POLITECNICO
MILANO 1863

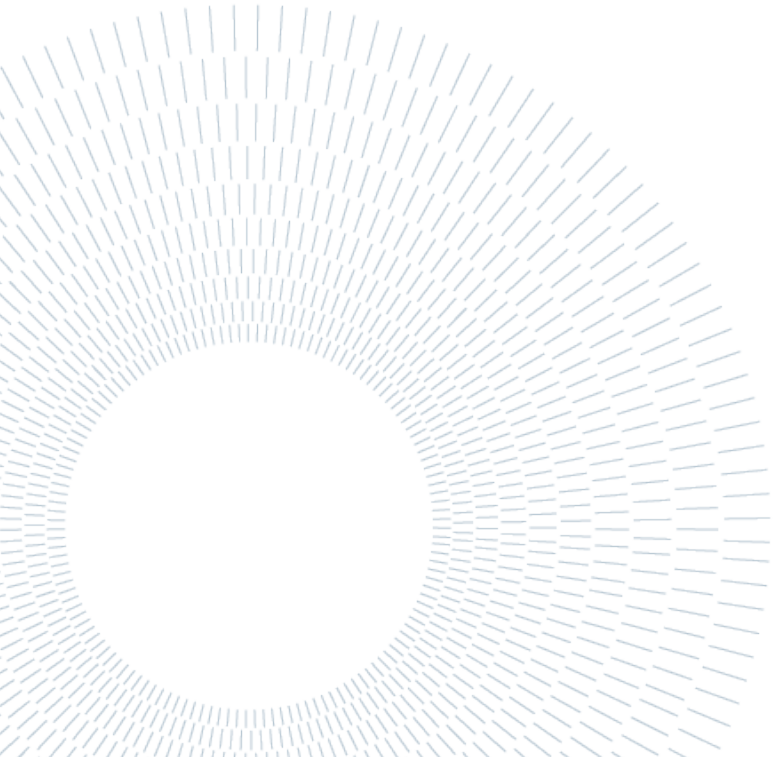
SCUOLA DI INGEGNERIA INDUSTRIALE
E DELL'INFORMAZIONE

Animal-free culture medium and bio-ink formulation for 3D bioprinting of cultured meat

TESI DI LAUREA MAGISTRALE IN
CHEMICAL ENGINEERING
INGEGNERIA CHIMICA

Author: **Elisa Rossi**

Student ID: 10864979
Advisor: Davide Moscatelli
Co-advisor: Filippo Bracco
Academic Year: 2022-23



Abstract

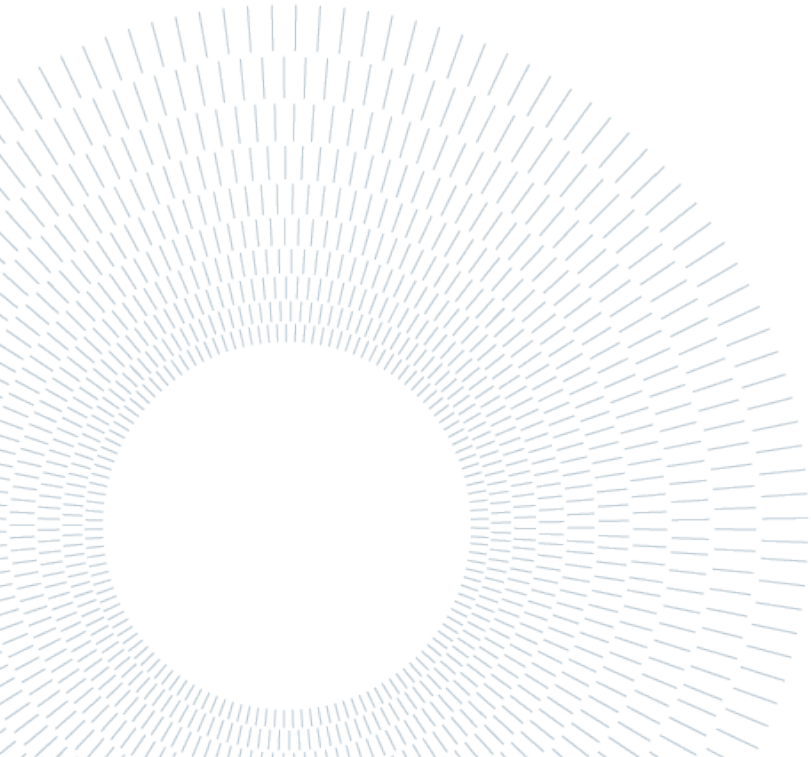
Livestock farming is one of the main causes of Green House Gasses emissions, water depletion, and land use. The emerging sector of protein alternatives aims at substituting the consumption of meat from livestock origin. Among the three different types of protein alternatives, cultured meat (CM) is the only one with the potentiality to substitute completely the livestock meat but proposing a product with the same properties. The production of cultured meat follows different procedures, and 3D Bioprinting (3DBP) represents one of the most promising. However, few research is dedicated to the development of this technology, mainly due to the low availability of materials that satisfy the constraints of edibility, biocompatibility, economic feasibility, and printability. This thesis aims at presenting the first attempt to define a process for the production of printed cultured meat that is completely animal-free: starting from the exclusion of fetal bovine serum (FBS) from the cell culture medium to the exclusion of gelatin from bio-inks for the 3D bio-printing of the product. The focus will be on replacing FBS and gelatin, from the cell substrate and bio-ink respectively, with protein hydrolysates derived from waste products.

Key-words: cultured meat, bioprinting, FBS, culture medium, protein hydrolysates, gelatin, alginate, wheat.

Abstract in italiano

L’allevamento del bestiame è una delle principali cause delle emissioni di gas serra, dell’esaurimento delle acque e dell’uso del suolo. Il settore emergente delle alternative proteiche mira a sostituire il consumo di carne di origine animale. Tra i tre diversi tipi di alternative proteiche, la carne coltivata è l'unica con la potenzialità di sostituire completamente la carne bovina ma proponendo un prodotto con le stesse proprietà. La produzione di carne coltivata segue diverse procedure e il 3D Bioprinting rappresenta una delle più promettenti. Tuttavia, poche ricerche sono dedicate allo sviluppo di questa tecnologia, principalmente a causa della scarsa disponibilità di materiali che soddisfino i vincoli di commestibilità, biocompatibilità, fattibilità economica e stampabilità. Questa tesi si propone di presentare il primo tentativo di definire un processo per la produzione di carne coltivata stampata che sia completamente privo di animali: partendo dall'esclusione del siero bovino fetale (FBS) dal mezzo di coltura cellulare fino all'esclusione della gelatina dal bioinchiostro per la stampa 3D del prodotto. L'attenzione del progetto sarà focalizzata sulla sostituzione di FBS e gelatina, rispettivamente dal substrato cellulare e dal bioinchiostro, con idrolizzati proteici derivati da prodotti di scarto.

Parole chiave: Carne coltivata, bioprinting, FBS, mezzo di coltura, idrolizzati proteici, gelatina, alginato, frumento.



Contents

1 Introduction

1.1. Cultured meat framework

Our current food system has a serious impact on the environment and animal agriculture is identified as one of the main causes of the climate crisis: currently, farming uses about 30% of the land and approximately 8% of fresh water, generating about 17% of the planet's total greenhouse gases. Large-scale breeding of animals for slaughter is currently the only solution to the population's demand for meat, but it has serious environmental limitations.

The plant-based choice is linked both to ethical and health reasons but also to the reduced environmental impact that this entails and to its potential to mitigate emissions, which are fundamental in the context of the climate crisis we are facing.

The environmental impact of breeding, the forecasts on the need for meat, combined with the ethical implications associated with animal sacrifice, have prompted us to explore the possibility of making a transition to a more sustainable food system. The excluding animal products from the diet is a choice linked both to ethical and health reasons but also to the reduced environmental impact that this entails and to its potential to mitigate emissions (Figure 1), which are fundamental in the context of the climate crisis we are facing [1].

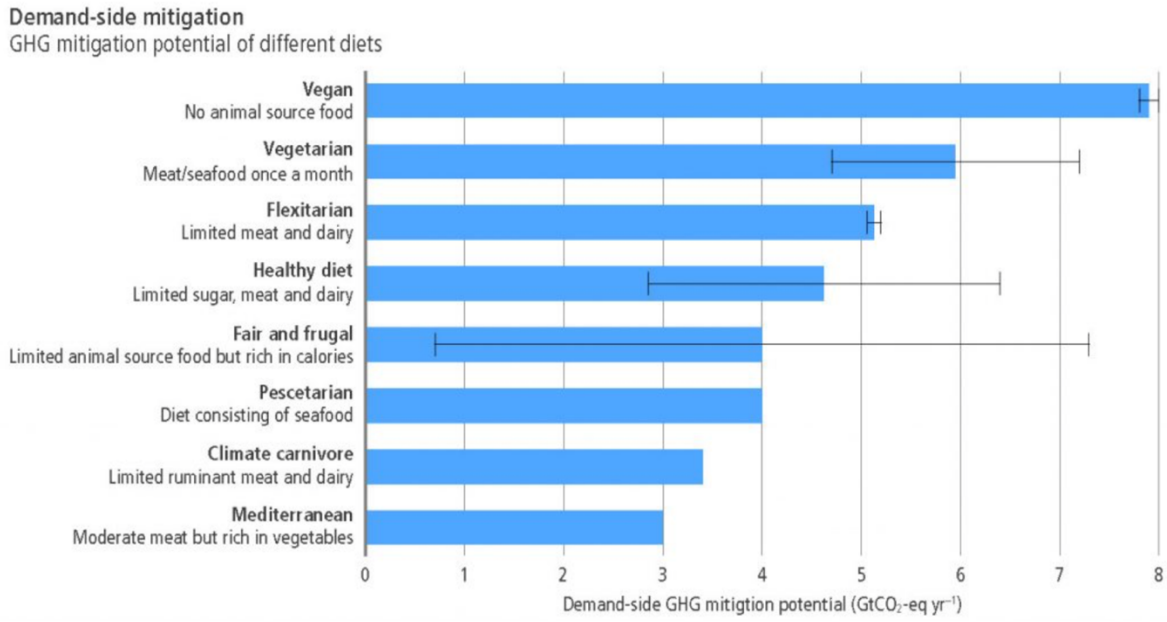


Figure 1 Demand-side GHG potential of different diets [1].

The International Panel on Climate Change (IPCC) in its latest report recommends a transition that sees plant production and cellular agriculture at the centre, recognizing that emerging food technologies such as cellular fermentation, cultured meat, plant-based alternatives to animal-based food products, and controlled environment agriculture can bring substantial reduction in direct greenhouse gas (GHG) emissions from food production [2].

1.1.1. Cell agriculture and cultured meat

A new kind of agriculture is born. The process is the same: a substrate has to be prepared, then there is the seeding phase, then it's necessary to guarantee the needed nutrition to optimize the growth of the seed, and then there is the harvest phase. But in this kind of agriculture the protagonist are not plant-seeds but animal cells that have to growth, proliferate and differentiate into adipose and muscle tissue.

In other words, cellular agriculture involves the production of authentic animal products without the need of animal breeding, rearing, or slaughter: cells or proteins are cultivated directly rather than receiving them from full animals. Research and methods from the fields of tissue engineering, regenerative medicine, synthetic biology, and fermentation are used in cellular agriculture.

While large-scale animal cell culture has been used for decades to produce materials for biological research and medicinal cures, food is a relatively recent application for animal cell culture.

Cell agriculture is giving in fact the chance to providing humanity with nutritional, safe and healthy food while optimizing the resources used, such water, land, energy, minimizing the emissions and reducing the individuals being killed every year for our food system. The newest product of cell agriculture is the so called "cultured meat", that is not the meat of a slaughtered animal but a muscle derived from the differentiation of animal cells cultivated in a laboratory. All that is necessary to its production is in fact a painless biopsy performed to take the cells from the animal, then the cells will be seeded in a preformulated substrate to guarantee them all the needed nutrients that favor their growth, proliferation and differentiation in muscle and adipose tissue that has the same organoleptic characteristics of that grown by the animal organism.

Starting to 2D cell cultivation in small flask in a laboratory scale system, to obtain the final muscle tissue and make the large-scale process more economically sustainable, there are different types of 3D cell cultivation technologies.

There are two approaches for the tissue biofabrication and maturation, the first one implies the production of cell-laden construct using materials containing cells and biological material. This approach permits to fabricate different structures and to control the spatial arrangement of cells. 3D bioprinting is a biofabrication approach based on additive manufacturing (AM) that allows an accurate cell deposition, a control on the cell density, on the geometry of the structure developed and on the ratio between various population of cells that led to the possibility to have a 3D multicellular contructs.

An alternative to this approach is the use of a scaffold, a 3D biodegradable or edible construct characterized by a determined porosity, texture architecture, and mechanical and chemical properties that are suitable for particular cell types. These scaffolds, that can be fabricated or obtained from decellularized tissues, are seeded with cells that adhere on it and proliferate.

Cells inside the 3D scaffolds for the maturation are suspended in bioreactors, unit process that permits to reduce the need of resources, time and handling steps giving the cells the optimal environment in which proliferate, as the bioreactors provide precise control over relevant variables such as temperature, oxygen concentrations, pH, and cell density. Several types of bioreactors are used, the most common being static culture, spinner flask, and perfusion bioreactors.

In particular the cells are suspended in a culture media which include glucose, inorganic salts, amino acids, vitamins, growth hormones, antibiotics, and antifungals, that are essential for ensuring cell proliferation.

1.1.2. Environmental impact of cultured meat and traditional meat

An article published in Nature which focuses precisely on the environmental impact of alternative foods, like cultured meat, cites various studies in which cell-based meat (CBM) proves to be responsible for less water and soil consumption, and seems to emit less waste than animal alternatives.

These advantages are based on assumptions of having a targeted tissue cultivation, so reduced by-products; higher production rates and vertical production systems. In 2011 a first relevant LCA states that CBM would reduce energy consumption by 45%, greenhouse gas emissions by 96%, land use of 99% and water use of 96% compared to animal-based meat.

Soil exploitation, in another research published in the same journal, is then further reduced by 30% with the implementation of cell culturing techniques [3].

As also described by the IPCC this year, in addition to the reduced consumption of water and nutrients, as well as soil, Future foods offer a better possibility of resilience, reduction of the use of pesticides and antibiotics, as well as the reduction of zoonoses [2].

Furthermore, wanting to compare the nutritional values of traditional animal proteins with those of the the alternatives, from the point of view of the impact on land use, an evident favor for vegetable products and derivatives from cellular agriculture can be found (Figure 2).

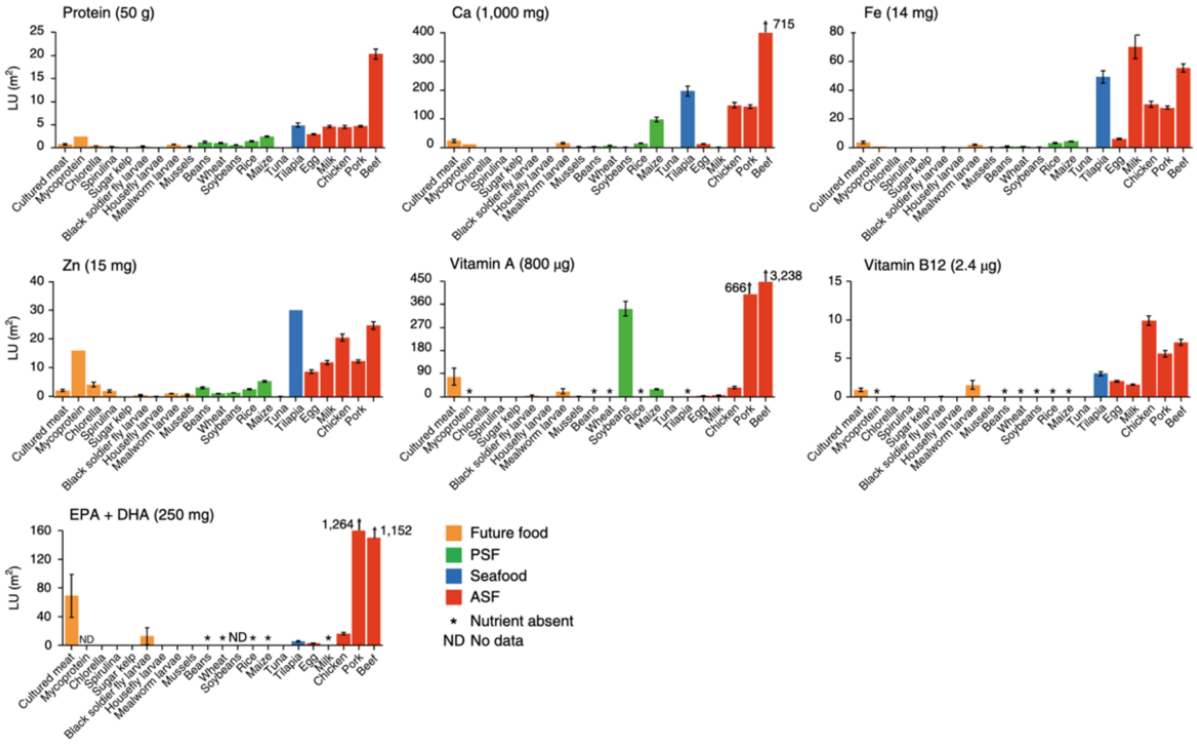


Figure 2 Land use of various produce expressed per daily recommended intake of each essential nutrient [2].

Similarly, the same results are obtained if the focus is on the GHG emissions (Figure 3).

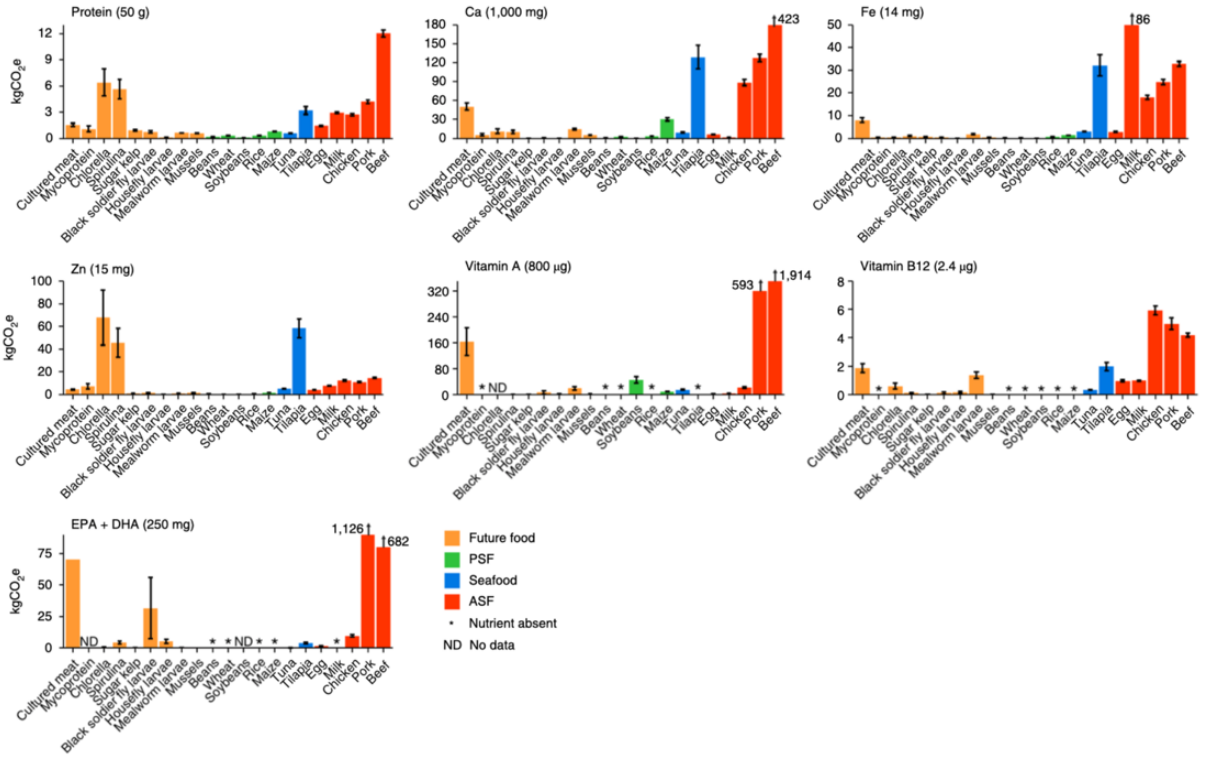


Figure 3 GHG emissions of various produce expressed per daily recommended intake of each essential nutrient [2].

It is therefore clear that as regards the protein intake for the same emissions, cultured meat is considerably favored compared to traditional meat.

In particular future foods like cultured meat show similar GHG intensities per unit of protein comparable to milk, eggs and tuna. As the main source of GHG emissions from cellular agriculture foods is energy consumption, is stated that their GHG intensity improves with increased use of low-carbon energy [2].

However, CBMs seem to have a lower ozone depletion potential, acidification potential and energy consumption than chicken farming for example, even if they are in any case superior, after the plant-based and insect-based alternatives, regarding feed conversion efficiency (Figure 4) [4].

In the latest IPCC report it is stated that the emissions linked to these new technologies are instead comparable with the poultry industry [2].

In any case, in the same study it is written that the introduction of vegetable alternatives or derivatives from cellular agriculture would make it possible to reduce the number of animals raised and therefore make farms more sustainable and reduce animal suffering, something alternatively impossible in a historical moment in where

the demand for meat is constantly growing and involves the birth of more and more intensive farms.

The energy consumption of these products should not be underestimated, but consists of the CO2 emissions linked to the reference energy sources, which depend on the use of fossil fuels [4].

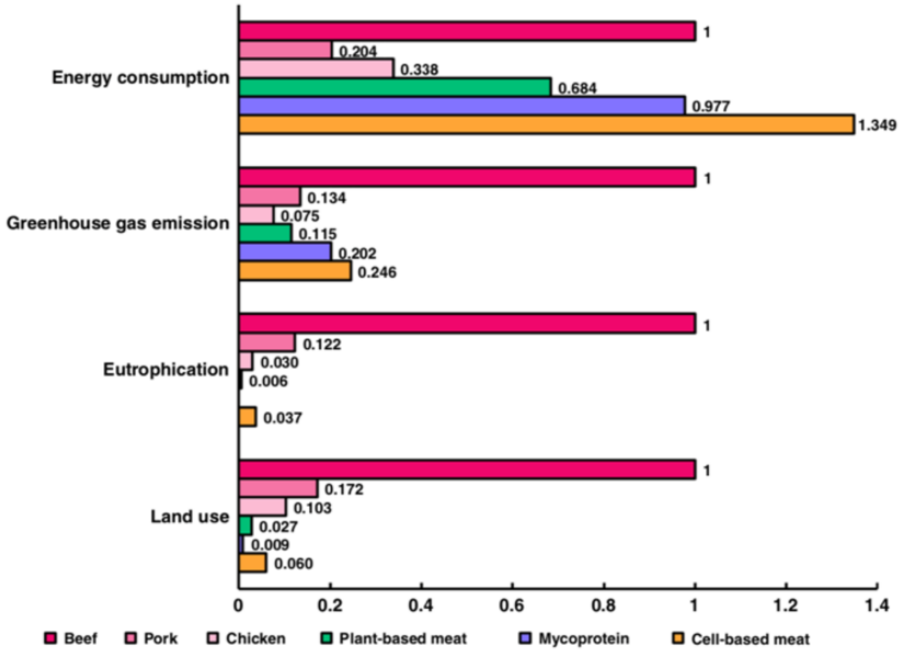


Figure 4 Comparison on the environmental impact of meat and meat analogs [4].

The emissions of climate-changing gases from the production of cultivated meat are linked to CO2 emissions, while the emissions of GHG from the livestock industry, which are far higher than those of the alternative, consist mainly in methane. The latter does not accumulate in the atmosphere as does CO2, which can remain for a millennium if not captured, but warms the atmosphere about 100 times more than carbon dioxide. A big problem given the urgency we have to stay within one and a half degrees in the coming decades.

If, thanks to the mandatory decarbonization we have to face, the plants that produce cultivated meat will reduce their CO2 emissions by switching to renewable energies, the livestock industries will not be able to do the same with the important emissions of methane and nitrogen, linked to the physiology of the animals and to the fertilizers used in monocultures [3].

Therefore it is evident that cellular agriculture has great potential, not only for the possibility of making the current food system more sustainable but also for what concerns the reduction of the environmental impact of the production process of the cultured meat itself.

1.1.3. Ethical concerns of the traditional meat production

Every year more than 70 billion land-raised animals are slaughtered all over the world [5], while the number of fish killed cannot be calculated, given that due to the enormous amount of individuals caught every year, one can at most speak in terms of weight of the catch (Figure 5).

