Cultured meat: meat industry hand-in-hand with biomedical production methods

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Abstract
Meat is the main protein source of the human diet in many cultures. Because of the increasing population growth and welfare, the conventional meat industry cannot follow consumer demands worldwide. Besides, some of the environmental, sustainability-related, and ethical concerns associated with the traditional meat industry have directed scientists to developed new strategies to tackle these negative effects. Culturing meat from cell culture is an emerging bioprocess that will revolutionize the industrial animal agriculture. Many tissue engineering techniques can be utilized for this rising field, although its further development faces important cell culture challenges as well as scale-up limitations. The invention of innovative tools for large-scale *in vitro* meat production will concurrently advance the technology for biomedical and therapeutic applications. This review highlights vital factors and fundamental cell biology parameters for designing a bioprocess to produce an environmentally friendly meat product that will be accepted by consumers. New applications of current biomedical products and concepts will form the groundwork for future academic research and novel designs enabling large-scale production of cultured meat.

Keywords: cultured meat; skeletal-muscle tissue engineering; culturing conditions; growth stimulation; bioreactor; nutritional value

1. INTRODUCTION

Meat is an essential part of the human diet in many cultures, and because of the increasing population growth and welfare, the meat industry cannot follow consumer demands worldwide. Additionally, limited land resources and several adverse effects of conventional meat production (e.g., the poor nutritional value of meat, food-borne diseases, extensive use of antibiotics, and greenhouse gas emissions) have compelled scientists to develop innovative techniques that will tackle the negative consequences of traditional meat production through livestock (Arshad et al. 2017; Langelaan et al. 2010; Post 2014a). The meat industry also results in substantially increased land and water use, as livestock farming also requires farmland to produce animal feed (e.g., corn, soy, etc.). To further illustrate (**Fig. 1**), according to research from Bard College, production of beef (per calorie) requires 160 times more land than potatoes, wheat, and rice (Shepon et al. 2018). Aside from that, the production of popular protein sources results in the formation of about 11 times more greenhouse gases than the production of rice. Having the above-mentioned in mind, a switch to cultured or engineered meat, referred to also as lab-grown or clean meat, from its "traditional" production, would lessen or even detain the effects of global warming. Furthermore, since this technology does not rely on the "sacrifice" of animals to produce food, it would also be more acceptable from the ethical point of view for the well-being of animals.
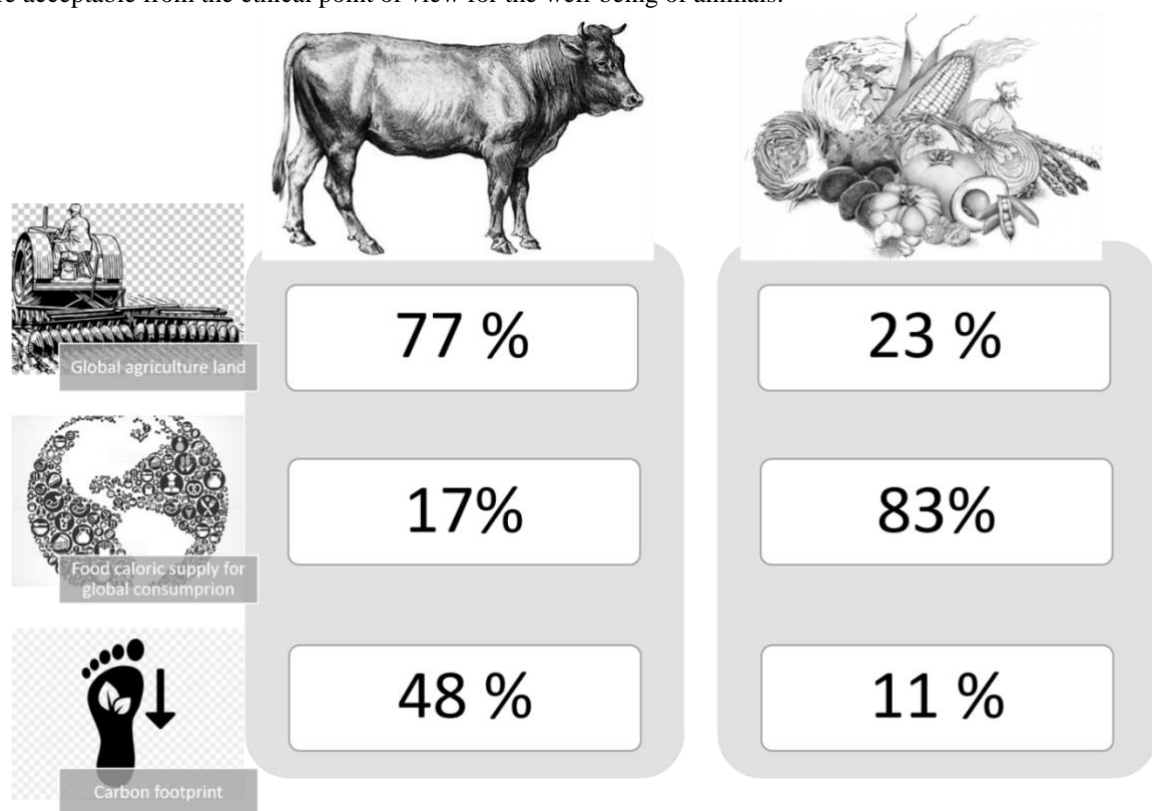

Fig. 1: Nutritional and environmental consequences associated with feed-to-food conversion between meat production and plant-based alternatives. Livestock takes up about 77 % of global agriculture land yet produces 17 % of the world's supply of calories. Crops occupy less than 25 % of global agriculture land for production of 83 % global food caloric supply. Farm-based meat production contributes 4-times more to the total greenhouse gas emissions than plant-based food

Many of the crucial technologies for the realization of large-scale cultured meat production are the same as those that have been pioneered for other large-scale cell culture applications (e.g., antibody therapeutics, cell-based therapy, regenerative medicine) (Rischer et al. 2020; Specht et al. 2018). For the most part, edible animal meat is derived from skeletal muscle tissue, *in vitro,* cultured meat production relies on techniques and strategies developed in skeletal muscle tissue engineering. Research conducted in the mentioned field has elucidated multiple fundamental mechanisms of skeletal muscle repair and identified various types of cells and regulatory factors, which play a crucial role in muscle regeneration (Qazi et al. 2015). Those findings can also be applied for creating

artificially engineered meat *in vitro*. Proof of concept for the feasibility to grow meat *in vitro* (beef patty) was provided in 2013 (Post 2014b). However, it was not a consumer-available product. Although the science behind skeletal muscle tissue engineering is steadily increasing (Boonen et al. 2011; Langelaan et al. 2011; Post and Hocquette 2017), the technology behind the production of cultured meat is still in its infancy (Allan et al. 2019; Post 2014a; Specht et al. 2018). Several technical challenges are facing its industrial-scale production before it can be introduced into the market in an appreciable quality, quantity, and at a reasonable price (Allan et al. 2019; Datar and Betti 2010; Edelman et al. 2005; Specht et al. 2018; Stephens et al. 2018).

2. SKELETAL MUSCLE TISSUE ENGINEERING FOR MEAT PRODUCTION

The process of growing tissue-engineered meat starts with the acquisition of desired cell or tissue types. Because mature skeletal muscle cells lack proliferation capability, stem cells (mesenchymal) are the most common primary source of myoprogenitors (Post and Hocquette 2017). Despite exhibiting some limitations regarding their regeneration potential, which is restricted only to minor damages (Rodriguez and Larkin 2018; Wang et al. 2015; Witt et al. 2017), the regenerative abilities of these stem cells (satellite cells) and their potential for proliferation and differentiation, present important foundations for skeletal muscle tissue engineering.

Skeletal muscle tissue (**Fig. 2**) comprises aligned myofibers formed through myoblasts fused into elongated multinucleated myotubes. The centralized position of their nuclei characterizes newly formed myotubes. As the myotubes mature to shape myofibers, their nuclei settle on positions at the cell's periphery. Each myofiber is surrounded by extracellular connective tissue, and numerous myofibers are assembled to form skeletal muscle. Connective tissue provides a supportive framework that maintains muscle shape and enables synergic contraction of myofibers during movement. Specific alignment of myofibers dictates force generation, and on top of that, connective tissue helps maintain muscle shape and allows myofibers to contract synergically during movement (Catts and Zurr 2002; Qazi et al. 2015; Wang et al. 2015).
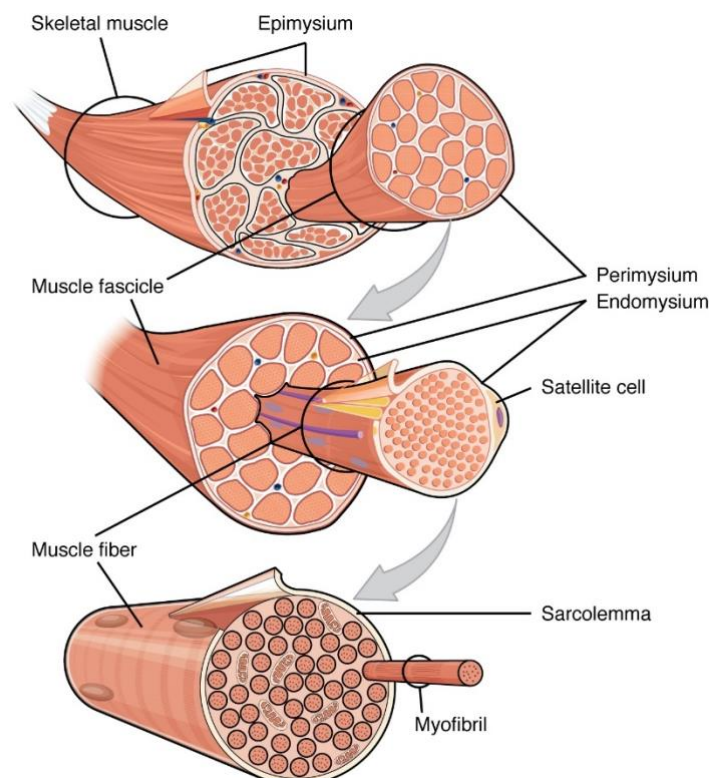

Fig. 2: Skeletal muscle tissue. Skeletal muscle consists of muscle fibers that are arranged in regular bundles enclosed by a dense connective tissue epimysium. From epimysium, a thin septum of connective tissue extends inward (i.e., perimysium), wrapping each fascicle of fibers. Individual muscle fibers (elongated multinuclear

cells) is surrounded by a delicate connective tissue, the endomysium, composed of a basal lamina synthesized by the muscle fiber, and reticular fibers and fibroblasts (J. Gordon Betts 2013)

In general, the skeletal muscle can regenerate in response to damage by activating satellite cells, which repose beneath the basal lamina of adult skeletal muscles. As part of the normal physiological response to trauma and injury, satellite cells proliferate and differentiate into myoblasts, which subsequently fuse to form multinucleated myofibers. They usually remain quiescent in the basal lamina until various growth factors and signaling pathways activate them. These primary cells are identified through the expression of Pax7, a transcriptional factor responsible for the regulation of myogenic proliferation. They can be harvested from adult muscle and successfully grown *in vitro*. Myogenic differentiation is regulated by Myf5 and MyoD, which are transcription factors expressed by myoblasts (and thus, both are identification criteria for them); at this stage, the cell is "committed" to become a muscle fiber (Rodriguez and Larkin 2018). To use tissue engineering either for various applications in regenerative medicine or for cultured meat production, some requirements need to be met. A cell source that can proliferate indefinitely, while it simultaneously has the potential to differentiate into functional skeletal muscle tissue, needs to be embedded (and precisely positioned, as well as oriented) in a three-dimensional (3D) matrix. The latter must allow muscle growth while at the same time, facilitates the delivery of oxygen and nutrients, as well as enables cell waste removal. To obtain mature, functional muscle fibers, muscle cells need to be cultured in a bioreactor that provides constant (and appropriate) biochemical and biophysical stimuli (Bach et al. 2004; Langelaan et al. 2010).

