the greenhouse gas effect. If greenhouse gas level in the atmosphere decrease, it will help reduce some of the effects of climate change

including melting of the polar ice caps, the rising of sea levels, powerful storms, and drought conditions.

6.2.3 Societal Influence

There will definitely be some individuals who are not in support of lab-grown meat. This is typically due to multiple reasons including 1) the individuals do not commonly eat meat, 2) the technology is new and different, and 3) it is too expensive. These are all challenges that need to be overcome if lab-grown meat is to become a popular alternative to conventional meat. It is possible that some vegetarians or vegans may be in support of Leef Jerky™ because it does not have the health risks associated with conventional meat and is sourced without harming animals. While the project team certainly hopes Leef Jerky™ is supported by this group of individuals, meat eaters are the target demographic because they is the potential to have a larger impact on the environment.

6.2.4 Political Ramifications

There is the potential for political blowback regarding lab-grown meat and Leef Jerky™. If lab-grown meat becomes a popular alternative to conventional meat, there will be a significantly reduced need for animals and many farmers and factory workers will be unemployed. Because of this, most farmers and factory workers will likely oppose lab-grown meat. “Big Ag”, comprising large-scale agricultural and slaughter factory companies, could be the biggest challenge facing lab-grown meat. “Big Ag” has significant political and lobbying power which could be used to delay the production of lab-grown meat or entirely outlaw it.

6.2.5 Ethical Concerns

Leef Jerky™ and other lab-grown meat products are more ethically-sourced than conventional meat. Cells are taken directly from living cows without the use of slaughter, hormones, or antibiotics. Significantly fewer animals would be required to supply to world with meat, and animals would have better treatment and more space to roam. It will also be easier to track and stop farm animal abuse. One ethical concern that could arise from lab-grown meat is the use of cell lines. Cell lines are genetically modified to make them immortal. Cell lines are viewed by many as unsafe, and others view genetic modification as unethical. Leef Jerky™ uses primary cells taken directly from the animal, so this would not be an issue.

6.2.6 Health and Safety Issues

Leef Jerky™ and other lab-grown meat products are significantly more safe and healthier for consumers. Leef Jerky™ can be made using without the slaughter of animals and the contamination risk presented by conventional slaughterhouses. In addition, Leef Jerky™ can be made without hormones, antibiotics, or other additives which can be harmful. Bacterial resistance is a potential global problem, and Leef Jerky™ helps to reduce it. Leef Jerky™ is also made without any sort of genetic modification to the primary cells which are isolated from the animal. Lab-grown meat is also safer than conventional meat because contamination can be easily identified during the cell culture process and the meat can be discarded. The labs which develop lab-grown meat (including Leef Jerky™) face strict cleanliness guidelines as well.

6.2.7 Manufacturability

During the entire design process of Leef Jerky™, manufacturability and scalability were a major focus. The entire bulk decellularization process is easily scaled to industrial-sized vats which provide continuous fluid flow through the leaves using a stirring mechanism. The reagents used during decellularization process can also be recycled and reused to save resources and money. The process of growing and seeding cells can be adapted using the tackle box alternative design. Cell media and water used during the growing process can also be recycled. It is important that Leef Jerky™ 's processes are scalable so it can be manufactured at a large-scale.

6.2.8 Sustainability

The process of growing Leef Jerky™ and other lab-grown meat products is extremely sustainable and friendly to the environment. Leef Jerky™ requires significantly less land and water than conventional meat. The process of creating Leef Jerky™ is "vertical", while the process for growing and slaughtering animals is "horizontal". In addition, the reagents and materials used throughout the decellularization and growing process can be recycled and reused. Few animals would be required to develop Leef Jerky™, as a sesame seed-sized biopsy from a living animal can likely be used to create thousands of kilograms of meat.

6.3 WPI Campus Survey

To determine the overall opinion of the WPI campus on both Leef Jerky™ and the clean meat industry as whole, a survey was conducted in the Rubin Campus Center. Prior IRB approval was achieved. A total of 219 participants took the survey. Survey questions included information on demographics, buying preferences, and opinion on clean meat products (Appendix K).

The most popular demographic was age 18-24 (94%) and female (64%). 86% of the audience stated that their diet was meat and vegetables (Figure 36).

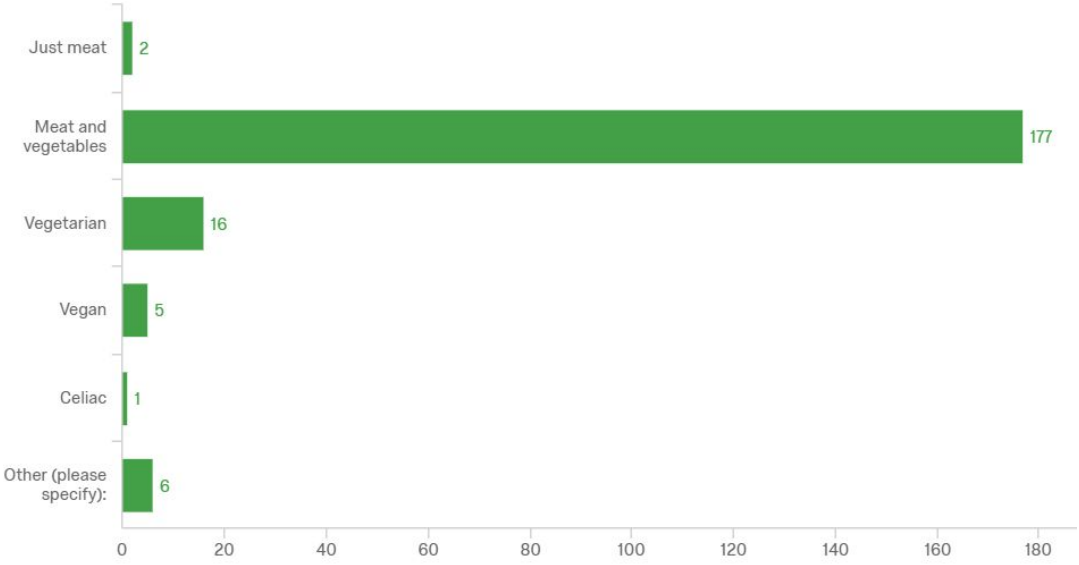

Figure 36: Survey Question 3: Dietary Preferences

61% of respondents stated that they were a food purchaser for their household, while 39% did not (Figure 37-A). For those who were food purchasers for their household, 81% purchased food on a weekly basis (Figure 37-B).

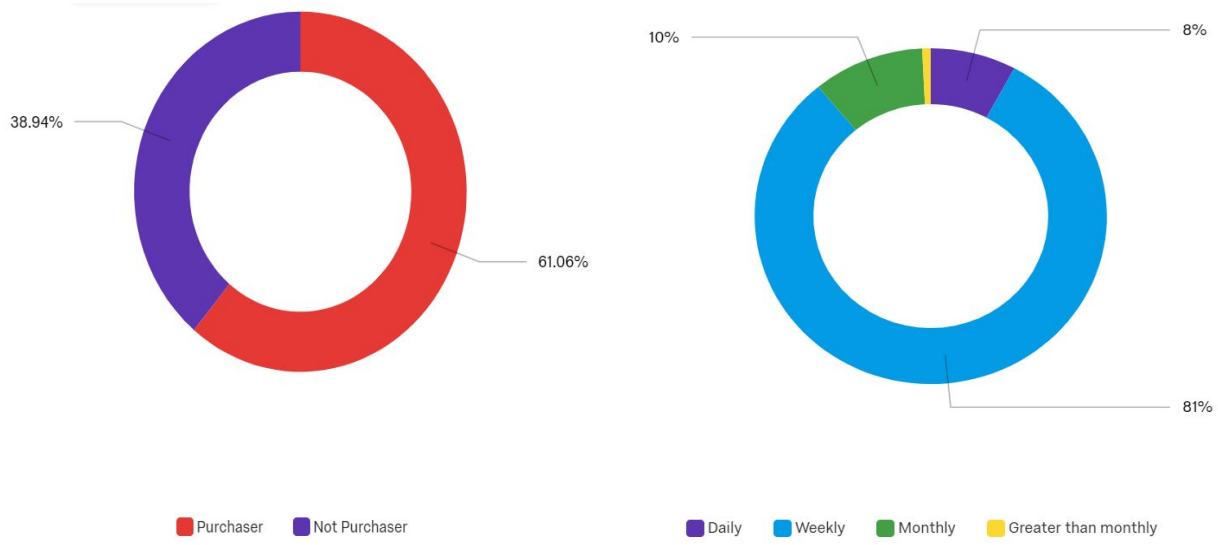

Figure 37A (left) and Figure 37B (right): Question 4: Food Purchasing

Participants were then asked to rank the importance of various factors when buying a food product. A score of 5 is represented as an extremely important buying consideration, while a score of 1 is represented as an unimportant buying consideration. Price, health benefits, and recognizable ingredients were regarded as the most important of the six (Figure 38).

