Assuring the Safety of Cultivated Meat:

HACCP plan development and application to a cultivated meat target-product

Cultivated Burger: Mosa Meat

gfi / Brazil℠

Credits

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Abbreviations and Acronyms

Buffer-ACK
Ammonium-Chloride-Potassium Lysing Buffer.

aw
Water activity.

ADI
Acceptable Daily Intake.

ANVISA
Brazilian Health Regulatory Agency

ATP
Adenosine Triphosphate.

BSE
Bovine Spongiform Encephalopathy.

BSA
Bovine Serum Albumin.

CAC
Codex Alimentarius Commission.

CCP
Critical Control Point.

CDC
Center for Disease Control and Prevention.

CIP
Clean-in-place.

CRISPR-Cas 9
Clustered Regularly Interspaced Short Palindromic Repeats/associated protein 9.

DMEM
Dulbecco's Modified Eagle Medium.

DMSO
Dimethyl sulfoxide.

DNA
Deoxyribonucleic acid.

DO
Dissolved oxygen.

EDTA
Ethylenediaminetetraacetic acid.

EGF
Epidermal growth factor

EMA
European Medicines Agency.

FACS
Fluorescence-Activated Cell Sorting.

FAO
Food and Agriculture Organization.

FBO
Food Business Operator.

FBS
Fetal Bovine Serum.

U.S. FDA
The United States Food And Drug Administration.

FGF
Fibroblast Growth Factor-Basic.

FSIS
Food Safety and Inspection Service.

gRNA
Guide of ribonucleic acid.

GAP
Good Agricultural Practices.

GCCP
Good Cell Culture Practices.

GHP
Good Hygienic Practices.

GMP
Good Manufacturing Practices.

GRAS
Generally recognized as safe.

HACCP
Hazard Analysis and Critical Control Point.

HSA
Human Serum Albumin.

IARC
International Agency for Research on Cancer.

ICMSF
International Commission on Microbiological Specifications for Foods.

IGF-1
Insulin-like Growth Factor 1.

JECFA
Joint FAO/WHO Expert Committee on Food Additives.

LLDPE
Linear Low-Density Polyethylene.

LOG
Letter of Guarantee.

mAbs
Monoclonal antibodies.

MAPA
Brazilian Ministry of Agriculture, Livestock, and Food Supply.

MCB
Master Cell Bank.

MWCB
Manufacturer's Working Cell Bank.

PBS
Phosphate buffered saline.

PGA
Polygalacturonic Acid.

pH
potential hydrogen.

PS
Penicillin-Streptomycin.

PTFE
polytetrafluoroethylene.

RASFF
Rapid Alert System for Food and Feed.

SFD
Staphylococcal food-borne disease.

SIP
Sterilized-In-Place.

STEC
Shiga-Toxigenic E. coli.

STR
Stirred Tank Reactor.

UNICAMP
University of Campinas

USA
United States of America.

USDA
United States Department of Agriculture.

USFDA
United States Food and Drug Administration.

WCB
Working Cell Bank.

WHO
World Health Organization.

Definitions

Allergen cross-contact

Unintentional incorporation of an allergenic food or ingredient into another food that is not intended to contain that allergenic food or ingredient (CAC, 2020).

Batch

A specific quantity that is intended to have uniform character and quality within specified limits and is produced according to a single manufacturing order during the same manufacture cycle. (FDA, 2023).

Bioreactor

A device where cells growth under closed and controlled conditions. (Allan, De Bank and Ellis, 2019).

Cell banking

Cell bank system consists of two tiers: a master cell bank (MCB); and a working cell bank (WCB), sometimes called a manufacturer's working cell bank (MWCB) (EMA, 1998).

CCP Decision Tree

A sequence of questions to assist in determining whether a control point is a CCP. (USFDA, 1997).

Control Measure

Any action or activity that can be used to prevent, eliminate or reduce a significant hazard to an acceptable level (CAC, 2020).

