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EXECUTIVE SUMMARY OF THE THESIS

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

TESI MAGISTRALE IN CHEMICAL ENGINEERING – INGEGNERIA CHIMICA

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1. Introduction

The environmental impact of animal agriculture, the forecasts on the need for meat, combined with the ethical implications associated with animal slaughter, have prompted us to explore the possibility of making a transition to a more sustainable food system. The exclusion of 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, which are fundamental in the context of the climate crisis we are facing [1]. Indeed, the International Panel on Climate Change (IPCC) in its latest report recommends a transition that sees plant production and cellular agriculture at the core, recognizing that emerging food technologies such as cultured meat can bring substantial reduction in direct greenhouse gas (GHG) emissions from food production [2].

In this framework a new type of agriculture has born. 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. 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 latest result of cell agriculture is the so called "cultured meat", *i.e.* a muscle derived from the differentiation of animal cells cultivated in a laboratory. The production of cultured meat strarts from a painless biopsy performed on the animal to take the cells. These are then 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 from 2D cell cultivation in small flasks in a laboratory-scale system, the final muscle tissue is obtained through different 3D cell cultivation technologies. Among them, 3D bioprinting is extremely appealing, as it allows an accurate cell deposition, a good control on the cell density, on the geometry of the structure developed and on the

ratio between various population of cells leading the possibility to have a 3D multi-cellular constructs [3].

Although cultured meat is a product created mainly to meet the needs of environmental and ethical sustainability, as well as economic and food safety, currently its production does not yet exclude ingredients of animal origin, first and foremost fetal bovine serum and gelatin. For this reason the aim of this project is to define completely animal-free formulation of the culture medium and the bio-inks that can possibly being used in the production of cultured meat, using economical ingredients that fit into the circular economy [4].

2. Methods and materials

Materials

The cell cultures were seeded in multiwell M96 using as basal medium the Dulbecco's Modified Eagle medium F-12 (DMEM F-12, from ThermoFisher) with 1% Penicillin-Streptomycin, 1% L-Glutamine. The additives to this medium were: 10% fetal bovine serum (FBS; ThermoFisher) for control experiments; soy peptone, wheat peptone and yeast hydrolysate from Thermo Fisher. For cell counting the Trypan Blue method was used. The sample was mounted on Luna Counting Slides and the images were captured with CELENA® S Digital System.
The MTT assay protocol was performed using MTT kit provided by Thermo Fisher, the fluorescence was measured with the TECAN and the data analysis was performed with the support of an Excel spresdsheet.
For the printability analysis, different bio-inks were formulatyed using alginate, gelatin (from Sigma-Aldrich) and the wheat peptone. The 3D bioprinter BIO X ™ provided by Cellink was used and the images captured during the experiment were analysed manually and the data was studied with the support of an Excel spresdsheet.
Live/Dead assay was performed with a Live/Dead from Thermo Fisher, the images were captured with CELENA® S Digital System and were studied with an algorithm implemented in the MATLAB environment for counting live cells and dead cells.
The cytotoxicity assay was performed with the Invitrogen™ CyQUANT™ Cytotoxicity Assay Kit and the data was collected with the TECAN and studied with the support of an Excel spresdsheet.

Design of the experiment

A preliminary phase of the experiment was focused on the investigation of the social acceptance of cultured meat with an anonymous survey given to a sample of 2.600 people.
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, with the MTT Assay protocol, the best performing culture medium by excluding FBS in its standard concentration of 10% and substituting it with an optimal concentration of either soy, yeast or wheat peptone.
The third step of the analysis saw the formulation of a bio-ink that excluded components of animal origin using the concentration of peptone identified as best in the previous phase and the evaluation of its printing performance comparing it with the bio-ink of alginate, using that as standard bio-ink, and that of alginate and animal gelatin.
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.

3. Results and discussion

The sample examined for the cultured meat acceptance analysis was made up of more than 2,600 people, and was divided into three specific sections. The first section was intended to get to know the person answering the survey: 30% of the people who answered had a high school diploma and 50% had a 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 survey regarding the impacts on sustainability and ethics of their eating habits: 60% of the people who

answered were omnivores and 40% 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.
The third and final section of the survey 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 (Figure 1): 50% of people have heard more of this new product referred to as "synthetic meat", 30% of people who replied to the survey said they would replace traditional meat with the cultivated one, and the majority of the people that has answered said that they would by the product for their pets.