Just in Europe, at the end of 2021, there were 142 million head of pigs, 76 million heads of bovine animals and 71 million head of sheep and goat.

The majority of livestock population is concentrated in just a few countries, like Spain, France, Germany and Italy.

For example, Spain has one quarter of pigs farmed in EU and about the 25% of sheep population: the same count for Greece's goat population. France achieved the 22.9% of EU's bovine population.

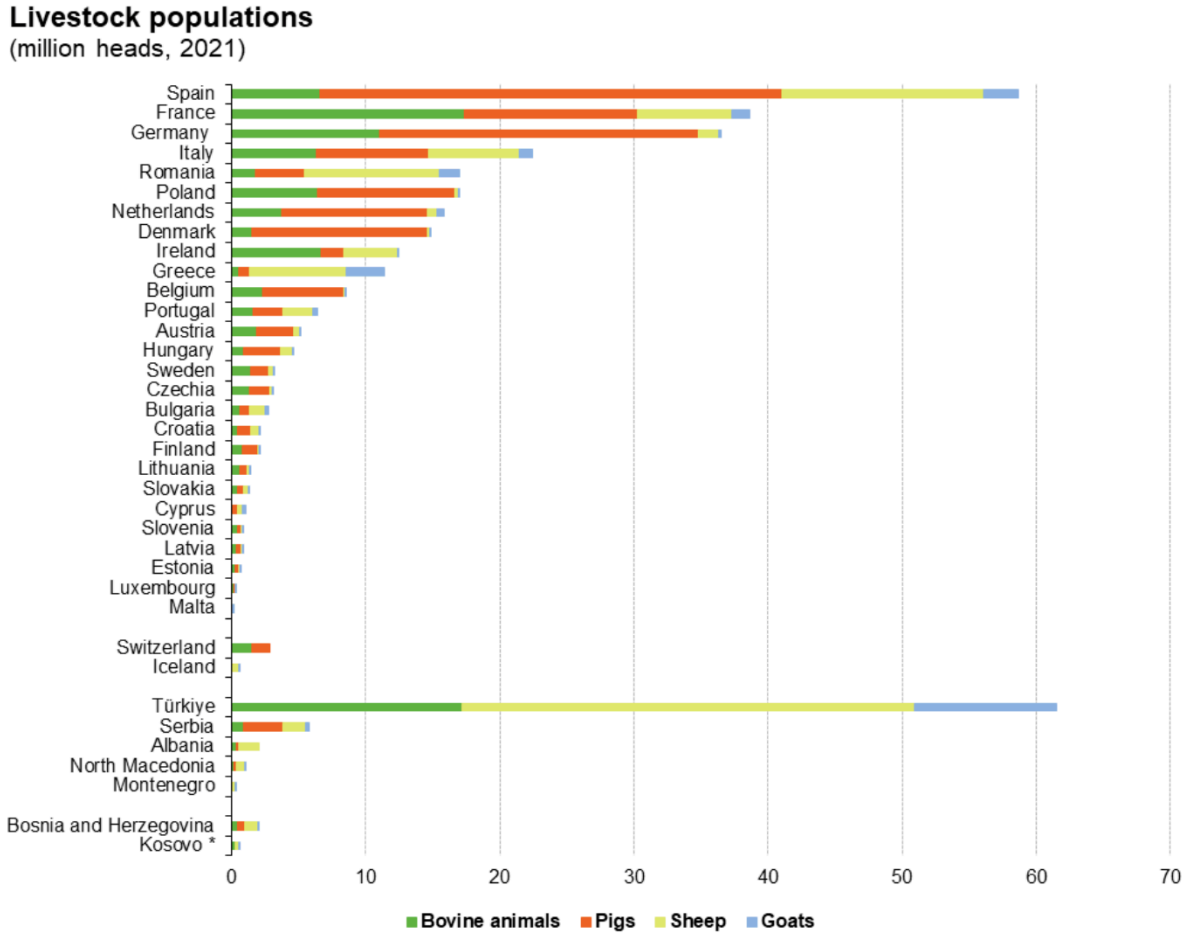


Figure 5 Europe's livestock population [5].

Across the EU, cattle numbers have declined over the previous two decades. Between 2001 and 2021, the number of head in each animal population decreased: the biggest decreases in percentage terms were observed for sheep and goats (both around 20%), while pig numbers declined at the slowest pace (about 8%).

In greater detail, between 2020 and 2021, the number of bovine animals in the EU reduced by 1.1%, while sheep (down 1.7%), goats (down 2.6%), and pigs (down 2.9%) declined at a faster pace. In 2021 Italy was one of the main poultry meat producers in the EU, covering the 10,4% of the production, with 1.4 million tonnes (Figure 6).

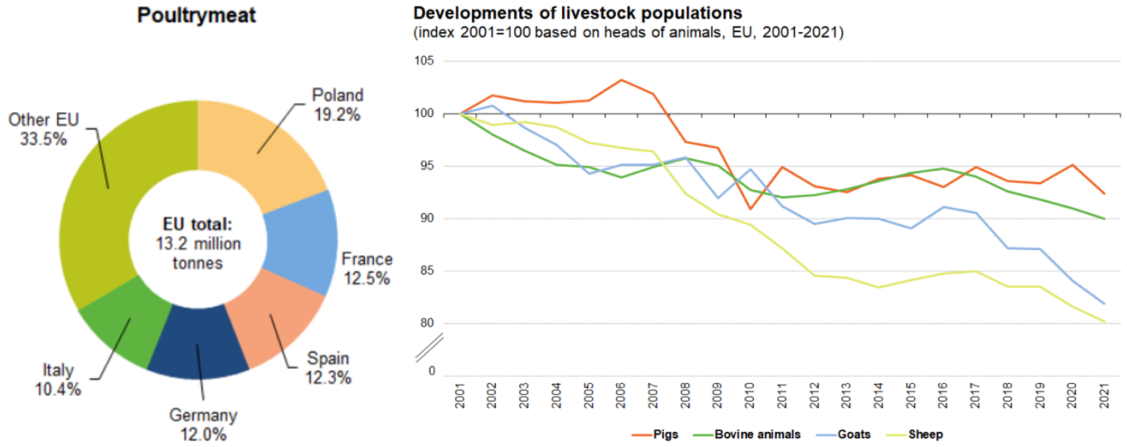


Figure 6 EU poultry meat productions and developments of livestock populations [5].

The EU produced 23.4 million tonnes of pig meat in 2021, a little increase (+1.6%) over 2020 and a new high (Figure 7). To put this into perspective, output in 2021 will be 2.0 million tonnes more than in 2006.

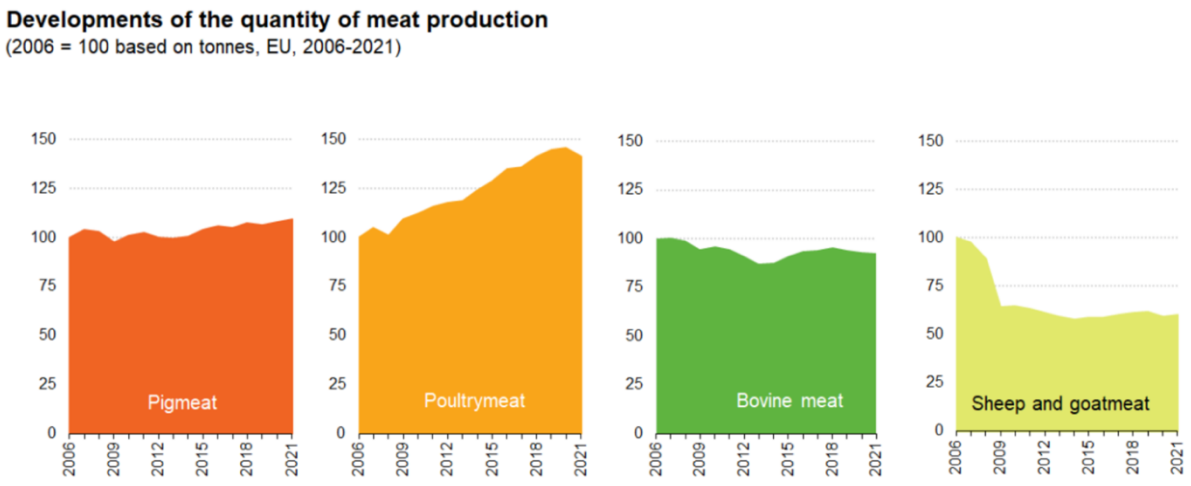


Figure 7 Developments of the quantity of meat production (2006 = 100 based on tonnes, EU, 2006-2021) [5].

Farms in the EU are many and diverse; they range in size, in terms of what is produced or animals raised, are managed differently, and are located in places with varying geologies, topographies, and temperatures.

Between 2005 and 2016, the number of poultry and animal farms in the European Union declined. In this time period, there was a drop of 3.4 million farms; if all sorts of farms are included, the overall decrease was 4.2 million units. During the same time period, however, the average number of animals per single firm grew: for example, the number of pigs went from 374 in 2005 to 688 in 2016, and the number of poultry increased from 2,941 to 5,555. Data that, when coupled with earlier ones, show a tendency toward intensification of European farms and indicate that small and medium-sized agricultural firms, in particular, have remained out of the market (Figure 7) [5].

The described meat production pattern results into a reduction in farm animal welfare. The animals, who have become nothing more than numbers and capital for the firm, are pushed to achieve maximum production in the shortest amount of time feasible, even at the expense of their ethological requirements. For instace, every year, more than 300 million animals in Europe alone are forced to live in cages that prevent them from expressing even the most basic natural behaviors, such as moving or turning around. Rabbits, chickens, quails, ducks, sows, and calves are still lawfully kept in cages in the European Union today (Figure 8).

Figure 8 Calves of a few days old, born from cows bred for milk production, separated from their mothers and closed in single pens. Photo taken by the author of the project.

Moreover, after a life spent on a farm, every animal destined for human consumption ends up at the slaughterhouse, but first, is forced to undergo the transport phase: every year 1.5 billion animals bound for slaughterhouse travel in and out of the Europe.

In 2017-2021, cross-border transport of animals (both in terms of number of animals and weight) consisted of 86% of intra-EU animal movements, 13.5% of movements to non-EU countries and 0.5% of animal imports from countries non-EU. 1.6 billion live animals were transported between EU Member States and to/from non-EU countries (Figure 9).

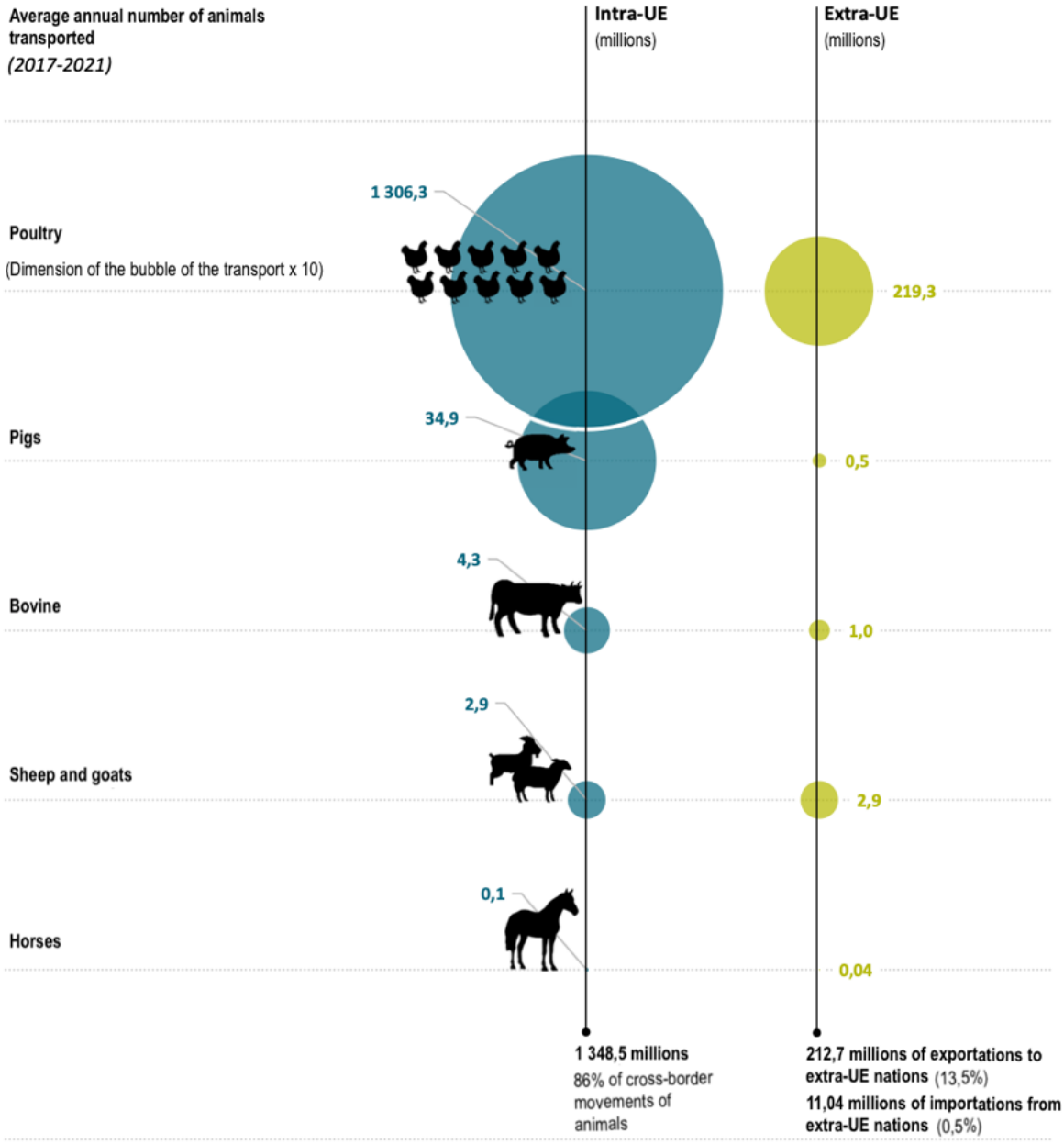


Figure 9 Cross-border transport of animals from 2017 to 2021 [6].

Animals can be transported by car, boat, plane, or train. Transport, regardless of mode, is a cause of stress for them and, as such, can have a detrimental influence on their welfare [6]. Animals are stressed during loading and unloading; they may suffer from hunger, thirst, heat, lack of space, and loss of rest. The welfare of the animals is affected by the distance and duration of the voyage, as well as the conditions of the journey: for example, available space, microclimatic and road conditions, and the amount of concern displayed by drivers (Figure 10) [7].

Figure 10 Photos taken in front of the Pini ITALIA slaughterhouse in the province of Cremona by the author (A). The animals arrive at the facility in uncomfortable conditions (B).

In recent years, EU citizens have become increasingly concerned about animal welfare and non-governmental organizations (NGOs) have highlighted the poor conditions suffered by animals on farms and during transport. All of this has not only raised questions about the effectiveness of EU rules, but also about the justification for using some farming practices and transporting live animals for long periods of time or over long distances.

Proof of this is, for example, the European citizens' initiative "End The Cage Age" launched in 2020 and which collected more than 1.6 million signatures and led the European Commission to table, by the end of 2023, a legislative proposal to phase out, and finally prohibit, the use of cage systems for all animals mentioned in the Initiative**.** Along with this, there are also various petitions against the transport of live animals that have reached thousands of signatures over the years.

This is due to the numerous investigations conducted on the streets and in farms, even showing only the legal and routine practices that have led them, which have resulted in an ever greater awareness of the population provided by an increased understanding of the entire food production process.

People are less and less inclined to neglect the farming methods and to accept that other individuals have to suffer in order for a particular one to be sold. More and more people recognize the singularity of each of those billions of animals killed and would like their right to a free existence to be recognized, perhaps also thanks to the philosopher Peter Singer, who laid the foundations of the modern anti-species movement, a social justice movement and therefore philosophical, political and cultural, which opposes the discrimination of species thanks to which the oppression of non-human animals, considered inferior, is legitimized.

1.2. Cultured meat research and development over the years

Although the technologies were not yet sufficiently developed for a concrete project of cell cultivation for food purposes, the idea of producing cultured meat was in the minds of the most progressive scholars as early as the 1930s: John Burdon Sanderson Haldane discussed it in his 1927 publication 'Possible Worlds and Other Essays' [8].

Russel Ross presented a research [9] in 1971 in which he testified that he had grown guinea pig smooth muscle cells for roughly 8 weeks and that the myofibrils were clearly identifiable within the cell layers after half the time needed.

Over time, NASA academics became interested in food production through cell cultivation as they were concerned about providing people in space with safe, healthful, and extremely nutritional diets. On December 5, 2002, a study [9] was published that described how the researchers were able to develop fish muscle cells in vitro on an FBS substrate with good results.

The Dutch government financed the first of two three-year research projects on adult and embryonic swine cell culture in 2005, and three years later opted to spend $4 million to this study field. In 2013, Dutchman Mark Post spent $330,000 to create the first edible beef burger in vitro. Memphis Meat, a US firm, introduced the first meatball manufactured from cultured beef in 2016, promising to generate a comparable product using chicken and duck cells by 2021 [8].

Produced the first dishes that imitated the goods derived from minced beef, the research horizons were geared towards the manufacture of steaks, to suit the public's organoleptic expectations. Aleph Farms, an Israeli startup, created the distinctive muscle tissue of a steak in its research laboratory in December 2018. In November of the following year, researchers from China's Nanjing Agricultural University obtained a piece of cultured meat weighing 5 g for the first time in the country's history [8].

The first marketing window for this product opened in 2020, thanks to the startup Eat Just, which created and released the first cut of cultivated chicken flesh in Singapore. The next year, Aleph Farms developed the first piece of rib using 3D bioprinting (Figure 11) [8].

In 2021, Upside Foods, old Memphis Meats, and Blue Nalu, which specialized in fish flesh, also debuted in line for the commercialization of its own laboratory products.

The Dutchman of Mark Post's firm, Mosa Meat, released an article [11] in 2022 that highlighted the potential for replacing the FBS used as a cell growth substrate.

The Dutch Parliament approved taste testing in March 2022, which are necessary for responding to European Food Safety Authority (EFSA) demands. Good Meat has received final U.S. Department of Agriculture approval to sell lab-grown meat. The United States become the second nation, after Singapore, to allow the sale of cultivated meat.

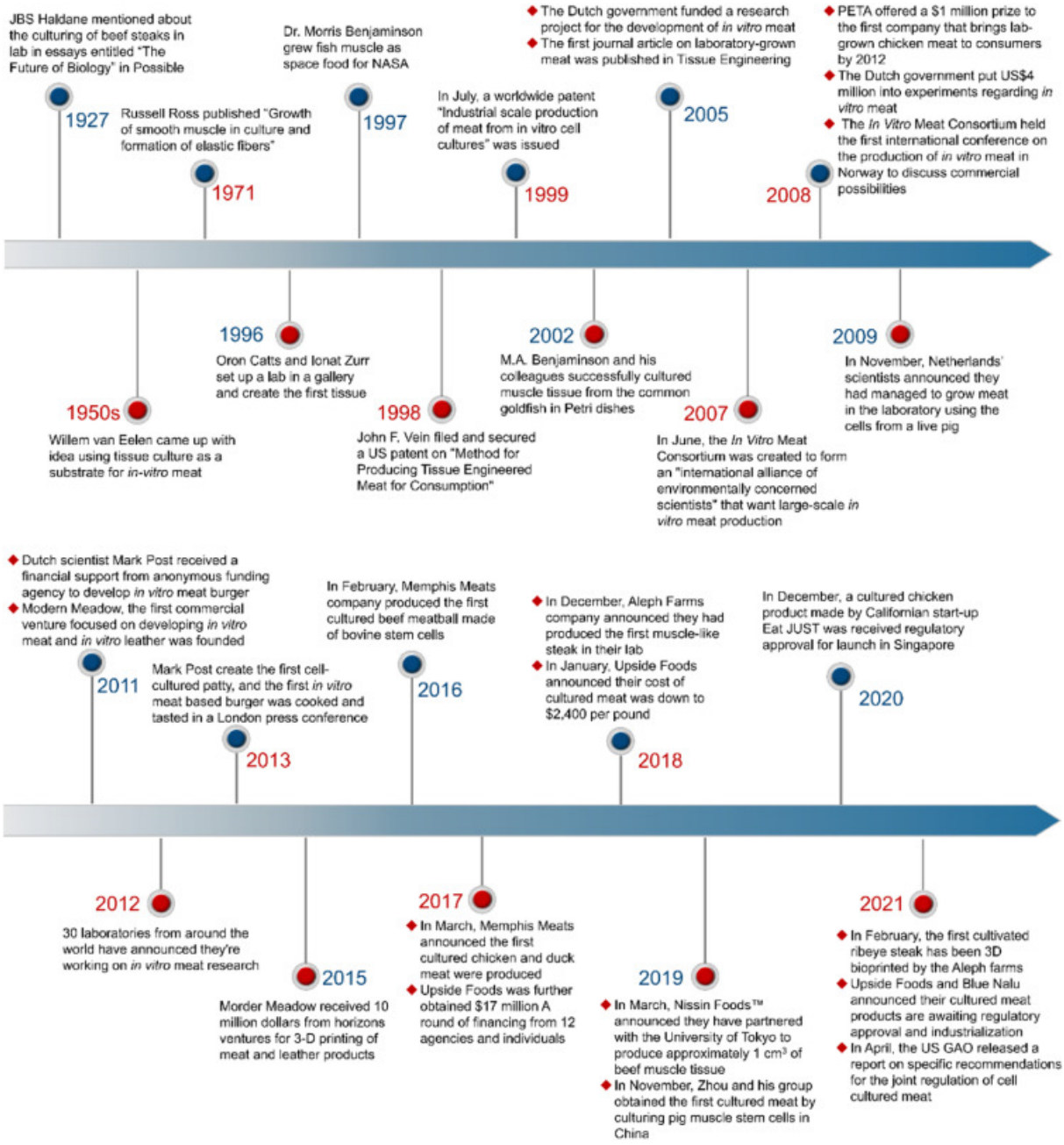


Figure 11 Timeline of events related to cellular agriculture through 2021 [8].

1.3. Cultured meat market

Since 2015, there has been a sharp rise in the number of businesses that deal with cultured meat. This growth has included businesses like Cell Farm Foods that characterize and stabilize proteins and cell lines as well as those that develop culture media like Multus Media, Heuros, Luyef, Biftek, Future Fields, and Cultured Blood.

Particularly, the number of cultured meat startups in Europe has significantly increased since 2017 and now accounts for 80% of all businesses (Figure 12) [8].

Figure 12 Emerging cultured meat startups [8].

Most of the startups that engage in the development of these laboratory products are located in Europe (40%) and in North America (34%), another important portion of the market is centered in Asia (15%), followed by South America, Australia and Africa. Similarly, research is also strongly oriented and sees 46% of efforts committed to the development of beef, almost 30% of resources instead are concentrated on the production of fish, followed by research on poultry and pork (Figure 13) [12, 13].

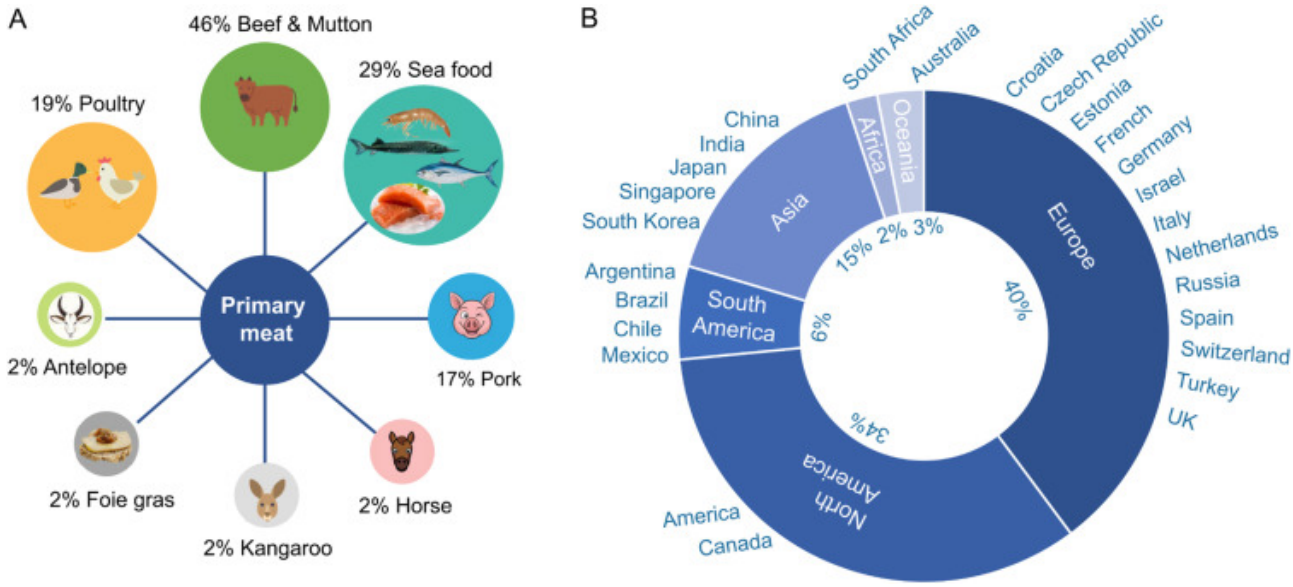


Figure 13 Geographical arrangement of cultured meat startups and product diversification [8].