Corrective Action

Procedures followed when a deviation occurs to re-establish control, segregate and determine the disposition of the affected product, if any, and prevent or minimize the reoccurrence of the deviation (CAC, 2020).

Critical Control Point (CCP)

A step at which a control measure or control measures, essential to control a significant hazard, is/are applied to a HACCP system (CAC, 2020).

Critical Limit

A maximum and/or minimum value to which a biological, chemical or physical parameter must be controlled at a CCP to prevent, eliminate or reduce to an acceptable level the occurrence of a food safety hazard (USFDA, 1997).

Deviation

Failure to meet a critical limit (CAC, 2020).

Downstream

Downstream processing refers to the transformation of the active ingredient into the final products. It includes steps such as cell harvesting, concentration, and product formulation. (Allan, De Bank and Ellis, 2019).

Food Business Operator (FBO)

Entity responsible for operating a business at any step in the food chain. Includes primary producers, importers, manufacturers/processors, food warehouse/logistics operators, food service operators, retailers and traders (CAC, 2020).

Food Additive

Any substance not normally consumed as a food by itself and not normally used as a typical food ingredient, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packaging, transport or holding of such food results, or may be reasonably expected to result, (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include "contaminants" or substances added to food for maintaining or improving nutritional qualities (CAC, 2021).

Food safety

Assurance that food will not cause adverse health effects to the consumers when it is prepared and/or eaten according to its intended use. (CAC, 2020).

Food Safety Plan

A Food Safety Plan consists of the primary documents in a preventive control food safety system that provides a systematic approach to identifying food safety hazards that must be controlled to prevent or minimize the likelihood of foodborne illness or injury. It contains a collection of written documents that describes activities that ensure the food safety during manufacturing, processing, packing, and storage (FDA, 2016).

Good Agricultural Practices (GAP)

Basic environmental and operational conditions necessary for producing safe and wholesome food. Good Management Practices (GMP) or Good Handling Practices (GHPs) are general practices to reduce microbial food safety hazards. The term may include both "good agricultural practices" used in growing, harvesting, sorting, packing, and storage operations and "good handling practices" used in sorting, packing, storage, and transportation operations (USDA, 2009).

Good Cell Culture Practices (GCCP)

Guidance document that presents some principles intended to support best practice in all aspects of using cells and tissues in vitro. (OECD, 2018).

Good Hygiene Practices (GHPs)

Fundamental measures and conditions applied at any step within the food chain to provide safe and suitable food (CAC, 2020).

Growth factors

Comprise molecules that can stimulate cellular events such as growth, differentiation, migration, morphological changes during the development and healing of tissues. (Seeger; Paller, 2015).

HACCP

Hazard Analysis and Critical Control Point is a science-based and systematic approach that identifies specific hazards and measures for their control to ensure food safety (CAC, 2020).

HACCP Plan

Document or set of documents prepared in accordance with the HACCP principles to ensure control of significant hazards in the food business (CAC, 2020).

HACCP System

Development of a HACCP plan and the implementation of procedures according to that plan (CAC, 2020).

HACCP Team

Group of people who are responsible for developing, implementing and maintaining the HACCP system (USFDA, 1997).

Hazard

A biological, chemical or physical agent in food with the potential to cause an adverse health effect (CAC, 2020).

Hazard Analysis

Process of collecting and evaluating hazard information identified in raw materials and other ingredients, the environment, in the process or in the food, and conditions leading to their presence to decide whether or not these are significant hazards (CAC, 2020).

Ingredient

Any substance, including a food additive, used in the manufacture or preparation of a food and present in the final product, although possibly in a modified form (CAC, 2009).

Ionizing radiation sterilization

Low-temperature sterilization method used for medical products and food.

Letters of Guarantee (LOG)

Legal documents that protect facilities from penalties if a supplier provides adulterated or misbranded food additives, raw materials, packages, etc.

Master cell bank (MCB)

Represents a collection of cells of uniform composition derived from a single source prepared under specific culture conditions. (EMA, 1998).