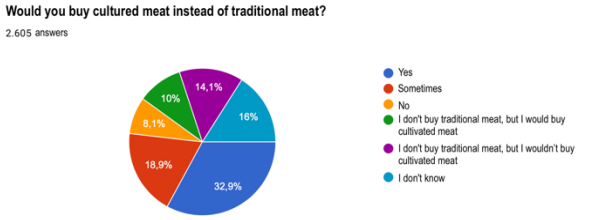

Figure 1 Survey responses on the possibility that the consumer replaces the purchase of traditional meat with that of cultured meat.

To evaluate the best seeding density for the experiment the first cultivation was performed in a M96 filled with 200 µL of medium each well and 3 different sowings were carried out, one of 2,000, 3,000 and 5,000 cells, for three different days of experimentation. Through an appropriate trade-off, the need to sow 3,000 in each well of the M96 was assessed, as already on the second day of experimentation those sown with 5,000 cells had already reached too advanced stage of confluency, while those seeded with only 1,000 cells showed less cell proliferation when examinated at the the CELENA® S Digital System (Figure 2).

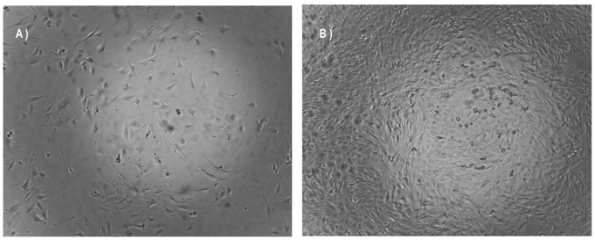

Figure 2 Images captured with SELENA®S Digital System of a 1k seeded well (A), and 5k seeded well during the second day of experiments.

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, the viability tests were performed using 6 different concentration of peptone hydrolysates and FBS: 5%, 2.5%, 1%, 0.5%, 0.1% and 0.05%. The absorbance of the solubilized formazan was measured at a wavelength between 600 nm with TECAN. 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. 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: additive was tested maintaining a final concentration equal to 0.05% with a contribution of 0.025% from both hydrolysates (Table 1, Figure 3). The MTT result confirms the best performance of wheat in the concentration of 0.05%. It's performance is however lowered if this is combined with soy.

Table 1 Schematization of control, treatments and reference with the different concentrations of additives to organize the M96 multiwell in view of the MTT assay.

		Concentration in the medium
Control		10% FBS
Treatment	T1	0.05% W
	T2	0.05% S
	T3	0.025% W + 0.025% S
Reference		10% FBS

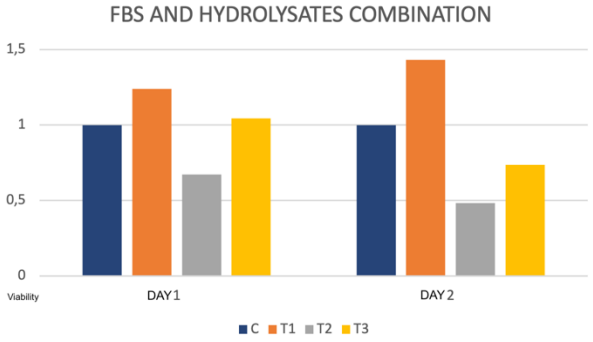

Figure 3 Outcome of the MTT assays to test cell viability using a culture medium additivated with 10% FBS (C), 0.05% of wheat (T1), 0.05% soy (T2) and 0.05% (T3) for soy and wheat.

A two-factor factorial design of experiment was used to investigate the printability of the suggested bio-inks. The eight pressure levels

considered were 5, 10, 15, 20, 25, 30, 35, 40 [kPa] and the scanning speed levels that were taken into account were 5, 10, 15, 20, 25 and 30 [mm/s]. The printed filaments were evaluated 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 1).

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

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 2).

$$c = \frac{p}{m} = 66 \quad (2)$$

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 2). For the printing of the Alginate and Gelatin based filament, the optimal pressure is 30 kPa, while the printing speed is optimal between the values of 5 mm/s and 15 mm/s. For the printing of the Alginate-based filament, it is highlighted that the optimal pressure range goes from a pressure of 5 kPa to a pressure of 10 kPa, while the printing speed is optimal between the values of 25 mm/s and 30 mm/s.