From 2015 to early 2020, cultured meat companies announced approximately $320 million in investments, of which $242.29 million was spent on land-based meat production and $49.5 million on seafood. Many government agencies are also investing in the sector, for example, Singapore Food Agency (SFA) announced a US$108 million fund for cultured meat research, while the Indian government has spent US$600,000 on the Agriculture Center of Excellence mobile phone, a similar investment was made by the Japanese Agency of Science and Technology. The European Commission has followed global trends by investing 3 million in the Dutch startup Meatble, Belgium has instead offered 3.6 million for cultured meat research, a similar amount was invested in 2020 by the National Science Foundation to support the same sector. The following year, China established a special fund of 93 million dollars for the 'Green Biological Manufacturing' project which includes studies on cellular agriculture [8].

1.4. Technological challenges

To make lab-grown meat affordable, one of the focal points is to reduce the cost of industrial-scale production. It is believed that a stirred tank type reactor of at least 5,000 L is necessary to create around 8x1012 muscle cells, however these quantities have not yet been thoroughly researched in the field of tissue engineering and cell proliferation in general [14]. Furthermore, in order to maintain good cell vitality, certain parameters must be accurately defined on a laboratory scale but are not applicable on an industrial scale. It is necessary to standardize the temperature, the dissolved O2, the pH value, and appropriate levels of nutrients agitation, which, if as vigorous as that required on a large scale, would end up damaging the cells. As a

result, the size of the reactors employed to date do not yet allow for substantially lowered final product pricing.

Another significant obstacle is the effective growth of stem cells to the necessary quantity and differentiation of these cells to create the desired tissue. The most often employed cell lines in cell culture are embryonic, muscle, and mesenchymal stem cells. Pluripotent embryonic cells are used because they multiply rapidly, although their capacity to develop directly into muscle cells is unknown. Similarly, muscle cells are problematic since they have a growth limit of 40 to 60 cells. Some firms, such as Meatble and Mosa Meat, have said that they have addressed these challenges but have not shared the technique.

Another difficulty is that cell survival severely restricts the tissue thickness achievable using scaffolds, particularly when the thickness of the formed structures surpasses 200 μm because criticalities associated to oxygen transport occur. As a result, research is focusing on the study of three-dimensional biological scaffolds made of various types of hydrogels, collagen, and biocompatible materials that promote natural cell growth by stimulating the formation of blood vessels and channels that facilitate the diffusion of oxygen and nutrients required to avoid cell death [14]. The industrial scale problem is clearly seen in this setting.

Another important criticality of the process is that it needs animal-based products, first of all fetal bovine serum (FBS). Fortunately, a sufficient number of studies have been reported which demonstrate how much research in this direction is progressing: alternative culture media have been studied which ensure good cell viability, but are extremely not very versatile and increase the sensitivity of cells to environmental changes [15].

Without a culture substrate free of animal serum, the possibility of marketing the finished product would be far away due to the stringent regulations in force and the environmental and ethical promises of cultured meat would certainly not be respected [16].

1.5. The fetal bovine serum problem

In Europe, 4% of cows destined for slaughter are pregnant, globally this percentage would seem to double, therefore the FBS appears to be a waste of this industry. In particular, the serum is extracted from the fetus of a calf during the last two thirds of the gestation period.

Serum extraction first of all involves the removal of the fetus from the mother's womb and the subsequent collection of non-coagulated blood directly from the calf's beating

heart using a special syringe: obviously it is not excluded that the newborn will feel pain during the procedure.

A single liter of FBS requires at least three fetuses: about 2 million of these are used annually to produce approximately 800,000 liters of FBS. After extraction, the whey is filtered and undergoes a quality control procedure which studies possible microbial or viral contamination, the presence of endotoxins, the immunoglobulin content and the total amount of protein present, before being sold at a price which, in 2019, was about $1,000 USD per liter.

Fetal bovine serum is a chemically undefined medium, in fact it contains thousands of different components and the concentration of these has not yet been adequately described. Furthermore, the composition varies according to the period of gestation in which the extraction is placed and to the maternal nutrition: in fact, the diet of cattle varies drastically at a geographical and even seasonal level, moreover, due to the various international regulations, also the administration of hormones and antibiotics to these non-human animals varies widely.

Another problem is certainly that of contamination by organisms such as mycoplasma, viruses and bovine spongiform encephalopathy. In particular, despite the appropriate filtrations, the absence of mycoplasma in the serum cannot be guaranteed, which is a parasitic bacterium that involves the variation of gene and metabolic expressions on cell lines and infects about 11% of cultures. The strict regulations to which the FBS must respond have led to the highlighting of 8 different viruses present in the serums deriving from each geographical region of origin, the same goes for encephalopathy.

The presence of these possible contaminants affects the experiments and bioprocesses in which the whey is used, adding to the costs.

In terms of the economic weight of FBS use, there is also the fact that the demand for whey for cell agriculture destined for cultured meat competes with that of more mature industries, such as those focusing on cell therapy research, which are currently sufficient to exceed the product's availability.

The replacement of fetal bovine serum will therefore be driven, in addition to ethical, safety and reproducibility reasons, by its limited availability and cost, which drastically increase the final prices of the finished product.

1.6. State of art on FBS-free cell cultures

1.6.1. Publication trends

To analyze the growth phenomenon that research on cultivated meat is facing, it is possible to study online databases that allow highlighting publications on the subject.

The Good Food Institute, for example, identifies 1187 companies that currently focus on the production of protein alternatives for consumers, many of which are plant-based or derived from cellular agriculture. The same database identifies 57 relevant publications about the attempt to find a substitute for animal serum as a culture medium.

Scopus constitutes a database of online publications and is a useful tool through which it is possible to analyze the trend of scientific research on a particular topic. For the characterization of the research, Boolean formulas are also available which allow to direct the analysis in a more precise way, the most used are 'AND' and 'OR' and allow to select, among all the publications present, those which have different combinations of your chosen keywords.

Searching for "cultured meat" as keywords, the database shows well over 568 results. Looking at the publication history, it can be seen that in 2022 there is a peak with more than 220 articles written, double the number of articles written a year earlier. If the keyword "cultured meat" is combined with the boolean 'AND NOT' followed by "FBS", the search obtains 513 results, with 197 articles published in 2022.

A similar trend occurs if the field is restricted to scientific reviews only, which in 2021 were around 24 and which doubled the following year. Research articles appear to account for the majority of publications overall, followed by review articles (Figure 14).

Figure 14 Scopus database search results for cultured meat related publications and related publication categories.

Similarly, studies in this area appear to be more flourishing in the area of agricultural and biological sciences, with more than 375 publications, followed by that of

biochemistry, genetics and molecular biology and then by the sector of environmental sciences (Figure 15).

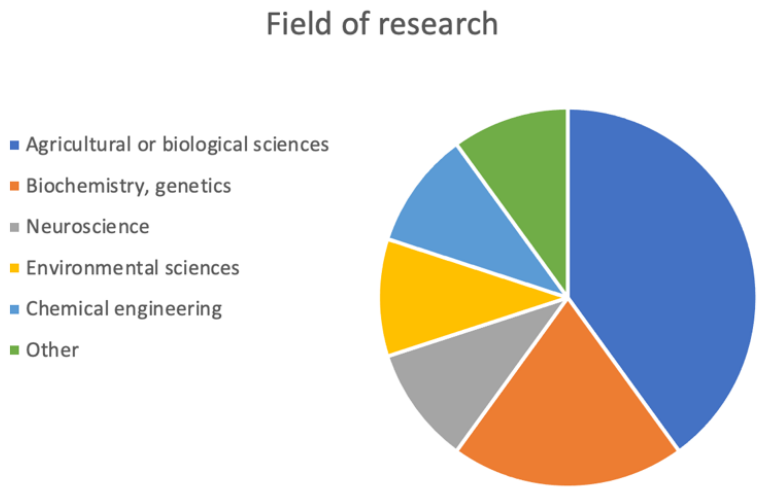


Figure 15 30 Scopus database search results for cultured meat related publications and publication scope.

Performing an analysis with the words "cultured meat" and "serum free" with the operator 'AND', the search leads to 48 results, most published between 2020 and 2022 (Figure 16).

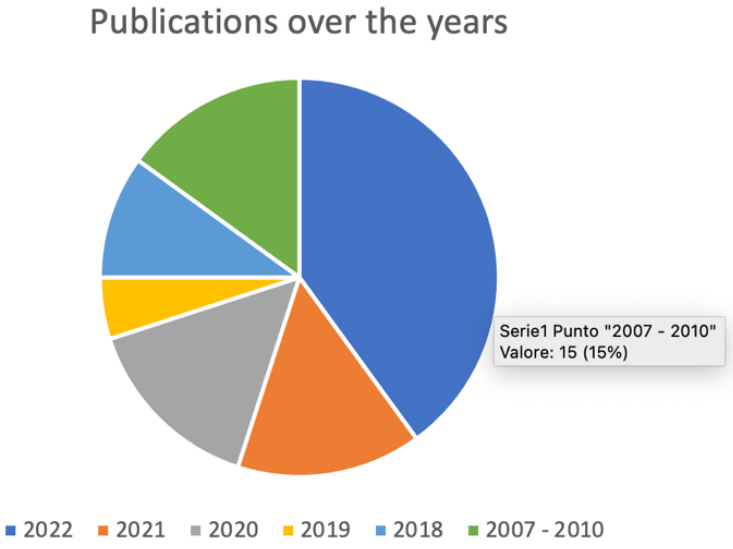


Figure 16 Scopus database search results for cultured meat related publications and publication.

1.6.2. Publications

In terms of the economic weight of FBS use, there is also the fact that the demand for whey for cell agriculture destined for cultured meat competes with that of more mature industries, such as those focusing on cell therapy research, which are currently sufficient to exceed the product's availability.

The alternatives to serum currently studied in the field of cell proliferation and differentiation are currently very complex, difficult to reproduce, not comparable in terms of production efficiency and economic sustainability compared to FBS, and have in any case required the use of animal derivatives for their realization. In the analysis of the literature it also emerges that these alternatives fail to satisfy the need to maintain a single culture medium for all stages of the process.

Gawlitta et al. published in 2008 a study [16] in which different differentiation substrates for engineered skeletal muscle C2C12 cells were compared. In particular, for research as a substitute for the typical serum used in the medium, containing precisely 2% horse serum (HS), the Ultroser G was selected which is characterized by various substances that favor differentiation (among all, the insulin, epidermal growth factors, fibroblast growth factors, IGF-I, thyroxine, bovine serum albumin). Good results have been obtained using the enriched Ultroser G (Table 1), the fact remains that however the use of this substitute does not completely eliminate the problem of the use of animal derivatives.

Table 1 Synthesis of the effects of different means on cells. Positive or negative symbols indicate how much a medium made a positive or negative contribution to creatine kinase levels, total protein levels, histological findings, and metabolic activity. IGF is an insulin-like growth factor [16].

Differentiation medium	CK	Total protein	Hystology	Metabolic activity
Horse serum	+	+	-	-
Horse serum plus IGF-I	++	+	+	-
IGF-I	-	-	-	-
Ultroser G	+	++	+	+
Ultroser G plus IGF-I	+	++	+	+

In 2009 by M. Das et al. studied [17, 18] the role of different growth factors capable of enhancing the formation of myotubes, which were used for the substrates used for the differentiation of muscle cells: a formulation for a culture medium was found (Table 2).

Table 2 Serum-free substrate formulation [17].

Components	**Amount**
L15	375 mL
M199	125 mL
Vitronectin	50 µg
B27	10 mL
Basic FGF	10 ng/mL
CT1	10 µg
GDNF	10 µg
BDNF	10 µg
NT3	10 µg
NT4	10 µg

Gottipamula et al. in 2013 published a review [19] on serum-free cell culture media of human stromal mesenchymal cells. In particular, they collect, in a very complete analysis of the literature, the research results carried out up to then and analyze the means on the market (Table 3).

Table 3 Commercially available serum-free media (2013). AF, no animal components; Cat no., catalog number; CD, chemically defined; CG, clinical grade; PE, ex vivo preclinical use only; PF, no protein; SF, whey-free; XF, xenon free [19].

Serum-free media	**Media type**
BD MosaicTM hMSC serum-free medium	DC, SF
CellGRO	SF, PE
HEScGRO	CD, SF, ACF
Mesechymal stem cell growth medium DXF	CD, SF, XF
MesenCult-XF	CD, SF, ACF
MesenGRO	CDM
MSC Qualified Plus	XF
MSC-Gro	SF, CG
MSCGS-ACF	SF
mTeSR	SF
PRIME-XV MSC Expansion SFM	SF
RS-Novo and GEM-Novo	CD, SF, ACF
SPE-IV	SF
Stemline MSC expansion medium	CD, SF,AF
StemPro MSC SFM	CD, SF

StemPro MSC SFM-XF	CD, SF, AF
StemXVivo	SFM
STK2	CD, SF, AF
TheraPEAK MSCGM-CD	CD, SF, AF
Ultrasor G	SF

In 2015, researchers at the NanoScience Technology Center of the University of Central Florida published an article [20] in which they analyze the non-animal-derived substrate used for the functional growth of myotubes from adult rat satellite cells. For cell proliferation a serum-free substrate was used a particular serum formulation, adding more medium after an incubation of 45 minutes.

After 8 days of proliferation the substrate was slowly replaced with another suitable for cell differentiation in order to promote cell fusion and alignment. The latter were then kept in an incubator at 5% CO2 and half of the medium was replaced every 3 or 4 days.

Table 4 Composition of serum-free cell differentiation medium per 500mL [20].

Components	**Amount**
Neurobasal	250 mL
L15	250 mL
EGF	50 ug
IGF	5 ug

The initial substrate was altered by spiking bFGF, which promotes cell proliferation, L15 medium (Table 4), which ensures increased myoblast life expectancy, and adding a calcium source in the form of CaCl2.

After a second time that the cells were placed in the respective dishes, a higher percentage of myoblasts and an increase in cell viability were seen to occur.

In 2019 a study by A. M. Kolkmann [21] came out in which the culture media on the market were analyzed, studying them in the context of the proliferation of myoblasts of bovine origin. FBM/DMEM and Essential8™ are found to have the potential to yield cell proliferation efficiency comparable to that obtained with media containing animal serum. But the researchers say further insights into cell attachment and viability during the first few days of culture and the combination of growth factors are needed.

In 2019 Kou et al. published a research [22] in which the use of an extremely cheap alternative serum for the growth of human pluripotent stem cells is described. The

substrate created is the result of the study of the previously used medium, E8 (Table 5).

Table 5 Composition of serum E8 [22].

Components	**Amount in E8**
DMEM/F12 with HEPES	1
Insulin	20 μg/mL
Ascorbic acid 2-phosphate	64 μg/mL
Transferrin	10 μg/mL
Sodium selenite	14 ng/mL
FGF2	100 ng/mL
TGFβ1	2 ng/mL
Sodium bicarbonate	1743 μg/mL

After comparing different commercial products currently in use, they used Matrigel or Trevigen/Gibco matrices for their experiments at a concentration of 2.5 μg cm-2 (dilution 1:800). They then followed a protocol that dissociated approximately 75% of hiPSCs into single cells using TrypLE, then seeded the cells into 12 dishes with the medium to be tested, allocating approximately 10,000 cells to each. The medium was changed every day and after five days the cell viability was studied with the PrestoBlue technique, for the first 24h the cells were immersed in a Rho kinase inhibitor.

The E8 study led to the implementation of serum B8 (Table 6) in a five-phase study. In particular, it was discovered that the insulin component is essential and in the final formula it was used at a concentration of 20 μg mL-1, but it can be replaced by IGF1 LR3 (at doses higher than 150 μg mL-1) even if this it is not economically comparable; acrobic acid 2-phosphate is not essential but increases cell growth capacity at high doses, therefore it was used in a concentration of 200 μg mL-1, similarly, although not essential, transferrin promotes growth when used in large percentages, has been used in 20 μg mL-1, and is inexpensive. The selenium component is essential for cells, therefore selenite sodium was used in a concentration of 20 ng mL-1, while FGF2-G3 was optimal at a concentration of 40 ng mL-1 and TGF-β3 protein if taken in 0.1 ng mL-1, as well as NRG1. In the study it was demonstrated that the addition of albumin does not influence the performance of the medium since its antioxidant properties were compensated by the high values of ascorbic acid. A percentage of activin A can be introduced if the concentration of TGF-β is lowered because they are not easily compatible.

The B8 greatly promotes cell growth and reduces the days in which the serum needs to be changed. The costs are reduced thanks to the ease with which the main components of the medium are reproducible in the laboratory, in particular a price of about $16/L has been reached which can greatly decrease with the optimization of the scale up process, while the substrates typically containing 20% FBS used for satellite cells cost about $200-500/L.

Table 6 Serum composition B8 [22].

Components	**Amount in B8**
DMEM/F12 with HEPES	1
Insulin	20 μg/mL
Ascorbic acid 2-phosphate	200 μg/mL
Transferrin	20 μg/mL
Sodium selenite	20 ng/mL
FGF2-G3	40 ng/mL
TGFβ3	0.1 ng/mL
NRG1	0.1 ng/mL
Sodium bicarbonate	2438 μg/mL

Stout et al. in their 2022 article [15] they explain how, starting from B8, the Beefy-9 medium for the culture of bovine satellite cells (BSCs) was synthesized. Also this substrate does not contain any animal derivatives. The addition to this of recombinant human albumin, expressed in rice, makes the medium efficient for cell expansion in vitro and the growth of BSCs is comparable to that verified in cultures involving the use of 20% FBS (BSC-GM). To lower the cost of B8 and Beefy-9, the concentration of FGF-2 can be reduced, in particular by reducing it up to 5ng/mL and 1.25ng/mL respectively. Unfortunately, the use of B8 alone has shown that the concentration of FBS can be reduced by 87.5%, but it has not been possible to eliminate it.

Numerous experiments were performed by combining the two mediums (Figure 17). The BSCs were planted in different dishes with B8, Beefy-9 and with BSC-GM respectively on day zero, on day 1 the cells were rinsed with phosphate buffered saline (PBS) and the medium was changed to Beefy-9. On day 3, at 70% confluency, cells were transferred using TrypLE and placed in a dish containing B8 and peptide adhesion agents. After one day the medium was changed back to Beefy-9. The proliferated cells were finally analyzed for adhesion, growth and myogenicity.

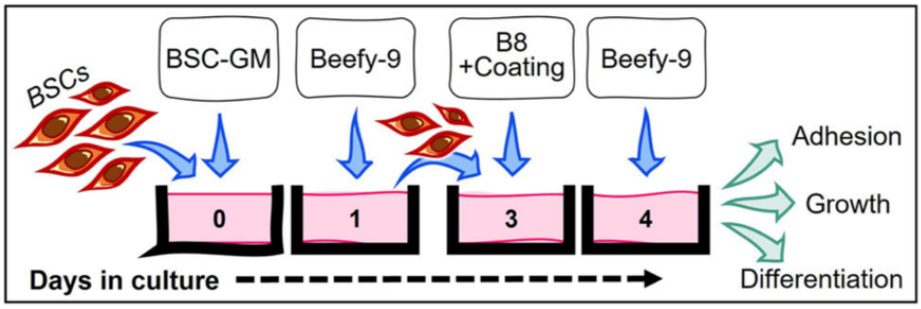


Figure 17 Procedure scheme of the experiments [14].

Beefy-9 substrate with high or low percentages of FGF-2 was found to be very effective in the long term but further optimization is needed to increase the growth rate over multiple passages. In addition, Beefy-9 demonstrates the ability to maintain cellular identity and myogenicity in a manner comparable to media containing serum. One highlighted issue is that cells cultured without whey accumulate lipids due to insulin resistance which is quite high in both B8 and Beefy-9, but this may not be entirely a problem considering that fat is an important organoleptic component for the meat.

At the beginning of 2022, Mosa Meat researchers published a study [11] in which they testify that they had induced the differentiation of bovine satellite cells thanks to a serum, chemically synthesized, without the condition of serum-starvation and the expression of strasgenes .

From the analysis on the serum-free differentiation medium (SFDM) performed, the complexity of the metabolic role of insulin and glucagon for in vitro culture can be deduced and further investigations are therefore promised to improve the results. It is also highlighted how LPAR1 favors the migration of activated SCs thanks to the stimulation of sphingosines.

By adding LPA at a concentration of 10 μM it was found that myogenicity was favored, considering that in the literature we read that higher values led to inhibition of differentiation for C2C12 cells. Furthermore, no relevant effects were found regarding the addition or not of oxytocin and ACh, even if the latter led to the formation of myotubes in bioartificial muscles (BAM): it is deduced that the addition of these maturation factors could give a significant contribution in the expression of genes that characterize the terminal phase of differentiation.

1.7. Economical raw material

Other very interesting studies concern the use of hydrolysates for animal cell culture, the first attempts of which date back to the early 1970s. These compounds are able to promote cell growth and protein production very effectively and, being often derived from waste products of low economic value, they fit into the context of the circular economy, helping to reduce waste. The objective of reducing the cost of the cultured

meat production process implies the search for a more economically convenient whey than FBS, in particular agricultural wastes meet this need, but generally have highly different compositions (Table 7) [23].

Table 7 Approximate composition of agricultural waste used for industrial microbial fermentation [16].

Hydrolysates are mainly composed of a complex mixture of amino acids, peptides, free acids, carbohydrates which result from partial or complete protein hydrolysis, also contain vitamins, lipids and inorganic acids which can support cell growth. Many types of hydrolysates have been studied in this regard, comparing their composition with that characteristic of animal serum (Table 8) [24].

Components		**Beet molasses**	**Corn steep liquor**	**Cottonseed embryo**	**Bacto peptone**	**Yeast extract**
Amino acids [%]						
	Aspartic acid	1.5			5	5.1
	Glutamic acid	1.5			8.1	6.5
Vitamins [mg/100g]						
	Folic acid	0.025	0.05			
	Biotin		0.01			0.14
Minerals [%]						
	Potassium	6.4	4.5	1.72	0.2487	0.04
	Calcium	0.21		0.25		0.04
	Magnesium	0.12		0.74	0.0017	0.03
	Phosphorus	0.03		1.31		0.29
	Sodium	1.6	0.2		1.8127	0.32
	Iron	0.03	0.03		0.00078	
Sugars [%]						
	Sucrose	48.9				
	Glucose	0.5	2.5		0.629	

Table 8 Approximate concentration of components present in sera of animal origin [17]

In particular, different amounts of different hydrolysates can be added to the basal substrate, the main ones being those of starch, soy or yeast, whose variability must be reduced using ultrafiltration techniques or by screening to highlight problematic components.

Steven C.L. et al. in 2016 published an article [25] analyzing the cell viability obtained using hydrolysate supplements to the basal culture medium. In particular, the two

Components		**Beet molasses**
Proteins and polypeptides		
	Albumin	20-50 g/L
	Transferrin	2-4 g/L
	Protease inhibitors	0.5-2.5 g/L
	Globulins	1-15 g/L
Growth factors	EGF, PDGF, IGF-1 AND 2, FGF, IL-1, IL-6	1-100 μg/L
Amino acids		0.01-1 μM
Lipids		2-10 g/L
	Cholesterol	3.867 mg/L
	Linoleic acid	2.805-28.05 μg/L
	Phospholipids	0.7-3-0 g/L
Carbohydrates		1.0-2.0 g/L
	Glucose	0.6-1.0 g/L
	Pyruvic acid	2-10 mg/L
Polyamines	Putrescine, spermidine	8.815-88.15 μg/L
Inorganic ions		
	Sodium	3.10-357 g/L
	Calcium	160.3-280.6 mg/L
	Phosphate	189.9-474.9 mg/L
	Potassium	195.5-586.5 mg/L
	Iron	0.03
Hormones		
	Insulin	5.778-577.8 mg/l
	Hydrocortisone	3.625-72.49 μg/L
Vitamins		10μg – 10 mg/L

substrates used are PowerCHO2 and CD FortiCHO with the addition of 6mM L-glutamine and other specific feeds for that cell line. 5g of hydrolysates were separately added to 1L of basal substrate, specifically those taken into consideration are: ultrafiltered yeast hydrolysate, peptone from soy glycine, Ex-Cell CD hydrolysate

fusion and HyPep 4601 hydrolysed protein derived from flour gluten. Exponential cell growth, total integral viable cell density (IVCD), and specific antibody productivity were then evaluated.