Monitor

The act of conducting a planned sequence of observations or measurements of control parameters to assess whether a control measure is operating as intended (CAC, 2020).

Prerequisite Programs

Programmes including good hygiene practices, good agricultural practices and good manufacturing practices, as well as other practices and procedures such as training and traceability, that establish the basic environmental and operating conditions that set the foundation for implementation of a HACCP system (CAC, 2020).

Processing Aid

Substance or material, not including apparatus or utensils, and not consumed as a food ingredient by itself, intentionally used in the processing of raw materials, foods or its ingredients to fulfill a certain technological purpose during treatment or processing and which may result in the non-intentional but unavoidable presence of residues or derivatives in the final product. (CAC, 2018)

Raw material

All materials which are in the final product (PAHO, 2005).

Significant hazard

A hazard identified by a hazard analysis, as reasonably likely to occur at an unacceptable level in the absence of control, and for which control is essential given the intended use of the food (CAC, 2020).

Step

A point, procedure, operation or stage in the food system from primary production to final consumption (CAC, 2020).

Serial subculture

The sequential transferring of some or all cells from previous cell culture to a new cell culture containing a fresh growth medium. (GIBCO, 2020).

Satellite cells

Multipotent cells found in mature muscle. Satellite cells are precursors to skeletal muscle cells, able to give rise to satellite cells or differentiated skeletal muscle cells. (Asakura; Komaki; Rudnicki, 2001).

Upstream

Upstream processing refers to the first part of bioprocessing where the target product is produced, i.e., the synthesis stage. It includes steps such as cell isolation or cell line development, media preparation, cell banks, seed train (inoculum production) and bioreactor production. (Allan; De Bank; Ellis, 2019).

Validation of control measures

Obtaining evidence that a control measure or combination of control measures, if properly implemented, is capable of controlling the hazard to a specified outcome (CAC, 2020).

Verification

The application of methods, procedures, tests and other evaluations, in addition to monitoring, to determine whether a control measure is or has been operating as intended (CAC, 2020).

Working cell bank (WCB)

A collection of cells derived from one or more vials of cells from a master cell bank and expanded by serial subculture. (EMA, 1998).

Executive Summary

Cultivated chicken sandwich: UPSIDE Foods

Of rapid development in recent years, cellular agriculture technology has emerged as an alternative to solve many food system problems. The successful combination of knowledge from fields such as biotechnology, tissue engineering and molecular biology enables the production of food products using cell culture, like cultivated meat. As the technology advances, so does the challenge for regulatory authorities. Regulatory agencies must obtain reliable technical-scientific information to base their regulatory authorization of different products in an environment whose technology advancements are disruptive, varied and most often protected within companies. At the same time, entrepreneurs and scientists require more precise guidelines to conduct the development of their products properly and swiftly. In this scenario, a key issue for regulation and technology development concerns the food safety of cultivated meat products.

Attesting food safety is a prerequisite for product commercialization and the first concern of regulatory agencies. Hence, after suggestion of the Brazilian Health Regulatory Agency (ANVISA) and the Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA), the Good Food Institute (GFI) Brazil in collaboration with the University of Campinas (UNICAMP) has developed the present study.

This study aimed to establish a Food Safety Plan for a cultivated meat target product and contribute toward assessing the safety aspects of cultivated meat production by employing the Hazard Analysis and Critical Control Points (HACCP) approach.

Hazard analysis was performed according to FAO (1998), FDA (2022), and Codex Alimentarius (CAC, 2020) guidelines. Through interactive meetings, the study team developed a HACCP plan for the cultivated meat burguer. Besides the process flow diagram and worksheets used, we also present the research priorities identified during the study.

Lastly, we hope the information presented here may provide the basis for future food safety studies, indicating some steps toward ensuring the food safety of cell culture food products and assisting all stakeholders interested in assuring safe cultivated meat production.