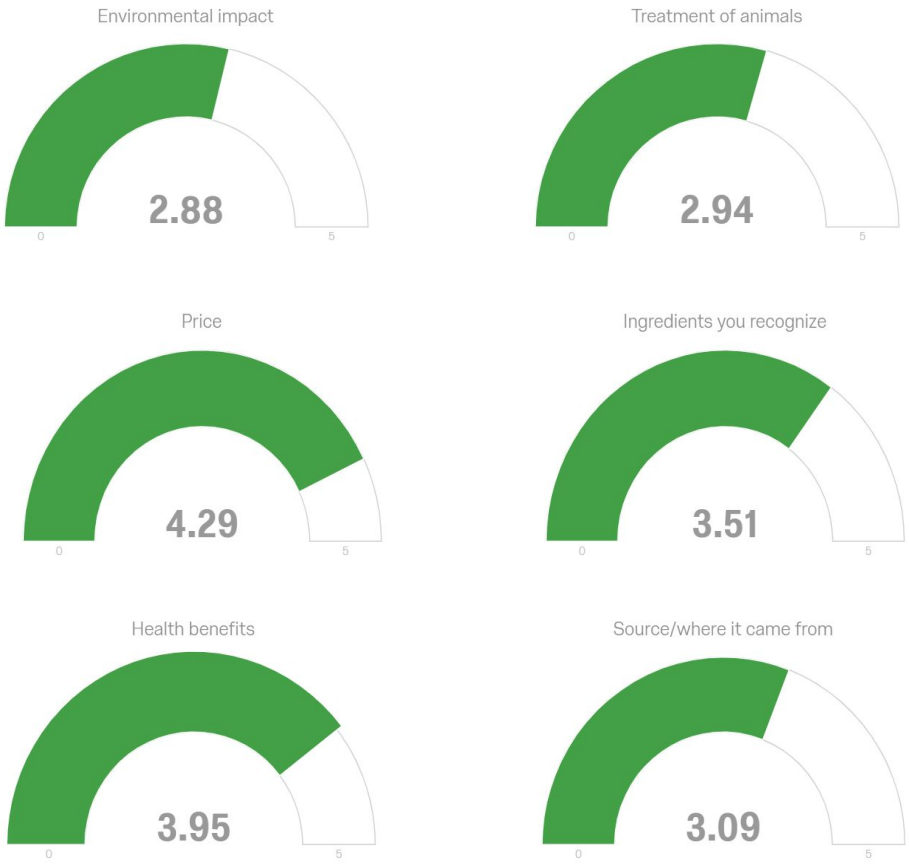

Figure 38: Question 5: Purchasing Factors

When asked how likely they would be to spend more money on an environmentally friendly and ethically-sourced food product than one that is not, 65% stated that it would be somewhat or extremely likely (Figure 39).

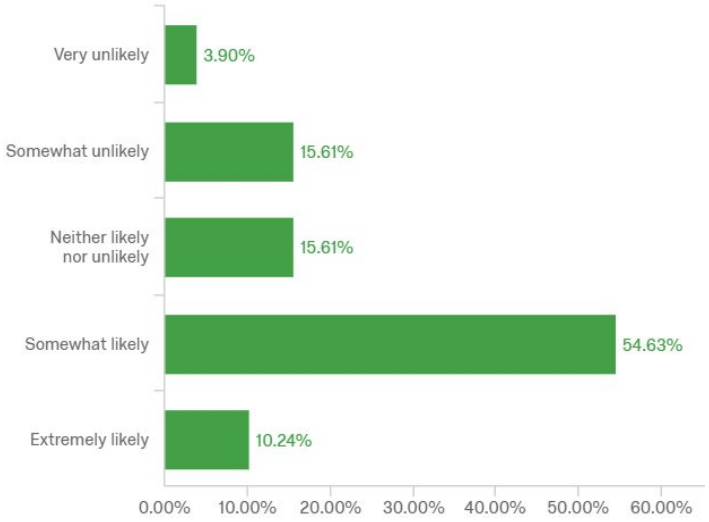

Figure 39: Question 6: Likelihood of Buying Environmentally Friendly and Ethical Food Products

The participants were then asked about their opinion on the following three terms, all of which are used interchangeably in industry: lab-grown meat, cultured meat, clean meat, and cell-based meat. Most participants stated that they thought clean meat sounded safer, healthier, and less mysterious than the other names. 58% of participants were familiar with at least one of the terms. 43% of participants are familiar with at least one of the terms from an internet article or video, 25% from someone they know, 16% from television, and 11% from a course topic.

73% of participants stated that they would be willing to try Leef Jerky™ at the grocery store when showed a packaging design. Many participants stated that the product's minimal impact on animals and the environment encouraged them to want to try it. Participants also thought the colors were vibrant and friendly. Some participants were opposed to trying it due to being a vegetarian or vegan, and thought the packaging made the product look expensive.

6.4 Interviews

As part of the WPI I-Corps spring 2019 cohort, we reached out to many potential customers and stakeholders involved in the clean meat space. For the purpose of confidentiality, only some of the information learned from these interviews will be discussed. Table 10 lists the people that we interviewed.

Table 10: Persons Interviewed

Name	Position	Organization	Industry
Jeffrey Foley	Senior Meat Buyer	Shaws Supermarket	Grocery
Steve Bares	CEO	Memphis Bioworks	Incubator
Geraldine Paulus	Associate	The Engine	VC/Incubator
Reed Sturtevant	General Partner	The Engine	VC/Incubator
Aisha Naibyeva	R&D	Ahold Delhaize	Grocery
Stephanie Wallis	CSO	Higher Steaks	Alternative Meat
Eva Sommer	Biotechnologist	SuperMeat	Alternative Meat
Rich Kelleman	CEO	Bond Pet Food	Alternative Meat
Paul MacDonald III	Store Director	Shaws Supermarket	Grocery
Pete Nelson	President	Aglaunch	Incubator
Natalie Rubio	Fellow	New Harvest	Research Institute
Amanda Murphy	Private Chef	Self-Employed	Restaurant
Chris Murphy	Private Chef	Self-Employed	Restaurant
Chris Maender	Director of Manufacturing	Axiom Space	Space
Justin Kolbeck	CEO	Wild Type	Alternative Meat
Peter Brewer	President	Southwick's Zoo	Zoo
Karen Hanner	Vice President of Manufacturing Partnerships	Feeding America	Hunger Organization
Ron Shigeta	CSO	Wild Earth	Alternative Meat
Peter Verstrate	CEO	Mosa Meat	Alternative Meat

Nicole Farhadi	Research Chemist/Project Officer	U.S. Army Natick Soldier System Center	Government
Steven Rothstein	Executive Director	JFK Library	Education
Robert Kirch	Master's Student	WPI	Research Institute
Jon Chorzepa	Bank Associate of Life Sciences	JP Morgan-Chase	Banking
Al Darzens	Independent Representative	Nanogas	Gas and Oil Technology
Gilda Barbino	Professor	City College of New York	Research Institute
Paul Shapiro	CEO	The Better Meat Co.	Alternative Meat
Grant Anderson	President & CEO	Paragon Space Development	Space

From the interviews with these individuals, we learned a significant amount about the current state of the clean meat industry, challenges facing clean meat companies, and how to be successful as a start-up company and attract investors.

6.4.1 Current State of Clean Meat

From our conversation with experts, we learned that there are about 35 different clean meat companies currently in existence. Of these companies, many are located in Europe, while many are also centered in California on the U.S. west coast. The people we spoke with generally believed that the world is ready for clean meat. Earth cannot supply the growing demands of meat consumption and the environment is being irreversibly damaged by today's meat climate. Clean meat is a way to reverse these trends and most people are willing to try it. Because the costs for lab equipment, technology, and data acquisition are low, more and more entrepreneurs are looking into the clean meat space. Because the future of clean meat is bright, there are no current products on the market, and there are many potential applications of the technology, more venture capitalists are willing to invest. Mosa Meat, one of the global leaders in the lab-grown space, projects that their clean meat products will be in some high-end restaurants by

the end of 2020. A few years later, Mosa Meat projects to have their process scaled to the point of large-scale manufacturing.

6.4.2 Clean Meat Challenges

Over the next few years, clean meat companies will face many difficult challenges. One of the biggest challenges is government regulation. In the U.S., the FDA and USDA will have joint-monitoring of clean meat facilities. Many states are already in the process of passing legislation regarding clean meat. Political opposition could also be a problem. Cattle farmers and "Big Ag" will likely oppose clean meat because it reduces the need for livestock and conventional slaughterhouses and factories. Because meat is such as large global industry (~1 trillion) and its supporters are extremely powerful, they could use their lobbying power to hurt clean meat companies.