The most straightforward *in vitro* tissue engineering approach to generate mature and contractile muscle constructs is to culture cells on a biomaterial substrate until they have evolved into a functional tissue. However, this approach faces several critical obstacles: (1) fabricating a large muscle construct while maintaining high myofiber packing density and alignment, (2) providing sufficient vascularization to maintain cellular viability in such a large construct for longer periods, and (3) fabricating engineered tissues that can generate physiologically relevant contraction forces. Even though the cells may be the protagonist for skeletal muscle tissue engineering, the important role of biomaterials should not be neglected. By combining various features, like the choice of biomaterials, scaffolds 3D architecture, chemical composition, surface functionalization, and bioactivity modulation of cell behavior can be achieved (Qazi et al. 2015).

3. CELL SOURCES

As outlined above, a vital component of skeletal muscle tissue engineering is myoprogenitor cells that can directly or indirectly re-form muscle tissue. The stem cell technology utilized for the production of engineered meat (which, as aforementioned, is a skeletal muscle) requires the following steps: (1) harvesting stem cells, (2) expansion of stem cell numbers, (3) their differentiation into myoblasts and myofibers, and (4) assembly into the final meat product. Each cell type comes with advantages over others; sourcing considerations, cost, and degree of characterization of the cell type are all relevant factors to contemplate. Since mature muscle fibers do not possess any proliferation potential, they cannot be used as a cell source for tissue-engineered constructs. Precursor cells and native regenerative cells are reliant upon numerous external factors to promote adhesion, proliferation, differentiation, and maintenance of the desired phenotype (Post 2014a; Rodriguez and Larkin 2018).

One of the main objectives of bringing cultured meat to a consumer market is the long-term cultivation of cell lines. The strains utilized for fermentation in brewing will likely be used as sample guidelines for these cell lines: cultures can be used continuously for some number of passages, but periodically they are re-seeded from frozen stocks to avoid genetic drift (Specht et al. 2018). Numerous strategies have been introduced to maintain stemness in continuous cultures. These, among others, include the exposure of cells to hypoxic conditions (Basciano et al. 2011) and modulating parameters like scaffold stiffness (Lu et al. 2014).

3.1 Cell lines

There are two possible cell sources for the production of *in vitro* meat, namely primary cells isolated from the original tissue, or established cell lines that are derived directly from native tumor tissue or artificially generated

from primary cells *in vitro* (Ramboer et al. 2014; Ramboer et al. 2015; Stephens et al. 2018). The latter can be achieved in two ways. A typical strategy involves genetic or chemical induction, which reprograms the cells to proliferate indefinitely (Ramboer et al. 2014; Ramboer et al. 2015). Another method is to select cells that went through a spontaneous transformation and became immortal and then further culture only the selected sub-clones (Stephens et al. 2018). These immortalized cells (i.e., continuous cell lines) have already proven to be a useful alternative source for fresh tissue samples, and they could be valuable tools to increase the speed of proliferation and differentiation (Ramboer et al. 2015; Stephens et al. 2018).

Nevertheless, since they have undergone significant mutations to become immortal, potential implications of these processes need to be evaluated with extreme care. Besides, these cells can change genetically over multiple passages, leading to phenotypic differences among isolates and potentially misidentification. One general limitation may also be that they are not always representative of primary cells. For example, they may exhibit different growth rates; hence cell data should be interpreted cautiously (Stephens et al. 2018).

Once cell lines for *in vitro* meat production have been established, best practices of cell banking, derived from the use of stem cells in biomedical applications, can be utilized to enhance the stability, reproducibility, and long-term maintenance of cell stocks for cultured meat (Specht et al. 2018).

3.2 Stem cells

Stem cells are considered the most promising cell source since they have characteristic capabilities to retain themselves in the undifferentiated form for a specific number of population doublings. Different types of stem cells can be used to create *in vitro* meat and meat-products (Arshad et al. 2017; Datar and Betti 2010; Langelaan et al. 2010). Two favorable options among them are embryonic stem cells and satellite (i.e., myosatellite) cells from native muscle tissue.

In theory, after the embryonic stem cell line is set up, its unlimited regenerative ability eliminates the need to harvest more cells from embryos. Despite their high proliferation and differentiation capacity, these cells must be specifically stimulated to differentiate into myoblasts and may inaccurately restate myogenesis. Moreover, the cell yields from harvests are usually meager (Datar and Betti 2010; Edelman et al. 2005). For embryonic stem cells to become muscle fibers, the first requirement is their differentiation into myogenic progenitor cells. One of the major challenges at this stage is achieving direct differentiation into myoprogenitor cells without the development of any other lineages. It seems that inducing myogenesis in embryonic stem cells *in vitro* is a tougher nut to crack than the *in vivo* counterpart. Zheng and colleagues demonstrated that myogenic precursor progeny from human embryonic stem cells effortlessly form myofibers when transplanted *in vivo* in mice after muscle damage (Zheng et al. 2006).

On the other hand, *in vitro* formation of myofibers from the cells has proven to be strenuous. Authors presumed that *in vitro* system lack some important *in vivo* niche elements (Zheng et al. 2006). Apart from the risk of uncontrolled proliferation and differentiation, an additional issue with using embryonic stem cells as a cell source for cultured meat production, are ethical concerns (Langelaan et al. 2010).

The most promising and practical cell types for skeletal muscle tissue engineering, as well as for *in vitro* meat production, are (myo)satellite cells. However, having the drawback of being a rare muscle cell type (they make up <5 % of the total nuclei in the skeletal muscle fiber of adult rodents and humans), they are the main contributors to muscle repair and regeneration, they recapitulate myogenesis (**Fig. 3**) more closely than the immortal myogenic cell lines. In the event of muscle damage and subsequent transmission of biochemical signals, activated satellite cells migrate to the injured site, proliferate, undergo myogenic differentiation, and fuse to form myotubes (Qazi et al. 2015). It has been shown *in vivo* that satellite cell fusion causes remodeling in adult fibers. Subsequently, their competence to proliferate and fuse with adjacent fibers in uninjured muscle has been revealed, providing a mechanism for the addition of nuclei to growing fibers. The inclusion of new nuclei to growing fibers alleviates further growth in fibers length and perimeter (Benjaminson et al. 2002). On the other hand, numerous studies have demonstrated that the expansion and culturing of satellite cells *in vitro,* even for a few days, can significantly limit

their proliferative properties. Usually, the satellite cell proliferation rate decreases with each passage until a stage, known as proliferative senescence, is reached. At this point, the cells cease to divide, plausible because of the loss of telomeric DNA that occurs with each cell division (Renault et al. 2002). A related key finding has been made by Gilbert et al., who showed that satellite cells could maintain their *in vivo*-like self-renewal properties when cultured on elastic substrates (Gilbert et al. 2010). Namely, when these primary cells are cultured on appropriate biomaterials (with Young's elasticity modulus of 12 kPa), they retain their characteristic proliferative and regenerative features.
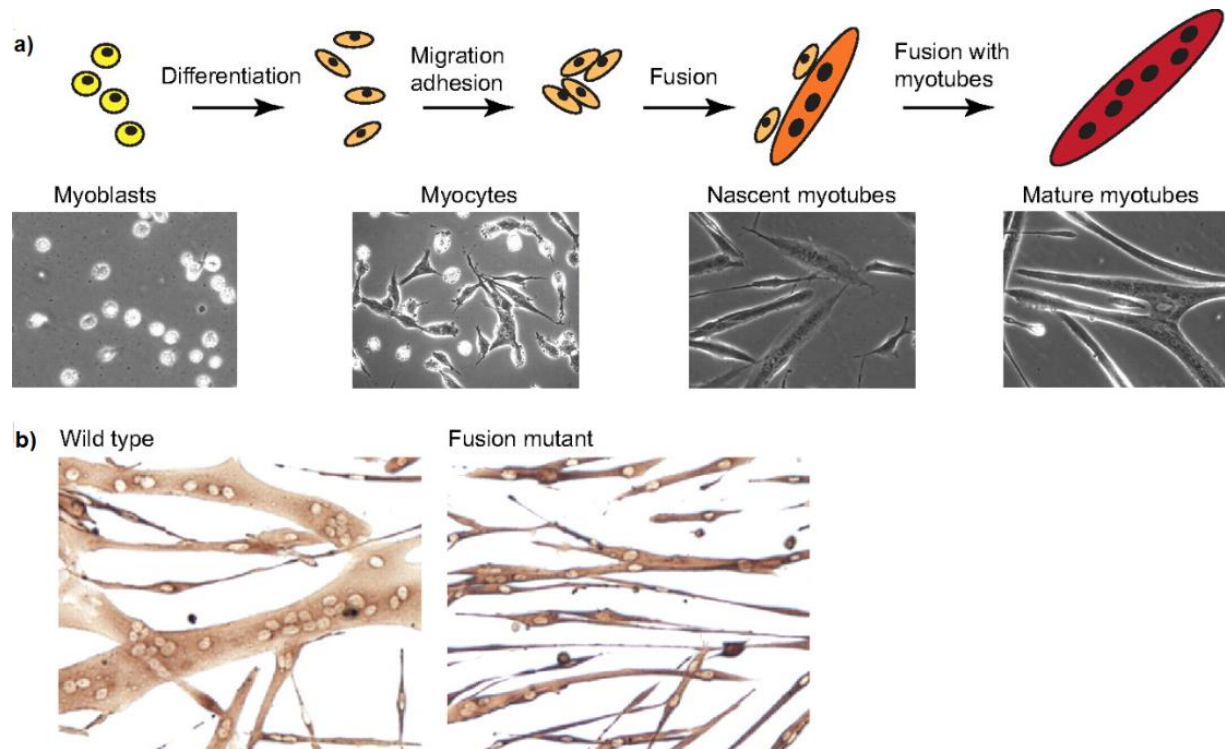

Fig. 3: Myogenesis. A) The development of skeletal muscle tissue begins when myoblasts align and fuse to make longer multinucleated myotubes. Myotubes continue differentiating and synthesize proteins to form functional myofilaments, where the nuclei are displaced against the sarcolemma. Part of the myoblast population does not fuse and differentiate but remains as a satellite cell located on an external surface of muscle fibers. Satellite cells proliferate and produce new muscle fibers following muscle injury. B) Example of mouse-tissue derived myotubes (Abmayr and Pavlath 2012)

Satellite cells have been until now successfully isolated and characterized from the skeletal muscle tissue of cattle (Dodson et al. 1987), chickens (Yablonka-Reuveni et al. 1987), fish (Powell et al. 1989), lambs (Dodson et al. 1986), pigs (Blanton et al. 1999; Wilschut et al. 2008) and turkeys (McFarland et al. 1991). Cells from respective animal species' have their benefits and limitations. Thus satellite cells isolated from different muscles have different capabilities to proliferate, differentiate, or be regulated by growth modifiers that mimic the extracellular matrix (ECM) environment encircling muscle cells (e.g., proteoglycans, growth factors, steroid hormones, etc.) (Burton et al. 2000; Datar and Betti 2010).