CHAPTER 1

Introduction

Cultivated burger: Ivy Farm

1. Background

Bioreactors: UPSIDE Foods

Cultivated meat is an alternative protein produced via animal cell culture under controlled conditions that can potentially replicate the sensory and nutritional profile of conventional meats (Porto; Berti, 2022). This technology can expand the frontiers and ways in which meat is produced and consumed, reducing the environmental impacts caused by human food systems, supporting the increasing global demand for protein to human consumption, and tackling global food insecurity. Cultivated meat production can also become an alternative to mitigate ethical and health concerns associated with traditional livestock agriculture based on raising and slaughtering animals.

In recent years, cultivated meat companies announced the construction of pilot production facilities, indicating an approaching commercial-scale production (Swatz, 2023). Rapid advances in cultivated meat technology have been overcoming the bottlenecks preventing industrial production, such as scaling cell production and developing an animal-free and cost-effective culture medium. At the same time, food safety competent authorities are challenged to follow these advances and create the basis for regulatory authorization flexible enough to cover the innovative and varied methods used in cultivated meat production while considering future scientific advances.

The present study was elaborated in a context where the cultivated meat industry is still in its early stages of development. Although progress in cultivated meat technologies occurs fast, policy and regulatory landscapes are still under elaboration. Only Singapore and the USA have approved cultivated meat products for commercialization, for example.

While governments worldwide are working to develop regulatory standards to ensure cultivated meat safety, multilateral organizations such as The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) are initiating discussions around the use of cell culture for food production aiming to identify ways to ensure its safety.

Working to advance the fundamental scientific knowledge in alternative proteins, the Good Food Institute (GFI) presents this technical report to contribute to future food safety analyses for cultivated meat products.

2. Overview of Food Safety Aspects of Cultivated Meat

Despite the cultivated meat industry's growth and the pace at which technologies are moving from research and development settings to commercialization, cellular agriculture as a research field has been slower to develop due to the lack of dedicated funding from government funding agencies (Ong *et al.*, 2021). This field of research is just beginning to be recognized as an academic research activity, and it has a long way to go regarding basic science generation.

Similarly, open-access scientific studies involving the food safety of cultivated meat products are still scarce. Most comprise review/opinion articles proposing safety and regulatory aspects for producing cultivated meat, indicating research priorities and contributing to compile information on the first steps toward building the fundamental scientific basis for ensuring the safety of cultivated meat (Ketelins; Kremers; De Boer, 2021; Ong *et al.*, 2021; FAO, 2022). Recently, numerous relevant data for the safety of these products has been generated from reports issued by regulatory agencies[1] and from a series of FAO/WHO technical documents, including hazard analyses, country case studies and terminology issues, putting us one step ahead in terms of ensuring food safety for cultivated meat (FAO; WHO, 2023a). However, as knowledge gaps on this topic abound, answering safety questions to gain insights and further develop effective control measures to ensure cultivated meat products' safety and safeguard public health.

Broadly speaking, microorganisms such as bacteria, viruses, and yeast are the primary cause of biological hazards in food safety. Transmission sources can range from poor hygienic practices, contamination of raw materials, processing failures, or environmental contamination. Some bacteria, such as *Staphylococcus* aureus and pathogenic types of *Escherichia coli*, may produce exotoxins that can suppress the consumer's immune response and cause illness. Presence of any chemical residue (e.g., veterinary drugs, antibiotics, reagent residue, sanitizers, and growth factors) in food products can lead to chemical hazards, and should either be found in acceptable levels set by the regulatory authorities or not be found entirely in the food

[1] FDA Completes First Pre-Market Consultation for Human Food Made Using Animal Cell Culture Technology; FDA Completes Second Pre-Market Consultation for Human Food Made Using Animal Cell Culture Technology.