Table 2 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

The previously tested three-dimensional bio-ink-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.
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. The alginate and gelatin-based bio-ink 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 (Figure 5).

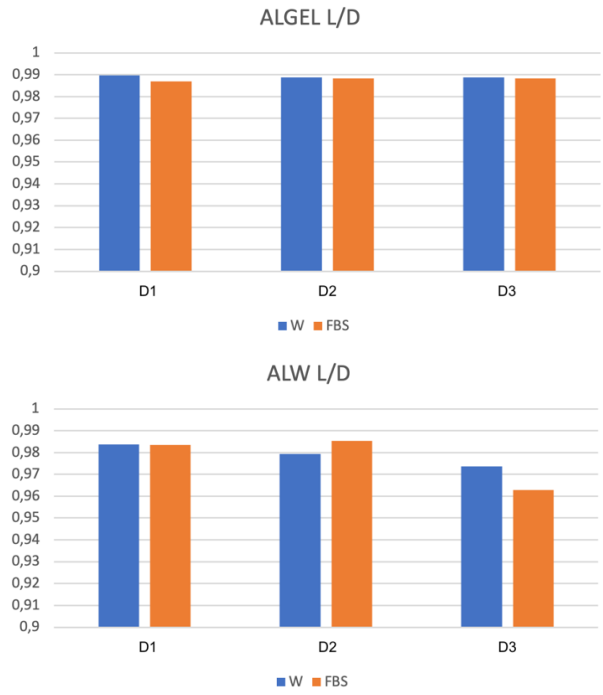

Figure 4 Results of the Live/Dead analysis in three different days on the bio-ink based on alginate and gelatin and on the one based on alginate and wheat, studied measuring the fluorescence (the vertical axes of both graphs).

The second plate was read in fluorescence every 24 hours according to the G6PD assay. 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. Observing the results obtained with the G6PD protocol, the cells in the different bio-inks appear less deadly from the second day. In particular, on the second and third day, the cells in the alginate-based bio-ink appear more vital than the other even if they present significant differences (Figure 6).

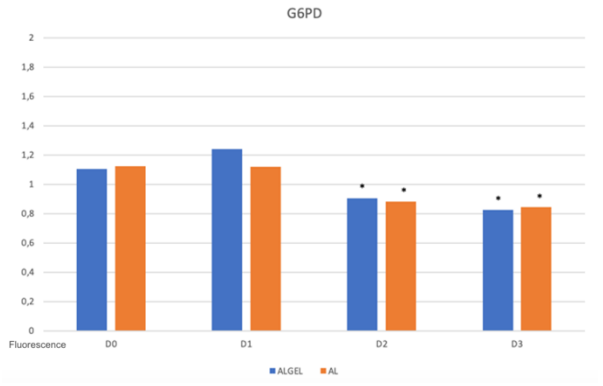

Figure 5 Normalized values of the fluorescence, analyzed by TECAN and evaluated following the G6PD assay, on each ink immersed in the medium with wheat compared to the average of the control in FBS of the respective day.

4. Conclusions

Starting with the results of the anonymous survey 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, and even if 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, there has been a great curiosity around the product and its entrance in the market seems to be waited.

Evaluated the optimal cell density of 3,000 cells for experimentation on cell viability of 2D cultures in which a hydrolysate-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 is possible to conclude that 0.05% hydrolysate 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.

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 procedure: the first made with alginate, the second with alginate and gelatin, and the third, with alginate and wheat as a substitute for gelatin. In particular, the alginate and wheat-based bio-ink demonstrated to make the printability parameters range wider, the most suitable parameters saw the optimal extrusion pressure between 20 and 25 kPa, while the scanning speed between 20 and 25 mm/s.

In addition the cell viability measured on the alginate and wheat-based bio-ink is comparable with that of the bio-ink formulated with animal derived products that was 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 bio-ink has been developed that is capable of

maintaining a vitality comparable to that containing animal derivatives.

5. Future developments

Interesting future developments of the study may include the implementation of the wheat-based medium with additional additives made of growth factors and vitamins to increase cell viability in both 2D and 3D.

References

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6. Acknowleggements

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