Adding the hydrolysates to the medium resulted in an increase in osmolality, which can increase productivity but hinder cell growth. The results show that yeast contributes significantly to increasing the performance of the process, while wheat hydrolysates increase cell growth.

Jian Yao Ng et al., in 2020 publish a study [26] in which, an aqueous extract of the alga Chlorella vulgaris is used as a growth factor (CGF) for cell proliferation.

Research seems to demonstrate that the growth of the chosen cell line (CHO) in the absence of animal serum is not comparable with that obtained using the same (Figure 18).

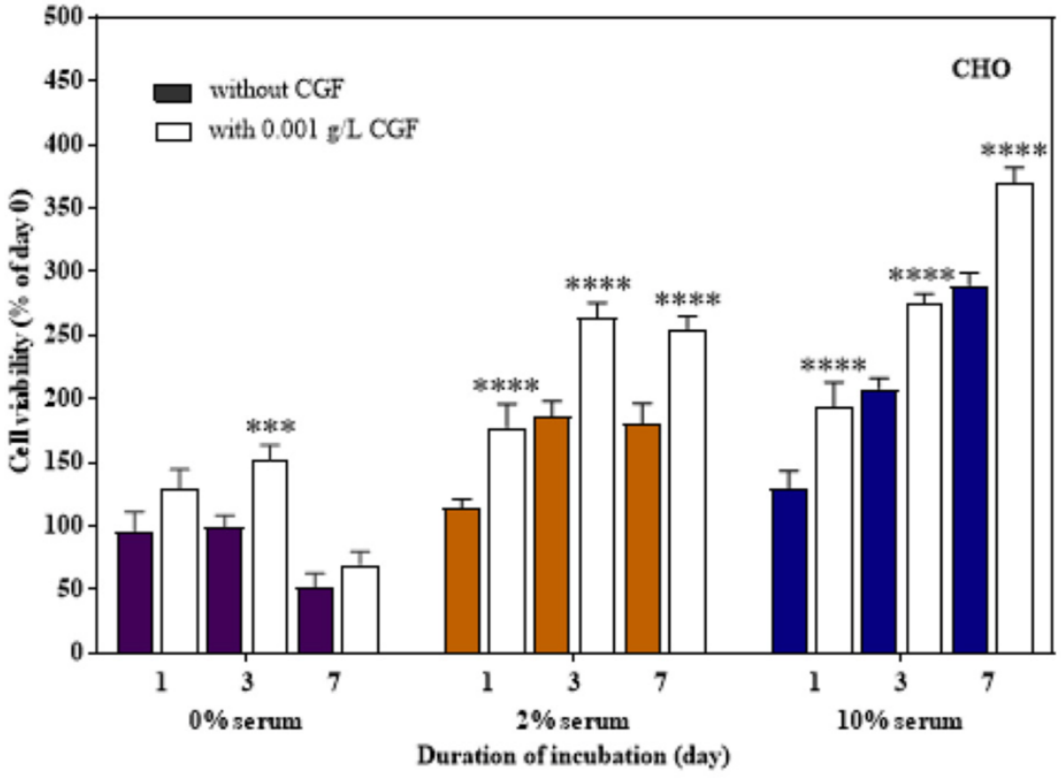


Figure 18 Effect of CGF on CHO viability [26].

However, it certainly highlights how the extract, at low concentrations, substantially increases cell proliferation and therefore can be a good supplement to the culture media.

1.8. State of art of the 3D bioprinting

1.8.1. Publications trends

Santoni et al. in 2021 [27] conducted a review on the current literature, specifically from 2000 to 2020, by retrieving 9314 scientific papers and 309 international patents. The researchers found a steady rise of the number of publication in the last 10 years and evidence the 143% increase of the number of scientific papers only in 2016 (Figure 19).

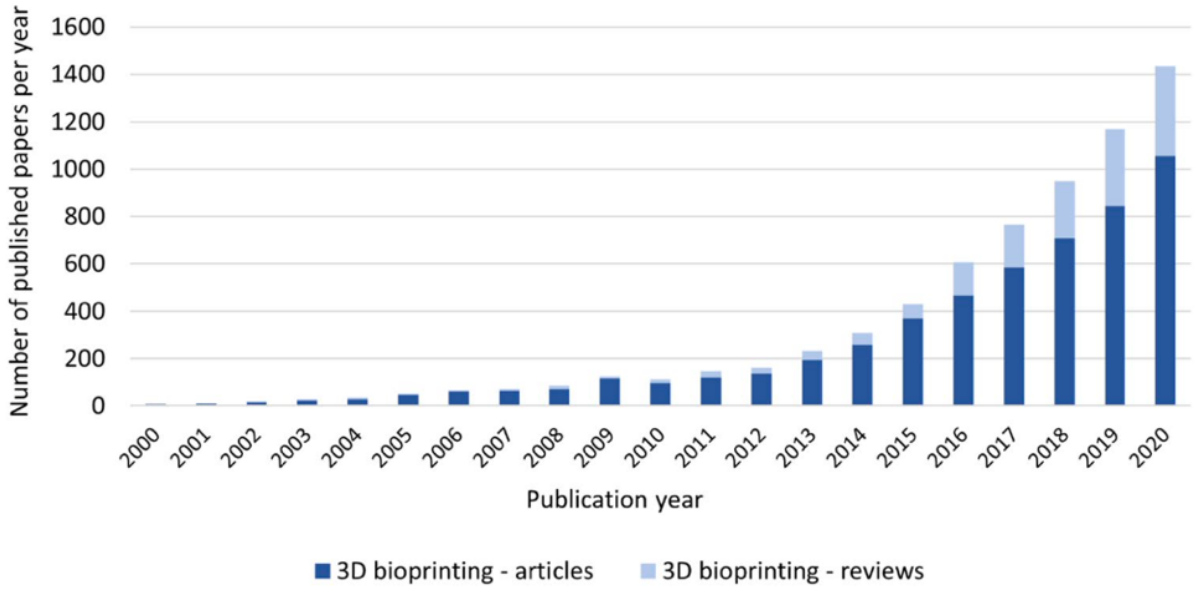


Figure 19 3D bioprinting publications by year. Blue bars correspond to the articles, while the light blue ones represent the reviews [27].

The most productive journals in this area appear to be Biofabrication, with 319 publications, followed by Biomaterials, with 184 publications, and Acta Biomaterialia, with 162 publications (Figure 20). The top-focalized journals was evidenced by studying the percentage of publications regarding bioprinting with respect to the overall number of papers from 2000 to 2020 [27].

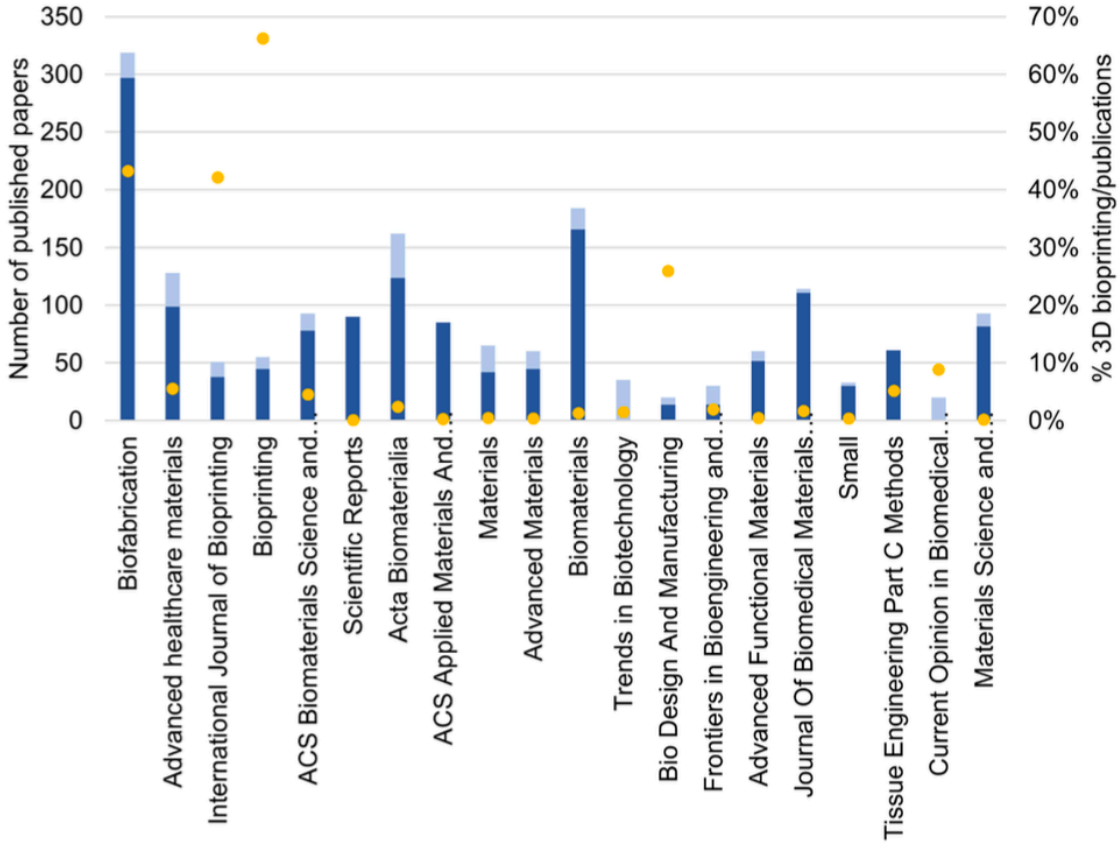


Figure 20 Top twenty journals focusing on 3D bioprinting. the bars represent the number of publications retrieved from Scopus.com while the yellow dots represent the percentage of publications focusing on 3D bioprinting with reguards to the total number of publications. The examined time interval is 2000 - 2020 [27].

The most widespread techniques studied are extrusion-base bioprinting, then the vat photopolymerization one and the lasts are the inkjet and laser-assisted bioprinting (Figure 21) [27].

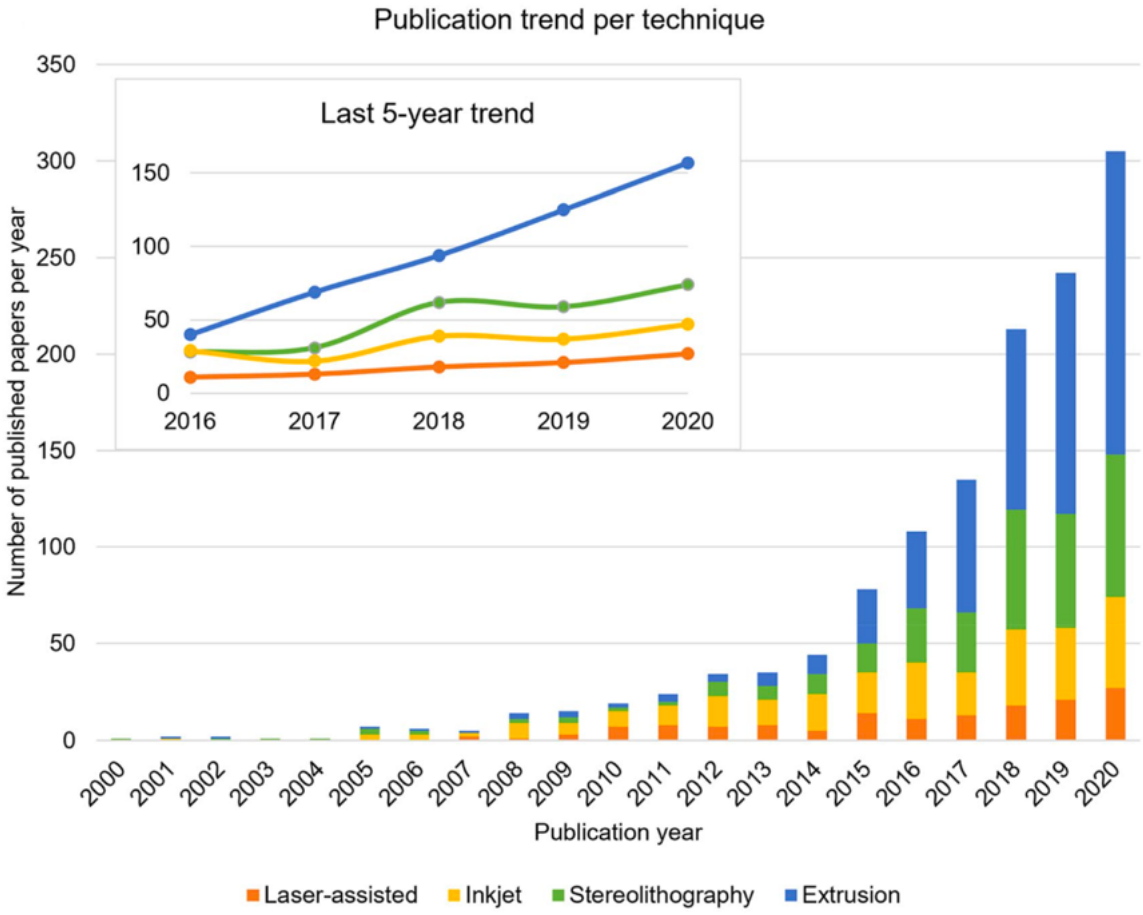


Figure 21 Number of pubblications for each bioprinting technique (extrusion, stereolithography, laser-assisted and inkjet) for publication years 2000 to 2020; inset: 5-year pubblication trend for 2016-2020

The researchers [27] clustered papers published since 2000 based on the text analytics keywords and shown them with its evolution over time in (Figure 22).

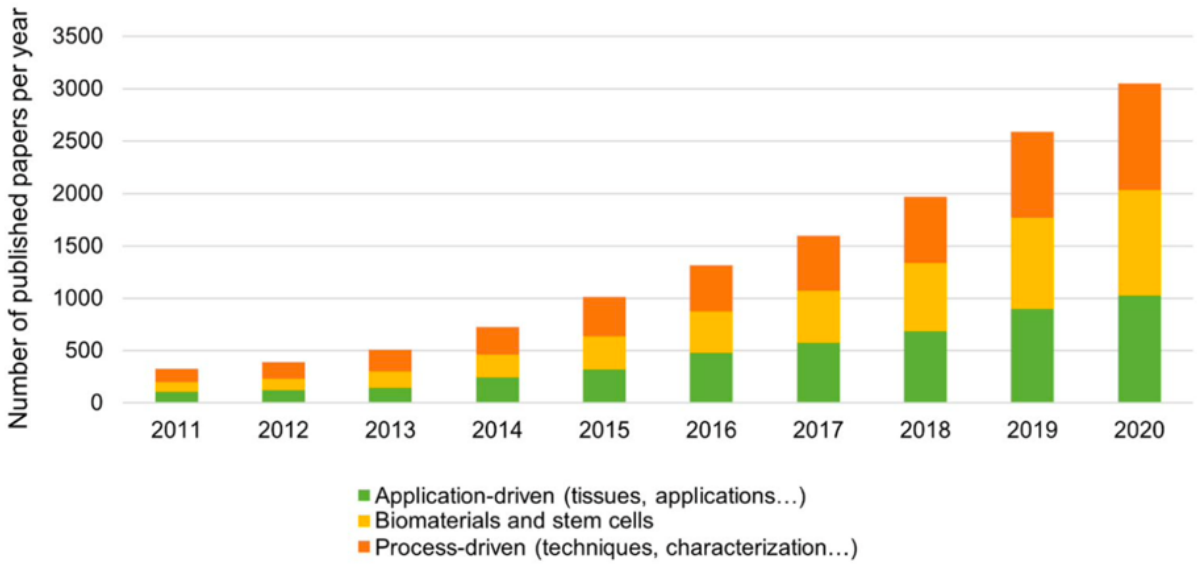


Figure 22 Trends of publication topics on 3D bioprinting over years. The number of publications relative to each topic are shown over time. The graph was created by counting at most one keyword in each topic class for each publication while having an average of two topics of interest in each publication [27].

The authors highlight (Figure 23) that even if there are some polarizing countries, the companies that develop and commercialize bioprinting technologies are relatively dispersed across nearly all continents [27].

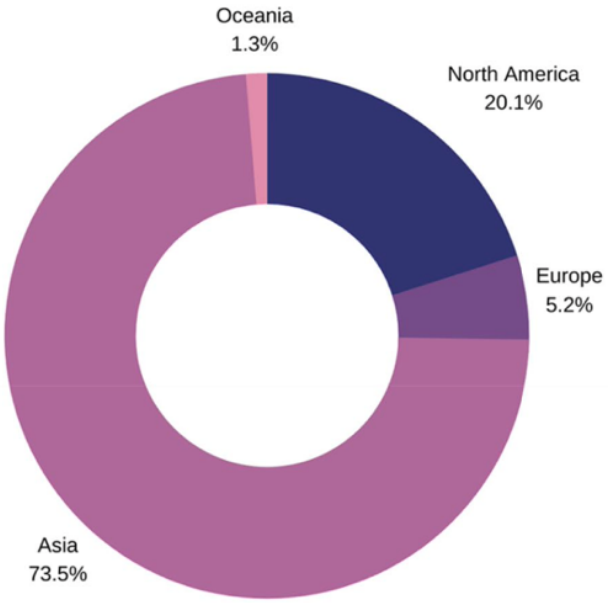


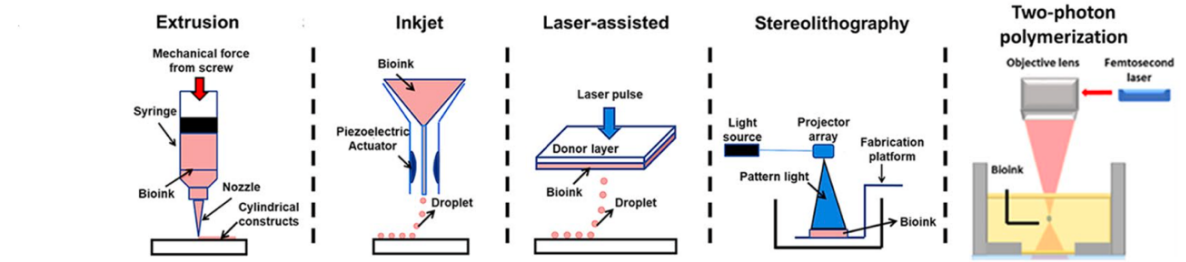
Figure 23 3D bioprinting market composition by continent [27].

1.8.2. Publications

In recent years, biotechnologies have oriented cellular agriculture towards 3D cultures, i.e. systems in which cells can grow, proliferate and differentiate in an environment that is no longer two-dimensional and therefore more similar to the organic one from a spatial point of view.

In the field of tissue engineering applied to biomedical and regenerative medicine in particular, a very promising approach is 3D bioprinting, focused on establishing biomimetic and functional tissue-like constructs in three-dimensions, manipulating cells together with growth factors and biomaterials based on a pre-designed computer model [28, 29].

The most common technologies used for printing biological material are injection, microextrusion and laser printing, and then the stereolithography and the two-proton polymerization techniques (Figure 24) [30].



	Extrusion	Inkjet	Laser-assisted	Stereolithography	Two-photon polymerization
Speed	Slow	Fast	Medium	Fast	Very fast
Cost	Moderate	Low	High	Low	Very High
Cell viability	85-95%	80-95%	<85%	>90%	>80%
Cell density	High	Low	Medium	Medium	Medium
Resolution	100 - 500 µm	100 - 500 µm	20 -100 µm	20 -100 µm	0,1 - 10 µm
Viscosity	6-30 × 10^7 mPa·s	<10 mPa·s	1-300 mPa·s	No limitation	No limitation
Applications	Tissue models for basic cell research, drug testing and	Supplementary to other technologies	Precision cell deposition	Scaffolds and complex structures with channels	Vascularized and high precision models

Figure 24 Different procedures for 3D bioprinting [30].

There are two main class of bioprinting technologies: the optical-based one, as stereolithography or vat photopolymerization 3D bioprinting, that is becoming a prominent bioprinting method for complex tissue, both in its traditional setting and the two-photon polymerization version.

Then there are extrusion-based technologies, as injections or microextrusion bioprinting.

Injection printers are the most used for printing biological and non-biological material, they allow you to print a controlled volume of liquid in predefined areas and are able to print at a very high resolution. An advantage of this type of printing is the possibility of introducing gradients of cells, materials and other grow factors into the three-dimensional structure.

Microextrusion is based on nozzle-deposition, which can have different printing resolutions and speed depending on the bioprinting head, the nozzle diameter and the droplet formation mechanism. This type of printing works thanks to a control of the temperature and of the material to be deposited by means of a robotic system that can execute the extrusion on the three axes x, y and z in different layers. Many materials are compatible with this type of printing, such as hydrogels, biocompatible polymers and cells. Extrusion-based bioprinting is the most studied approach as it is the least expensive technology, allows the use of a large range of bioinks.

Cell viability after microextrusion is lower than that given by injection molding and is around 40-86%, decreasing with the increase in extrusion pressure and that of the nozzle section: this is due to the greater stress of shear that cells undergo in the viscous fluid that is deposited.

In particular, the 3D bioprinting technique used for cell cultures consists in encapsulating cells in a bio-ink which ideally replicates the extracellular matrix and subsequently in extruding the latter with the assistance of a program which allows to customize the geometry of the structure to be printed.

The bioink can be composed of different edible or biodegradable materials: hydrogels are the most often used because they can offer a sustainable microenvironment for cell adhesion, growth, and proliferation [31].

Hydrogels can be natural or synthetically made. Among the former, the most used are alginate, chitosan, agarose, hyaluronic acid (HA), collagen, fibrin and dECM (decellularized extracellular matrix) [32]. Among the latter, however, there are polyacrylic acids and their derivatives, polyethylene glycol and others. Natural hydrogels are more beneficial in terms of biological properties, in fact they promote biocompatibility and cell encapsulation (Figure 25) [32].

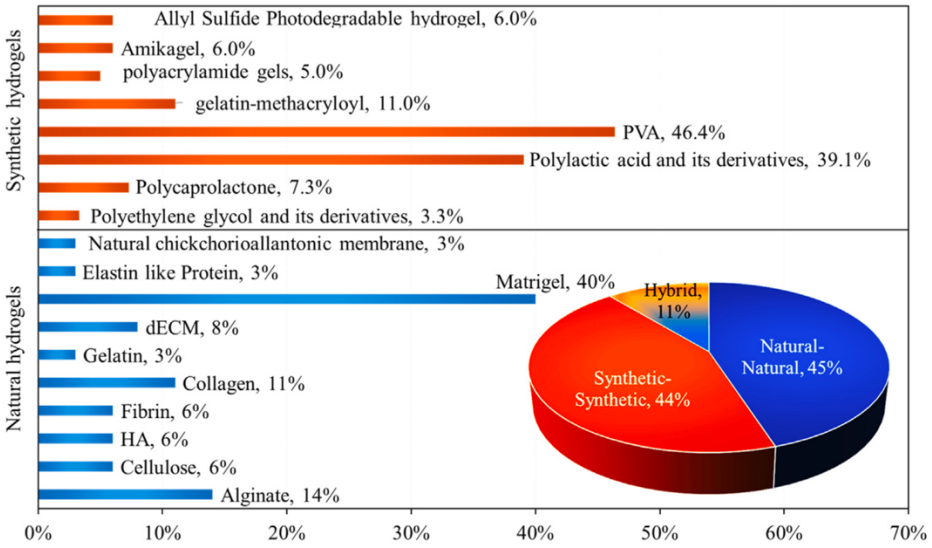


Figure 25 Percentage usage of hydrogels for biomedical applications [32].

The bioink is collected in a chamber, which pressurizes the bioink, causing the material to be expelled from the extrusion nozzle according to the specific geometry, which can also be multilayered [33, 34]. In particular, the printing parameters such as pressure, extrusion speed and temperature significantly influence the printing result, as well as the size of the nozzle, which must be suitable for that of the particles, to ensure a good stability of the bioink deposited and the cellular viability in it. The formulation of the ink is also fundamental both for printing and for the organoleptic properties of the product. In particular, gelling agents are essential and are necessary to promote efficient expulsion. These gelling agents can be made up of many types of compounds, among the most used ones we have: guar gum, a type of hydrocolloid capable of forming gels and retaining water, reducing the shear stress to which the cells are exposed during extrusion; gelatin or biopolymers such as starch, the latter being the most sustainable alternative and excluding the use of animal derivatives for the formulation of the bioink [35, 36].