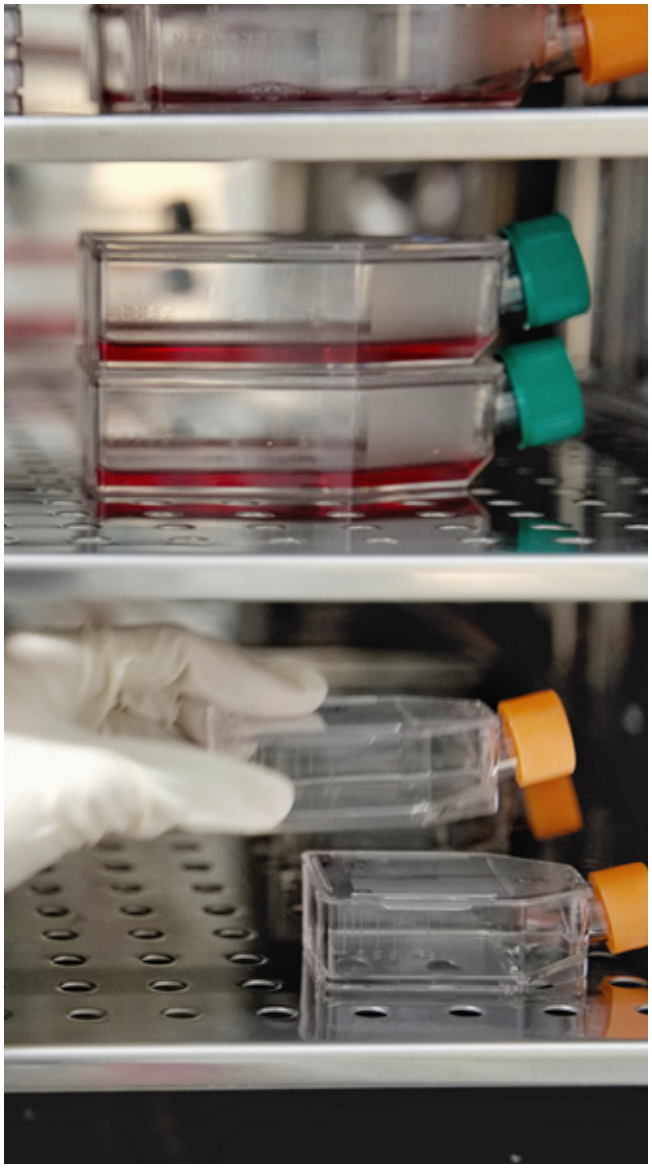
product. Physical hazards include the discovery of foreign materials such as broken glass, plastic, stones, wood, or metal (Malik; Krishnaswamy; Mustapha, 2021). Below we describe some of the safety aspects raised for cultivated meat from a centralized perspective.

Risk of microbial contamination constitutes one of the main safety concerns regarding cultivated meat. While meat contamination by pathogenic microorganisms in traditional livestock agriculture is frequent due to the use of feedlots, animal features, and failures across the meat processing chain (Cassin *et al.*, 1998, Møller *et al.*, 2016, Han *et al.*, 2022, Brashears; Chaves, 2017), cultivated meat is produced under well-controlled sanitary conditions and using cells that were previously subjected to rigid screening procedures for microbial contamination (FAO, 2022, Ong *et al.*, 2021). Moreover, the entire cultivation process must be conducted in bioreactors, equipment and processing premises under rigid quality control practices and proper monitoring for microbial contaminants. By controlling cell origin and implementing strict hygienic measures during processing one can ensure that cultivated meat is less prone to contamination by pathogenic microorganisms commonly associated with foodborne disease outbreaks linked to meat consumption such as *Salmonella* spp., pathogenic *Escherichia coli*, *Listeria monocytogenes*, among others (FAO, 2022).

Another core safety concern of cultivated meat is potential allergenicity. As animal cells are employed to produce cultivated meat, it is expected that any allergies to meat obtained by traditional animal agriculture could also be observed when cultivated meat is consumed. Hence, cultivated meat producers will need to assess the product's potential allergenicity and properly inform consumers through labeling, for instance.

On the other hand, cultivated meat technology can help mitigate risks related to traditional livestock agriculture, such as reducing the use of antibiotics in animal husbandry. This practice is a significant contributor to the rise of antibiotic-resistant microorganisms, which is detrimental to treating human infections (Wallinga *et al.*, 2022, Xu *et al.*, 2022, Wu *et al.*, 2023). Cultivated meat production is expected to require much lower use of antibiotics or none at all, potentially contributing to reduce the occurrence of antibiotic-resistant microorganisms in the food systems.