Cost is another challenge posed by clean meat production. Clean meat is inherently more expensive than conventional meat alternatives because it requires a process void of all animal-based serums, coatings, and other products. Developing plant-sourced components for the clean meat process is difficult, but companies are making progress and the cost is gradually going down year by year.

Getting people to try clean meat is another major hurdle. The industry experts we spoke with were not overly concerned with getting people to try clean meat, but acknowledged it as a potential issue. We were recommended that our team use social media or a "Pepsi Challenge"-type activity to raise awareness of our product and to show people that it tastes and looks like regular meat, just without the slaughter and environmental effects. They also recommended that we target the conventional meat eaters rather than the vegans or vegetarians because they will be more likely to try our product and we can have a larger global impact.

6.4.3 Start-up and Investor Tips

The experts we spoke with recommended that we assemble a qualified and diverse team if we decide to form a company because it will make us more attractive for investors. Because venture capitalists have a lot of risk investing in a clean meat company, showing them that your team is capable of success and is different from everybody else will lower the risk for investment. When trying to attract investors, building a social media presence and a brand is a must. By getting other people excited about your company and building a positive brand for your products, venture capitalists are more likely to invest.

6.4.4 Potential Markets

From our almost 30 interviews, we identified many promising markets that we could explore with our lab-grown meat technology.

A. Space

There is a clear need for a renewable and continuous food supply during long-term space travel. During long-term space travel, astronauts are unable to pack large amounts of food due to volume and weight restrictions. While there is research ongoing regarding growing plants and meats in space, the results have been very poor. Lab-grown meat technology could be beneficial for this application because meat can be grown continuously with minimal equipment. In addition, cell media, water, and decellularization reagents can be recycled throughout this process. Space creates a unique challenge regarding fluids, as they act differently in space. We also learned that scaffolding is not required in space, as cells tend to self-assemble into structures in zero gravity.

B. Restaurant and Grocery

We spoke with representatives from both the restaurant and grocery industry. For the most part, grocery representatives seemed skeptical of the idea of lab-grown meat, and they do not envision lab-grown meat products being in their stores anytime soon. As a result, grocery stores do not appear to be a viable entry market. On the other hand, restaurants seem to be more promising. Many lab-grown meat companies consider restaurants to be an ideal entry market because of high costs and low scaling of processes initially. With a restaurant, lab-grown meat companies can charge more money for the product and don't have to produce product as quickly. In addition, lab-grown meat may gain popularity if the meat product becomes a cultural fad or if celebrities start trying it.

C. Department of Defense

There was some interest in using lab-grown meat technology as an MRE (meal ready to eat). Soldiers need to have high protein, high calorie, and low-weight food sources during combat and stationed abroad. Lab-grown meat accomplishes all of these. Leef Jerky™, especially, could also have an extended shelf life because it is void of fat, which causes spoiling. Lab-grown technology can also be used to grow meat in combat areas to eliminate the need to ship MREs.

D. Hunger Organizations

Hunger organizations, such as Feeding America, have expressed interest in lab-grown meat as a sustainable food source for developing or impoverished countries. These organizations would definitely be in support of our technology but stated that cost would be the biggest consideration. Until the process of growing meat is scaled and the cost is comparable to that of conventional meat.

E. Animal Feed and Pet Food

Another potential application of lab-grown meat is for animal feed and pet food. Lab-grown meat may be an attractive alternative to conventional meat for pet or animal or animal owners. These owners are becoming increasingly concerned about what their pets are eating and the quality of the ingredients. Lab-grown meat can be slaughter, antibiotic, and hormone-free, and can be altered to have a higher nutritional content. Zoos and animal shelters could be potential customers for this application as well.

F. Cellular Agriculture Partnership

Other cellular agriculture companies have shown interest in the decellularized plant leaf technology because it allows for vascularized fluid flow and promotes structural alignment and differentiation of muscle cells. Such as scaffold is key towards surpassing the nutrient diffusion limit and growing thick, structured meat. As a result, it may be appropriate to come up with a licensing agreement with these companies.

G. Other Tissue Engineering Applications

The decellularized plant leaf technology also has many other tissue engineering applications. Such applications would require the development of 3-D tissues that require proper cell alignment and vascularization to promote the diffusion of nutrients. The Gaudette and Pins Lab have already started using such applications to grow skin and heart muscle, but there are truly limitless other opportunities in the field of tissue engineering.

7 Discussion

The purpose of this chapter is to discuss and interpret the results and information presented in Chapters 5 and 6 and to investigate some of the reasons for experimental success and failure. In addition, limitations and areas for improvement will be discussed. There will be a section dedicated to each facet of the development of Leef Jerky™: isolating the cells, decellularizing the scaffolds, seeding onto the scaffolds, and the processing of the final product.

7.1 Isolation and Contraction

A total of five different bovine skeletal muscle cell isolations were conducted, three which succeeded and two which failed. There are a variety of reasons why two of the isolations resulted in failure.

The isolation is a complex process with little room for error, and the project team was not completely prepared at first. It is very time consuming and there are many steps where contamination can occur. The failed isolations were performed individually and without the use of surgical gloves, which likely increased the contamination risk. The meat sample was left sitting for too long before the isolation process was started as well. The longer the meat sample sits, the longer the bacteria have to grow and infect the inside of the sample. It is also possible that contamination occurred because the same surgical tools were used when handling the outside and inside of the meat sample. The inside of the meat sample is typical contaminant-free, while the outside contains excessive bacteria, fungus, and yeast. Handling the inside and outside of the meat sample should be done using separate tools to limit the spreading of contamination. Another potential issue that might have been completely out of the project team's control was construction going on in the lab at the time of the isolation procedure. There were numerous other complaints of yeast contamination by other lab persons during the construction process as well.

During the isolation procedure, the goal was to extract as many myosatellite and myoblasts cells as possible from the meat sample. Because myosatellite cells and myoblasts have the potential to form myocytes, they are extremely valuable when creating lab-grown meat product. Fibroblasts, on the other hand, are not as important in a meat product. While fibroblasts play a key role in tissues by producing collagen and extracellular matrix, a meat product exclusively made of fibroblasts will not form functional myocytes. This will alter the taste and texture of the final product.

Most successful isolation attempts required patience and time before the cells started growing rapidly. Many different approaches were investigated to maximize the amount of myosatellite cells and myoblasts present in the culture. During a media change, the cells floating in the media were harvested rather than aspirated. The project team hypothesized that the floating cells were likely myosatellite cells and myoblasts, while the adhered cells were fibroblasts. During passaging, both floating and adhered cells were plated onto non-tissue treated well plates to further separate out the fibroblasts. Fibroblasts tend to adhere to all types of surfaces, even non-treated ones.

Once the team was able to properly isolate bovine skeletal muscle cells from a butcher sample, the cells were stimulated with a C-PACE machine. The stimulation of muscle cells was important to determine if any of the muscle cells had differentiated into myocytes, and to see if the myocytes were functional. Myocytes are an important component of structure meat. The muscle from cows that are slaughtered contains fully functional myocytes. In order to achieve proper structure and texture of Leef Jerky™, the isolated cells must have the potential to form functional myocytes. Upon stimulation with electrical current, there was evidence of contracting myocytes on the 6 well plate. These results were promising for the team. The next objective for the project included developing a suitable scaffold to grow the muscle cells.

7.2 Decellularization

Growing thick, structured meat products is a major challenge facing lab-grown meat. Tissue engineering scaffolds can be utilized to overcome these challenges, but few are edible, vascularized, and drive cellular alignment and differentiation. Decellularized spinach, leek, and iceberg lettuce were chosen as scaffolding materials for growing meat, all of which meet these desired characteristics. The process of decellularizing plant leaves was pioneered by the Gaudette lab, and featured a cannulation process to decellularize plant leaves. This process, while effective, is not ideal because only leaves with stems can be decellularized, it takes up a significant amount of benchtop space compared to the small amount of leaves that can be decellularized, utilizes non-food-grade materials, and is not easily scaled to an industrial size. To overcome these disadvantages, a completely new method of decellularization was created: the bulk decellularization apparatus.

The bulk decellularization apparatus features a continuous flow system that can be easily scaled to an industrial level. The device utilizes a stirring mechanism which directs the flow of chemicals throughout the vasculature of the leaf and a protector plate which prevents the leaves from being destroyed. Leek, iceberg lettuce, and spinach were all successfully decellularized using this system. The system required four days to completely decellularized the leaves. One

day in SDS, one day in Tween 20 + Bleach, one day in D.I. H2O, and one day in Tris Buffer. Tween 20 was substituted for Triton-X100 due to it being a common food additive in manufacturing processes.