3.2.1 Adult stem cells

Myosatellite or satellite cells are one of the representatives of an adult stem cell type with multilineage potential. Adult stem cells, also known as progenitor cells, are preferred sources for cultured meat production, independent of their *in vivo* origin. They have been obtained from pigs (Kues et al. 2005; Zeng et al. 2006) and cattle (Kook et al. 2006). Alas, at present, the proliferative ability of adult stem cells does not match that of embryonic stem cells, mainly because they tend to differentiate spontaneously *in vitro* (Arshad et al. 2017; Langelaan et al. 2010).

3.2.2 Adipose tissue-derived adult stem cells

Another cell type, relevant to *in vitro* meat engineering, are adipose tissue-derived adult stem cells. As the name suggests, this unique population of multipotent cells is located in adipose tissue. These highly expandable cells can be relatively non-invasively obtained from subcutaneous fat, and further transdifferentiated to myogenic, osteogenic, chondrogenic, or adipogenic cell lineages (Kim et al. 2006). The most significant worry regarding their usage remains their proneness to the malignant transformation in long-term cultures. Adipose tissue-derived adult stem cells can be immortalized at high frequency and can undergo a rapid transformation in long-term culturing; however, to-date, researchers have been unable to reproduce some of the reported spontaneous transformation events (Rubio et al. 2005). For *in vitro* meat production, re-harvesting of adult stem cells might be necessary to minimize the risk of spontaneous transformation. Thus, harvesting adipose tissue-derived adult stem cells from subcutaneous fat is far less invasive than collecting satellite cells from muscle tissue via biopsy. More importantly, from the ethical point of view, these samples can be obtained from certain animals without causing considerable harm (Datar and Betti 2010).

3.2.3 Mesenchymal stem cells

Another cell type being deemed ever more usable for skeletal muscle tissue engineering is mesenchymal stem cells (MSCs). These are multipotent cells, which possess the ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Also, MSCs can undergo myogenic differentiation, provoking the formation of myotubes that can contribute to muscle growth. It has been reported that this cell source can be differentiated towards the myogenic lineage by expressing muscle-specific markers, regardless of their limited myogenic potential. However, it is still unclear how reproducible the transformation of MSCs into skeletal myoblasts is, especially having in mind their multipotency combined with the uncertainty of collecting them from different sources such as bone marrow, adipose tissue, synovial membrane, and umbilical cord blood (Qazi et al. 2015; Witt et al. 2017). One of the main disadvantages of using MSCs is the decline in their regenerative properties over time, which is further compromised with *in vitro* expansion (Qazi et al. 2015). Although myogenic differentiation of MSCs alone might not be satisfactory, they can still represent a promising cell source for co-cultivation with myoblasts; they can fuse myoblasts, and therefore play a part in the muscle regeneration process. Moreover, it is known that MSCs secrete several growth factors involved in the muscle regeneration process, as well as stimulate myoblast migration, proliferation, differentiation, and cell survival upon co-cultivation (Witt et al. 2017).

3.3 Co-cultures

The simplest approach for the production of a cultured meat system is to use a single myogenic cell line from an animal, or co-culture it with other cells that are beneficial for the whole system. Once primary cells are differentiated into myoblasts, these cells, amongst producing some components of the extracellular matrix (ECM) are specialized in synthesizing contractile proteins. The ECM is the non-cellular part present within all tissues and organs which fills the space around the cells, and it is arranged in a unique 3D organization. Its precise composition and structure vary from tissue to tissue, depending on its particular functional need. Therefore, the ECM not only provides a mere structural scaffold for cells, but it is also one of the key regulators of cellular activities. Its active role involves modulation of many cellular functions in different ways. Moreover, through varying the degree of stiffness of the matrix components, it acts as a mechanical stimulator and thus directly influences cell differentiation (Fuoco et al. 2016).

To engineer functional skeletal muscle, it is necessary to combine different cell types. This is experimentally and technically challenging because the number of culture parameters that need to be carefully considered exponentially increases with an increasing number of different cell types. Furthermore, cell types are very specific in their nutritional and stimuli needs (Post and Hocquette 2017). Co-culturing myoblasts with another (lone) cell population (*e.g.,* fibroblasts, mesenchymal stromal or endothelial cells) has been shown to influence myoblast differentiation and proliferation capability, as well as alignment. Within a muscle, the main "factory" of EMC is fibroblasts. One of the concerns regarding co-culturing fibroblasts and myoblasts involves the risk of fibroblasts overgrowing the myoblasts, due to the difference in growth rate (Benjaminson et al. 2002; Cerino et al. 2016; Langelaan et al. 2010). Previous studies in monolayer cultures revealed that stromal cells positively impact the

expansion of myoblasts; an enhanced proliferation of myoblasts was present in the first 24 h of contact co-culture and then gradually decreased and became negligible after 72 h (Sassoli et al. 2012). These findings in two dimensional (2D) co-cultures have been partially reproduced in 3D systems. Co-culture with stromal vascular fraction (SVF) cells (i.e., non-expanded MSCs from adipose tissue) promoted proliferation and differentiation of myoblasts, resulting in muscle-like constructs. Besides, SVF cells deposited an increased amount of ECM and formed organized endothelial cell-structures (Cerino et al. 2016).

The skeletal muscle has an abundant blood vessel supply, so one of the major challenges in engineering thick, complex tissues like muscle, is to vascularize the construct *in vitro*. The introduction of vascular networks *in vitro* could aid in maintaining cell viability during tissue growth and induce structural organization. Levenberg et al. hypothesized that embryonic endothelial cells in the appropriate 3D environment could be employed to promote the formation of endothelial vessel networks *in vitro* engineered skeletal muscle tissue (Levenberg et al. 2005). When both myoblasts and endothelial cells were cultured on scaffolds, the endothelial cells organized into tubular structures amid myoblasts and throughout the structure, assembling vessel networks within the cultured muscle tissue *in vitro*. The inclusion of embryonic fibroblasts under appropriate culture conditions into the mentioned co-cultures strongly advanced vascularization of the engineered muscle; their addition promoted stabilization of vessel organization over time. Their study highlights the importance of multicell cultures in providing relevant signals for vascular structure development in skeletal muscle tissue. Furthermore, co-cultures with endothelial cells may also be important to prompt differentiation of engineered tissues, because embryonic endothelial cells are pivotal for the earliest stages of organogenesis of muscle tissue (Levenberg et al. 2005). Namely, the formation of microvascular networks is essential to generate suitable conditions for adipogenesis and to affect the maturation of nearby ECM secreting cells (Ben-Arye and Levenberg 2019). Recent studies have demonstrated that bovine stromal vascular cells (SVC) can promote both angiogenesis and adipogenesis *in vitro*, owing to their heterogeneity (Ben-Arye and Levenberg 2019; Ma et al. 2018). Albeit vascularization is one of the crucial factors in functional muscle tissue development, production of the cultured meat of lower cell plurality can be achieved without them, as blood vessels may be a negligible component of meat taste and texture (Ben-Arye and Levenberg 2019).

4. SCAFFOLDS

Myoblasts are attachment-dependent cells, capable of spontaneous contraction. For proliferation and differentiation of myoblasts to take place, they require an appropriate substratum or scaffolds. Scaffolds made from biocompatible materials present an effective tool to control and guide tissue development locally. Scaffolds can play an essential role in providing a suitable environment also for myogenesis; they can act as a mimicked ECM for cells and can provide desired mechanical, as well as biochemical stimuli to the cells (Rodriguez and Larkin 2018; Qazi et al. 2015). An ideal scaffold would have a large surface area for attachment and growth, be flexible to enable contractions, maximize media diffusion, and be easily removed from the meat culture. Besides, scaffold and its by-products must be non-toxic or even edible, if they are not removed after cell culturing and may be of non-animal origin (Datar and Betti 2010; Edelman et al. 2005). The best choice would be a scaffold that closely mimics *in vivo* conditions in the skeletal muscle. It has been reported that myotubes best differentiate on the substrates with tissue-like stiffness (Engler et al. 2004). Moreover, to achieve the optimal *in vivo* cell niche, a 3D muscle construct that leads to uniform cell alignment and reproducible architecture is needed. The advantage of 3D matrices with tubular pores over patterned 2D substrates is that large quantities of cells can be concentrated into a small scaffold volume and can be simultaneously stimulated to form myotubes. Furthermore, physiologically relevant 3D models permit longer culture times and more significant developmental maturation. In a 3D setting, scaffolds material mechanical properties, forces generated by cells and constructs mechanical behavior (e.g., deformation), also seem to have an impact on cell survival, organization, and differentiation (Khodabukus and Baar 2016; Langelaan et al. 2010; Qazi et al. 2015). Cutting-edge micro and nanofabrication techniques have facilitated the development of novel biomaterial substrates and 3D scaffolds that can, through their unique architecture, promote alignment and fusion of myoblasts *in vitro*. An example of such a model that is being developed in our laboratories is shown in **Fig. 4**.

Fig. 4: Schematic depiction of a possible cultured-meat scaffold design. The Red tubes represent perfusable channels enclosed with muscle fibers, layered on top of each other in parallel with interstitial layers of adipose and fibroblast cells

4.1 Myofiber alignment

Myofiber alignment is one of the specific requirements of muscle cells, and thus should be investigated in scaffold design. Native muscle fibers are either aligned along the longitudinal axis or are oriented at an angle to the axis (i.e., pennation angle). The texturized surface of scaffolds can contribute to myotube alignment. Despite numerous strategies to recreate skeletal muscle tissue, the reality in the majority of cases is that myoblasts were embedded in bulk hydrogels where they assembled in a "chaotic-like" 3D lattice (Mozetic et al. 2017). Without surface markers or patterns, myotubes cannot align. Therefore, they create a branched network. As a result, misaligned networks may generate contractile forces in opposing directions, contrary to those in native muscle fibers, reducing the overall contractility of the construct, and hence hinder the desired continuous protein production. In the absence of surface stimulation, external electrical and mechanical cues (including applied passive tension) can boost alignment. Furthermore, it has been shown that increased fiber alignment is associated with enhanced differentiation as well as upregulation of contractile proteins and the presence of advanced sarcomeric structures. Myoblast elongation and alignment are generally encouraged by utilizing a micropatterned surface (Rodriguez and Larkin 2018). Findings from various studies have shown that scaffold topology has a significant impact on the organization of aligned myofibers. By slight modifications of parameters, the degree of alignment can be altered (Chen et al. 2015). Myofiber organization dictates the functional characteristics of muscle, and therefore also the texture properties of meat (Datar and Betti 2010).