Another beneficial contribution is a potential decrease in the risk of zoonotic diseases, i.e., diseases transmitted from animals to humans, as using properly screened animal cells grown under controlled conditions to produce cultivated meat will require reduced or no slaughtering. Lastly, due to the strict safety and quality control measures assigned to cultivated meat production, the occurrence of physical hazards associated with meat obtained from traditional livestock agriculture should be mitigated (Cavalheiro *et al.*, 2020, Iko Afé *et al.*, 2021, Smulders; Rietjens; Rose, 2019, FAO, 1998).



Considering all these aspects, a crucial step to ensure food safety for a new product and one of the requirements to acquire regulatory approval is identifying potential hazards in food production. Given the potential presence of chemical, biological, and physical hazards in cultivated meat (FAO, 2022), the Hazard Analysis and Critical Control Point (HACCP) approach can be used to manage food safety.

HACCP is a systematic preventive tool that can be used to identify and control hazards in food production chain, in addition it is also adopted by the Codex Alimentarius Commission to assess hazards and establish control systems focusing on prevention rather than relying on end-product testing. Developing a HACCP plan may require changes in raw materials, processing parameters, manufacturing technology, end-product characteristics, and distribution method employed in the intended use or in the prerequisite program applied. Any HACCP system should be capable of accommodating change, such as advances in equipment design, processing procedures, or new technology (CAC, 2020).

Cultivated meat production's potential to become a safer and more sustainable option than traditional meat production will likely be achieved if these and other safety concerns are properly assessed. Addressing them will require bridging research gaps and cooperation between producers, regulatory agencies, consumers, researchers, and other stakeholders in designing, validating, implementing, and monitoring proper and strict measures to ensure the production of high-quality and safe cultivated meat.

3. Scope of this study

• This study presents a Food Safety Plan for a hypothetical product, the cultivated meat burger. A process flow diagram of the process for obtaining the target product was modeled and cross-checked by international experts in the cultivated meat field, thus enabling hazard analysis;

• The process modeled includes 24 steps, from selection of the animal donor to product storage and distribution. Upstream processing includes immortalization and expansion of bovine satellite cells using 500L Stirred Tank Reactor (STR) bioreactors in parallel. Downstream processing covers unit operations to harvest cell biomass, which is added with plant-based ingredients, shaped into the target product, stored and distributed;

• The multidisciplinary study team, consisting of ten scientists, held discussion sessions to elucidate questions and decisions regarding every study stage and to complete the hazard assessment. To ensure as much as possible complete hazard identification, safety assessment considered all ingredients, processing aids, raw materials, equipment, and packaging used in the process;

• Despite the study's relevance in contributing to ensure cultivated meat food safety, limitations should be highlighted;

• This study was proposed in the context of the few regulatory processes on cultivated meat publicly available. Moreover, cultivated meat products can be manufactured using a wide variety of ingredients, raw materials and bioprocess designs, most of which are currently developed under the intellectual property protection of companies;

• Hence, the modeled process proposed here is only representative of how a cell culture-based food production can be carried out, as well as how its inputs are applied and steps conducted. Our hazard analysis, therefore, does not intend to exhaust all potential hazards and appropriate control measures for all cultivated meat products, nor to meet regulatory requirements of any particular country or region. Food safety assessments should always be product-specific.

CHAPTER 2

Methodology to develop HACCP Plan

Cell expansion in scaffolds: UFMG (Federal University of Minas Gerais - Brazil)

1. Approach to accomplish the preliminary tasks

Given the technical complexity of producing cultivated meat and the need to conduct a robust hazard analysis, we assembled a multidisciplinary study team of ten scientists from five different institutions with specific knowledge and expertise in the fields of food safety, food microbiology, quality control, processing technologies for conventional meat, cultivated meat, tissue engineering, cellular and molecular biology, material engineering, biotechnology and bioprocessing.