The challenge is to identify the printing parameters and the characteristics of the bioink in order to make a trade-off between the needs related to cell viability and those related to the stability of the printed geometry (Figure 26).

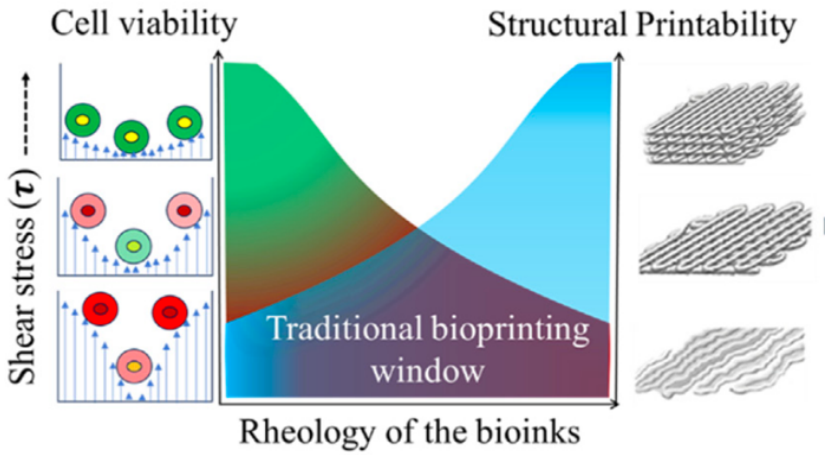


Figure 26 Trade-off of the reology of the bioink to maximise the cell viability and the printability of the hydrogel [36].

The bioink before or after being deposited in the pre-established shape, undergoes crosslinking to ensure the stability of the printed crosslink. Bioink rheology plays a significant role for filament extrusion, therefore, the type of crosslinking adopted has a significant impact on the printed constructs. Furthermore, the duration of the crosslinking can also alter the properties of the ink.

In particular Naghieh et al. in 2018 demonstrated [37] that the increase in the elastic modulus of the bioink is proportional to the duration of the crosslinking. In fact, alginate has excellent mechanical and rheological properties, and its viscosity depends on the molecular weight, composition, pH and temperature (Figure 27).

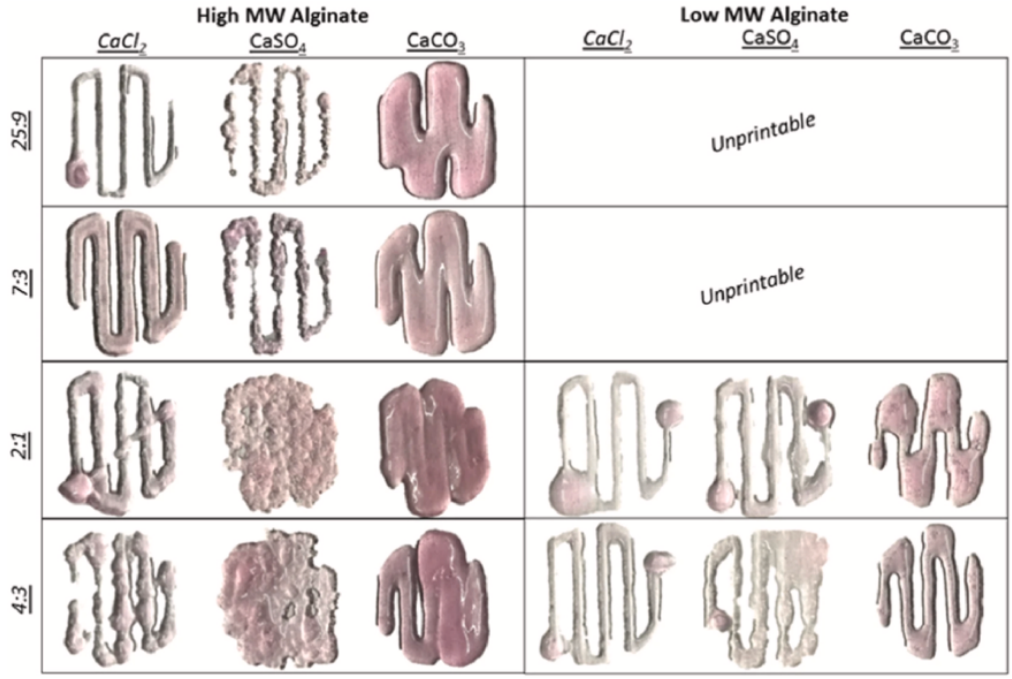


Figure 27 Printability of alginates bioinks with different molecular weight and temperature. Numbers to the left show crosslinking ratios [37].

Reakasame et al. studied [38] a crosslinking with calcium or barium ions on an alginate-based bioink, finding greater vitality using the first crosslink, while greater stability with the second (Figure 28).

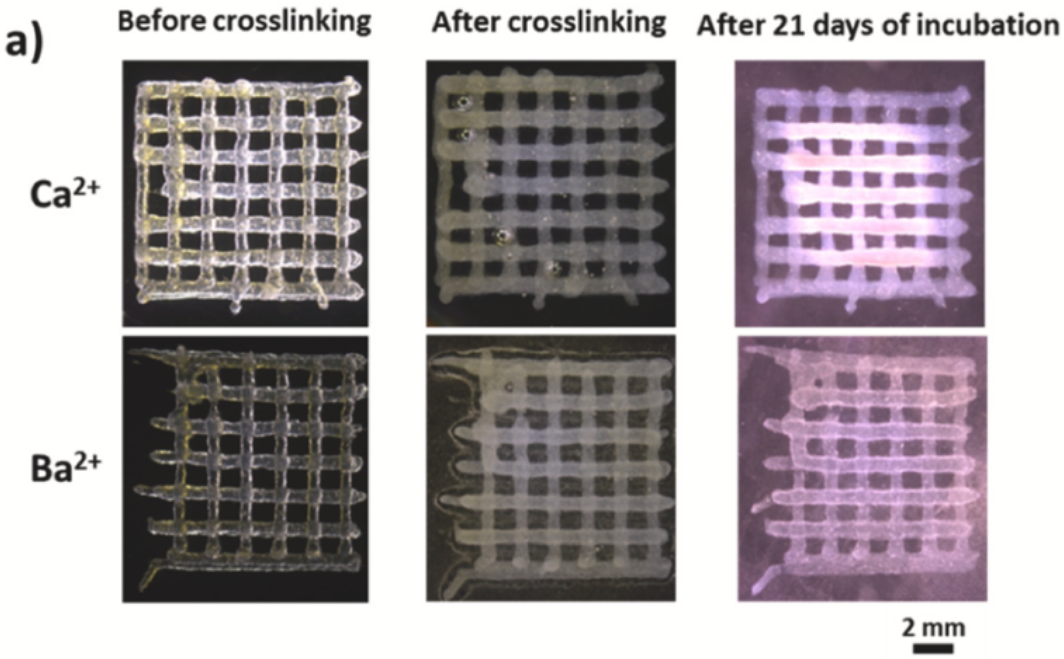


Figure 28 Different crosslinking effects [38].

Sbrana et al. published in 2021 a study [39] in which is described the use of the Bio X CELLINK 3D printer loaded with a particular hydrogel for CLL cell culture. The hydrogel was tested by setting a speed of 7mm/s and a pressure of between 11 and 14 kPa as printing parameters. In particular, from the Live/Dead assay it resulted that 75% of the cells printed in the scaffolds resulted to be viable (Figure 29).

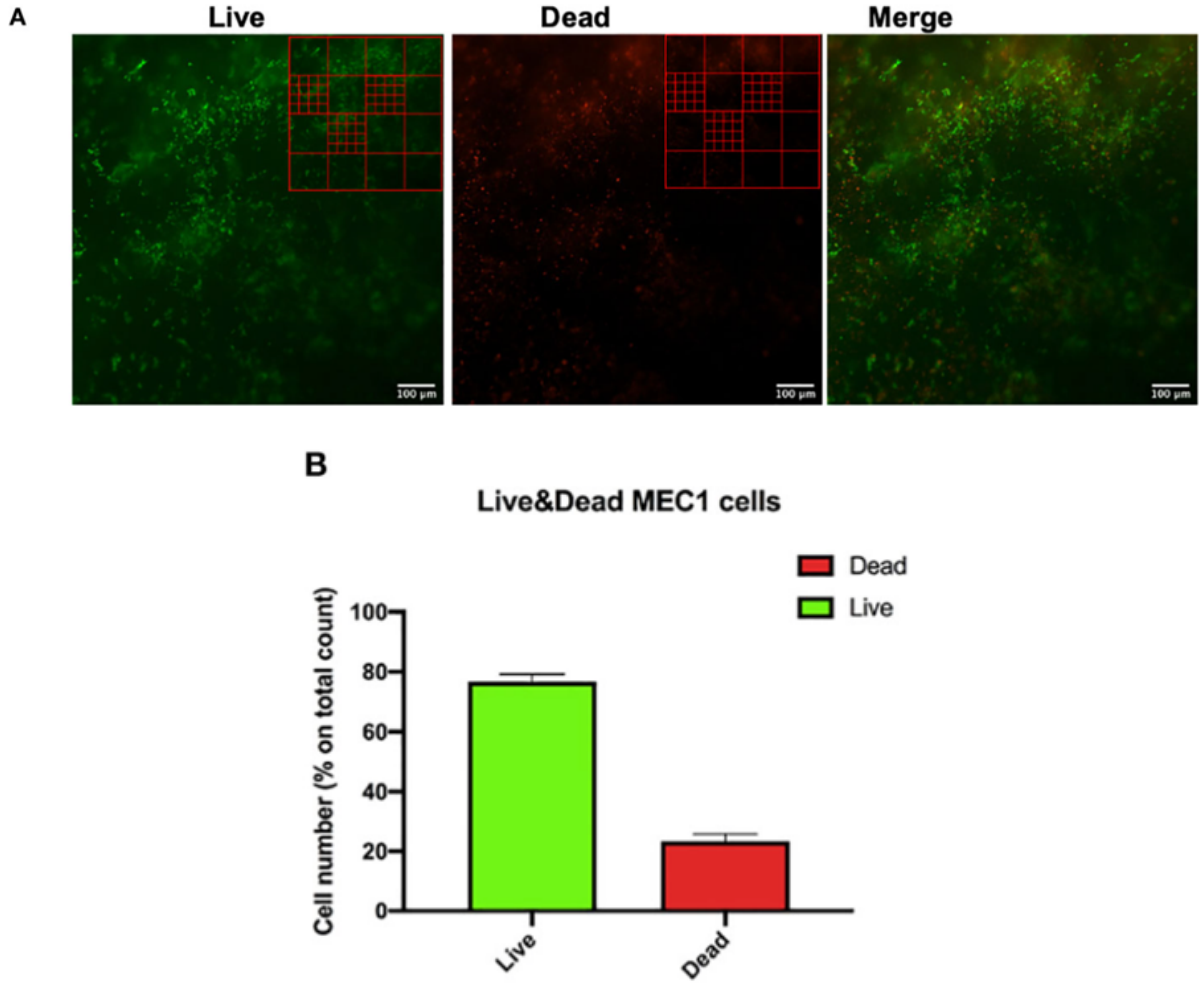


Figure 29 Viable and well-distributed cells in the printed construct (a) with a viability of 75% (b) [39]

Ianovici et al. demonstrated in research [40] published in 2022 that the alginate formulation spiked with soy and pea proteins results in a bioink suitable for cell culture and 3D printing (Figure 30).

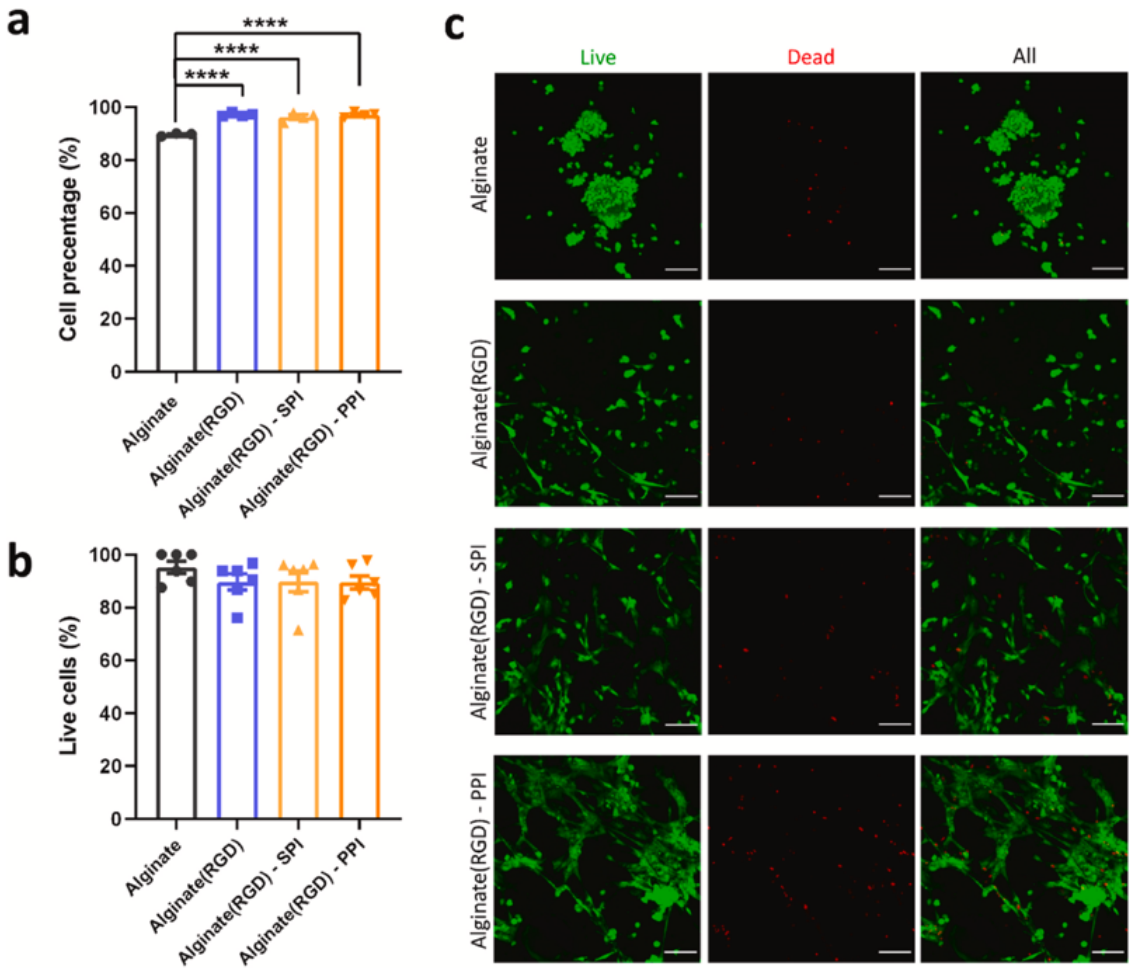


Figure 30 Cell viability with different types of alginate-based bioinks [40].

2 Research question

This study aims to identify, using economical plant ingredients derived from food or forest waste, a formulation of a culture medium and a printing bioink completely free of animal origin that can compete with those currently used today with the aim to lay the foundations for a cultured meat production process that excludes animal derivatives at every stage of production and which is cheaper on the market.

3 Materials and methods

3.1. Methods

3.1.1. Cultured meat acceptance analysis

The analysis of the acceptability of cultured meat was performed with an anonymous questionary on Google Forms to predict the outcome of the product's market entry.

The questionnaire, titled "Cultured Meat" had three different sections: the first was used to place the person who answered the questionnaire in a specific category defined mainly by age, place of birth, training and profession; the second part of the questionnaire investigated the individual person's interest in issues relating to sustainability and ethics in the context of the consumption of foods of animal origin; the third and last part deepened the knowledge of the person answering on the subject of cultured meat and the possibility of introducing this new product into the family diet.

3.1.2. Liquid nitrogen storage

C2C12 cells, a myoblast cell line that is a subclone of the mouse myoblast cell line, were stored in a growth medium supplemented with 10% (v/w) DMSO in 1 ml aliquots of approximately 1 x 10^6 cells.

3.1.3. Cell culture

1 mL aliquot of cryopreserved cells are thawed in water bath at 37°C. Cells are transferred in a 50 mL falcon with 24 mL warm media, that consist of Dulbecco's Modified Eagle medium (DMEM, from ThermoFisher), a basal medium that supports the growth of different type of mammalian cells; supplemented with 10% fetal bovine serum (FBS; ThermoFisher); 1% Penicillin-Streptomycin, a ready-to-use antibiotic mic of penicillin and streptomycin; 1% L-Glutamine, an essential amino acid required by mammalian cells grown in culture.

The solution was gently mixed. Cells were then plated in a T-75 Flask at a density of 100,00 cells/cm^2. After 24 h of incubation at 37°C with 5% CO_2 the medium was removed, cells were washed with PBS and then fresh media was added. Cells were cultured in the incubator to a maximum of 70% confluence.

3.1.4. Cell counting

Cells was mixed with an equal volume of 0.4% Trypan Blue (ThermoFisher) and the solution with cells was then transferred on Luna Counting Slide. Cells was counted with a CELENA® S Digital System with the DEFAULT protocols with Autofocused Counting activated (Figure 31).

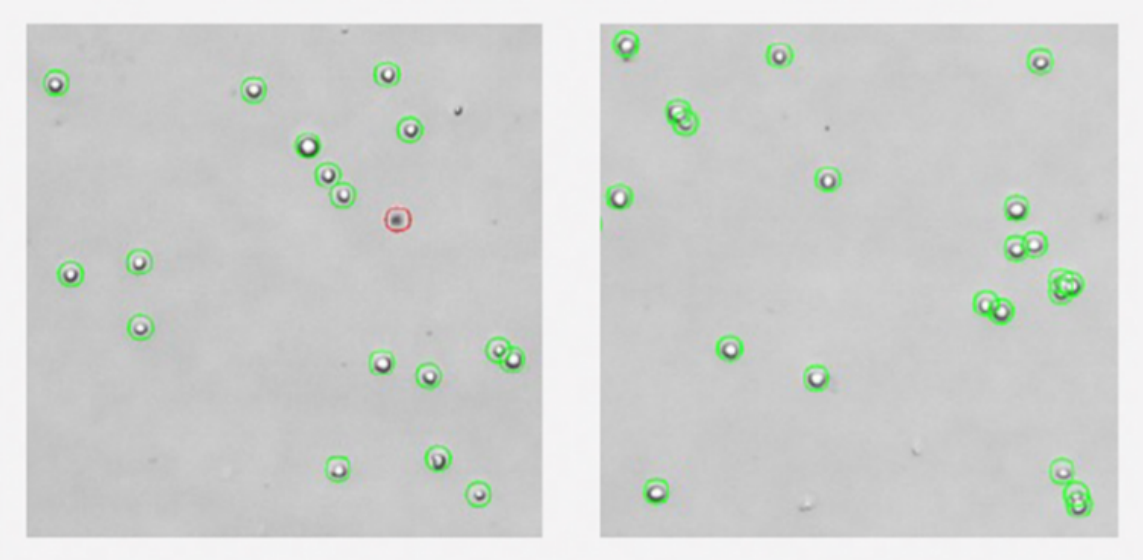


Figure 31 Cell images captured and analyzed with the automated cell counting feature of the CELENA® S. Live and Dead cells are labeled with green and red circles, respectively.

There was performed 3 counts (C) for each side of the LUNA counting chamber and the average (A) of the results was used to plate the wanted number of cells in a T-75 Flask (Equation 1).

$$\frac{\Sigma C}{6} = A \qquad (1)$$

With the summatory of C that goes from i = 1, 2, 3, 4, 5, 6 and A is the average of the count.

$$\frac{5}{m} = s \qquad (2)$$

Having 5 on the top of the fraction as a single T-75 Flask need to contain 500,000 cells. s are the mL of cell solution necessary to plate the exact number of cells in the Flask that was filled with 15 mL - P of medium too.

3.1.5. Splitting

Reached the 80% of confluence the cells were splitted. Cell medium was removed, the Flask was washed with PBS and then cells was incubated with trypsin for 5 min at

37°C. After that time the doubled volume of fresh media was used to neutralize the action of trypsin.
Detached cells were then splitted from the first flask in two different ones and maintained in line. The experiments were performed in multiwell M96 (Figure 32).

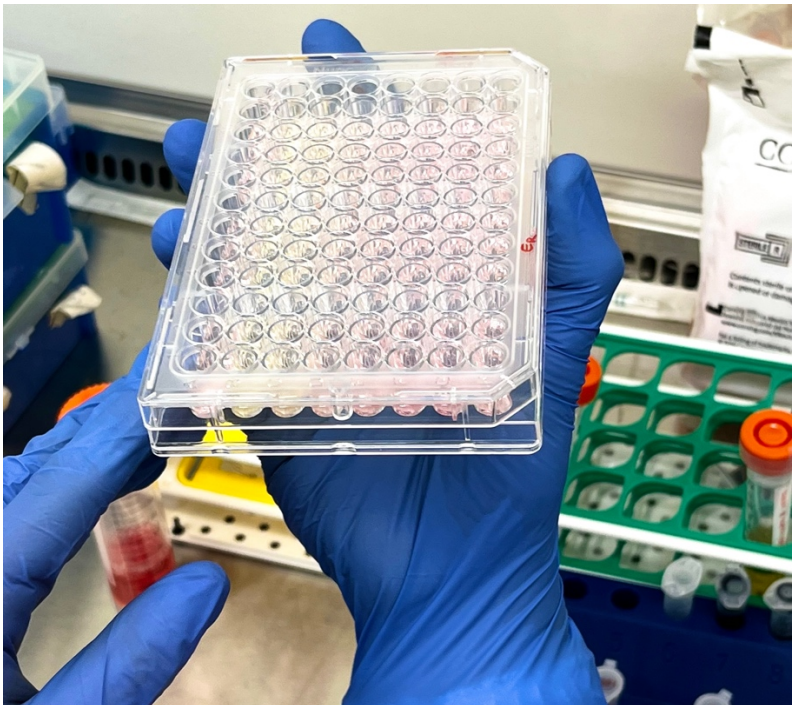


Figure 32 Multiwell from 96 in which cell viability was being tested in cell culture with FBS-free medium. Photo taken by the author.

3.1.6. MTT assay and data analysis

The MTT assay protocol [41] was performed using MTT kit provided by Thermo Fisher. This test is based on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. Viable cells with active metabolism convert MTT into formazan. Dead cells lose this ability and therefore show no signal (Figure 33). This color formation serves as a useful and convenient marker of only the viable cells. The measured absorbance at OD 590 nm with the TECAN is proportional to the number of viable cells.

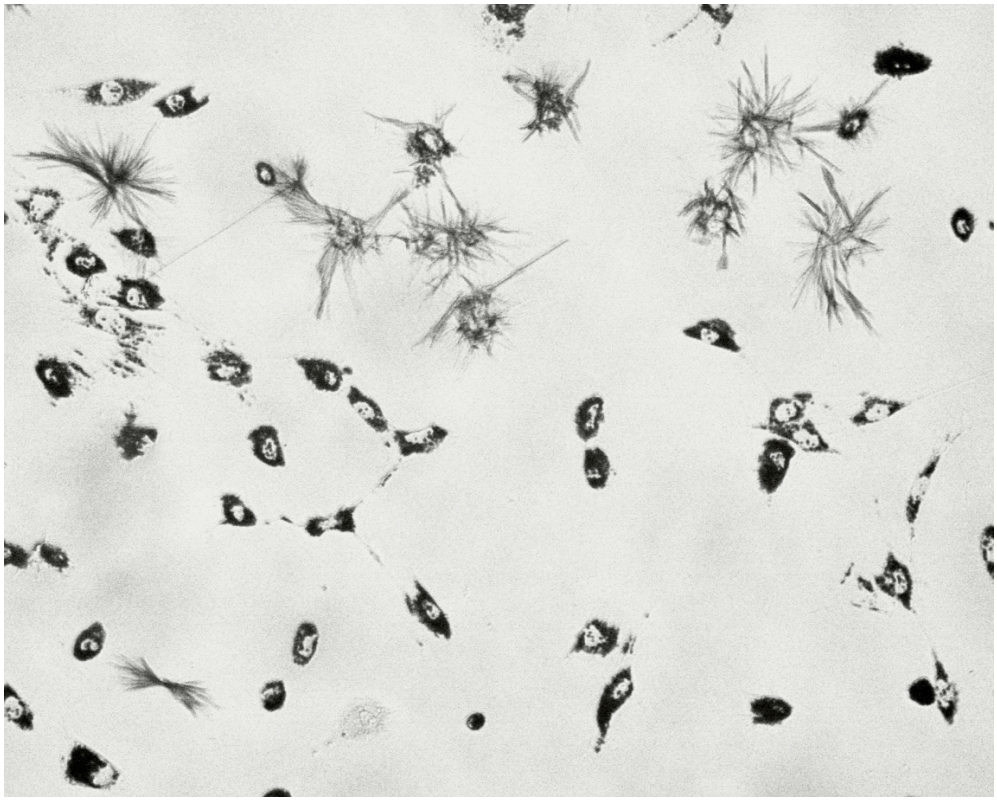


Figure 33 Image captured at the CELENA after half the incubation time of the wells containing the MTT solution.