4.2 Mechanical properties

To imitate native muscle tissue mechanical characteristics, an ideal scaffold should possess similar elasticity and mechanical stiffness as its native counterpart. The muscle is an elastic tissue (Young's modulus of ~ 12 kPa) (Engler et al. 2004; Gilbert et al. 2010). Therefore a suitable scaffold elasticity is a prerequisite to resemble the contraction and relaxation abilities of native muscle fascicles. An improper elasticity can reduce force transmission, which eventually leads to motion hindering. In tissue engineering, unsuitable stiffness of a construct can cause a mechanical mismatch between the construct and native tissue, resulting in the formation of stress concentrations (Rodriguez and Larkin 2018). Mechanical loading of the tissue construct has a beneficial influence on cell alignment, elongation, proliferation, and fusion (Vandenburgh and Karlisch 1989). Additional cyclic mechanical stretching can even increase protein synthesis and hypertrophy of *in vitro* engineered muscle (Vandenburgh and Kaufman 1979). Two main characteristics govern cell phenotyping, namely elasticity and stiffness. Cells receive mechanical cues from their surrounding that can either promote or inhibit both proliferation and differentiation. Under proper conditions, such cues can be applied *in vitro* as a means to amend and accelerate myogenic differentiation (Ansari et al. 2016; Gilbert et al. 2010; Liao et al. 2008). The most straightforward and scalable format to obtain "self-anchored" muscle fiber is letting them grow in a ring around a central column of elastic material. Using this strategy, the maturation period of a muscle fiber takes about three weeks (Post 2018). As described above, Gilbert et al. showed that, when cultured on soft polyethylene glycol hydrogels with an elasticity of 12 kPa, skeletal muscle stem cells exhibit *in vivo*-like self-regenerative properties (Gilbert et al. 2010).

Another research group reported that alginate hydrogels with an elasticity between 10 and 16 kPa displayed the highest potential for myogenic differentiation as evidenced by upregulation of myogenic genes (myogenin, MyoD, and Myf5) (Ansari et al. 2016). While static mechanical stimulation is largely associated with induction of myogenic differentiation, there have been inconsistencies concerning the role of cyclic mechanical strain on signaling and myogenic marker expression (Heher et al. 2015). In early stages of myogenesis, uniaxial passive tension is superior to cyclic strain as it more closely reflects the natural situation in muscle development and growth (Heher et al. 2015; Vandenburgh and Karlisch 1989). However, cyclic tension might play a role as an upregulatory stimulus for muscle hypertrophy and maturation of myotubes at later stages of myogenesis (Moon du et al. 2008; Powell et al. 2002). The continuous static strain on skeletal muscle, caused by bone growth and elongation during embryogenesis and neonatal development, affects muscle weight, muscle length, and myofilament organization. Bone growth can be simulated by using ramp stretch. The mixed outcomes (with beneficial (Vandenburgh et al. 1991) and negative effects (Boonen et al. 2010) of combined stretch protocols from various studies are likely linked to the amount of stretch that the myotubes undergo (Khodabukus and Baar 2016).

4.2.1 Electrical stimulation (contraction)

Neural stimulation (i.e., regular contraction) presents an essential environmental signalization during embryogenesis in adult muscle since it promotes differentiation and healthy myofiber morphology. In a prolonged absence of it, muscle is subjected to atrophy (Datar and Betti 2010; Gielen et al. 1984). Muscles *in vivo* are innervated, which enables regular and controlled contraction. It was found that subjecting scaffolds to mechanical stretching can fulfill the requirement of providing contraction. However, this approach is less effective than electrical stimulation for optimal skeletal muscle development (Datar and Betti 2010). Incorporation of external electrical cues induces contraction internally, and under *in vitro* culture conditions, can be used to simulate part of the *in vivo* niche in the muscle (Boonen et al. 2011). Living cells create electrical forces in the form of membrane (i.e., action) potentials. Studies have shown that aside from the impact on muscle cell phenotype (Dusterhoft and Pette 1990), myosin expression (Naumann and Pette 1994; Wehrle et al. 1994) and contractile sarcomere assembly (Fujita et al. 2007), electrical stimulation can also modulate fiber type switch (Pette et al. 2002), as well as induce contractility in differentiated myotubes (Kaji et al. 2010). By incorporating electroactive materials directly into scaffolds, an alternative strategy can be used to imitate the natural cellular microenvironment. Inclusion of electrically conductive polymers (e.g., polyaniline, polypyrrole, polythiophene) (Breukers et al. 2010; Goldspink et al. 1995; Rangarajan et al. 2014), coupled with externally applied electrical stimuli, can promote tissue development (as evident by increased alignment and differentiation) on account of the delivery of electrical signals to cells via conductive polymers. According to literature, external electrical cues can trigger the action potential in *in vitro* cultures due to the difference in electrical resistance between culture media and intracellular fluid (Yamasaki et al. 2009). Consequently, calcium ions are released from the sarcoplasmic reticulum, and myotube contraction is triggered. On the contrary, when the applied electrical forces are too strong, they can cause membrane damage, decrease force generation, and prevent the increase in force generation after electrical induced contraction (Khodabukus and Baar 2016). Boonen et al. have observed a complex interaction between electrical stimulation, surface protein coating, and substrate stiffness (Boonen et al. 2009). The response of muscle progenitor cells to electrical stimulation was most efficient on substrates with an elasticity of 21 kPa, which is close to the physiological stiffness of differentiated myoblasts (Boonen et al. 2009; Collinsworth et al. 2002). In another study, they showed that electrical induced contractions of cells on different coatings could activate different pathways of maturation (Boonen et al. 2011). These effects were also reproduced in a 3D culture system, in which myotube maturation was even faster (Langelaan et al. 2011). The authors confirmed the advantages of cell cultures grown in a 3D construct through histological analysis, which showed that the myotubes were organized in the direction of stress. Uniform alignment of myotubes is important for the final construction of engineered muscle tissue, as it supports maximal force generation upon contraction. However, the strength and duration of the electric stimulus require careful fine-tuning to result in active contractions of the myotubes (Langelaan et al. 2011). Electrical stimulation has been successfully applied for the expansion of myogenic progenitor cells in 3D scaffolds without loss of myogenic potential. This is an useful method, especially for expanding satellite cells, which are known for their loss of regenerative potential after expansion in "standard" cell cultures (Serena et al. 2008).

5. CULTURE CONDITIONS AND GROWTH MEDIA COMPOSITION

Despite recent advances in tissue engineering and consequent rapid development of novel engineered muscle models, with each new model utilizing a diverse range of culture conditions, there is still no established "gold standard" for growing muscle cells *in vitro* (Khodabukus and Baar 2016). In the process of building engineered muscle tissue, which could be used in regenerative medicine and cultured meat production, it is necessary to develop a native-like tissue architecture that possesses the ability of active force generation. Regardless of the efforts and breakthroughs in this field, it remains a challenge to overcome muscle cell's inability to fully mature within engineered muscle constructs. Biochemical cues have the main role in the initial differentiation process, whereas (bio)physical stimuli have been proven to be pivotal in maturation towards fully functional and mature engineered tissue (Langelaan et al. 2010). Moreover, to replicate the biological processes of the muscle tissue formation at an industrial scale raises several technical issues (Hocquette 2016; Warner 2019). Due to the complexity of the development of a mature muscle fiber, the step-wise production of a meat tissue will encompass the critical decision points (Warner 2019).

5.1 "Base" growth media

To compete with the conventional meat industry, large-scale production of cultured meat would need to employ a low-cost media system. Aside from affordability, such media should contain essential nutrients, which are readily available (mostly through diffusion) for the cultured myoblasts and associated cells. Refinements in the composition of commercially available cell culture media have improved the cultivation of various types of animal cells (Edelman et al. 2005). For culturing mammalian cells *in vitro* on an industrial scale, serum-free media is a more realistic option, because it reduces both operating costs and process variability while lessening the potential source of infectious agents. Serum-free media have been developed for culturing satellite cells from turkey, sheep, and pig. Variations among different serum-free media outline the fact that satellite cells, derived from different animal origins, have different requirements, and also respond variously to certain additives (Dodson et al. 1996). The animal serum is usually added to primary media formulations to supply an undefined assortment of growth factors, hormones, and other additives that promote cell survival, proliferation, and differentiation. The proportion of serum supplementation is one of the key parameters to induce myogenesis (Goto et al. 1999) as it guides skeletal myoblasts either towards proliferation or differentiation. In general, fetal bovine serum (FBS) is added in high levels (10-20 % of final concentration) into media to encourage proliferation (Arshad et al. 2017; Cerino et al. 2016; Khodabukus and Baar 2016). Based on previous studies, myogenic differentiation can be achieved *in vitro* by using lower levels (2-10 %) of serum content in culture media (Cheng et al. 2014; Cerino et al. 2016; Khodabukus and Baar 2016; Lassar et al. 1994; Lawson and Purslow 2000). Reduced concentrations of serum are needed for cell cycle withdrawal, which is a key factor for the onset of differentiation (Lassar et al. 1994). When using animal-derived serums, lot-to-lot variations and serum origin need to be considered as both were shown to significantly affect spontaneous contractility and force production (up to 3-fold), as well as influence growing muscle cell phenotype (Khodabukus and Baar 2014). To develop commercially available serum substitutes that will replace FBS, some research groups have already designed animal-free serums. In their attempt to create an *in vitro* and edible muscle protein production system, Benjaminson et al. noticed that mushroom extracts were comparable to animal-derived serum in promoting explant surface area expansion (Benjaminson et al. 2002). Aside from chitin, cellulose, and melanin, mushrooms yield high-quality amino acids that can be applied as a rich serum supplement for an *in vitro* meat production. Plant-derived serum surrogates seem to be the ideal solution. However, their potential allergenicity should be considered (Datar and Betti 2010).

5.2 Antibiotics

Extensive use of antibiotics in traditional livestock farming is an important risk factor for cultivating resistant bacteria (e.g., *Salmonella*, *Campylobacter*, *Listeria,* and *Escherichia coli*), which could be eliminated in cell culture conditions in a strictly controlled industrial environment. In this context, it is expected that cultured meat production will reduce the incidence of animal-borne infections (i.e., zoonosis) and other emerging diseases (Datar and Betti 2010). In particular, the high degree of environmental control will likely prevent problems associated with foodborne diseases by reducing the risk of foodborne pathogens and consequently improve food safety (Ben-

Arye and Levenberg 2019; Warner 2019). In general, antibiotics are added into growth media as prophylaxis to prevent microbial contamination of cultured cells. Typically, used antibiotics in cell culture are penicillin and streptomycin. Streptomycin has a negative impact on skeletal muscle culture; it decreases protein synthesis and developmental maturation of myotubes due to inhibition of spontaneous contractility. In static cultured muscle tissue, the addition of streptomycin resulted in a 3- to a 4-fold reduction of force generation and a decline in frequency and intensity of spontaneous contractions (Khodabukus and Baar 2015). Based on these trends, it has been advised to eliminate streptomycin from culture media to minimize variability and maximize maturation and functionality of *in vitro* skeletal muscle, and rather increase the concentration of penicillin instead (to 100 IU/ml) (Khodabukus and Baar 2016). As an alternative, sodium benzoate could be added to growth media to protect cells from yeast and fungus infection (Warner 2019). Sodium benzoate does not pose an additional risk to consumer health since it is generally used as a preservative in processed meat products (Hoang and Vu 2016). Even though cultured meat is produced under sterile culture conditions with the possible use of antibiotic-free media, it is mandatory to investigate the magnitude of their use in-depth. Promoters of cultured meat production advocate that the process can be achieved with a notable lower level of antibiotics compared to current use in the conventional meat industry (Ben-Arye and Levenberg 2019; Specht et al. 2018) or even without their use (Van Eelen 2007; Warner 2019). At this point, providing the required sterile environment during production represents a great financial burden and is very difficult on a larger scale. Since the sterile environment can be difficult to conserve long term at lab level, there is a liability for the addition of chemicals and antimicrobials in the production process. However, it remains unknown whether antibiotics, antimicrobials, or chemicals will be routinely or occasionally requisite during muscle cell culture. On the contrary, the benefits of establishing the cultured meat production will reduce the exposure to noxious chemicals (e.g. pesticides and fungicides) in traditional animal-agriculture industry (Warner 2019).