Due to this broad and multidisciplinary background, we deemed it necessary to conducted an initial training with all experts involved to standardize the basic knowledge on the core topics required to develop the food safety plan, namely: HACCP system, the microbiology of foods and meat, food business hazards, good manufacturing practices, cell culture techniques, large-scale bioprocessing, HACCP application in the pharmaceutical industry, and HACCP application in the meat industry.

The target product of the study is the cultivated meat burger. This product was chosen because it can be representative of the first generation of cultivated meat products developed by the industry. In other words, processed foods (e.g., hamburgers, sausages, ham, and nuggets) made of a mix of animal cells and plant-based ingredients and intended to be sold in restaurants. Likewise, during process conception we considered using some inputs applied by the industry at is early stage (such as FBS and other animal-derived inputs). However, those inputs have been progressively replaced by more suitable versions that meet industry demands for ingredients at lower costs and environmental impacts. When describing the product's intended use, we considered features of similar conventional products and the current status of the cultivated meat industry (Table 1), The team also included an intermediate product used as an ingredient for burger production—bovine muscle cell biomass.

2. Approach to Process Flow Diagram Construction

The flow diagram is the systematic representation of the sequence of steps used in food production (CAC, 2020). Its purpose is to provide a detailed outline of the steps involved in the process in order to work as the basis for hazard analysis and the application of the other HACCP principles.

Typically, a HACCP team would develop the flow diagram and perform an on-site review of the operation to validate its accuracy and completeness, but as this is a case study based on a theoretical product, the study team built the flow diagram using available scientific data describing methodologies and steps used in cultivated meat production (Bomkamp et al., 2022; Bodiu et al., 2020; Bradley, 1978; Brasil, 2004; Brasil, 2015b; USHHS, 2022; Ding et al., 2018; Rodríguez Escobar et al., 2021; Geraghty et al., 2014; USHHS, 2010; Hanga et al., 2020; Hanga et al., 2021; USHHS, 2009; Humbird, 2021; Ianovici et al., 2022; Joo et al., 2022; Kang et al., 2021; Kern et al., 2016; Inamdar et al., 2012; Flecknell, 2009; Letti et al., 2021; Li et al., 2021; Pereira; Oliveira, 2020; Post et al., 2020; Marga et al., 2015; Melzener et al., 2021; Melzener et al., 2022; Messmer et al., 2022; Ramezani et al., 2019; Sart; Agathos, 2016; Shit; Shah, 2014; Skrivergaard et al., 2021; Ben-Arye et al., 2020; Verbruggen et al., 2018; Genovese, 2017). The experts' previous experience establishing bioprocesses was also valuable to this outlining.

Once finalized, we submitted the flow diagram and its process description for extensive reviews by international experts from seven companies based in different countries operating in cultivated meat and one academic researcher working in the field, all performed anonymously. Pertinent suggestions were considered to ensure that the methodologies used as a model complied with the current practices and challenges of cultivated meat production.

3. Approach to HACCP plan development

Cultivated burger: Mosa Meat

Work dynamics for developing the Food Safety Plan included synchronous and asynchronous work, including offline activities. During the discussion sessions held, the study team deliberated on queries and decisions regarding every stage until reaching an agreement. Due to the limited scientific data on several aspects of cultivated meat safety the team used a conservative approach to decide and reach a consensus during the HACCP plan development. Below we describe details of how the team addressed each HACCP principle.

3.1. Conduct a hazard analysis (Principle 1)

Hazard analysis was performed according to FAO (1998), U.S.FDA (2022), and Codex Alimentarius (CAC, 2020) guidelines. Qualitative risk assessment of each hazard or set of hazards should be conducted according to the severity of the adverse health effect caused by the hazard and the likelihood of it occurring. According to FAO (1998), low-probability and low-severity hazards should not be tackled by the HACCP system, but rather through Good Manufacturing Practices (GMPs) and Good Hygiene Practices (GHPs).