MTT analysis was performed to:

- Evaluate the optimal concentration of cells that was needed to be seeded in a single well of a M-96 Multiwell.

- Evaluate the optimal concentration of additive that has to be integrated in the basal medium to have the maximum of cell viability.

  The test was conducted to identify the minimum concentration of FBS, as well as to investigate the possibility of replacing some protein hydrolysates with FBS.

  Since soy, wheat, and yeast hydrolysates have been found to be more effective in the literature, those in different concentrations were used.

Day 0 – After the cells detachment it is necessary to count them to have a solution with de desired cell density to plate 100 µL in every well.

Day 1 – The medium was aspired from every wells, leaving the cells attached.

Then the wells were filled with the same amount of DMEM/F12 cell medium form Thermo Fisher, a completely animal-free medium, with 1% of L-Glutammine and 1% Penicillin-Streptomycin, with a constant 10% of FBS in the medium but with different

cell densities, or with different concentration of FBS and hydrolysates was stocked with the optimal cell density highlighted.

Day 2, 3, 4 – From the wells representative of 24 h, 48 h and 70 h of treatment the medium was removed and the MTT solution was introduced. After 3,5 h of incubation at 37°C with 5% of CO_2 it was possible to read the results of the experiment at the TECAN, individuating the viability of the cells in the different wells thanks to the proportion with the value of absorbance.

The data analysis was performed on Excell developing an algorithm that executes the mean of all the values obtained from the control, the treatment and the reference (no-cell control), and a normalization of those means with respect to the control values.

All the graph was obtain from the results developing an Excel algorithm to highlight the trends of the cell viability during the days of the experiment.

3.1.7. Stock solution for the printability

An 8% w/v alginate stock solution was prepared dissolving sodium alginate in sterile PBS. To achieve the complete hydration of the sodium alginate powder, with a heated magnetic stirrer the solution was continuously stirred at 50°C for 4 h. The same procedure was adopted to produce the 6% Alginate + 4% Gelatin stock and the Alginate + 0.05% of hydrolysate stock, taking care in not trespassing 30°C for the gelatin stock solution in order to avoid the degradation of gelatin. The same inks were produced with viable cells inside.

3.1.8. Bioprinting

The 3D bioprinter BIO X ™ provided by Cellink was used. It has a printhead able to scan along the x-y plane and print bed moving along the z axis and it is possible to insert in it either a pneumatic extruder with or without temperature control, an inkjet or a piston of a filament extruder and an HD camera. The bioprinter is also capable of controlling the temperature of the nozzle and print bed.

The bioprinter was equipped with a pneumatic extruder with temperature control together with the HD camera. Bio-inks and cells are gently mixed at a concentration of 1 million per mL, following the protocol provided from Cellink. A single droplet in a single well was printed with an extrusion nozzle of 0.410 mm diameter. The printing was performed in a sterile environment.

3.1.9. Image analysis

The images were gathered by the bioprinter HD camera tool, and imported to a PC from the BIO X. The image analysis was performed manually and the data analysis was supported with Excel spreadsheet.

3.1.10. Live/Dead assays

Live/Dead assay is a very common cell staining procedure. It was performed using the Live/Dead kit from Thermo Fisher. Live cells are stained with calcein (2μM in medium) and generate green fluorescence upon excitation of their cytoplasm while dead cells are labeled with Propidium Hydride (PI, 0.5μM in medium) which binds to their DNA and fluorescence red. The images of the wells with the drops of gel-containing cells and fed in the optimized culture medium were captured with the appropriate CELENA® S Digital System option.

The Live/Dead experiment was completed by studying the images captured under an optical microscope with an algorithm implemented in the MATLAB environment for counting live cells and dead cells.

3.1.11. Cytotoxicity assay

To study the amount of damaged or dying cells the Invitrogen™ CyQUANT™ Cytotoxicity Assay Kit was used following the protocol [42]. It provides a method that monitors release of a cytosolic enzyme, glucose 6-phosphate dehydrogenase (G6PD), from damaged cells into the surrounding medium through a two-step enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin.

The resulting fluorescence signal is proportional to the amount of G6PD released into the cell medium, and this release correlates with the number of dead cells in the sample.

The assay was performed by seeding the M96 multiwell with 30,000 cells in a 50 μm volume reserving three sets of triplicate wells for a reference, that consists of a no-cell control, untreated cells and completely-lysed cells, used to determine the total amount of cellular G6PD.

The cytotoxic agents were added to the experimental wells and then the M96 was incubated for 24 h.

The Reaction Mixture Solution formulated in the protocol [42] was combined with the 2X Resazurin Stock Solution and the Reaction Buffer and 50 μm of the solution was added to each well. 1 μm of 100X Cell-Lysis Buffer was added to the completely-lysed control wells only. The M96 was then incubated at 37°C for 30 minutes. The fluorescence was measured at 5-minutes intervals to determine the optimal time point for the particular experiment.

The values of untreated control cells were subtracted to those of experimental cells and the completely-lysed control cells. The relative cytotoxicity was determined for each experimental condition dividing the corrected fluorescence of the experimental cells by the corrected fluorescence of the completely-lysed control cells.

3.2. Design of the experiment

The objective of this thesis project, which is part of the study of the production of cultured meat, was to exclude the use of animal derivatives from 2D and 3D cell cultures. For this reason the study was divided into two main phases: the first was carried out to investigate the possibility of creating a formulation for the cell culture substrate without FBS, evaluating the performance of alternative formulations that are economical and included in a concept of circular economy using protein hydrolysates derived from food or forest waste. In particular, the study initially focused on the evaluation of three different hydrolysates, one soy-based, one yeast-based and one wheat-based.

The first step of the experiment focused on defining the optimal seeding cell density for 2D cultures of the cell line studied. Based on the time the cells reached confluence, a better specific cell density was identified.

The second step of the experimentation focused on identifying the best performing culture medium by excluding FBS in its standard concentration of 10%. The performance of the three protein hydrolysates selected as an alternative to FBS was evaluated in terms of cell viability by studying different concentrations of the hydrolysates themselves in the basal medium. The combination of the two best hydrolysates in the culture medium was then studied to verify whether the performances were increased by using the hydrolysates together or whether it was better to use them individually. In this way, the optimal formulation of medium with a specific concentration of hydrolysate was identified which was able to guarantee cell viability comparable to that obtained with the medium having the standard FBS concentration.

The third step of the analysis saw the formulation of a bio-ink that excluded components of animal origin using the concentration of hydrolysate (ALW) identified as best in the previous phase and the evaluation of its printing performance comparing it with the bio-ink of alginate (AL), using that as standard bio-ink, and that of alginate and animal gelatin (ALGEL). In particular, the optimal ranges of pressure and printing speed were identified by evaluating the acceptable size of the printed filaments.

The fourth step of the experiment had the aim of evaluating the cell viability that the bio-ink based on alginate and peptone hydrolysate can guarantee compared to that containing gelatin, using the one with a formulation free of animal derivatives as a culture medium.

In this way it was possible to study the possibility of carrying out cell cultures with which to create cultured meat without the use of animal derivatives in the manufacturing process.

3.3. Data collection

The data that were collected during the phase of the project that concerned the analysis of the acceptability of cultured meat by consumers was analyzed using graphs prepared by the Google Forms software and indicated the percentages of individuals who chose a certain answer to each respective question.

The data collected during the cell density phase of the project was collected under the CELENA light microscope by comparing cell confluence.

The data that were collected during the phase of the project that concerned the identification of the optimal concentration of FBS replacement additives in the medium was collected by measuring the fluorescence intensity using the TECAN.

Each additive was evaluated with three different iterations. The data collected from these repetitions was averaged. The mean of each reference, which corresponds to the cell-free medium, was subtracted from that of the treatments on the respective days and the result was normalized by dividing by the mean of the control, which corresponds to the medium with 10% FBS (Equation 3).

$$\frac{\bar{x}_i - \bar{r}}{\bar{c} - \bar{r}} = t_i \qquad (3)$$

The data were put on an histogram-type graph which highlighted the intensity of the fluorescence, proportional to the cell viability, on the different days of experimentation, for each of the treatment.

The data that were collected during the phase of the project that concerned printability was collected with the HD camera of the bioprinter and the areas of the bioinks printed were identified by a confront to the known dimension of a 1 cent coin.

During the phase of the project that concerned the cell viability on the bioprinted construct the data were collected firstly with the optical microscopy by capturing images of the wells containing the constructs cells in the two different mediums.

In particular, using the Live/Dead kit with Calcein, PI and DAPI, under the microscope different colors can be found which correspond to cell viability or non-viability. The fluorescence evaluated under the optical microscope can appear green or red: the cell cytoplasm has green fluorescence, while the part of the nucleus has red fluorescence. The prevalence of red over green indicates a high cell mortality, while if the cells appear green they are alive. The images were analyzed with a special algorithm on the Matlab work environment developed for this type of cell count.

The data regarding cytotoxicity were collected by TECAN daily by averaging the values reported in the area of each construct present in the wells of the multiwell. The data corresponding to the wells in which the medium spiked with wheat was used were normalized, for each day, with respect to the respective controls, those containing the medium with FBS. The T-Test statistical test was performed to verify the

concreteness of the results obtained by TECAN in the three repetitions carried out for each ink, in each medium.

For the data obtained from the G6PD assay collected by TECAN, an average was made.

The fluorescence values of each construct cultivated with medium containing wheat, day by day, were normalized with respect to the respective controls and a statistical test of the T-Test type was performed to evaluate the concreteness of the values obtained in the three repetitions.

A subtraction was made from each construct grown in wheat-containing medium, of the reference, which consisted of the respective ink grown in the presence of FBS. With the subtraction result, a cytotoxicity ratio was calculated (Equation 4), with respect to each ink cultured with medium containing FBS, day by day.

$$\frac{\bar{t}-\bar{c}}{\bar{c}} \cdot 100 = r_i \qquad (4)$$

4 Results and discussion

4.1. Cultured meat acceptance analysis

The sample examined, made up of more than 2,600 people, had to answer various questions divided into three specific sections. The sample, was reached through a social media campaign, and through private sharing of the link containing the anonymous survey.

The first section was intended to get to know the person answering the questionnaire.

50% of the answers were given by people aged between 20 and 40 who live in the city. While 60% of the answers came from people who identify themselves with feminine pronouns.

30% of the people who answered had a high school diploma and 50% had scientific training, while 40% of the answers came from students and 20% from office workers.

The second section was drawn up in order to identify the diet of the sample examined and the sensitivity of the people who answered the questionnaire regarding the impacts on sustainability and ethics of their eating habits.

60% of the people who answered were omnivores.

more than 1100 people declared that they eat meat less than seven meals a week and 50% explained the fact that they consume meat for taste reasons, while 40% are out of habit.

Nearly 50% of people who say they have reduced their meat consumption have done so for environmental reasons.

40% of the sample buys meat in supermarkets. Furthermore, the meat consumers who responded stated that, when they are buying it, they first consider their health, then the farming methods and finally the environmental impacts of the product. Furthermore, they would agree to pay up to 20% more than the basic price if the product was firstly certified for a better environmental and health impact, and subsequently for better methods rearing of slaughtered animals (Figure 34).

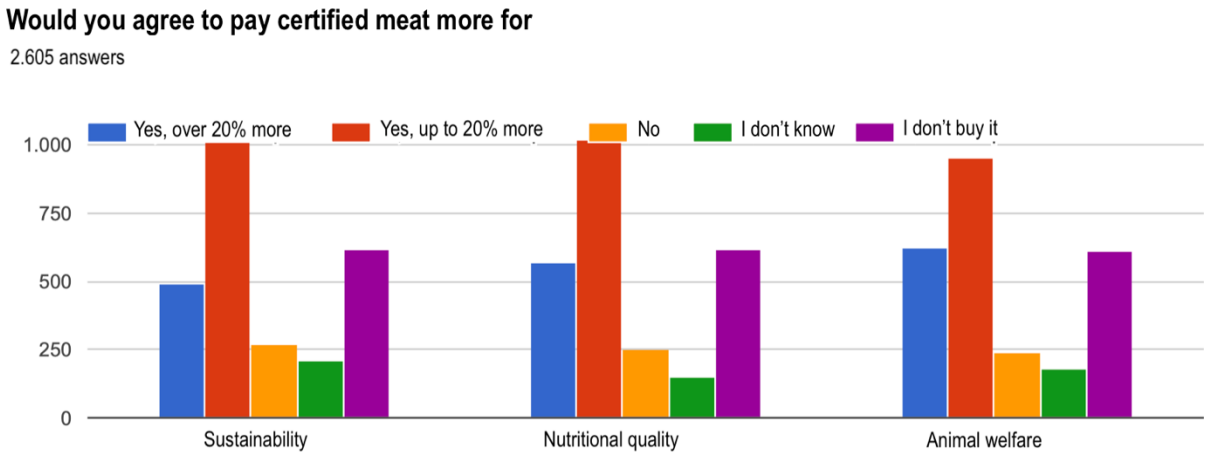


Figure 34 Survey responses on consumers' predisposition to pay more for a product with certain certifications.

The third and final section of the questionnaire had the intention of understanding the knowledge of the sample examined on the subject of cultured meat and the possibility of the people who answered to include the new product in their family's diet.

45% of the sample stated that they know how to explain what cultured meat is, but not precisely, and 30% of the people who answered get information on the subject through social media.

In addition, about 50% of people have heard more of this new product referred to as "synthetic meat", compared to only 20% who have heard it referred to as "cultured meat" (Figure 35).

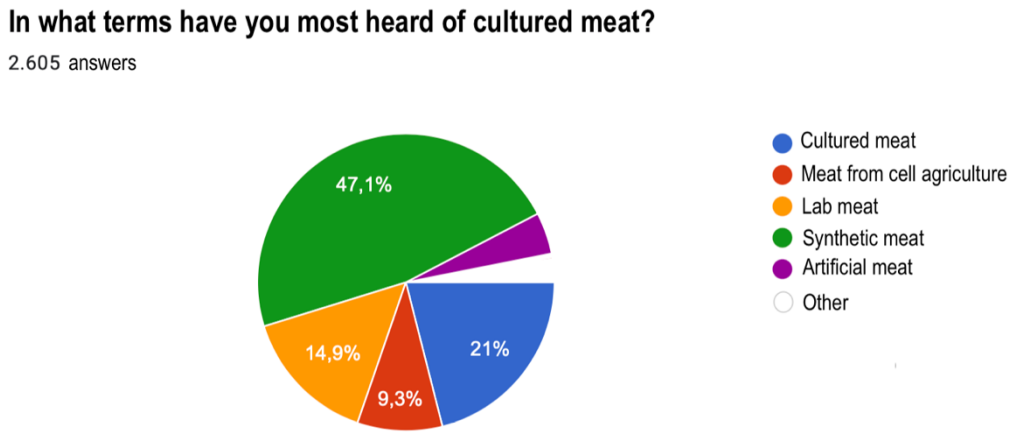


Figure 35 Survey responses about how the product is known by the public.

More than 2000 people answered that, in their opinion, cultured meat compared to traditional meat has a positive impact on the environment and the life of farmed animals, while 40% of the answers indicated that cultured meat will cost more than traditional one and 30% of people believe that the product will be similar to the traditional one in terms of taste.

The analysis proceeded by evaluating the opinion of the sample regarding the research on cultured meat, its production and marketing: an average of 2,000 people said they were in favor of all three things (Figure 36).

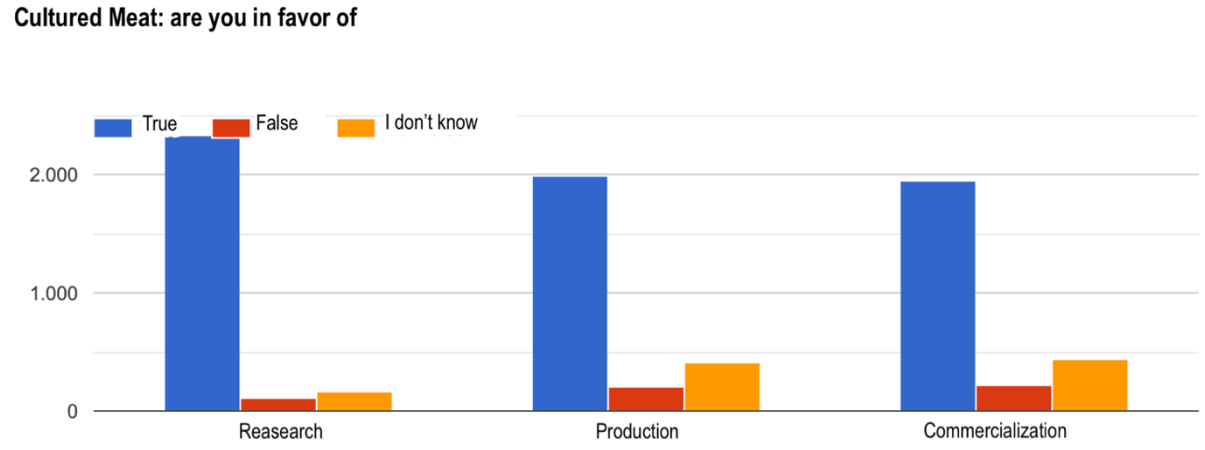


Figure 36 Survey responses about public favor on cultured meat research, commercialization, production.

Nearly 80% of the sample responded positively to the question "Would you taste cultured meat?" (Figure 37).

30% of people who replied to the questionnaire said they would replace traditional meat with cultivated one. The majority of the people that has answered said that they would by the product for their pets.

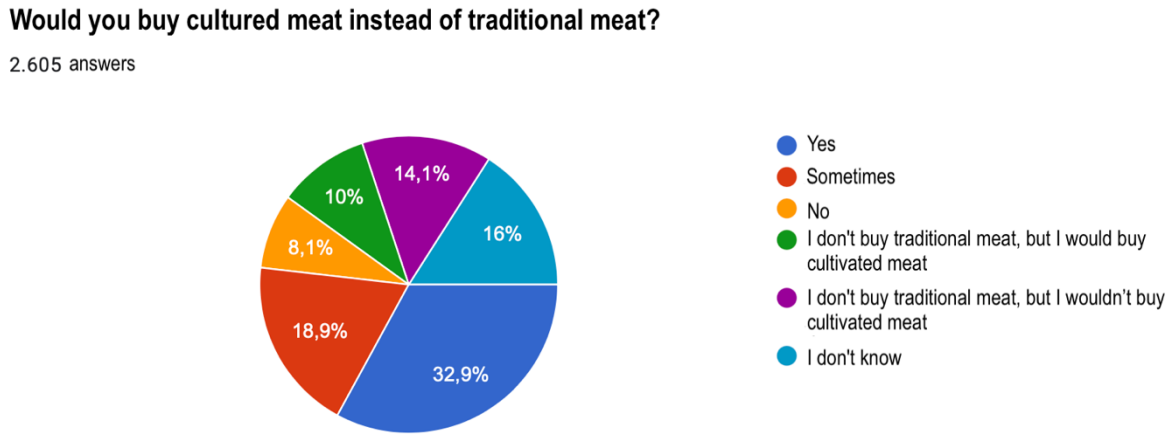


Figure 37 Survey responses on the possibility that the consumer replaces the purchase of traditional meat with that of cultured meat.

4.2. Cell seeding density

In the context of cellular agriculture, sowing density is fundamental as it influences the growth and trend of cell vitality over time. Having decided to carry out the viability tests on Multiwell M96, the respective sowing densities suggested in the literature were analyzed and it was decided to carry out a preliminary test to identify the optimal sowing density to be maintained in subsequent experiments dedicated to identifying the optimal concentration of soul-free additives.

The best densities found in the literature are 2,000 and 5,000 cells in each well filled with 200 µL of medium. For this reason, 3 different sowings were carried out, one of 2,000, 3,000 and 5,000 cells, for three different days of experimentation (Table 9).

Table 9 Scheme of the contents of the Multiwell to study cell optimal density.

Control			
Cells [thousands]	M. with cells [µL]	Medium [µL]	Treatment [µL]
1	10	190	-
5	50	150	-
10	100	100	-
Treatment			
1	10	90	100

5	50	50	100
10	100	0	100
Reference			
0	0	100	-

The M96 was divided vertically into three sections: the first hosted the control, made from cells seeded in DMEM medium, 10% FBS, 1% Glutamine and 1% PenStrep; the second hosted the treatment, made by cells seeded in DMEM medium, 10% hydrolyzate (Moscatelli), 1% Glutamine and 1% PenStrep; the last one hosted the reference and the wells were filled with DMEM medium, 1% Glutamine and 1% PenStrep, but did not contain cells.

Each section was then further divided into three areas, so that the viability trend was analyzed for the 3 different cell densities on three different days using the MTT assay (Figure 38).

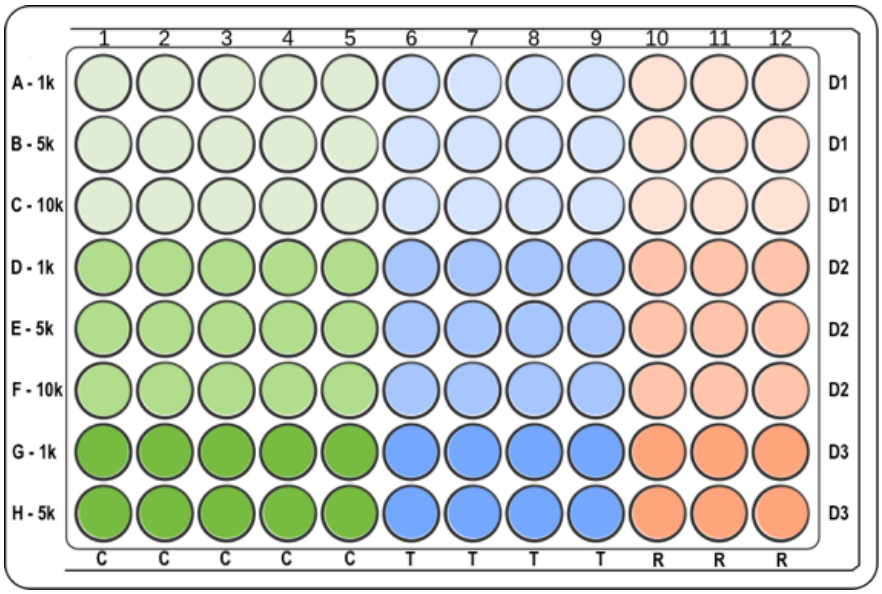


Figure 38 The organization of the M96 and the control, treatment and reference (no-cell control) of the experiment used to evaluate the optimum cell density for the seeding.

After seeding, the cells were incubated for 24 h at +37°C and 5% CO2. At the end of the incubation, 100 µL of medium containing a final concentration of 0.5 mg/ml of MTT Reagent were added to each well. The plate was subsequently placed again in the incubator, under the same conditions, for 3 h.

After the incubation time the medium with the reagent was removed and 100 µL of DMSO was added to each well.

It was checked that the solubilization of the violet formazan crystals was complete, then the absorbance of the samples was measured with a microtiter plate reader, the TECAN. The absorbance of the solubilized formazan was measured at a wavelength of 570 nm.

The hydrolysate used in this preliminary test resulted cytotoxic, but the experimentation was sufficient to establish, through an appropriate trade-off, the need to sow 3,000 in each well of the M96 as already on the second day of experimentation those sown with 5,000 cells had already reached too advanced a confluency, while those seeded with only 1,000 cells showed less cell proliferation when viewed on the SELENA® S Digital System (Figure 39).