5.3 Regulatory biological/growth factors

Stimulation with different supplements, such as growth factors, potentiates myoblast proliferation and differentiation capacity. To ensure high rates of cell growth, the formulation of the growth media needs to contain not only the appropriate level of nutrients but also the appropriate level of the myogenic regulatory growth factors and hormones (Warner 2019). In addition to promoting myoblast proliferation and differentiation, different members of the transforming growth factor-β (TGF-β)superfamily, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) also stimulate migration and cell survival upon co-cultivation (Maley et al. 1995; Witt et al. 2017). Both TGF-β and bFGF reduce myoblast recruitment and differentiation (under independent mechanisms) by increasing myoblast proliferation, with the difference being that bFGF is more stimulatory in its action (Guthridge et al. 1992; Langelaan et al. 2010). Myostatin, a member of the TGF-β superfamily that is highly expressed in skeletal muscle, is a negative regulator of skeletal muscle growth. Loss of myostatin function is associated with an increase in muscle mass in mice, cows, and humans (Ben-Arye and Levenberg 2019; Elkasrawy and Hamrick 2010; Ismaeel et al. 2019; Kim and Lee 2017). Similar action is prescribed to TGF-β1, another member of the TGF-β superfamily, which inhibits the proliferation of myogenic cells (Kamanga-Sollo et al. 2005). The isoform TGF-β1 promotes ECM preservation by enhancing matrix protein synthesis and, at the same time, suppressing the activity of ECM degradation proteins, resulting in an increased overall quantity of the ECM (i.e., connective tissue) in muscle cross-section (Ismaeel et al. 2019). In the case of the bFGF role, Guthridge et al. (Guthridge et al. 1992) proposed a model whereby intracellularly produced bFGFs regulate myogenesis by autocrine and paracrine action. The complex mechanisms involving both paracrine and autocrine regulation dictate the proliferation capability of myoblasts. A bFGF positive-feedback loop, which is initiated by an exogenous factor, maintains myoblast proliferation. Disruption of the auto-stimulatory loop by inhibition of FGF synthesis or by inhibition of FGF export leads to the terminal differentiation of myoblasts (Guthridge et al. 1992). In other words, when a sufficient level of bFGF is present in growth media surrounding cells, most myoblast will proliferate. When the growth factor in growth media is depleted, the myoblasts cease division and fuse into multinucleated myotubes. It is well known that HGF primarily induces the proliferation of satellite cells by binding to the c-met tyrosine receptor and stimulates different downstream targets. The majority of circulating IGF-1 is bound to specific IGF-binding proteins (IGFBPs), and its function, among many others, is to activate proliferation as well as differentiation of satellite cells. Although stimulation with HGF and IGF-1

induces upregulation of different myogenic markers, it was discovered by Witt and colleagues that it is probably not essential for myogenic differentiation. IGFBPs participate during myogenic differentiation, differing amongst culture and stimulation conditions (Witt et al. 2017). It is important to note that the specific selection of suitable extrinsic regulatory factors must be based according to the chosen cell type and animal species, as they respond differently to the same regulatory factors (Burton et al. 2000). Besides, increasing the number of cells (i.e., proliferation) is feasible only at the satellite cell stage and the myoblasts stage (Warner 2019). Regarding a serum supplement ratio in growth media, it is very likely that the combination of growth factors should be changed over the course of culturing; one particular combination of growth factors may be beneficial for the proliferation period, whereas the differentiation and maturation period may require a different mixture (Datar and Betti 2010).

6. BIOREACTORS

Animal cell culture has its roots in cell-based therapy, which has become viable on a commercial level and thus provides important guidelines for the transfer of cultured meat technologies to the industrial scale (Specht et al. 2018). It is generally believed that a sufficiently advanced tissue bioreactor system will enable the development, growth, and maintenance of mature living muscle (Dennis et al. 2009). For the most part, some basic functions are common to many tissue engineering bioreactors: (1) maintaining an aseptic environment, (2) controlling environmental parameters such as temperature and pH, (3) controlling nutrient delivery, and (4) offering controlled experimental interventions, including online sensors. In addition, a system intended for developing skeletal muscle tissue generally requires mechanical and/or electrical stimuli that mimic the *in vivo* environment. However, the complete process from single-cell proliferation and differentiation to the maturation of large and well defined structured cuts of meat probably requires several stages taking place under different conditions. Thus, the construction of a technological blueprint for a muscle bioreactor system is a complex engineering endeavor (Allan et al. 2019; Dennis et al. 2009), let alone the scaling up to an industrial level, which will require substantial effort to achieve sustainable and cost-effective large-scale production of cultured meat (Bodiou et al. 2020; Warner 2019; Zhang et al. 2020). Also, decisions related to the type, size, and the number of bioreactor units are influenced by several factors, such as seeding density, final cell number or density, and passaging steps. The obtained cell density will differ for suspension systems that use microcarriers for anchorage-dependent cells compared to single-cell suspension systems. Importantly, when designing a bioreactor, engineers should note the fact that the "working volume" of a bioreactor does not dictate the process of media requirements. Due to cell metabolic requirements over the entire duration of the culture period, the media requirements will be much greater than the bioreactor working volumes (Allan et al. 2019).

6.1 Bioreactor system

Companies currently working on scalable cultured meat products do not disclose information about their bioprocess design. However, a theoretical model of a bioreactor system, which could fulfill all of the criteria mentioned above, was presented in the work of (Specht et al. 2018), as shown in **Fig 5**. In essence, tissue development would take place in two stages: a) for cell proliferation (*e.g.*, in a stirred tank bioreactor) to provide a sufficient amount and density of cells, as well as b) for tissue development (*e.g.,* on a porous scaffold in a perfusion bioreactor) where a structured and large scale cut of meat would grow and mature (Post 2012; Specht et al. 2018). Ideally, both stages should allow precise control over the bio-physicochemical parameters of the culture, continuous production, and efficient medium recycling. Although the production of cultured meat from self-replicating satellite cells derived from a biopsy is a simple concept, one of the main problems to overcome is its scalability (Bodiou et al. 2020; Warner 2019). Satellite cells are anchorage-dependent, meaning that they need a surface for attachment, as well have to be expanded *in vitro*, to obtain large cell numbers. When the required cell density is reached, the differentiation process is initiated, resulting in the formation of myotubes and the expression of proteins characteristic to functional myocytes (Bodiou et al. 2020).
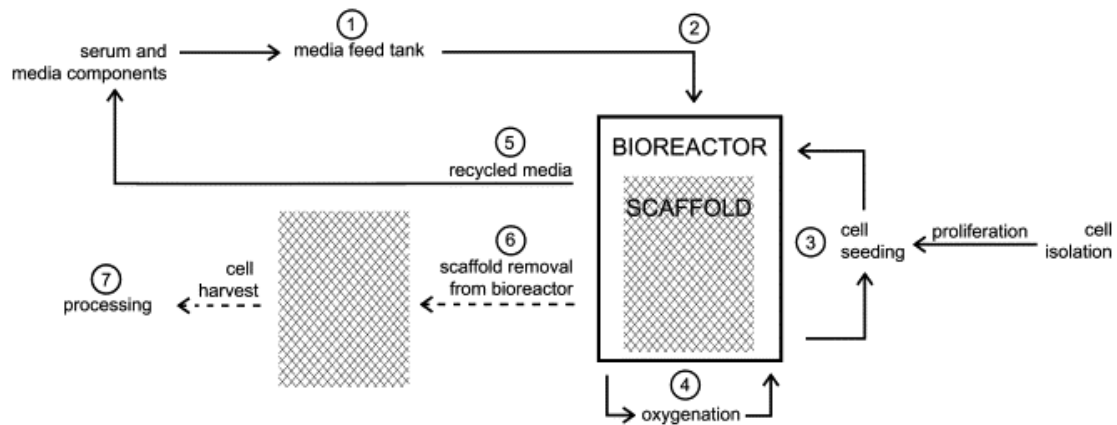
Fig. 5: Overview of a possible scaffold-based preparation of cultured meat. It starts with the isolation of myosatellite cells from muscle and their proliferation. The sequence of the following steps includes: 1) A formulated media is kept in the feed tank before being deposited 2) into a bioreactor with a scaffolding system. Media perfusion enables expanded cells to be seeded 3) onto a scaffold within a bioreactor. During the culturing process, media is being constantly replenished and oxygenated in an external fluid loop 4). After the culturing process is completed, media can be recycled 5), while the scaffold and cultured tissue are removed from the bioreactor system 6). Further processing includes tissue removal from the scaffold (Datar and Betti 2010)

6.1.1 Proliferation and differentiation stage

Present conventional planar cell culture systems have notable limitations related to their low surface-to-volume ratio, the lack of pH, gas, and metabolite concentration control, offering to produce up to 10^{11} cells (Bodiou et al. 2020; Rowley et al. 2012). To meet the demands for large-scale production, generation of a significantly higher amount of cells (in comparison 10^{12} - 10^{13} cells, correspond approximately to $10 – 100$ kg of meat) is favored while using limited space, time, amount of resources and requiring minimal handling (Bodiou et al. 2020; Moritz et al. 2015). Stirred tank bioreactors remain the most prevalent type of bioreactors in the biotechnology industry. However, possible cell damage due to the shear forces caused by the impeller, alternatives such as airlift bioreactors is approaching the market (O'Mara et al. 2018). Due to increased production efficiency and consequently lower requirements of volume per output, the biopharmaceutical sector has recently also begun adopting single-use bioreactors (SUBs), which eliminate the need for cleaning and validation, while preventing cross-contamination (O'Mara et al. 2018). With the advent of smaller culture vessels, new approaches to agitation can be deployed. Thus SUBs can be adapted to several stirring mechanisms, such as orbitally shaken or wave bioreactors(O'Mara et al. 2018). The described approaches provide new possibilities for process optimization in the experimental phase of cellular agriculture. For continuous production on an industrial level perpetual supply of nutrients, and biological regulators (e.g., growth factors, hormones), as well as a supply of oxygen and removal of waste products, is integral in the culture media (Warner 2019). Therefore, the development of cultured meat can be seen as a finite-state machine (**Fig. 5**), meaning that every development stage is a stable condition that depends on its previous condition and the present values of its inputs. At each development stage, the growing muscle tissue (i.e., meat) will require a variety of inputs to reach the next level of development. Important development stages encompass (1) terminal differentiation of myoblasts to myocytes, (2) fusion of myoblasts to form primary (weak and low excitability) myotubes, (3) growth of secondary (stronger, more excitable) myotubes, and (4) expression of adult muscle proteins, and (5) formation of the target product (Dennis et al. 2009). Alongside the units for proliferation and differentiation, a muscle bioreactor system should incorporate additional components such as media storage tanks, media heat exchangers, and suitable controllers to maintain a constant temperature within the system. In addition, an advanced bioreactor should also include units for automatic and frequent monitoring of muscle tissue development without affecting it, and thus aiding in assessing suitable quantity and duration of applied stimuli to the tissue in real-time (Allan et al. 2019; Dennis et al. 2009).