In some instances, the decellularized plant leaves did not come out as desired, as some cellular and other plant material was left on the leaves. Some of the reasons for this are a result of the bulk decellularization device being a prototype and requiring further modification. If the stirring mechanism is set to be too fast or an excessive number of leaves are placed into the container, some of the leaves may rip or may not decellularized completely. This can be remedied using less leaves, a lower rpm, and an additional day in SDS. The SDS step is important for washing away excess oils and other contaminants on the leaf surface, so soaking in SDS for an extra day will result in better decellularization of the leaves,

7.3 Seeding Experiments

Results from the seeding experiments supported several key design objectives for using decellularized plant material as scaffolding for producing structured meat. From the initial seeding experiment, the results demonstrated that lettuce, leek, and iceberg lettuce were all able to support proliferation to grow a confluent monolayer of aligned cells without the use of adherent protein. This success was very important because adherent protein coatings and the general functionalization of scaffolding materials is normally achieved using animal by-products such as collagen IV and fibronectin, which conflict with the process of developing slaughter free meat.

The MF20 seeding experiments had varied success, while they were able to show development of myocyte formation on the scaffolding, the majority of imaged cells were not myocytes and did not show precursory signs for myocyte formation. The most likely factor for the lack of myocyte differentiation on the scaffolds is a result of poor myoblast population following the initial protein isolation from the bovine skeletal muscle sample. Leading to a primary fibroblast population. However, results did demonstrate that the scaffolding materials of leek, iceberg lettuce, and spinach were able to support cell differentiation in multiple experiments.

The final experiments involving seeding multiple layers of cells supported the same results of the MF20 experiments. More precursory formation of myocytes was visible. However, this was likely as a result of a highly increased cell density. Furthermore, imaging using fluorescent microscopy was difficult due to the high amount of autofluorescence occurring from the oversaturation of nuclei containing Hoechst 33342. Future experiments could improve visibility by using a confocal microscope for imaging.

7.4 Post-Processing

The results of the post processing experiments showed that the scaffolding could reproduce the desired coloring of conventional dried meat products. However, due to not working within a food grade process or facility, nothing that was produced in this project was tested due to health and safety concerns. Conducting experiments to determine the proper flavoring, cooking, and packaging methods are considerations for future iterations of this project.

8 Conclusions and Recommendations

In the face of growing meat consumption across the globe significant land, water, and ethical constraints are pushing the development of new food technologies that can satisfy the increasing appetite for meat. This project developed new cellular agriculture technology for the production of a dried meat product using food safe and plant materials while still being an authentic meat product. The result was Leef Jerky™, a sustainable, slaughter-free meat product for a better future (Figure 35). Leef Jerky™ utilizes patented bulk decellularization technology to produce large volumes of plant based scaffolding material required to grow structured meat. In addition, in developing Leef Jerky™ key proof of principle experiments showed that the scaffolding could support the adherence, proliferation, and differentiation of functional muscle tissues. Shown in Figure 40 is the final logo design for the product. This logo was designed and created by Michelle Weaver, a graphics designer for the Massachusetts Dental Society.

Figure 40: Leef Jerky™ Final Logo Design

Leef Jerky™ benefitted from the professional consult of over 30 industry executives and scientists in markets including the department of defense, other lab grown meat companies, pet foods, space exploration, and others. These professionals expressed market potential for Leef Jerky™, pending further research and development. In light of this, the project group will continue to work with developing the product and the processes necessary to procure the funding necessary bring Leef Jerky™ to market in the future. These areas of research will likely include, but are not limited to, demonstration that the scaffolding can provide the oxygen diffusion limit of 100-200 microns in cell layer thickness, and experiments that demonstrate the ability to layer the structures on top of each other to increase overall thickness of the product to resemble the real size and nutritional content of conventional dried meat products. Other research may be

conducted on different types of structured meat products can be produced using the bulk decellularized plant leaf technology.

9 References

[1] S.Šimčika. Is The Percentage of Vegetarians and Vegans in the U.S. Increasing? https://bit.ly/2VcI4nf

[2] Per Capita Red Meat and Poultry Disappearance: Insights Into Its Steady Growth. https://bit.ly/2K68sgd

[3] How Is Land in the United States Used? A Focus on Agricultural Land. https://bit.ly/2I8yWMQ

[4] W.J. Ripple. Status and Ecological Effects of the World's Largest Carnivores. Science, vol 343, pp. 124148, January 2014.

[5] Global Greenhouse Gas Emissions Data. https://bit.ly/2Ib4QIk

[6] J.T. Houghten et al. Climate change 2001: the scientific basis. The Press Syndicate of the University of Cambridge, 2001.

[7] C.A. Schlosser. The Future of Global Water Stress: An Integrated Assessment. Earth's Future, vol. 2, pp. 341-361, May 2014.

[8] N. Stephens et al. Bringing cultured meat to market: Technical, socio-political, and regulatory challenges in cellular agriculture. Trends in food science and tech., vol 78, pp. 155-166, August 2018.

[9] Where's the Beef? Check the Snack Aisle. http://bit.ly/2IbmkUZ

[10] Gershlak, Joshua R. et al. Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds. Biomaterials, vol. 125, pp. 13-22, May 2017.

[11] Substances Added to Food (Formerly EAFUS). https://bit.ly/2FH95bK

[12] C. Radnitz *et al*. Investigation of lifestyle choices of individuals following a vegan diet for health and ethical reasons. *Appetite*, vol. 90, pp. 31-36, 2015.

[13] H Willer *et al*. BIOFACH Session. https://lucris.lub.lu.se/ws/files/17879851/With_Care_for_Cows.pdf

[14] TLT Nguyen *et al*. Environmental costs of meat production: the case of typical EU pork production. *Journal of Cleaner Production*, vol. 28, pp. 168-176, 2012.

[15] A. Reisinger *et al*. How much do direct livestock emissions actually contribute to global warming? *Global change biology*, vol. 24, pp. 1749-1761, 2018.

[16] D. Caro *et al*. Global and regional trends in greenhouse gas emissions from livestock. *Climate Change*, vol. 126, pp. 203-216

[17] KJ Bonnedahl and P Heikkurinen. *Strongly sustainable societies: Organising human activities on a hot and full earth*. Routledge, 2018.

[18] WF Ritter *et al*. Impact of animal waste lagoons on ground-water quality. *Biological Wastes*, vol. 34, pp. 39-54, 1990.

[19] A. Potts. Meat Culture. https://lucris.lub.lu.se/ws/files/17879851/With_Care_for_Cows.pdf

[20] S. Sharma *et al*. In vitro meat production system: why and how? *Journal of Food Science and Technology*, vol. 52, pp. 7599-7607, 2015.

[21] EB Richi *et al*. Health Risks Associated with Meat Consumption: A Review of Epidemiological Studies. *International Journal for Vitamin and Nutrition Research*, vol. 85, pp. 70-78, 2015.

[22] A Pan *et al*. Red Meat Consumption and Mortality Results From 2 Prospective Cohort Studies. *Archives of internal medicine*, vol. 172, pp. 555-563, 2012.

[23] Livestock, Dairy, and Poultry Outlook: January 2018. https://www.ers.usda.gov/publications/pub-details/?pubid=86848

[24] PD Edelman *et al*. Commentary: In vitro-cultured meat production. *Tissue Engineering*, vol. 11, pp. 659-662, 2005.

[25] M Wilks *et al*. Attitudes to in vitro meat: A survey of potential consumers in the United States. *PloS one*, vol. 12, pp. E0171904, 2017.

[26] R Cheung *et al*. Back to grass: the market potential for US grass fed beef. *Stone Barns Center for Food and Agriculture, Armonia LLC, Bonterra Partners, SLM Partners*, 2017.

[27] Memphis Meats Makes The Cover Page. https://www.memphismeats.com/press

[28] T Hayes. Why We Are Investing in Alternative Proteins. https://www.tysonfoods.com/the-feed-blog/why-we-are-investing-alternative-proteins

[29] C. Hawks. How close are we to a hamburger grown in a lab? https://edition.cnn.com/2018/03/01/health/clean-in-vitro-meat-food/index.html

[30] Frequently Asked Questions. https://www.mosameat.com/faq

[31] CEA Jochems *et al*. The use of fetal bovine serum: ethical or scientific problem. *ATLA-NOTTINGHAM- 30*, pp. 219-228, 2002.

[32] Cultured Meat. https://www.futurefood.org/in-vitro-meat/index_en.php

[33] I Datar *et al*. Possibilities for an in vitro meat production system. *Food Science and Emerging Technologies*, vol. 11, pp. 13-22, 2010.

[34] The Scientists Making the Future of Food. https://www.cell.ag/scientists-making-the-future-of-food/

[35] The Science Behind Lab-Grown Meat. http://elliot-swartz.squarespace.com/science-related/invitromeat

[36] N Berenstein. How scientists are hijacking plant skeletons to make lab-grown steaks. https://newfoodeconomy.org/cell-cultured-meat-plant-scaffold/

[37] E. Byrd. Clean Meat's Path to Your Dinner Plate. https://www.gfi.org/clean-meats-path-to-commercialization

[38] MS Arshad *et al*. Tissue engineering approaches to develop cultured meat from cells: a mini review. *Cogent Food and Agriculture*, vol. 3, pp. 1320814, 2017.