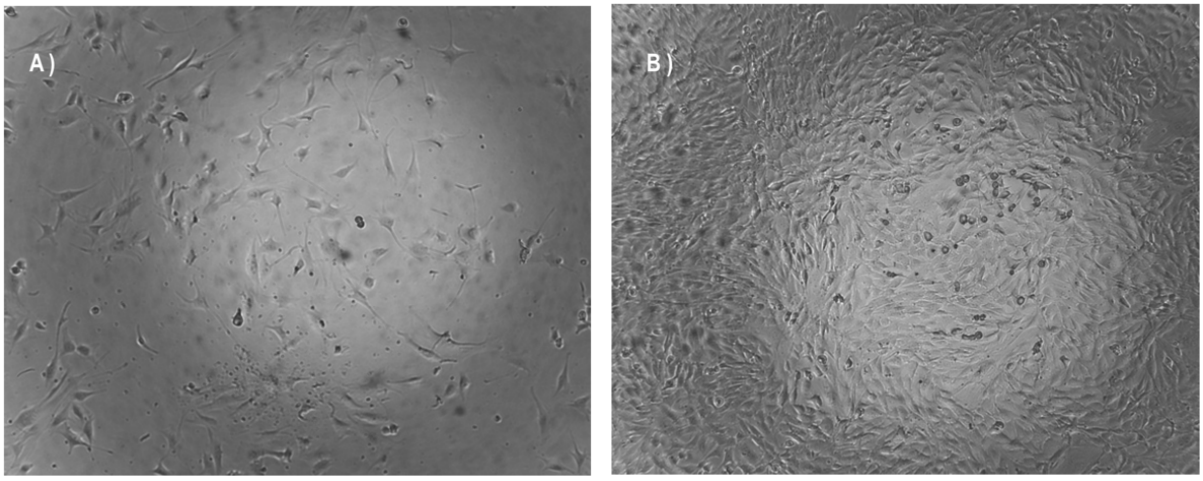


Figure 39 Images captured with SELENA®S Digital System of a 1k seeded well (A), and 5k seeded well during the second day of experiments.

4.3. Optimal concentration of hydrolysates and FBS

The objective of the next phase of the experimentation was to identify an optimal concentration of additive that could replace 10% of FBS in the medium, guaranteeing a proliferation and differentiation capacity analogous to the standard medium.

Having decided to perform the viability tests on multiwell M96 and having identified the seeding density in the preliminary experiment, we selected the hydrolysates which in the literature appeared to be more efficient for cell cultures and the concentrations of these which gave viability results better.

The selected hydrolysates had to have to be edible, and to be a cheap and waste products of the agricultural or food industry.

The hydrolysates with these qualities that have shown the best performance in the literature are those derived from soy, wheat and yeast. The selected concentrations were, for the hydrolysates: 5%, 2.5%, 1%, 0.5%, 0.1% and 0.05% (Table 10).

In parallel, a test was also performed with decreasing FBS concentrations to minimize the amount of serum to be used, in particular concentrations of: 5%, 2.5%, 1%, 0.5%, 0.25%, 0% were used (Table 10).

Table 10 Schematization of control, treatments and reference with the different concentrations of additives to organize the M96 multiwell in view of the MTT assay.

		FBS Level [%]	Hydrolysate Level [%]
Control		10	-
Treatment	T1	5	5
	T2	2.5	2.5
	T3	1	1
	T4	0.5	0.5
	T5	0.25	0.25
	T6	0	0.05
Reference		10	-

The M96 was divided horizontally into three sections: the first hosted the control, made up of cells seeded in DMEM-F12 medium, 10% FBS and 1% PenStrep; the second hosted the different levels of the treatment, made from cells seeded in DMEM-F12 medium, the respective percentage of hydrolyzate and 1% PenStrep; the latter hosted the reference and the wells were filled with complete medium, i.e. DMEM-F12 and 1% PenStrep, but did not contain cells.

Each section was then further subdivided into three areas, so that the trend of vitality was analyzed on three different days by the MTT assay (Figure 30).

The M96 was divided horizontally into three sections: the first hosted the control, made up of cells seeded in DMEM-F12 medium, 10% FBS and 1% PenStrep; the second hosted the different levels of the treatment, made from cells seeded in DMEM-F12 medium, the respective percentage of hydrolyzate and 1% PenStrep; the latter hosted the reference and the wells were filled with complete medium, i.e. DMEM-F12 and 1% PenStrep, but did not contain cells.

Each section was then further subdivided into three areas, so that the trend of vitality was analyzed on three different days by the MTT assay (Figure 40).

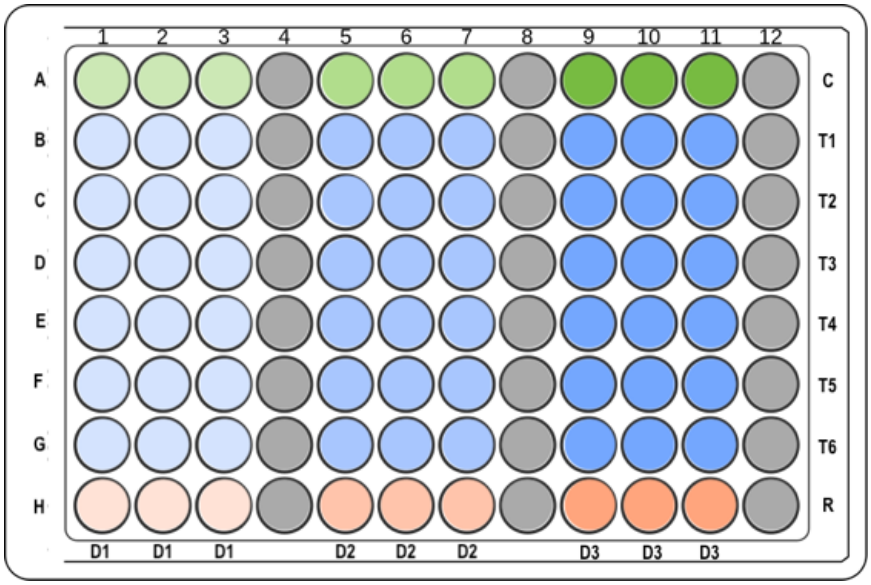


Figure 40 The organization of the M96 and the selected concentrations of FBS and hydrolysates for MTT assay viability analysis.

During day 0 the cells were sown in the previously selected density by filling each well, excluding those belonging to the reference section without cells and with only the complete medium, with 100 µL of medium with cells. The plate was then incubated for 24 h at +37°C and 5% CO2.

Experimentation day 1 began with the removal of the medium, with the aim of leaving in the wells only the cells that had taken root during the hours of incubation.

The medium was replaced with 100 µL of complete medium, with and without FBS or additive, in the wells belonging to the control, treatment and reference areas. At the end of the incubation, 100 µL of medium containing a final concentration of 0.5 mg/ml of MTT Reagent were added to each well. The plate was subsequently placed again in the incubator, under the same conditions, for 3 h.

After the incubation time the medium with the reagent was removed and 100 µL of DMSO was added to each well.

It was checked that the solubilization of the violet formazan crystals was complete, then the absorbance of the samples was measured with a microtiter plate reader, the TECAN. The absorbance of the solubilized formazan was measured at a wavelength between 600 nm (I don't remember the number, in the literature there is this), with a reference wavelength greater than 650 nm.

Satisfactory results were obtained by observing the viability trend in two days with the use of FBS, as concentrations of less than 10% of FBS led to excellent cell viability, compared with the control.

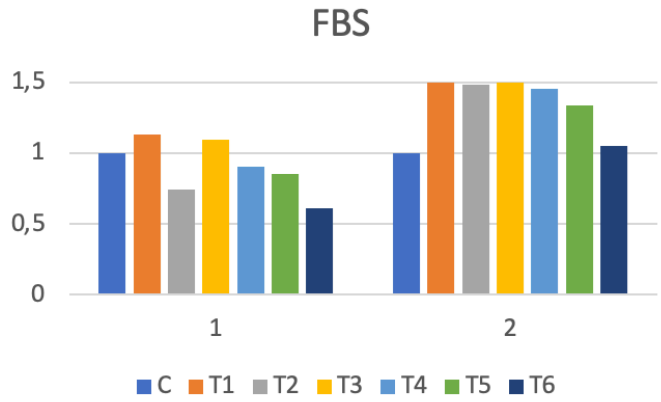


Figure 41 Outcome of the MTT assays for two days for different concentration of FBS. The value of absorbance measured is normalized on the control average.

The results of the MTT assay on the plates with the various hydrolysates showed that wheat at the lowest concentration has a better result, even outperforming the control. Then there is the soybean and finally the yeast (Figure 42).

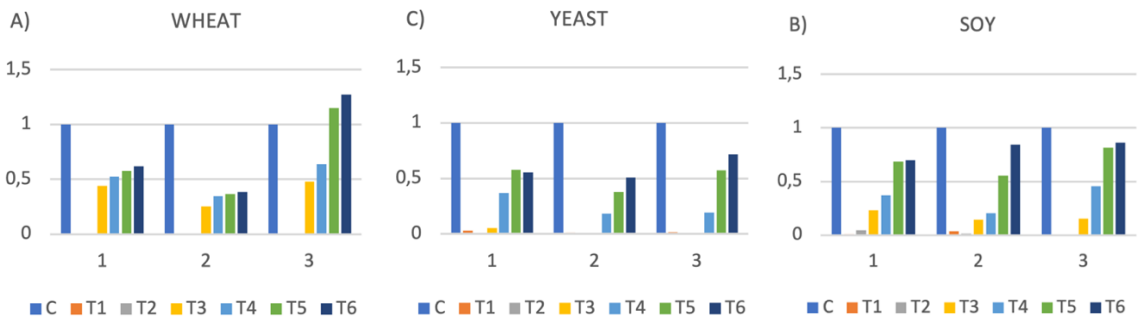


Figure 42 Outcome of the MTT assays for the three different hydrolysates: A) Wheat; B) Soy; C) Yeast in three different days of experiments.

Net of this last experimental outcome, the subsequent experiment saw a combination of the best hydrolysates to possibly evaluate whether the culture medium formulation could be further improved. In particular, the plate was organized by defining a control and a reference like the previous ones, furthermore the lower concentrations of soy and wheat hydrolysate were tested again; and a new additive was tested originating from the combination of the two best hydrolysates maintaining a final concentration of additive equal to 0.05% with a contribution of 0.025% from both hydrolysates (Table 11).

	Level

Control		10% FBS
Treatment	T1	0.05% W
	T2	0.05% S
	T3	0.025% W + 0.025% S
Reference		10% FBS

The MTT result confirms the best performance of wheat, which is however lowered if this is combined with soy (Figure 43).

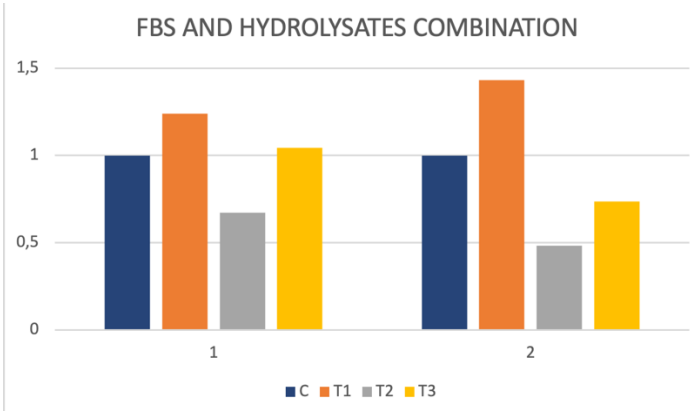


Figure 43 Outcome of the MTT assays to test cell viability using a culture medium additivated with 10% FBS (C), 0.05% of wheat (T1), 0.05% soy (T2), 0.05% for soy and wheat (T3).

4.4. Printability

A two-factor factorial Design of Experiment (DoE) was used to investigate the printability of the suggested bioinks. The pressure of extrusion of the pneumatic extruder (P) and the velocity of scanning of the print head (v) were the two factors chosen (Figure 44). It was decided to investigate 6 levels for each bioink over three replications, with randomized data collection. The pressure, indicated in kPa, specifies the entire force delivered by pressurized air to the syringe-like cartridge back, divided by the syringe's cross section area. The greater the pressure, the greater the projected flow rate of bioink extruded via the nozzle.

The lower the pressure, on the other hand, the lower the predicted flow rate, with a low limit value of pressure below which the shear stress caused by the nozzle inner walls will overcome the compressed air pressure, and no extrusion will occur.

The eight pressure levels considered were 5, 10, 15, 20, 25, 30, 35, 40 [kPa].

The range is between 0, which represents the critical case in which no pressure is applied to the bioink, leading to the absence of extrusion, and 40, which represents a pressure value over which it has been observed from prior experiments that the amount of ink extruded is excessive, leading to the problem of over extrusion as well as the risk of fatally damaging the cells because they are sensitive to shear stresses.

The extruded filament will be thinner at high scanning speeds because the same amount of material is spread over a wider area, and thicker at low scanning speeds because the same amount of material is spread over a smaller area.

The eight scanning speed levels that were taken into account were 5, 10, 15, 20, 25 and 30 [mm/s]. The range lies between 0, the crucial point at which the print head would be motionless and no build would form, and 30, the point at which the printer will experience excessive oscillations.

Throughout the whole experiment, the nozzle's temperature was maintained at 22°C (room temperature). This value was chosen to reduce effort in maintaining it, resulting in energy savings especially in the optic of the required scalability of the process.

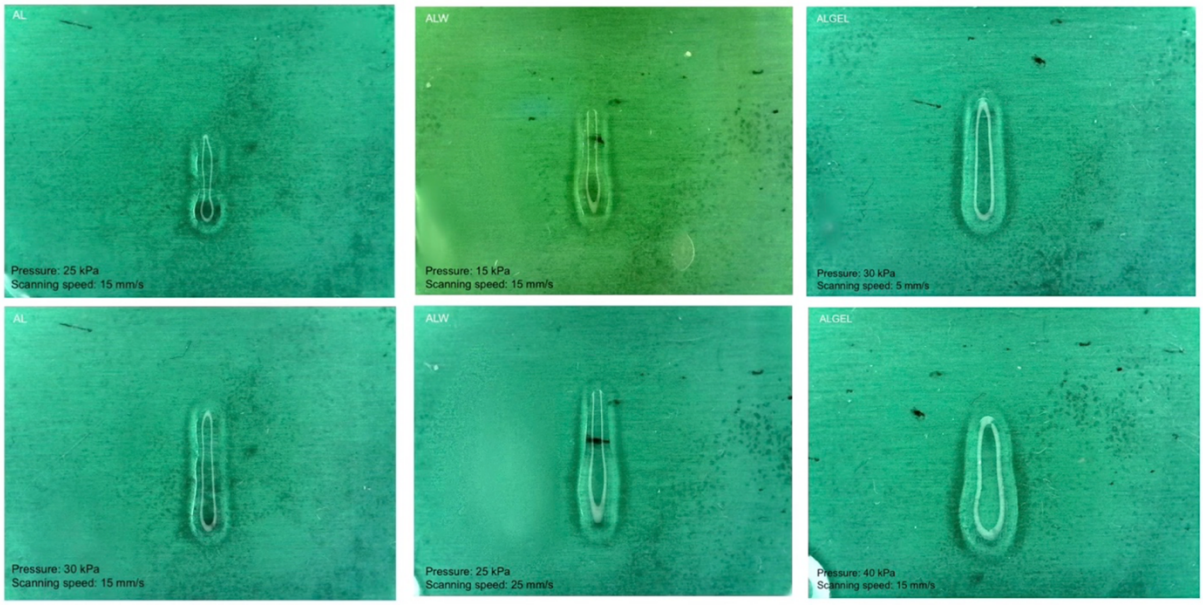


Figure 44 Photos obtained by the 3D printer chamber of the contructs printed at different pressure conditions and scanning speeds.

The printed filaments were manually measured based on the average width (L) over the length of the construct itself detected thanks to the images captured by the printer's HD camera. In fact, from the images, to define the width of the filaments, the number of pixels (p) corresponding to the longitudinal length of the filament were obtained and subsequently, the same detection was performed on an object of

known dimensions photographed by the same camera. The width calculation was performed using a correction factor obtained (c) from the study of the known object (Equation 3).

$$\frac{1}{c} \cdot p = L \qquad (3)$$

In particular, using a 1 cent coin as an object of known dimensions, the correction factor was calculated using a ratio of the dimensions measured in pixels (p) and those measured in millimeters (m) (Equation 4).

$$c = \frac{p}{m} = 66 \text{ pixels/mm} \qquad (4)$$

In this way it was possible to evaluate an acceptable range of pressures and printing speeds based on the dimensions of the filament obtained, defining an adequate filament size. An interval between 0.91 mm and 1.97 mm was chosen for the current work.

As regards the bioink formulated with alginate and wheat, was chosen to study a pressure range ranging from 5 kPa to 30 kPa and a printing speed from 5 mm/s to 30 mm/s.

Observing the numerical results obtained from the printing of the Alginate and Wheat based filament, it is highlighted that the optimal pressure range goes from a pressure of 15 kPa to a pressure of 25 kPa, while the printing speed is optimal between the values of 15 mm/s and 25 mm/s (Table 11).

Table 11 Numerical results obtained with the printing of Alginate and Wheat based filaments using different pressures and different printing speeds. In the table the acceptable dimensions of the construct in millimeters and the consequent ranges of the printing

ALW		Scanning speed					
		5	10	15	20	25	30
Pressure	5	0,00	0,00	0,00	0,00	0,00	0,00
	10	1,14	1,14	1,09	0,68	0,00	0,00
	15	2,50	1,52	1,35	0,91	1,02	0,88
	20	3,24	3,02	1,68	1,58	1,17	1,30
	25	5,38	3,67	1,97	1,67	1,52	1,41
	30	5,00	3,68	2,95	2,71	1,68	2,42

As regards the bio-ink formulated with alginate and gelatin, was chosen to study a pressure range ranging from 5 kPa to 30 kPa and a printing speed from 5 mm/s to 30 mm/s.

Observing the numerical results obtained from the printing of the Alginate and Gelatin based filament, it is highlighted that the optimal pressure is 30 kPa, while the printing speed is optimal between the values of 5 mm/s and 15 mm/s (Table 12).

Table 12 Numerical results obtained with the printing of Alginate and Gelatin based filaments using different pressures and different printing speeds. In the table the acceptable dimensions of the construct in millimeters and the consequent ranges of the printin

ALGEL		Scanning speed					
		5	10	15	20	25	30
Pressure	5	0,00	0,00	0,00	0,00	0,00	0,00
	10	0,00	0,00	0,00	0,00	0,00	0,00
	15	0,00	0,00	0,00	0,00	0,00	0,00
	20	0,00	0,00	0,00	0,00	0,00	0,00
	25	0,00	0,00	0,00	0,00	0,00	0,00
	30	1,67	1,03	0,91	0,00	0,00	0,00
	35	5,91	3,33	0,00	2,88	0,00	1,98
	40	0,00	0,00	2,23	0,30	2,27	2,20
	45	7,42	5,30	0,00	0,00	0,00	0,00

As regards the alginate- based, was chosen to study a pressure range ranging from 5 kPa to 30 kPa and a printing speed from 5 mm/s to 30 mm/s.

Observing the numerical results obtained from the printing of the alginate-based filament, it is highlighted that the optimal pressure range goes from a pressure of 25 kPa to a pressure of 30 kPa, while the printing speed is optimal between the values of 5 mm/s and 20 mm/s (Table 13).

Table 13 Numerical results obtained with the printing of Alginate based filaments using different pressures and different printing speeds. In the table the acceptable dimensions of the construct in millimeters and the consequent ranges of the printing parameters

AL		Scanning speed					
		5	10	15	20	25	30
Pressure	5	0,00	0,00	0,00	0,00	0,00	0,00
	10	0,00	0,00	0,00	0,00	0,00	0,00
	15	0,80	0,00	0,00	0,00	0,00	0,00
	20	1,23	0,94	0,82	0,00	0,00	0,00
	25	1,52	1,18	1,05	0,98	0,83	0,79
	30	1,85	1,44	1,11	0,91	0,89	0,89

From the results, it can be seen how the construct that has a percentage of wheat increases the range of printing parameters compared to alternatives based on Alginate alone or Alginate and Gelatin.

This lead to an increased acceptable area in the process parameters domain. Moreover, the gelatine-based bioink shows a narrow area due to its gelation at the temperature evaluated. In conclusion, alginate wheat-based bioink shows a more flexible behaviour while extruded.

4.5. Cell viability on bio-printed products

The previously tested three-dimensional bioink-printed constructs containing cells were cultured in different media, with the aim of verifying that the cell viability measured under three-dimensional conditions was in line with that previously experienced in 2D.

To print with the various bioinks containing cells, it was decided to deposit drops with a nozzle with a diameter of 0.410 mm in the wells of a multiwell M96 for each bioink tested (Figure 45).

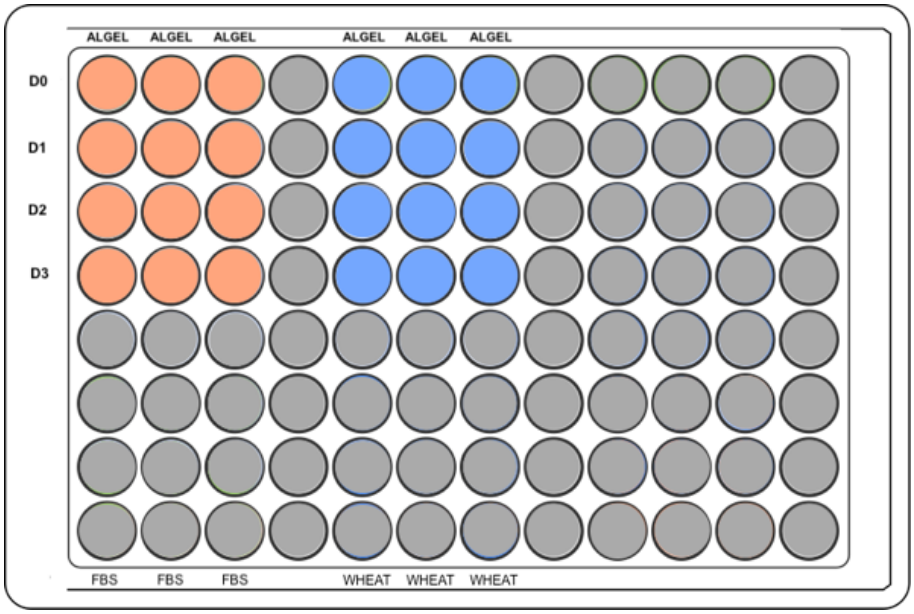


Figure 45 Schematic of the split multiwell for a 4-day study on the same ink but with two different culture media.

The multiwell was subdivided so that the experiment could be performed over 3 days, every 24 h. With each cell-containing ink formulation, one drop was deposited on selected wells during day zero. Based on the position of the drop in the multiwell

section, this drop was immersed in 100 µL of medium: in the left section of the multiwell the medium contained 10% FBS while on the right side 0.05% wheat.

Every 24 h the medium was removed and partially prepared in another plate for subsequent analysis with the G6PD assay and CELENA optical microscope images were captured in order to visualize dead cells and live cells in the printed constructs, following the Live/Dead assay protocol (Figure 46) [43].

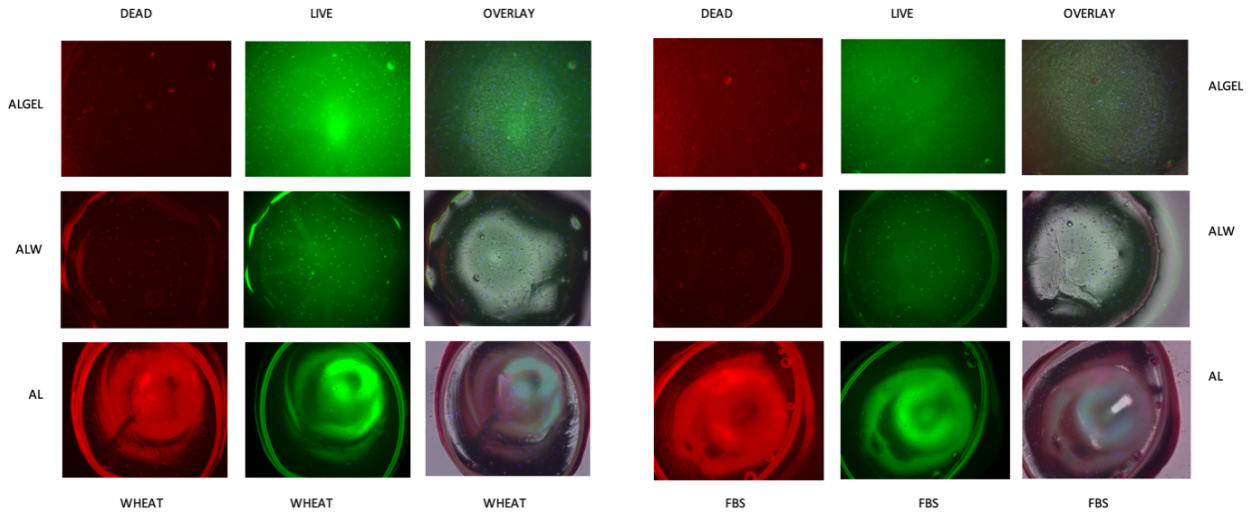


Figure 46 Images captured on the second day of experimentation.