6.1.2 Maturation stage – final product development

At the development stage of the final product, the bioreactor system should provide conditions for maturation and maintaining the engineered muscle tissue. To facilitate the development of a large and structured 'cut of meat,' scaffolds are likely to be included in the process to provide the cells with a well-defined 3D growth substrate, along with which the tissue will develop. Successful nutrient supply and removal of waste products inside large 3D structures is currently a major limiting factor in the scalability of cultured meat. Within the scope of realizing a highly structured product that mimics large cuts of meat (e.g., steak, chops, tenderloin), several research groups endorse the use of a perfusion-based bioreactor system with a 3D engineered tissue construct as the most suitable technique for the production of cultured meat (Zhang et al. 2020). To enable continued growth and long-term sustainability of viable 3D engineered tissue constructs, homogeneous and adequate oxygen supply is paramount to avoid a necrotic nucleus. Albeit, owing to the microporous structure of the scaffold material, media flow can only be laminar, restricting the heat and mass transfer efficiency. To maintain the optimal, replenishing rate across scales, it is vital that the flow rate increases linearly with the scale of the structure. This, in turn, contributes to high shear stress and a considerable pressure drop. To subdue such problems typical approach encompasses enlarging the pore size of 3D engineered construct to improve permeability (Zhang et al. 2020). However, this could limit control over the texture and consistency of the final product. Various approaches to sustaining continuous medium perfusion through the scaffold have been proposed, including stirred tank, rotating vessel, or direct perfusion bioreactors (Fig. 6). While a high mass transfer of medium flow is required, it is accompanied by a significant increase in shear stress, therefore determining a suitable flow rate is critical for successful and effective growth (Datar and Betti 2010; Martin et al. 2004). Direct perfusion bioreactors provide This type of bioreactor is mainly intended for scaffold-based myocyte cultivation and high-density, uniform myocyte seeding (Radisic et al. 2003). Cerino et al., 2016 demonstrated a one-step (proliferation and maturation) platform by the use of SVF co-culture and a 3D perfusion bioreactor, creating a complex biological model for engineering functional 3D uniform muscle tissue that resembles its native multi-cellular environment (Cerino et al. 2016). However, at this point, the proposed system is only suitable for small sample sizes with limited scalability. Despite the accomplishments in tissue engineering, in its current form, such a perfusion-based bioprocess system is unsuitable for upscaling the cultured meat production (Zhang et al. 2020). To ensure controlled tissue production, monitoring of its development is crucial. Biomarkers that can be used to evaluate the developmental stage of the muscle harmlessly are contractility (force and rate of contraction and relaxation), excitability (energy required to achieve stimulation), and metabolism, which can be, among other methods, determined by oxygen consumption (Dennis et al. 2009). Besides, several technologies for in-line, real-time monitoring of systems performance, culture conditions, and cell viability have been validated (Zhao et al. 2015), alongside with Raman-based strategies for *in situ* monitoring of chemical composition (Mehdizadeh et al. 2015). For all bioprocessing systems, three main sensor parameters, in particular pH, dissolved oxygen, and temperature, are prerequisites. Cell culture media normally contains buffer agents and sodium bicarbonate to maintain pH values within optimal working conditions. The optimal pH of cell media alters throughout the bioprocess; therefore, it must be carefully monitored at each stage of the process. Namely, even a (rather) small change of 0.1 pH units from the optimum can greatly affect cell viability and concentration (O'Mara et al. 2018). Dissolved oxygen is another critical parameter that must be closely monitored and optimized. This is crucial for the survival of aerobic cell types. However, high levels of dissolved oxygen levels can lead to the production of super-oxides or peroxides, which have a damaging effect on the cell membranes or even cause DNA breakdown. To ensure optimal cell viabilities and high product yields during bioprocessing, the temperature inside a bioreactor must be accurately measured. For the production of mammalian cells, the optimal temperature has been widely established for several years to be around 37 °C (like body temperature). Though recent studies demonstrated that at lower temperatures, in the range of 30-35 °C, the high production of some proteins can be achieved. Thus, depending on the desired product, the temperature sensors must operate accurately in the range of 30-40 °C as the process temperature will vary over time. Moreover, they must be highly sensitive in temperature variations to prevent loss in cell viability. The bioreactor's monitoring system can be further implemented for continuous measurements of the substrate and product concentrations, (viable) cell density, and metabolites (O'Mara et al. 2018).
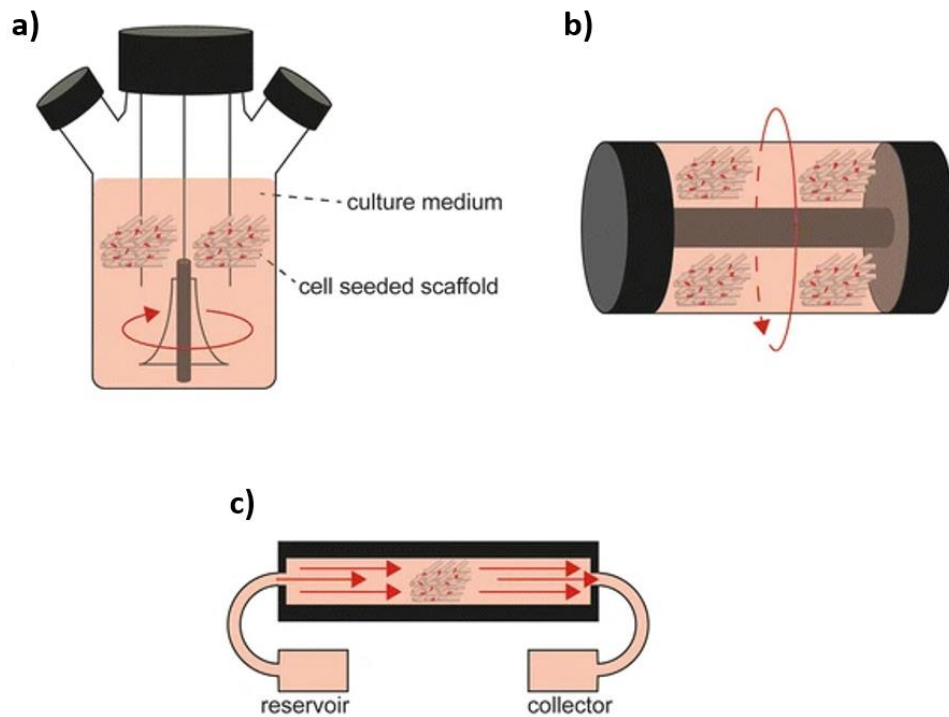
Fig. 6: Commonly used bioreactors for the production of 3D tissue-engineered constructs: a) spinner flask, b) rotating wall vessel, and c) perfusion bioreactor (Pirosa et al. 2018)

6.2 Microcarriers

Owing to a large surface-to-volume ratio, microcarriers (MCs) are the most promising candidates for large-scale implementation. The suspended MCs provide a 3D environment, but the cells still grow on a 2D surface, although the strong curvature of the bead surface affects cell attachment and proliferation. Since the microenvironment of the cells remains unchanged, the translation from the traditional monolayer culture to a suspension one is smoother. Moreover, depending on their buoyancy and density, they can be used in various types of vessels (e.g., stirred-tank, fluidized bed, packed bed, bubble column bioreactor) that are generally employed for scaling-up chemical and bioprocesses. As opposed to fixed-bed bioreactor cultures, such as hollow fiber or microplate, MC-based bioprocesses are easier to control and monitor, resulting in the quality and consistency of the products. A significant advantage of MCs is that the cell growth surface can be increased simply by adding new MCs to the culture. This is feasible due to a phenomenon referred as "bead to bead transfer," which describes the cell migration from bead to bead, and the population of newly added MCs (Bodiou et al. 2020; Hervy et al. 2014; Kong et al. 1998; Ohlson et al. 1994; Rafiq et al. 2013; Rafiq et al. 2018; Verbruggen et al. 2018; Zhang et al. 2020). Introducing a new surface area into satellite cell culture also is beneficial to avoid aggregation of MCs (Bodiou et al. 2020; Verbruggen et al. 2018). The use of MCs has long been instituted in the cell-based therapy industry and vaccine production for expansion of cells producing a molecule of interest (e.g., monoclonal antibodies, proteins, vaccines) (Phillips et al. 2008; Zhang et al. 2020). Verbruggen and colleagues (Verbruggen et al. 2018) provided a proof-of-concept experimental work of using MCs-based bioprocess for cultured beef production. The materials for such MCs should comply with food regulations (following good manufacturing practice, GMP, and hazard and critical points, HACCP, standards). MCs can be designed to serve as a temporary substrate for cell attachment and proliferation, or they can be embedded in the final product, and ergo need to be edible. Using edible materials can obviate the need for dissociation/degradation/separation steps and limit the risk of non-edible residues. Besides, they can be even tailored to enhance the sensorial and nutritional qualities if embedded in the final product. Towards that end, abundant, edible polymers, such as alginates, pectins, chitosan, that are prevalent in the food industry as stabilizers, thickeners, coatings, and emulsifiers (Ahmadi et al. 2015; Bodiou et al. 2020; Shit and Shah 2014), seems to be promising candidates for upscaling the process.

6.3 Oxygen carriers

Cell viability and density positively correlate with oxygen gradient in statically grown tissue cultures (Radisic et al. 2008). To overcome limitations in oxygen diffusion in tissue cultures and to maintain the high oxygen concentration in the bioreactor, oxygen carriers can be added into growth media. They can be divided into two varieties: hemoglobin-based oxygen carriers (HBOCs), which are mostly modified hemoglobin versions, and chemically inert, artificially produced perfluorocarbons (PFCs). Even though PFCs dissolve large amounts of oxygen and can perform the same functions as hemoglobin, they are extremely potent greenhouse gases on a per molecule basis (Datar and Betti 2010).

6.4 Recycling

Technical experiences from the established biotechnology industries (e.g., brewery, pharmaceutical, and recombinant/therapeutic protein industries, etc.) pose good parallels for upscaling the bioprocess of cultured meat manufacture. Downstream units require recycling operation with cell debris removal, media refinement, cell harvesting, and product formulation (Allan et al. 2019). The management of metabolic waste by recycling (as well as by disposal or upgrading) must satisfy the requirements of the HACCAP procedure (Warner 2019). If recycling is put into operation, the emissions from excess nutrients in the wastewater could be comparable with, or lower than, a poultry operation (Mattick et al. 2015; Warner 2019). One approach for media refinement is through the replenishment of utilized nutrients (e.g., glucose and glutamine) and removal of waste by-products such as lactate and ammonia, which, even in small quantities, are known to inhibit cell growth. One strategy for media replenishment is by perfusion mode, in which fresh media is continuously pumped at a given perfusion rate together with the removal of used media at the same rate. At the same time, the cells are retained in the bioreactor. This implantation of continuous perfusion processes has gained prevalence in the biopharmaceutical industry for large-scale production, as constant media refinement and removal of toxic by-products ensure high productivity and product quality. Moreover, for industry related to cell therapy, maintaining the concentration of essential nutrients and metabolic products at optimal levels may be crucial in the regulation of cell growth, differentiation, and other vital attributes (Abraham et al. 2017). It might be favorable to reuse a part of supplements in growth media (e.g., growth factors, cytokines) that were either added or produced by cells and therefore stimulate further cell growth (Moritz et al. 2015). Nath and colleagues (Nath et al. 2017) established a culture media refinement method using dialysis for the expansion of human-induced pluripotent stem cells (hiPSCs) in suspension culture. By exchanging fresh media only once, following the refinement of media with the proposed dialysis system, they removed toxic metabolites. At the same time, essential macromolecules of media (e.g., growth and autocrine factors) were recycled efficiently (Nath et al. 2017). Recovery of purified water is also feasible by using downstream separation units that comprise one or more of the following processes: membrane filtration, (electro)dialysis, precipitation, solvent extraction, and absorption systems (Allan et al. 2019).