[39] GA Villalona *et al*. Cell-Seeding Techniques in Vascular Tissue Engineering. *Tissue Engineering Part B: Reviews*, vol. 16, pp. 341-350, 2010.

[40] EM Bueno *et al*. Enhancing cell seeding of scaffolds in tissue engineering through manipulation of hydrodynamic parameters., *Journal of biotechnology*, vol. 129, pp. 516-531, 2007.

[41] D Wendt *et al*. Oscillating perfusion of cell suspensions through three‐dimensional scaffolds enhances cell seeding efficiency and uniformity, *Biotechnology and Bioengineering*, vol. 84, pp. 205-214, 2003.

[42] H Li *et al*. A scaffold cell seeding method driven by surface acoustic waves. *Biomaterials*, vol. 28, pp. 4098-4104, 2007.

[43] Y Chen *et al*. Characterization and Optimization of Cell Seeding in Scaffolds by Factorial Design: Quality by Design Approach for Skeletal Tissue Engineering. *Tissue Engineering Part C: Methods*, vol. 17, pp. 1211-1221, 2011.

[44] Formal Agreement Between The U.S. Department Of Health
And Human Services Food And Drug Administration And U.S.
Department Of Agriculture Office Of Food Safety.
https://www.fsis.usda.gov/wps/wcm/connect/0d2d644a-9a65-43c6-944f-ea598aacdec1/Formal-Agreement-FSIS-FDA.pdf?MOD=AJPERES

[45] S. Nix. How to Identify a Tree Using Leaf Shape, Margin, and Venation.
https://www.thoughtco.com/id-trees-using-leaf-shape-venation-1343511

[46] Plastics Sterilization Compatibility.
https://www.industrialspec.com/resources/plastics-sterilization-compatibility/

[47] Food Additive Status List.
https://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm091048.htm

[48] P Mozdziak. Overview of Animal-Derived Cell Culture Technology.
https://www.fda.gov/downloads/Food/NewsEvents/WorkshopsMeetingsConferences/UCM615605.pdf

[49] Scientific Opinion on the safety evaluation of the substance, 1,3,5-tris(2,2-dimethylpropanamido)benzene, CAS No. 745070-61-5, for use in food contact materials. https://www.efsa.europa.eu/en/efsajournal/pub/3306

[50] Imagine Real Meat Without Factory Farming. https://www.cleanmeatharvest.com/

[51] ISO/FDIS 11737-2. https://www.iso.org/standard/70801.html

[52] ISO 22000:2018. https://www.iso.org/standard/65464.html

[53] M Quick. Five grand ideas that will shape this century – in charts. https://bbc.in/2TTSc2H

[54] HL Tuomisto *et al*. Environmental Impacts of Cultured Meat Production. *The Journal of Environ. Science and Tech*., vol. 45, pp. 6117-6123, June 2011.

10 Appendix

Appendix A. Isolation of Primary Satellite Cells, Myoblasts, and Fibroblasts

The goal of the isolation is to isolate desirable cells from a butcher sample of bovine muscle. Once the cells are isolated, they are grown until they are ready to be seeded on a scaffold of choice. The biggest challenge with the isolation procedure is the high risk of contamination. All isolation steps must be followed with strict sterile technique. Extensive preparation is required as well. It is crucial that the isolation is performed as quickly and as efficiently as possible to mitigate the contamination risk.

Most of the isolation procedure will require two people. One person will remain inside the biosafety cabinet while the other will remain outside to retrieve materials if needed. This also helps limit contamination. During the isolation, small chunks of bovine muscle are harvested from the interior of the sample. It is important that 1) fatty samples are not harvested because they contain minimal muscle cells and 2) surface samples are not harvested because they are contaminated.

Once the small muscle chunks are harvested, digested, and filtered according to the isolation protocol, they must be observed and maintained daily. There are multiple cell types that are isolated.

Fibroblasts – Connective tissue cells. They love to adhere to surfaces, even non-culture treated ones.

Myoblasts – The precursors to muscle fibers. During the process of myogenesis, myoblasts fuse to form long, tubular cells called myocytes. Myogenesis occurs when growth factors are removed from the culture media or when myoblasts come into contact with each other. Myoblasts tend to float in the media, but like to adhere to laminin-coated surfaces.

Myosatellite cells – The precursors to myoblasts. They differentiate into myoblasts when growth factors are removed from the culture media. Myosatellite cells tend to float in the media, but like to adhere to laminin-coated surfaces.

During the isolation, it is important to maximize the number of myoblasts and myosatellite cells you end up with. Myoblasts and myosatellite cells have the ability to differentiate into myocytes during their lineage. The goal of any lab-grown meat product is to have lots of myocytes because

they provide muscle structure, striation, and contractility. After the cells are isolated, they will grow relatively quickly for many weeks (up to passage 12 or so).

Materials and Methods

The following section was adapted from the "Isolation of primary satellite cells, myoblasts, and fibroblasts from muscle" protocol. The materials and methods section can be broken down into three steps.

1. Preparation
2. Isolation
3. Culturing

1. Preparation

Before you can perform the isolation, there are multiple things you want to do in advance. First, you want to know the exact date and time you are picking up the meat sample. When the meat sample is picked up from the butcher, its surface is heavily contaminated. It is important that you coordinate when the sample is being picked up so it can be isolated as soon as possible. The longer the sample sits, the longer the bacteria have to colonize and infiltrate the center of the sample. You also want to ensure that you and your partner are free for about 5-6 hours or so to perform the isolation. The filtering steps can be done alone, but the dissection steps should be done together to reduce the contamination risk.

You will need to sterilize some tools in advance for the isolation. It is always good practice to have duplicate tools in case you contaminate one accidentally during the procedure.

(4) forceps (2 large, 2 fine)
(3) scalpels, multiple blades
(2) hemostats
(3) iris/curved scissors

The tools are sterilized using the autoclave. Tools that are used to cut or handle the surface of the sample should not be used when handling the inside of the sample. Use different tools when harvesting the inner biopsies.

You are also going to want to make sure you have the following lab materials as well (all sterile).

Multiple 5, 10, 25 mL serological pipettes and a pipette gun
Multiple aspirating pipettes
(3) 150 mm petri dishes
Multiple 15 mL and 50 mL conical tubes
(1) 100 μm cell strainer, (1) 70 μm cell strainer, and (3) 40 μm cell strainers
(1) T-75 tissue culture-treated flask
(2) pairs of surgical gloves

It also might be beneficial to prepare the mediums/solutions you will need for the isolation in advance (all sterile or sterile filtered).

Soaking Medium (DMEM/F12 (Ham's) and 1% Pen Strep)

The meat sample is first placed in soaking medium. It contains antibiotics which should help kill any surface contaminants. Soaking medium is made using 49.5 mL of F12 DMEM and 0.5 mL of Pen Strep. Make sure all components are sterile and are prepared in the biosafety cabinet.

Collagenase Type 1 Solution (1800 units/mL solution in HBSS)

Collagenase type I can be found in the fridge and must be weighed out on the benchtop. Collagenase is an enzyme that is used to break down collagen, a connective tissue protein. By breaking down the collagen, you will be able to more effectively isolate the cells. The collagenase type I is added to 5 mL of Hank's Balanced Salt Solution. Make sure you filter the solution in the biosafety cabinet because both components are not sterile!

Tissue Digestion Medium (DMEM/F12 (Ham's), 1% Pen Strep, 10% Collagenase Solution)

The tissue digestion medium is used to break down the collagen in the meat sample biopsies. Tissue digestion medium is made using 5 mL of the collagenase type I solution (prepared above), 0.5 mL of Pen Strep, and 44.5 mL of F12 DMEM. Make sure all components are sterile and are prepared in the biosafety cabinet.

Tissue Rinse Medium (DMEM/F12 (Ham's), 1% Pen Strep, 10% Fetal Bovine Serum)

The tissue rinse medium is used during the filtering steps of the isolation. Tissue rinse medium is made using 5 mL of heat-inactivated FBS, 0.5 mL of Pen Strep, and 44.5 mL of F12 DMEM. Make sure all components are sterile and are prepared in the biosafety cabinet.

Cell Culture Growth Medium (DMEM/F12 (Ham's), 1% Pen Strep, 10% Fetal Bovine Serum, 4ng/mL FGF2, 10ng/mL EGF, 2.5ng/mL, HGF, 5ng/mL IGF1)

Cell culture growth medium is used when culturing the isolated cells and encourages them to proliferate. Cell culture growth medium is typically prepared in a 500 mL F12 DMEM bottle. Remove 55 mL of DMEM from the bottle and place in the fridge. Add 5 mL of Pen Strep, 50 mL of heat-inactivated FBS, and four pre-aliquoted growth factors to the DMEM bottle. The growth factors are FGF2 (all 20 µl), IGF (all 25 µl), HGF (only 3.1 µl), and EGF (all 50 µl). Make sure all components are sterile and are prepared in the biosafety cabinet.