The study of cell viability on the printed constructs performed using the Live/Dead protocol involved the analysis through an automatic counting of the cells detected in the images obtained with the optical microscope. In particular, an algorithm in the MATLAB environment was used for the automatic counting of cells using green (calceine) and red (PI) fluorescence.

The results of the analyses were graphed in order to highlight the effectiveness of the treatment, i.e. the use of the medium with wheat hydrolysate and FBS-free on the three different constructs containing cells, printed with alginate-based bio-ink, based on alginate and gelatin and based on alginate and wheat (Figure 47).

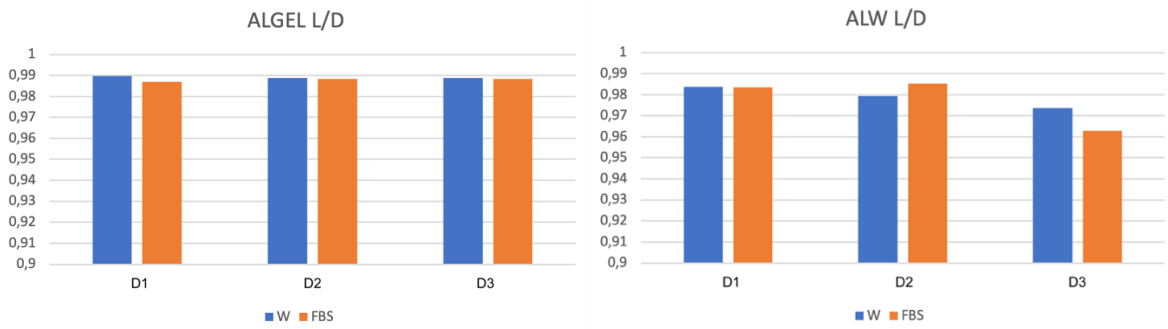


Figure 47 Results of the Live/Dead analysis on the bio-inks alginate-gelatin and on the one based on alginate-wheat. The value expresses the perchentage of the living cells counted over the total amount, for both treatment with FBS and for the two bio-inks.

The results of the analysis showed that the bioink formulated exclusively with alginate is not as performing as regards cell viability compared to those in which gelatin or wheat are added to the alginate, as expected from the literature.
In particular, the alginate and gelatin based bioink appears to have better performance than that with alginate and wheat, and maintains good vitality for three consecutive days, whether it is cultured with 10% FBS, or whether this is excluded and replaced with wheat.
The cell viability measured on the alginate and wheat-based bio-ink is however comparable with that of the other ink and on the third day higher values are obtained from the ink immersed in the FBS-free medium.

The media removed from each well were arranged neatly in a second subdivision plate so as to differentiate the different composition of the substrates and the different bioink droplets they coated (Figure 48).

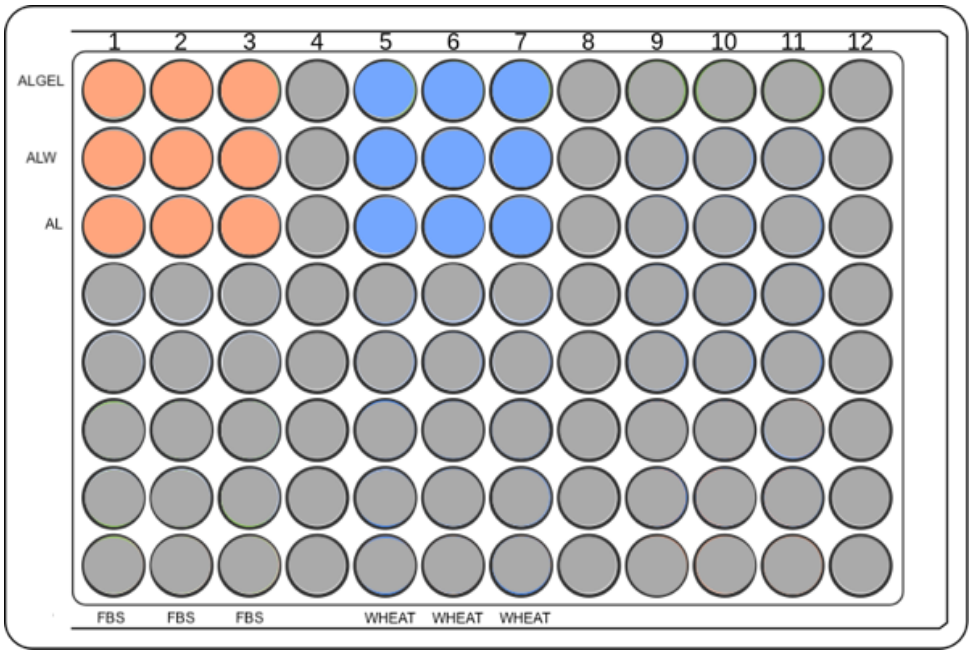


Figure 48 Scheme of the multiwell divided in order to differentiate the different composition of the substrates and the different drops of bio-ink.

The second plate was treated according to the G6PD assay and read in fluorescence every 24 hours.

Following the G6PD protocol another multiwell was organized dividing it in three main sections in which the medium without cells, the medium with cells and the medium with lysed cells was studied (Figure 49).

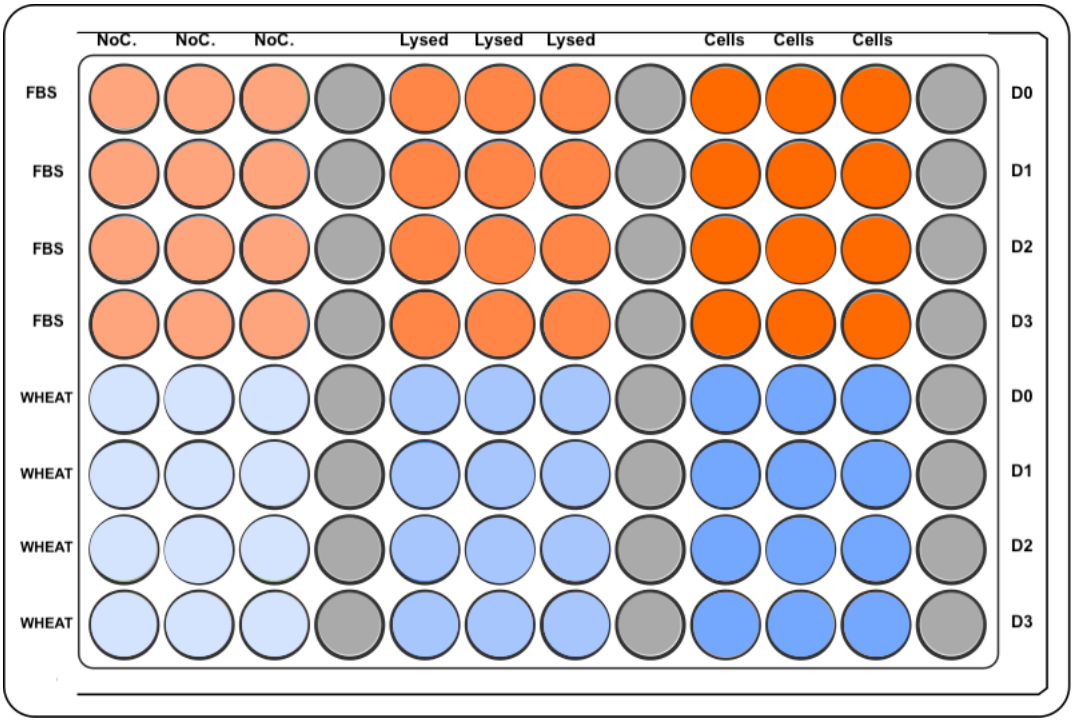


Figure 49 Scheme of the multiwell divided in order to study the medium without cells, the medium with lysed cells and the medium with cells.

The data for each bio-ink coated in wheat-containing media, were normalized with respect to the control, considered as the respective bio-ink grown with the FBS-containing medium.

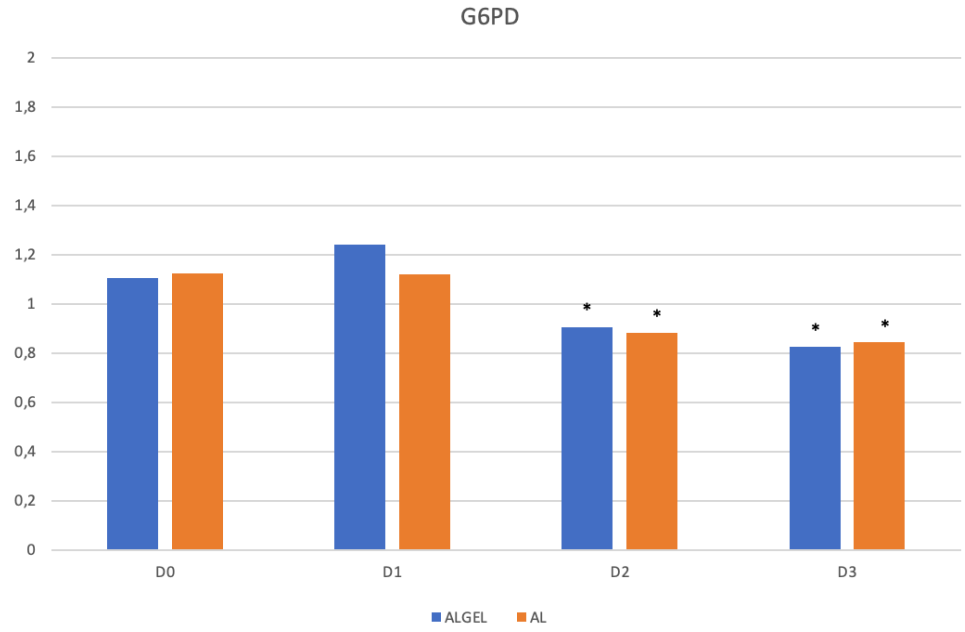


Figure 50 Normalized values of the fluorescence, analyzed by TECAN and evaluated following the G6PD assay, on each ink immersed in the medium with wheat compared to the average of the control in FBS of the respective day.

A T-test statistical analysis was performed on the data obtained from the G6PD protocol for each bioink and for each day to evaluate whether the treatment with wheat in the medium and FBS-cultured had significant differences. The average values obtained on the second day in the bioink formulated with the gelatin and the wheat component and those obtained on the third day in the formulated with the wheat component showed a p-value less than 0.05. Hence, wheat in the medium had shown in this case a better performance after 2 days of culture for all the inks.

Observing the results obtained with the G6PD protocol, the cells in the different bioinks appear less deadly from the second day. In particular, on the second and third day, the cells in the alginate-based bioink appear more vital than the other even if they present significant differences (Figure 50).

5 Conclusions

Starting with the results of the anonymous questionnaire on the subject of cultured meat, submitted to a sample of more than 2,600 people, it can be deduced that more and more people are interested in environmental issues, the impact that a product can have on human health and all 'ethics of the production process of this, even going so far as to pay more for the aforementioned product if it has appropriate certifications. In particular, the topic of cultured meat does not seem to be well known by most people, who have mostly heard of it in unscientific or incorrect terms. Beyond the knowledge on the subject, however, it is evident from the questionnaire that people are in favor of research on the subject, as well as the production and marketing of the product. In fact, these favorable responses were also given by the majority of people who personally would not buy the product or who would not buy it instead of traditional meat. In general, there has been a great curiosity around cultured meat, as the majority of people are prepared to taste or buy it.

Evaluated the optimal cell density of 3,000 cells for experimentation on cell viability of 2D cultures in which a hydrolyzate-based additive was substituted for FBS, it was possible to demonstrate that wheat can be a valid substitute for FBS in this type of cultivation. In particular, it was decided to evaluate minimum concentrations of hydrolyzate arriving at the conclusion that 0.05% hydrolyzate of the culture medium is the optimal concentration to maintain good cell viability, even higher than that guaranteed by the presence of FBS in the medium. The experiment was also performed with soy and yeast, but these did not have the same performance as wheat: yeast drastically increased mortality and was therefore excluded from subsequent experiments. We wanted to test a minimum combination of fument and soy composed of 0.025% of both, as an additive to put in the culture substrate instead of FBS, unfortunately, however, the addition of the minimum percentage of soy was worsening for cell viability. We therefore came to the conclusion that only wheat could be a good substitute for FBS in culture media for 2D cell cultures.

Having identified these positive performances of wheat in 2D cell cultures, we also wanted to test them in 3 dimensions. With the aim of excluding any animal derivative from the production process of cultivated meat, it was decided to consider 3 different bio-inks for printing: the first made with alginate, the second with alginate and gelatin, and the third, with alginate and wheat as a substitute for gelatin.

Therefore, the printability of the three different constructs under different printing conditions was first evaluated, identifying some optimal parameters for each one. In

particular, for the alginate-based bio-ink the most suitable parameters saw the optimal extrusion pressure between 20 and 30 kPa, while the scanning speed between 5 and 15 mm/s. For the one based on alginate and gelatin, an optimal extrusion pressure was found between 35 and 40 kPa, while the scanning speed between 10 and 15 mm/s. Finally, for the alginate and wheat-based bio-ink the most suitable parameters saw the optimal extrusion pressure between 20 and 25 kPa, while the scanning speed between 20 and 25 mm/s.

Once the printing parameters were evaluated, cell viability was then tested in the various constructs, using a medium with 10% FBS as a control and that with 0.05% wheat as a treatment. The cell viability measured on the alginate and wheat-based bio-ink is comparable with that of the others

bio-inks tested but on the third day higher viability was obtained from the samples immersed in the FBS-free medium.

In conclusion, the study carried out led to the definition of an animal-free culture medium alternative to the one usually used and containing animal derivatives, demonstrating that FBS can be replaced by cell culture medium. Similarly, a plant-based bioink has been developed that is capable of maintaining a vitality comparable to that containing animal derivatives.

Bibliography

[1] https://www.ipcc.ch/site/assets/uploads/2019/08/2f.-Chapter-5_FINAL.pdf

[2] https://www.ipcc.ch/report/ar6/wg3/downloads/report/IPCC_AR6_WGIII_Full_Report.pdf

[3] https://www.nature.com/articles/s41893-018-0189-7.epdf?author_access_token=NEyYP0K3x7HaKsFMh56EFNRgN0jAjWel9jnR3ZoTv0OtHgR33Yi4j7cy

[4] https://www.nature.com/articles/s41467-020-20061-y

[5] FAOSTAT, http://www.fao.org/faostat/en/#data/QCL

[6] Autorità europea per la sicurezza alimentare (EFSA), *Welfare of cattle during transport*, 2022, pag. 16.

[7] Direzione generale delle Politiche interne dell'Unione del Parlamento europeo (IPOL), *Patterns of livestock transport in the EU and to third countries*, 2021, pag. 7; EFSA, *Welfare of cattle during transport*, 2022, pag. 5.

[8] Y. Ye et al., *Commercialization of cultured meat products: Current status, challenges, and strategic prospects*, Future Foods 6 (2022) 100177

[9] R. Russel et al., Growth of Smooth Muscle in Culture and Formation of Elasti Fibers, The English Historical Review, Volume LXXXVI, Issue CCCXXXVIII, January 1971, Pages 172–173

[10] M. M. A. Benjaminson et al., In Vitro Edible Muscle Protein Production System (MPPS): Stage 1, Fish, Acta Astronautica Vol. 51, No. 12, pp. 879–889, 2002

[11] M. Post et al., An alternative animal protein source: cultured beef. Annal. New York Acad. Sci. 1328 (1), 29–33.

[12] Global Information Inc, 2021c,. Global cultured meat market - analysis by source (poultry, beef, seafood, pork), end use, by region, by country (2021 Edition): Market insights, Covid- 19 impact, competition and forecast (2021-2030).

[13] Global information Inc, 2019a. Global cultured meat market-2019-2026.

[14] N. Stephens et al., 2018. Bringing cultured meat to market: Technical, socio-political, and regulatory challenges in cellular agriculture. Trends Food Sci. Technol. 78, 155–166.

[15] Stout et al., 2022. Simple and effective serum-free medium for sustained expansion of bovine satellite cells for cell cultured meat. Commun. Biol. 5, 466.

[16] S. Gottipamula et al., 2013. Serum-free media for the production of human mesenchymal stromal cells: a review. Cell Prolif. 46 (6), 608–627.

[17] M. Das et al., Developing a *Novel Serum-Free Cell Culture Model of Skeletal Muscle Differentiation by Systematically Studying the Role of Different Growth Factors in Myotube Formation In Vitro*, Cellular & Developmental Biology. Animal, Vol. 45, No. 7 (Jul. - Aug., 2009), pp. 378-387

[18] W. Christopher, W. McAleer et al., *Functional Myotube Formation from Adult Rat Satellite Cells in a Defined Serum-Free System*, 2015, Wiley Online Library

[19] A.M.Kolkmann et al., *Serum-free media for the growth of primary bovine myoblasts, Cytotechnology*, 2019

[20] H. Kuo et al., Negligible-Cost and Weekend-Free Chemically Defined Human iPSC Culture, Stem Cell Reports (2019)

[21] T. Messmer et al., *A serum-free media formulation for cultured meat production supports bovine satellite cell differentiation in the absence of serum starvation*, Nature, Food, 2022

[22] Ho, Y.Y., Lu, H.K., Lim, Z.F.S. *et al. Applications and analysis of hydrolysates in animal cell culture*. Bioresour. Bioprocess. 8, 93 (2021). https://doi.org/10.1186/s40643-021-00443- w

[23] O'Neill EN, Cosenza ZA, Baar K, Block DE. *Considerations for the development of cost- effective cell culture media for cultivated meat production.* Compr Rev Food Sci Food *Saf*. 2021;20:686–709. https://doi.org/10.1111/1541-4337.12678

[24] Ho SC, Nian R, Woen S, Chng J, Zhang P, Yang Y. *Impact of hydrolysates on monoclonal antibody productivity, purification and quality in Chinese hamster ovary cells*. J Biosci Bioeng. 2016 Oct;122(4):499-506. doi: 10.1016/j.jbiosc.2016.03.003. Epub 2016 Apr 7. PMID: 27067279.

[25] Ng JY, Chua ML, Zhang C, Hong S, Kumar Y, Gokhale R and Ee PLR (2020) *Chlorella vulgaris Extract as a Serum Replacement That Enhances Mammalian Cell Growth and Protein Expression.* Front. Bioeng. Biotechnol. 8:564667. doi: 10.3389/fbioe.2020.564667

[26] Santoni, S., Gugliandolo, S.G., Sponchioni, M. *et al. 3D bioprinting: current status and trends—a guide to the literature and industrial practice.* Bio-des. Manuf. 5, 14–42 (2022). https://doi.org/10.1007/s42242-021-00165-0

[27] L. Ouyang, R. Yao, Y. Zhao and W. Sun, *Effect of bioink properties on printability and cell viability for 3D bioplotting of embryonic stem cells*, 2016, Biofabrication 8 035020, doi: 10.1088/1758-5090/8/3/035020

[28] Santoni, S., Gugliandolo, S.G., Sponchioni, M. *et al. 3D bioprinting: current status and trends—a guide to the literature and industrial practice.* Bio-des. Manuf. 5, 14–42 (2022). https://doi.org/10.1007/s42242-021-00165-0

[29] N. Paxton, W. Smolan, T. Bock, F. Melchels, J. Groll, *T*. Jungst, *Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability,* 2017Biofabrication9044107, DOI10.1088/1758-5090/aa8dd8

[30] Catoira, M.C., Fusaro, L., Di Francesco, D.et al.*Overview of natural hydrogels for regenerative medicine applications.* J Mater Sci: Mater Med **30**, 115 (2019). https://doi.org/10.1007/s10856-019-6318-7

[31] Devara Venkata Krishna, Mamilla Ravi Sankar, *Persuasive factors on the bioink printability and cell viability in the extrusion-based 3D bioprinting for tissue regeneration applications*, Engineered Regeneration, Volume 4, Issue 4, 2023, Pages 396-410, ISSN 2666-1381, https://doi.org/10.1016/j.engreg.2023.07.002.

[32] Devara Venkata Krishna, Mamilla Ravi Sankar, *Persuasive factors on the bioink printability and cell viability in the extrusion-based 3D bioprinting for tissue regeneration applications*, Engineered Regeneration, Volume 4, Issue 4, 2023, Pages 396-410, ISSN 2666-1381, https://doi.org/10.1016/j.engreg.2023.07.002.

[33] Kamalnayan Tibrewal, Prajakta Dandekar, Ratnesh Jain, *Extrusion-based sustainable 3D bioprinting of meat & its analogues: A review*, Bioprinting, Volume 29, 2023, e00256, ISSN 2405-8866, https://doi.org/10.1016/j.bprint.2022.e00256.

[34] Arianna Dick, Xiuping Dong, Bhesh Bhandari, Sangeeta Prakash, *The role of hydrocolloids on the 3D printability of meat products*, Food Hydrocolloids, Volume 119,2021, 106879, ISSN 0268-005X, https://doi.org/10.1016/j.foodhyd.2021.106879

[35] Saman Naghieh, Mohammad Reza Karamooz-Ravari, MD Sarker, Eva Karki, Xiongbiao Chen, *Influence of crosslinking on the mechanical behavior of 3D printed alginate scaffolds: Experimental and numerical approaches*, Journal of the Mechanical Behavior of Biomedical Materials, Volume 80, 2018, Pages 111-118, ISSN 1751-6161, https://doi.org/10.1016/j.jmbbm.2018.01.034.

[36] Supachai Reakasame, Dalia Dranseikiene, Stefan Schrüfer, Kai Zheng, Dirk W. Schubert, Aldo R. Boccaccini, *Development of alginate dialdehyde-gelatin based bioink with methylcellulose for improving printability,* Materials Science and Engineering: C,Volume 128, 2021, 112336, ISSN 0928-4931, https://doi.org/10.1016/j.msec.2021.112336.

[37] Sbrana FV, Pinos R, Barbaglio F, Ribezzi D, Scagnoli F, Scarfò L, Redwan IN, Martinez H, Farè S, Ghia P and Scielzo C (2021) *3D Bioprinting Allows the*

Establishment of Long-Term 3D Culture Model for Chronic Lymphocytic Cells. Front. Immunol. 12:639572. doi: 10.3389/fimmu.2021.639572

[38] Iris Ianovici, Yedidya Zagury, Idan Redenski, Neta Lavon, Shulamit Levenberg, *3D-printable plant protein-enriched scaffolds for cultivated meat development*, Biomaterials, Volume 284, 2022, 121487, ISSN 0142-9612, https://doi.org/10.1016/j.biomaterials.2022.121487.

[39] https://www.thermofisher.com/it/en/home/references/protocols/cell-and-tissue-analysis//vybrant-mtt-cell-proliferation-assay-protocol.html

[40] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018500_CyQUANT-LDH-Cytotoxicity-Assay-Kit_PI.pdf

[41] https://www.thermofisher.com/it/en/home/references/protocols/cell-and-tissue-analysis/protocols/live-dead-viability-cytotoxicity-kit-for-mammalian-cells.html

List of Figures

List if tables

List of equations

$$\frac{\Sigma C}{6} = A \tag{0}$$

$$\frac{5}{m} = s \tag{2}$$

$$\frac{1}{c} \cdot p = L \tag{3}$$

$$c = \frac{p}{m} = 66 \text{ pixels/mm} \tag{4}$$

List of symbols

Variable	Description	Unit
C	Counts of cells	Number of cells
A	Average of the counted cells	Number of cells
s	Cell solution to seed	mL
p	Dimension of the known object	pixels
m	Dimension of the known object	mm
c	Correction factor	pixels/mm
L	Dimension of filments	mm

List of Abbreviations

GHG, Greenhouse Gas

AM, Additive Manufacturing

CBM, Cell Based Meat

IPCC, International Panel on Climate Change FBS – Fetal Bovine Serum

EFSA, European Food Safety Authority

BSC, Culture of Bovine Satellite Sells

3DBP, 3D Bioprinting 3DBP

AL, Alginate-based bio-ink

ALGEL, Alginate and gelatin based bio-ink ALW – Alginate and wheat based bio-ink

PBS, Phosphate Buffered Saline

Acknowledgments

A big thank goes to the advisor Mattia Sponchioni and to Filippo Bracco, the co-advisor of this project and to Giovanni Zanderigo, who followed me throughout the process. Further thanks go to the colleagues of the 3D Cell laboratory and to all the people who helped me during this thesis. As well as the people with whom I share my daily battles for animal rights, and who have helped give me the motivation to delve deeper into the topic of cultured meat.