6.5 Production and supply at the regional level

As cultured meat production is generally believed to be most feasible at an industrial level, some companies like FM Technologies propose two conceivable scenarios where cultured meat production could proceed at (1) a small business or (2) individual consumer scale (Warner 2019). FM Technologies (https://www.future-meat.com/) portrays this as a "distributive approach to sustainable manufacturing". Their proposed model system centralizes stores of stem cells, which are periodically delivered to local communities. The latter would have small-scale bioreactors with the capacity to feed small villages or regions, and a central point will give the technological know-how. Models of the distributive approach present are purely theoretical and remain to translate into practice (Warner 2019).

7. NUTRITIONAL AND SENSORIAL ASPECTS

The reasoning behind cultured meat is that "traditional" consumers expect meat, which is as readily accessible and comparable (if not superior in nutritional value) to native meat and not a meat substitute with perceived lower quality (from the start). The resemblance should not be guided only by sensory factors (e.g., color, flavor,

tenderness), but also by nutritional and health values. In other words, the biochemical and structural composition of engineered meat needs to be like the natural counterpart. The key factors that govern cell and tissue cultures on the way to becoming a desired product are culture conditions, as well as the external dynamic environment (mechanical and electrical cues). With existing technology, all these conditions can be controlled and further optimized, with a focus on flavor (taste and aroma), texture, nutritional value, and food safety (Ben-Arye and Levenberg 2019; Post and Hocquette 2017; Post 2018). However, some differences will still be present; some nutrients that are not produced by myoblasts nor adipocytes, such as vitamin B12, are absorbed from the environment (*in vivo* from blood). The requirements of these "environmental" nutrients can be easily fulfilled by supplementing the growth media. For efficiency and safety reasons, future studies will guide the selection of nutrients, which are essential for the development of cultured meat, and at the same time harmless for consumers (Allan et al. 2019). Although alternatives to farm-grown meat (i.e., plant-based protein and cultured meat) have received considerable attention within academia and the popular press, consumers' willingness to adopt cultured meat is unclear even if it tastes the same. Nevertheless, the current market is less optimistic about a lab-grown version. It is important to note that consumers' preferences are amenable, and can be changed by new information govern by marketing campaigns or social norms (Bekker et al. 2017; Slade 2018). The abundance of vegetarian meat substitutes may "prepare the ground" for acceptances of cultured meat in the form of burgers and sausages, as well as the possibility to prepare meat variations from animals that are considered as a delicacy (Slade 2018).

7.1 Protein composition

The highly complex meat composition, which includes a wide variety of proteins, carbohydrates, fatty acids, and aromatic compounds, is the main contributor to its flavor (Mottram 1998). Muscle tissue is rich in proteins as it contains up to 6500 types of proteins with an extremely wide expression range of several orders of magnitude (Ohlendieck 2011). The most abundant proteins are myosin, actin, and titin, which together make up 75 % of cytoskeletal proteins of myoblast cells and represent between 40 % and 60 % of the total amount of protein in muscle fibers (total percentage depends on the type of muscle fiber) (Murgia et al. 2015). It is hypothesized that highly aligned and tightly co-expressed myosin and actin molecules have the most significant contribution to the meat's texture. Nutritional value, taste, and mouthfeel are co-determined by the amount and composition of the most abundant proteins (e.g., actin and myosin, respectively) in myoblasts. Therefore, the muscle proteome presents the foundation for the creation of meat tissue that consumers "perceive as meat." Optimizing protein composition focuses primarily on the proteins actin and myosin. However, it is also likely that scarcer proteins, such as heme-containing proteins (e.g., hemoglobin and myoglobin) possess specific components contributing disproportionally to taste and appearance (Post 2018).

The culture conditions that will lead to these improvements are well-known in medical tissue engineering but will need to be implemented in a scalable process for meat production. Current culturing methods are suitable for creating fully mature muscle tissue with typical cross-striation that indicates the development of sarcomeres (Boonen et al. 2009; Boonen et al. 2011; Langelaan et al. 2011). The overall protein content is 20 %, which is comparable to native muscle fibers (Post 2018). On the other hand, the previously mentioned electrical stimulation has not yet proven to be a resource-efficient method, and alternative ways for stimulating the muscles to produce higher amounts of relevant proteins will need to be developed (explored) to achieve the same result. Similarly, stimulation of myoglobin expression has been described and confirmed in many other muscle cells of vertebrates. It can be easily used in ways that are compatible with scalable production methods. For example, by reducing oxygen concentration during cell culturing, myoblasts started to show a 5-fold increase in myoglobin expression (Helbo et al. 2013; Kanatous and Mammen 2010). Sufficient myoglobin is important for color, nutritional value (heme iron), and taste of meat (serum-like taste and metallic mouthfeel of red meat) (Miller 2012).

7.2 Fat composition

To achieve complete meat flavor, ensure its expected nutritional value and mimic its natural texture, it is mandatory to add adipose tissue to the final meat product. It appears that a large number of small peptides are jointly responsible for taste (Claeys et al. 2004), while an equal amount of aromatics (originating mostly from adipose tissue) co-determine the flavor (Shahidi et al. 1986). Intramuscular fat (IMF) accounts for about 80% of the muscle

fat, while the remaining 20% is stored as lipid droplets within myofibers. Its amount and fatty acid composition are accounted for meat quality (e.g., juiciness, flavor, tenderness, and nutritional value). IMF is created via adipogenesis, in which stem cells differentiate into adipocytes, and lipogenesis, in which triglycerides are accumulated inside the adipocytes (Ben-Arye and Levenberg 2019; Listrat et al. 2016). Between muscle types, the phospholipids coverage is relatively constant, while the muscle triglyceride content is highly variable among the species (Listrat et al. 2016). Adipose tissue can be cultured from MSCs, from adipose-derived stem cells, or from myosatellite cells that entered an alternative differentiation pathway (resulting in the formation of intermuscular adipose tissue, IMAT) (Lepper and Fan 2010; Vettor et al. 2009). Adipogenic differentiation involves activation of several transcriptional factors including a peroxisome proliferation-activated receptor γ (PPAR-γ) that is a dominant regulator of white-adipocyte differentiation (Casteilla et al. 2007), and for which free fatty acids (FFAs) present natural ligands. In addition to their function as an energy source, some of those FFAs have been proven to induce adipogenesis in stem cells with mild and natural stimulation (e.g., cultivation in FFA-supplemented media) (Mehta et al. 2019). Maturation of adipose tissue into characteristic white adipose tissue takes approximately 2-3 months, whereas maturation of muscle tissue takes around three weeks. Despite the longer maturation period, the formation of fat tissue presents a less challenging process compared to the preparation of mature muscle fibers *in vitro*. The interactions between differentiating adipocytes, which are the basis to formation of a similar structure to the native tissue, are not as significant and complex as in the case of muscle fibers. Although the fat fraction is one of the main components of meats taste and texture, it may also be associated with cardiovascular diseases due to high content of saturated fatty acids and low levels of polyunsaturated fatty acids (PUFAs). In cultured meat, PUFA levels can be controlled with adjusting the composition of fat tissue culture media (Wood et al. 1999; Wood et al. 2004). In the interest of "not compromising" the taste of the engineered meat, the optimal amount of PUFAs will need to be derived from a combination of biochemical and sensory evaluations (Post 2018).

7.3 Tenderness

To mimic livestock derived meat in the majority of sensory aspects, the procedure of recreating conventional meat *in vitro* should also involve the usual aging process after native tissue's "death." Many studies have shown the complexity of meat tenderization. It is based on the extent of proteolysis of vital target cytoskeleton proteins within muscle fibers and the alteration of muscle structure due to the sequential actions of enzymes. After slaughtering the farm animals, muscle fibers undergo postmortem rigidity (*rigor mortis*) due to protein contractions, resulting in muscle toughness. The extent and mechanism behind *rigor mortis* are still rather poorly understood and likely vary for different harvesting conditions of the muscle. However, it is expected that mimicking only ischemic conditions in engineered meat cannot produce the same comparable effects on muscles as a sequence of death-related events *in vivo* (i.e., preslaughter stress and post-mortem muscle metabolism), such as the release of catecholamines, increase in blood lactate and cortisol concentration (Daskalova 2019; Dokmanovic et al. 2014; Post and Hocquette 2017). In the case of farm animals, the aging process is accompanied by a decline in aerobic metabolism due to decreasing oxygen supply. Subsequently, glycogen is converted into lactic acid, which induces a decrease in pH to 5.4-5.8 (Post and Hocquette 2017), activating enzymes responsible for tenderization and formation of aroma precursors (Fraeye et al. 2020). The aging period depends on the meat; for example, in beef that has a low amount of proteases, aging takes approximately 14 days. Aside from pH, temperature also has an impact on the palatability of meat. Hence both factors should decrease within a precise timeframe (Thompson 2002). The involvement of different groups of muscle peptidases that are responsible for the postmortem protein breakdown during cadaver storage is still a controversial topic. Among these peptidases, the calpain system accompanied by its inhibitors (e.g., calpastatin) was considered to be the predominant system accountable for meat tenderization (Lian et al. 2013). The extent to which proteolytic enzymes act is strongly influenced by the microenvironment conditions (e.g., pH, ionic strength, cellular oxidative, and nitrosylation status) (Lonergan et al. 2010). Hence, mechanisms that are responsible for *postmortem* degradation of structural proteins are under the scope of various studies, which aim to elucidate the underlying processes. Likely, intracellular conditions in cultured meat might overall differ from conventional meat, which would affect the rate and extent of tenderization and flavor development. (Fraeye et al. 2020). Considering this, further insights into the phenomena of postmortem degradation would enable a better prediction of the quality and resemblance to the conventional meat. Isoforms of actin and myosin in cultured muscle tissue were found to be rather neonatal or embryonic than adult (Fraeye et al.

2020; Thorrez and Vandenburgh 2019). This could affect the postmortem protein breakdown, resulting in altered postmortem transformations. In the case of their absence, the engineered muscle tissue is not transformed into the meat and, consequently, biochemically dissimilar (Datar and Betti 2010).

Moreover, particularly in cattle, connective tissue influences meat tenderness by its composition and structure, whereby collagen is seen as a major determinant of the shear force. However, there exist substantial differences between raw and cooked meat that are, in the case of raw meat, highly correlated with the collagen content. On the other hand, in cooked meat, the level of the relationship between the content, thermal solubility, or cross-linked level of collagen and meat shear force varies according to muscle type and cooking conditions (Listrat et al. 2016). All mentioned will aid the newly emerging industry of cultured meat to create a product that is as close an imitation of livestock meat as possible (Allan et al. 2019).