2. Isolation

Now you are prepared to do the isolation. You will need a partner and about 5-6 hours to perform the isolation carefully and entirely. You must do the isolation all at once. The entire isolation should be conducted in the biosafety cabinet using very strict aseptic technique. Once the biosafety cabinet is sprayed down with ethanol, all solutions and mediums have been heated in the water bath, and all materials are in the biosafety cabinet, you are ready to begin. Once the meat sample is brought into the biosafety cabinet, the person who is conducting the isolation cannot leave. The other person will be responsible for getting any forgotten materials outside.

Isolation Procedure:

1. Thoroughly spray down the bag containing the meat sample before bringing it into the biosafety cabinet. Normal nitrile gloves can be used for this part of the procedure. Ensure that your hands have been washed before putting the gloves on.

2. Open the bag in the biosafety cabinet and cut off a fist-sized chunk of muscle from the sample. Close the bag and put it to the side.

3. Place the muscle chunk in a petri dish filled with 50 mL of soaking medium. Soak for 10 minutes and flip it over after 5 minutes.

4. The person in the biosafety cabinet should put on surgical gloves. Do not touch the outside of the surgical gloves while putting them on. Take off the nitrile gloves before doing so.

5. Using a pair of forceps and a scalpel, cut into the sample like you are attempting to fillet it.

6. Once a couple of inches into sample, grab a new set of surgical tools. Carefully cut 10-20 interior penny-sized muscle biopsies from the meat sample and place in a petri dish filled with 50

mL of digestion medium. Cutting the samples as small as possible makes the digestion process more efficient.

7. Move the digestion medium dish into a 5% CO_2 incubator and incubate for 1 hour at 37°C. You can try freezing the excess meat for another isolation attempt, but it has never been attempted.

8. Swirl the dish every 15 minutes.

9. After 1 hour, bring the dish back into the biosafety cabinet and transfer its contents into a 50 mL conical tube. You do not require surgical gloves or a partner for these steps, but sterility is still crucial!

10. After letting the larger pieces settle, transfer the small tissue pieces and medium (supernatant) through a 100 µm cell strainer into a new 50 mL conical tube.

11. Centrifuge the contents for 5 minutes at 0.3 rcf.

12. Aspirate the supernatant and resuspend the cell pellet with 5 mL of tissue rinse medium and gently titrate until the pellet is resuspended.

13. Pass the suspension through a 70 µm cell strainer and transfer to a new conical tube.

14. Repeat spin/rinse/strain using a 40 µm cell strainer 3 times.

15. After the 3rd centrifugation, resuspend the cell pellet in cell culture growth medium.

16. Transfer the cell suspension into a T-75 flask (10-12 mL of volume) and put in the incubator.

17. Change the cell culture growth medium every 2 days and passage when approaching 70% confluency.

3. Culturing

Culturing the isolation is just as important as the isolation itself! The isolation should contain a mixture of fibroblasts, myoblasts, myosatellite cells, or even muscle chunks that might not have been strained. It is extremely important that you maintain enough myoblasts and myosatellite cells in the culture. These cell types will eventually differentiate into myocytes via contact with

each other or the removal of growth factors from the media. That's not to say you don't want fibroblasts in the culture; you just don't want exclusively fibroblasts.

Culturing cells in growth factor media tells them to proliferate. This is helpful because it speeds up the process of growing cells. Once you have enough cells and they are seeded on a scaffold, you can remove the growth factors from the media, causing differentiation of the myoblasts and myosatellite cells.

When changing the media from an isolation flask, NEVER aspirate the media (sometimes referred to as supernatant for simplicity). This supernatant likely contains myosatellite cells or myoblasts that have not yet attached to the bottom of the flask. Harvest these cells by sucking them out of the flask, spinning them down, aspirating the media, resuspending them in growth factor media, and plating them on a new flask.

During passaging you also want to make sure you keep both the supernatant (floating cells) and the adhered cells. The adhered cells are most likely fibroblasts because they are known to adhere to any surface they can grab onto. After spinning down the cells and resuspending the cell pellet, you should have two conical tubes: one that contains the cells that were originally floating in the media, and another than contains the cells that were trypsinized and adhered. You will want to follow a slightly different procedure when plating them.

Plating Isolation Cells After Passaging:

1. Grab a non-tissue cultured treated 6-well plate and bring it in into the biosafety cabinet.

2. Combine the contents of both conical tubes and add growth factor media until the total volume is 12 mL.

3. Add 2 mL of the suspension to each well of the well plate. Place in the incubator for five hours to allow the fibroblasts to attach to the bottom of the plate.

4. After 5 hours, remove the floating cells from each well and plate them in an appropriately sized flask. T-150 flasks are especially useful because they can hold double the volume of a T-75 and the cell population takes longer to become confluent. T-75 flasks typically are seeded with 500k cells, while T-150 flasks can be seeded with over a million cells.

5. Trypsinize the adhered cells again and repeat the process of passaging. Plate them on an appropriately sized flask.

Important: A couple of times during this procedure, there were tissue chunks floating around in each well after the 5 hour waiting period. In this case the tissue chunks should be treated separately.

Tissue Chunks Procedure:

1. Remove the tissue chunks from the well plate using a 10 mL pipette and a pipettor. It is okay to suck up a little media. Transfer to a conical tube.

2. Spin down the tissue chunks for 5 minutes at 0.3 rcf, aspirate out the media, and resuspend in 10 mL of trypsin. You can use a micropipette to gently separate the chunks mechanically. Don't be too rough.

3. Transfer the tissue chunks to a T-75 flask and place in the incubator for 15 minutes. Agitate the flask every 3 minutes or so.

4. After 15 minutes, repeat the process of spinning/aspirating/resuspending in trypsin again if needed. You can use a micropipette to gently separate the chunks mechanically. Don't be too rough.

5. Once satisfied, spin down the suspension, aspirate the trypsin, resuspend in growth factor media, and plate on an appropriately sized flask.

Appendix B. Feeding Primary Satellite Cells, Myoblasts, and Fibroblasts

Preparation:

1. Wash your hands when you enter the lab. General lab safety measure.
2. Put media in the water bath set to 37℃.

Media options:

- Meat Growth Medium (DMEM/F12 (Ham's), 1% Pen Strep, 10% Fetal Bovine Serum, 4ng/mL FGF2, 10ng/mL EGF, 2.5ng/mL, HGF, 5ng/mL IGF1)
- Meat Differentiation Medium (DMEM/F12 (Ham's), 1% Pen Strep, 10% Fetal Bovine Serum)

Verifying cells are healthy:

3. Take cell culture flask out of the incubator. Take care not to tilt the flask, the media should not enter the neck of the flask.
4. Inspect the media visually:
    a. Color: - should be dark pinkish red. If yellow / yellowish orange – immediate action – change media / discard cells.
    b. Transparency: Cells in good health show transparent, clear media. If cloudy à sign of contamination / aging / dying cell culture.
5. Microscopic examination: Examine the cell culture flask under an inverted microscope; first under low magnification (usually 4x) and then under medium magnification (usually 10x). Things to look for:
    a. Floating cells (dead or unhealthy), cellular debris, bacteria or fungi, other unidentifiable debris are a signs of unhealthy culture. Immediate action necessary:
        i. If contaminated – suction out all media into waste, spray the inside of the flask with alcohol, suction the alcohol, and then discard in biohazard.
        ii. If unhealthy, but not contaminated – change media with fresh, warm culture media. Examine again after 24 hours and feed with fresh media again.
    b. If cells are nicely spread out (adhered), look for vacuoles within the cells. Presence of too many vacuoles is an indication that the media needs to be changed.
    c. Confluence of culture à Look at at least 5 locations within the flask, usually four corners and the center. Calculate confluence and note it down. For 80 – 100 % confluence – passage the cells. For cultures older than 14 days – passage the cells,

irrespective of confluence. For the rest, replace old media with fresh media (which is referred to as "feeding the cells.")

Preparing the Hood:

6. Take cell culture cart from lab bench area to cell culture room, along with 1000 µL micropipette / pipette aid (depending upon flask size), corresponding sterile pipette tips and other items as necessary.
7. Put gloves on, spray hands with alcohol. (do this every time something unclean/not sterile is touched)
8. Spray the inside of the laminar flow hood (working surface and bottom third of the side walls) and wipe down. Make sure to clean the vacuum line also, by spraying both the outside and inside of the tube with the vacuum turned on.
9. Spray and wipe all objects that you intend to take inside the laminar flow hood. Be careful not to spray the cap of the flasks the cells are in.
10. Attach the Pasteur pipette on the vacuum tube and place it in a manner that the tip doesn't touch any object while you work in the hood. Set up all other items in the hood.
11. Bring warm media from the water bath and the cells from the incubator. WIPE DOWN MEDIA AND CELL CULTURE FLASKS THOROUGHLY WITH ALCOHOL, as the water bath and incubator are common sources of contamination.

Feeding:

12. Unscrew the flask cap and place it in a way that its inner side doesn't touch anything while you're working on the flask. Tilt the flask so as to accumulate the media in one corner of the closed end of the flask (opposite the open end through which you insert the Pasteur pipette).
13. Insert the Pasteur pipette slowly taking care not to touch any inner walls of the flask. Suction the media out from the cell culture flask taking care not to touch the bottom surface, where the cells are attached.
14. Add required volume of fresh, sterile, warmed media with a micropipette/pipette. Take care not to touch any part of the flask (especially inner surface of the neck) with the pipette tip while dispensing media. If the tip touches any surface accidentally, discard the tip and use a fresh one. It's important not to contaminate the stock solution of media, therefore use only a fresh, sterile tip to aspirate media from its storage bottle.
15. Cap the cell culture flask immediately after media is added screw the cap on tight. Return the cell culture flask to the incubator. (As a general rule, try keeping the cells out of the incubator for as short a duration as possible: mammalian cells like to be in a 37oC-environment – that of the incubator).

Appendix C. Passaging Primary Satellite Cells, Myoblasts, and Fibroblasts

1. Place media, trypsin in water bath at 37∘C.
2. Remove T-75 flask and verify cell viability and confluence with scope. Place in bio-safety cabinet.
3. Remove cap and aspirate media off cells with sterile Pasteur pipettes.
4. Add 5mL of trypsin to flask.
5. Put flask back in incubator and let sit for 5 min.
6. Remove flask and confirm cell detachment with scope. (Detached cells will float freely and appear round)
7. Add 5mL of 10% FBS in DMEM (or MSCGM) to T-75 flask. (This deactivates the trypsin)
8. Pipette contents of tube in 10mL pipette and place in a 15ml conical tube.
9. Centrifuge the 15ml conical tube for 5min @ 1000rpm making sure to balance the centrifuge.
10. Being sure to spray down the 15ml conical tube, reintroduce it into the sterile field and aspirate off the supernatant being sure not to disturb the cell pellet.
11. Resuspend the pellet in desired amount of media. (Varies between 0.5ml to 1ml based on pellet size)
12. Triturate the solution with a 1000ul pipette to ensure the solution is homogeneous.
13. Remove 30ul of cell suspension and add it to the 30ul of trypan blue stain.
14. Load 10ul of the cell+trypan blue mixture in each side of the hemocytometer.
15. Count enough boxes to achieve a count of 100 cells of greater. Once you begin counting a box you must count the whole box.
16. Use this formula to determine the cell density.

$$\#\ of\ cells\ counted \div \#\ of\ boxes\ counted * 2 * 10{,}000 * \#\ of ml = cell\ count \div 1\ ml$$

17. Either seed 500,000 cells per T-75 flask, with 10-12mL of media, or use cells for other intended purpose. Recommended seeding density of ≈7000 cells per cm2

Appendix D: Freezing Muscle Cells

Materials:

- MSCGM
- Sigma-Aldrich® DMSO (Dimethyl sulfoxide)
- Eppendorf Research Pipettes 1mL
- VWR™ tips for 1mL micropipette
- Eppendorf Research micropipettes 10µL
- RT-10F filtered tips for 10µL micropipette
- Drummond® Pipette Aid
- VWR™ Serological Pipettes 10ml
- Sterile BD Falcon™ culture flasks
- VWR 15mL Centrifuge Tubes with Screwcaps
- Beckman Coulter GS-6R Centrifuge with Beckman GH 3.8 (rotor)
- VWR International™ Low Temperature Freezer Vial (2.0ml or 1.2ml)
- ISOTEMP 210 Fisher Scientific (Water bath)
- NALGENE® Mr. Frosty™ Cryo 1°C Freezing Container, Cat. No. 5100-0001
- Thermo Forma Class II A/B3 Biosafety Cabinet
- VWR® CryoPro (tank) with liquid N_2
- 70% Ethanol spray bottle
- REVCO® -80°C Freezer

Freezing Cell Preparation:

1. Follow passaging cells protocol for a T-75 flask of hMSCs.
2. Count cells – want to freeze 500,000 cells in a 1.8ml cryovial.
3. Add desired volume of cell solution (for 500,000 cells) to a 1.8ml sterile cryovial. Add enough MSCGM to bring volume in cryovial to 900µL. Add 100 µL DMSO.
    a. Final freezing solution should be 10% DMSO:MSCGM
    b. Can make up freezing solution before passaging cells if you can estimate the count of cells. After trypsining and centrifuging cells aspirate media and resuspend pellet in the desired amount of freezing solution so that 500,000 cells are placed in each cryovial.
    c. Once DMSO is added IMMEDIATELY place the lid on the cryovial and place the cryovial in "Mr. Frosty" in -80C.
4. **Note:** Be careful not to spill or come into contact with skin with DMSO.
5. Store at -80C overnight and then transfer to Liquid Nitrogen cryotank.
    a. If cells are not needed for long-term storage can keep in -80C.

Appendix E: Thawing Muscle Cells

Materials:

- MSCGM
- Pipettes - 100µL, 1mL, 10mL serological pipette
- Sterile Culture flasks, 15mL conical tubes

Procedure:

1. Place media into the 37°C water bath
2. Spray surface of hood with 70% Ethanol, spray exterior of all containers before being placed into the hood, set up the necessary items inside the hood
3. Place 5mL of media into a 15mL conical tube and place into water bath
4. Retrieve cryovials from cryotanks/dry ice/-80°C freezer
5. Thaw cryovial rapidly by immersing into the water bath, do not completely submerge. Keep the water line below the cap. Gently agitate for approx. 1-2min until all ice crystals are melted
6. Remove cryovial and 15mL conical tube from water bath. Spray both with ethanol and reintroduce into the hood. With the 1mL pipette extract all cell solution from vial and **slowly/drop-wise** add to the **pre-warmed media** in the 15mL conical tube. Once all cell solution has been added triturate solution.
7. Place conical into centrifuge and spin @ 1000rpm for 5min
8. Aspirate supinate, suspend in known amount of media (Between 0.5-10mL). Perform cell count to determine cell concentration and cell viability.
9. After determining cell number plate in appropriate tissue flask, for optimum growth plate at a density of ≈7000 cells per cm^2
10. Place cells into incubator for seeding, allow for 24hrs
11. After 24hrs, confirm seeding. Replace media in flask with fresh pre-warmed media.

Appendix F: Protocol for Plant Decellularization via Cannulation

Materials:

- 27G Needles
- 0.38 ID 1.09 OD LDPE Medical tubing
- Hexanes
- PBS 1x
- DI H2O
- 1X SDS solution in DI H2O
    - For 2L of solution mix 200 mL of 10x SDS with 1800 mL of DIH20
    - 2L of 10x SDS Solution
        - Mix 200 g of SDS powder in 2L DIH20 until there are no more visible SDS pellets
        - Can be stored at room temperature until needed
    - 0.1% Triton-X/Tween 20 with 10% bleach (or Cl tablets) in DI H2O
        - 48 mL of concentrated Clorox bleach and 20 mL of Triton-X100/Tween 20 Solution are added to 2L of DiH20 and mixed until in solution
    - Tris buffer solution
        - 10 mM Tris Buffer (605.7 mg in 500 mL of DiH2O)
        - Buffered to pH 9.0

Note: Same protocol applies if using Gaudette Lab decellularization apparatus or custom set-up. Gaudette decellularization apparatus requires ~2-4L of each solution to function efficiently. Custom decellularization apparatus should use an appropriate amount of solution depending on size..

Decellularization Protocol:

1. Cannulate leafs and parsley stems via the stem, affixing cannulas with suture.
2. Once leaves are cannulated, **(Repeat 3x times)** Submerge in hexanes and wash vigorously for 2 minutes. Remove and rinse in 1x PBS for 2 minutes.
3. Affix plant materials to decellularization set up.
4. Attach 4 L of 1x SDS solution to the set up and begin flow. Monitor plant materials to ensure proper flow through the leaf vasculature, modifying flow rate to ensure a slow, steady drip.
    a. Rapid flow rate will deplete SDS too quickly, whereas too little flow runs the risk of dehydrating the plant material and damaging the plant structure.