7.4 Color

As mentioned above, the myoglobin, which contains heme, is "the main culprit" for the red color of meat, specifically its chemical state (Fraeye et al. 2020; Listrat et al. 2016; Post and Hocquette 2017). In deep muscles and meat stored under vacuum, myoglobin is in a reduced state, which gives the meat the purple red color. In oxygenated form (i.e., when exposed to oxygen conditions) as oxymyoglobin, it exhibits an attractive red color. During the meat storage and cooking process, oxymyoglobin is further oxidized into metmyoglobin that is displayed as a darker brown-red color. Muscles that are rich in myoglobin (e.g., from cattle, sheep, horses) are apt to metmyoglobin formation and decreased color stability (Listrat et al. 2016; Simsa et al. 2019). Due to the suppressed myoglobin expression at ambient aerobic conditions and, therefore, the absence of myoglobin, the engineered muscle tissues have a pale color (Fraeye et al. 2020; Gholobova et al. 2018; Post and Hocquette 2017). Thus, several strategies have been proposed to improve the myoglobin load of cultured meat. Briefly mentioned in the previous subsection ("7.1 Protein composition"), the first approach involves culturing myofibers under hypoxic conditions (Helbo et al. 2013; Kanatous and Mammen 2010; Post and Hocquette 2017). However, deeper insight is needed to determine whether a low amount of oxygen is sufficient enough, as well as any potential impact on culturing efficiency (Moritz et al. 2015). Namely, this can lead to media acidification, which is harmful to cell viability (Datar and Betti 2010; Fraeye et al. 2020; Kadim et al. 2015). Supplementation of culture media with additives, such as lipids and acetic acid could also stimulate myoglobin expression (Moritz et al. 2015). Only myoglobin protein synthesis is not enough for color development; the presence of a sufficient amount of iron in the cell is also of great importance. In general, cell culture media contain no iron (e.g., IMDM) or only low amounts of it in the form of ferric nitrate nonahydrate (e.g., DMEM: 0,1 mg/L) or ferrous sulfate heptahydrate (Ham's media: 0.8 mg/L). The addition of extra iron into culture media increases the iron content in the cultured cells. However, only part of the iron is uptaken. This indicates that while media can be formulated to affect nutritional coverage, there is a limit to the amount of this microelement the cells can incorporate (Rubio et al. 2019). Since the iron uptake is dependent on transferrin, a protein that binds iron and mediates transport in the cell (Kadim et al. 2015; Moritz et al. 2015), further investigations are necessary to determine the extent to which iron is then incorporate into heme (for good iron bio-accessibility) and myoglobin (for red color development) (Fraeye et al. 2020; Post and Hocquette 2017).

Recently, Simsa et al. (Simsa et al. 2019) proposed a second approach to implement the myoglobin content in cultured cells by direct addition of myoglobin into media, which could be of value for large-scale cell expansion. Authors discovered that the proliferation and metabolic activity of bovine muscle satellite cells were significantly increased when cultured in myoglobin-supplemented media, resulting in a more meat-like coloration of cultured muscle tissue. However, given the limitations of the proposed cultivation system, the myoglobin proportion in the cultured cells was much lower compared to fresh beef meat, and the resulting color had a more similar tone to cooked meat (Fraeye et al. 2020; Simsa et al. 2019).

7.5 Flavor

More than 750 compounds are accounted for the flavor of the meat. Upon the heating (i.e., cooking), complex thermally induced reactions ensuing in the formation of an enormous range of the volatiles that contributes to the

aroma and odor (Fraeye et al. 2020; Warner 2019). Meat derived from a whole muscle is a unique mixture of muscle cells, fat cells, and connective tissue that all together provide the whole experience of the taste of the meat. In cattle and lambs, increased content of myoglobin attributes not only to the color of the meat but also to the finest juiciness and flavor. This favorable effect can be assigned to the high phospholipid content, which are decisive factors of the flavor of cooked meat (Listrat et al. 2016). Other compounds involving in the taste development are lactate (sour taste) and inosine 5'-monophosphate (umami taste), both formed during postmortem metabolism (Fraeye et al. 2020). Furthermore, upon prolonged heating in suitable conditions, collagen (from connective tissue) breaks down to gelatin, which has a distinctive and attractive flavor. Also, it contains essential amino acids for human muscle function and health (e.g., glutamate) (Warner 2019).

Intracellular lipids, in particular phospholipids from membranes, are accountable for lipid degradation during the cooking process even in lean meat and meat products (Fraeye et al. 2020; Mottram 1998). Phospholipids generally have a higher proportion of PUFAs that are more susceptible to oxidation. The higher amounts of fat in meat tissue and, consequently, the contribution of these volatiles give the additional kick to the overall flavor (Mottram 1998). Albeit products formed upon oxidation promote the desirable aroma, they can also cause the unpleasant taste of meat and are often the causative agent of meat spoilage (Fraeye et al. 2020). The fat presence in cultured meat should be viewed from dissimilarity between whole cut and processed meat. In the case of the former, incorporating a fat fraction to cultured meat can be achieved by co-culturing of muscle cells with adipocytes. On the contrary, in finely minced meat products, fat is often added at the end of culture process, therefore the substitutes, such as cultured fat or plant-based fat (used in vegan diet), may replace animal-derived fat (Feiner 2006; Fraeye et al. 2020). If the cultivation of meat itself does not provide a satisfactory flavor in a final product, additional artificial flavor compounds similar to those at present used in plant-based meat alternatives might overcome this issue (Fraeye et al. 2020; Langelaan et al. 2010).

7.6 Texture
The texture of meat contributes to the full sensory experience (Fraeye et al. 2020; Warner 2019). In the conventional animal-derived meat, the texture depends on numerous factors, including myofibrillar structure as affected by *rigor mortis* and aging, the amount and structure of connective tissue in the muscle, and the proportion and composition of fat in the muscle (Feiner 2006; Fraeye et al. 2020; Listrat et al. 2016). The texture of meat changes during cooking because of fluid loss and proteins' response to temperature. Cooking losses from meat seem to be driven predominantly by thermal denaturation of proteins at different temperatures, causing shrinkage in the muscle at a macro- and microlevel and alterations in the gel matrix binding proteins together (Purslow et al. 2016; Warner 2019).

The production of ground cultured meat products, such as hamburgers or sausages, is more doable, as demonstrated by the cultured meat hamburger patty in 2013 (Post 2014b). Despite that, this prototype represents one of the milestones in biotechnology. From a culinary perspective, it did not accomplish a satisfactory level. Namely, its sensorial characteristics (e.g., taste, color, and texture) were different from conventional beef patty (Gaydhane et al. 2018). However, ongoing advancement in technology has enabled refinement in terms of sensorial traits of culture meat. From a textural point of view, the minced cultured meat products ease the complexity of the production process, since the scaffold materials for cell culturing can be omitted (Fraeye et al. 2020; Specht et al. 2018). Therefore, structure formation in such products strongly relies on technical approaches. Since the tissue fragments in beef patty from 2013 were significantly smaller compared to traditional ones, to bind these fragments and to provide the final product with akin texture addition of breadcrumbs, egg whites, and other binders are requisite. The resulting texture will be likely to resemble industrially processed burgers as opposed to fresh, high-quality burgers (Fraeye et al. 2020; Post and Hocquette 2017; Specht et al. 2018). In the case of cooked, processed meat products (e.g., sausages), the texture is affected by functional properties of dissolved proteins, in particular, the gelation of the myofibrillar protein actin and myosin during pasteurization. With the addition of fat, the proteins stabilize the fat through the formation of an interfacial protein film around the fat globules. The gelling and emulsifying properties of meat proteins are vital in the production of finely minced meat products (Feiner 2006; Fraeye et al. 2020). Given that the cultured and conventional meat contain muscle fibers, it is presumed that the

biochemical composition will be more or less the same (Bhat et al. 2019; Fraeye et al. 2020). As already mentioned, at the current stage, muscle fibers formed by *in vitro* cell culturing methods include only a small proportion of predominantly embryonic or neonatal isoforms of actin and myosin. Therefore, electrical and/or mechanical stimulation is needed to (1) increase myofiber diameter, (2) strengthen myotube structure, and (3) increase myofibrillar protein fraction. In terms of large-scale production, it needs to be determined whether such stimulation is scalable, economically manageable, and whether the dissolved protein will provide the gelling and emulsifying properties that are crucial in processed meat production (Fraeye et al. 2020).

8. FOOD SAFETY AND REGULATION

One of the hot topics regarding cultured meat production is food safety and regulation. From the public point of view, the safety concerns were linked to the perception of unnaturalness and a sense of scientific uncertainty (Bryant and Barnett 2018). If the cultured meat production proceeds, it is likely that it will supplement the current market of animal-derived meat proteins (Warner 2019). Since the high demand for meat protein and, consequently, the potential increased consumption in the developing countries, some predictions state that the animal agriculture industry will remain alongside the development of the cultured meat industry (Stephens et al. 2018; Warner 2019). The safety standards required to maintain the safety and guarantee consumer confidence would be at some level included in a standard industrial setting for cultured meat production. Albeit an extensive regulatory system is requisite to monitor and regulate the safety, there are certain benefits related to cultured meat production (Bhat et al. 2019). The use of sterile and aseptic environments will eliminate microbial infections and contaminations, and through a continuous monitoring system, any deviation from accepted standards will be reduced. Apart from that, continuous monitoring of production will also enable the detection of any contamination at each stage of the process, providing the capability to incorporate the correction measures on time (Bhat et al. 2019). All components needed for cultured meat production could also be used as food ingredients. Nevertheless that the culture media and its components will not be consumed, traceability of the components should be in place (Bhat et al. 2019; Specht et al. 2018), reducing the cost of post-processing.

To set the regulatory framework, the procedures for cultured meat production must be clearly outlined. Since this technology is still in its infancy, the regulatory framework is not yet established. Therefore, regulations will need to follow the development of document procedures. Since the final product will be regarded as a food product, the food safety authorities are most likely to supervise the cultured meat production system rather than medical authorities. Regulatory guidelines, HACCP plans, and auditing must consider some stages and aspects, encompassing governance at local, state, and federal levels (Bhat et al. 2019; Warner 2019).

9. CONCLUSIONS AND FUTURE REMARKS

Cultured meat is an alternative animal protein source that will ameliorate some of the environmental, sustainability-related, and ethical issues associated with the conventional meat industry. At this point, considering the knowledge established from cell-based therapy and tissue engineering techniques, it is currently only feasible to create *in vitro* minced meat with composition and taste, comparable to the "traditional farm-derived meat." Creating whole cuts of meat requires a more sophisticated tissue engineering approach, including (1) incorporating a high-density channel system that enables the perfusion of culture media through the entire growing tissue construct, (2) fabricating a more complex and larger 3D constructs, tailored specifically (in terms of morphology, biochemical and mechanical properties) for different cell type requirements and individual meat cuts, and (3) establishing optimized differentiation protocols for co-culturing myoblasts, adipocytes, and fibroblasts. Since these demands are shared with tissue engineering for applications in regenerative medicine, scientific advances in both areas will eventually give rise to an abundance of strategies that promise to increase the efficiency of scaling up and reduce cost expenses of culturing meat *in vitro*. Last but not least, an important consumer-oriented approach in culturing meat will certainly be the possible preparation of meat variations based on exotic animals (e.g., dinosaurs, panda bear) or supplemented variants with or without specific molecules (even drugs for specific therapies).

Conflict of interest

The authors declare no conflict of interest.

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