5. Maintain in SDS for 1 day in order to decellularize plant material, until leaves and stems become more transparent in appearance. Green coloration at this step is normal and not indicative of an unsuccessful decellularization.
6. Remove 1x SDS and add 4L of Triton-X/Bleach solution to set up.
7. Maintain set up in Triton-X/Bleach for 24 hours, can go longer if needed. Watch until leaves and stems have become clear/transparent. This solution should purge any remaining coloration from the plant matter.
8. Remove Triton-X/Bleach solution and attach DI H2O to set up. Perfuse leaves/stems in DI H2O for 24 hours.
9. Wash decellularized leaves on rotator in Tris buffer solution overnight.
    a. Replace the solution at least twice (usually after the first hour and then secondly in the morning)
    b. Tris buffer removes residual SDS that maybe left entrapped in the leaf
    c. Make sure the rotator moves the leaf gently
    d. Watch the stem as they become very fragile
10. Remove leaves and stems from solutions, freeze overnight in -20°C freezer
11. Lyophilize leaf/stem for 24 hours
12. Store lyophilized leaf scaffold at room temperature until needed
13. Please see the rehydration protocol for next steps

Appendix G: Protocol for Plant Decellularization using Bulk Decellularization

Materials:

- Plant material
- 1 L beaker
- Decellularization chemicals made according to the cannulation protocol (Appendix F)
    - SDS (Day 1)
    - Triton X-100 or Tween 20 + Bleach (Day 2)
    - DI H2O (Day 3)
    - Tris Buffer (Day 4)
- Stir bar
- Stir bar protector plate
- Stir plate
- Forceps

Procedure:

1. If dirty, wash the plant leaves with distilled water
2. Place a stir bar and a stir bar protector plate at the bottom of a 1 L beaker
3. Fill the 1 L beaker with SDS to the 800 mL mark
4. Put the plant leaves into the beaker
5. Depending on the amount of leaves and the type of stir plate, set the stir plate to an appropriate rpm. The rpm should be set so the leaves are moving around but are not being destroyed by the stir bar or the force of the flow.
6. Let the leaves soak in SDS for 24 hours
7. After 24 hours, replace the SDS with Triton X-100 or Tween 20 + Bleach
8. After 24 hours, replace the Triton X-100/Tween 20 + Bleach with DI H2O
9. After 24 hours, replace the DI H2O with Tris Buffer
10. After 24 hours in Tris Buffer, the decellularized leaves can be placed in the freezer for a few weeks until they are lyophilized
11. Remove leaves and freeze overnight in -20°C freezer. The leaves can stay in the freezer up to three weeks.
12. Lyophilize leaves for 24 hours
13. Store lyophilized leaf scaffold at room temperature until needed.
14. Please see the rehydration protocol for next steps

Troubleshooting:

If the leaves are not decellularized enough, increase the rpm and the time in SDS.
If the leaves are destroyed by the stirring mechanism, decrease the rpm and use less leaves.

Appendix H. Rehydrating Decelled Leaves and Seeding Muscle Cells

Preparing and rehydrating the leaves:

1. Cut leaves to desired shape and size
2. Place leaves into cell culture plate
3. Cover leaves in tris buffer solution; leave for 30 minutes on shaker plate
4. Aspirate and replace tris buffer with DI water; leave for 30 minutes on shaker plate
5. Aspirate and replace DI water with 70% ethanol
6. Spray plate with ethanol and move into biosafety cabinet; leave 30 minutes
7. Rinse with sterile PBS three times, waiting five minutes between each rinse
8. Move leaves into sanitized polystyrene container that fits the shapes of the leaves
9. Cover leaves in cell growth media and incubate overnight overnight

Seeding cells onto leaves:

1. Remove plates from incubator and put back into biosafety cabinet
2. If using cloning wells, place sterilized cloning wells onto leaves at this point
3. Passage and count your cell supply
4. Deposit desired amount of cells onto each leaf, depending on quantity of cells available
5. Deposit enough growth media to cover the leaf
6. Include a control plate of cells growing without a leaf scaffold
7. Incubate the plates
8. Check on the media daily and refeed every other day

Appendix I. Phalloidin/Hoechst Staining

Reagents:

- Phosphate Buffered Saline
- 4% Paraformaldehyde (Only needed for tissues/cells that have not been fixed);
- 0.25% Triton-X
- 0.25% V/V Triton-X in PBS
- 10 µL Triton-X in 3990 µL PBS
- 1% BSA
- 1% V (W)/V BSA in PBS
- 40 µL in 3960 µL PBS
- Phalloidin (AF 488 Phalloidin A12379 or FITC Phalloidin, Invitrogen)
- 2.5% V/V Phalloidin in PBS
- 50 µL in 1950 µL
- Hoechst
- 0.0167% Hoechst dye in PBS
- 0.5 µL in 3000 µL PBS

For unfixed sections/cells:

1. Rinse in PBS x2
2. Fix in 4% Paraformaldehyde for 10 minutes
3. Rinse in PBS x2
4. Follow directions for fixed sections

For fixed sections/cells:

1. Rinse with PBS x2
2. Triton-X solution for 10 minutes
3. Rinse with PBS x2
4. Block with BSA solution for 30 minutes
5. Phalloidin solution for 30 minutes
6. Rinse with PBS x2
7. Hoechst solution for 3-5 minutes (typically 3)
8. Rinse with PBS x2
9. Cytoseal and coverslip
10. Store frozen at -20 degrees C.

Results:

- F-actin is stained green if you used 488, red if you used FITC
- Nucleus is stained Blue

Appendix J. MF20 Staining (Myocyte Staining)

Reagents:

- 5% Normal Goat Serum
- In 5% goat serum
    - Primary mouse monoclonal MF20 1:30
- In 5% goat serum
    - Secondary antibody - 1:400 goat anti-mouse Alexa Fluor 488
- Hoescht
    - 0.0167% Hoescht dye in PBS
    - 0.5 uL in 3,000 uL PBS

Procedure (For fixed tissue sample):

1. Thaw tissue in PBS for 5 minutes
2. 0.25% Triton-X-100 for 10 minutes
3. 3 washes in PBS, 5 minutes each
4. Block with 5% Normal Goat Serum for 45 minutes
5. Leave goat serum on negatives but aspirate serum off the positives
6. Primary mouse monoclonal anti-myosin 1:30 for 1 hour at room temperature
7. 3 washes in PBS, 5 minutes each
8. Secondary antibody - 1:400 goat anti-mouse Alexa Fluor 488 for 1 hour at room temperature in the dark
9. 3 washes in PBS, 5 minutes each
10. Hoescht - 1:6000 in PBS for 5 minutes
11. 3 washes in PBS, 5 minutes each
12. Cytoseal and store frozen in -20 degrees C

Results:

- MF20: green
- Nuclei: blue

Appendix K. WPI Campus Survey Questions

Q1 – How old are you?

- Under 18
- 18-24
- 25-34
- 35-44
- 45-54
- 55-64
- 65-75
- Over 75

Q2 – Which best describes your gender?

- Male
- Female
- Prefer not to say
- Prefer to self-describe:

Q3 – Which of the following most closely describes your dietary preference?

- Just meat
- Meat and Vegetables
- Vegetarian
- Vegan
- Celiac
- Other (please specify):

Q4 – a. Are you the primary food purchaser in your household (ie. at WPI or at home)?

- Yes
- No

b. On average, how regularly do you purchase food for yourself? – <u>IF YES</u>

- Daily
- Weekly
- Monthly
- Greater than monthly

Q5 – Rank the following on a scale of (1) not important to (5) extremely important when you are considering buying a food product.

- Environmental impact
- Treatment of animals
- Price
- Ingredient you recognize
- Health benefits
- Source/where it came from

Q6 – How likely would you be to spend more money on an environmentally friendly and ethically-sourced food product than one that is not?

- Very unlikely
- Somewhat unlikely
- Neither likely nor unlikely
- Somewhat likely
- Extremely likely

Q7 – What comes to mind when you hear the following terms? (short response)

- Lab-Grown meat
- Culture meat
- Clean meat
- Cell-Based meat

Q8 – a. Were you familiar with, or heard any of these terms, before taking this survey?

- Yes
- No

b. How did you hear about these term(s)? Choose all that apply. - <u>IF YES</u> (short response)

- Television or news show
- Internet article or video
- Somebody I know
- Course topic
- Other (please specify):

Q9 – a. Based on the cardboard box design representing the packaging of a slaughter-free dried beef product (as it is shown below), would you buy it at the store?

- Yes
- No

b. Please explain why.


LEAF JERKY
"Still beef... but slaughter-free."
When compared to the leading slaughtered jerky...
SAME GREAT TASTE!
FAT FREE!
50% LOWER SODIUM!
ENRICHED WITH FIBER!
LOWER CHOLESTEROL
INGREDIENTS:
all-natural beef
lettuce
salt
seasoning
THAT'S IT!
NO SECRET INGREDIENTS!
18% of all greenhouse gas emissions are attributed from livestock (agriculture).
LOVE THE ENVIRONMENT!
LOVE YOUR BODY!
CHOOSE SLAUGHTER-FREE!