Despite knowledge of the severity around each biological hazard, probability of occurrence and the behavior of potential foodborne pathogens in cultivated meat products are unknown and can vary according to the process adopted by each food business operators (FBOs). Thus, in the present HACCP plan, hazard analysis was performed based on epidemiological scientific data on foodborne illness linked to conventional bovine burgers, and experts' opinions.

In our quest to adequately address possible concerns and provide the most accurate information to readers, we posed several questions during the HACCP plan development, such as:

- Is there a chance for product contamination with hazardous substances?
- Is there a chance of using ingredients above the critical limit allowed by local or international regulations?
- What hazards may result if the food composition is not controlled?
- Does the cultivated meat permit the survival or growth of pathogens in the product during processing?
- Is there a chance for biological or chemical cross-contamination during processing?
- Does the package include instructions for safe handling, storage, and food preparation by the end-product consumer?
- Is the packaging material resistant to damage, thereby preventing microbial contamination?
- Are there any potential allergens in the ingredients which should be included in the list of ingredients on the label?
- Can cleaning and disinfection affect the safety of the product?
- Would improper storage lead to microbiologically unsafe food?

Once the hazard analysis concluded, the study team listed some control measures, i.e., any actions and activities that can prevent, eliminate, or reduce a food hazard to an acceptable level, for each hazard. According to Codex Alimentarius (CAC, 2020), more than one measure may be required to control a specific hazard, and a specific measure may control more than one hazard.

3.2. Determine critical control points (Principle 2)

CCPs determination was performed according to Codex Alimentarius (CAC, 2020) and U.S.FDA (2022) guidelines, an aided by the Codex Alimentarius decision tree (CAC, 2020) (Appendix 02). CCPS were established at steps where significant hazards were identified during the hazard analysis (Table 3) and were essential to produce safe food. CCPs are shown in the HACCP worksheet (Table 4) and highlighted at the appropriate step on the flow diagram (Figure 1).

It is essential to emphasize whether a CCP step is multifactorial and varies between FBOs and from process to process. As more than one CCP may be applied to control a specific hazard, or a CCP may control more than one hazard (CAC, 2020), the CCPs presented are specific to the processes and formulation conditions described in this document.

3.3. Establish critical limits (Principle 3)

Critical limits should be measurable or observable, and separate acceptable from unacceptable products. We established critical limits for each step classified as CCP based on regulations or literature data. Given the technological novelty, a real process will require some of the critical limits as well as control measures to be previously validated to obtain evidence that they are capable of controlling the hazard and ensuring safe food.

3.4. Establish monitoring procedures (Principle 4)

According to FAO (1998), monitoring procedures for CCPs should be capable of timely detection of a deviation from an established critical limit to allow isolation of the affected products. The suggested monitoring procedures aim simply to illustrate the numerous possibilities available. As such, each FBO should consider routine procedures and particularities to establish the most suitable procedure and its frequency for CCP monitoring.

3.5. Establish corrective actions (Principle 5)

A corrective action plan must be enforced immediately after monitoring indicates a critical limit deviation. According to USDA (2021), a corrective action plan comprises four actions:

1. Product destination: Segregate all products processed after the last acceptable check until appropriate disposition is taken. Analyze processing parameters and products to determine whether the product must be discarded, reprocessed, or sent for by-product processing. Product destination depends on the sensory characteristics of the product, the magnitude and type of deviation, among others;

2. Deviation cause: Determine and eliminate the root cause of the deviation;

3. Re-establishment of CCP control: Take actions to bring the CCP under control;

4. Prevention of recurrence: Take measures to prevent future occurrences.

Since the corrective actions are deviation-specific, the present HACCP plan suggested no corrective actions as each company should perform a case-by-case analysis to establish the best corrective action to control CCP and prevent recurrence.

3.6. Establish verification procedures (Principle 6)

According to Codex Alimentarius (CAC, 2020), verification procedures ensure that the control measures effectively control the hazards as intended. The verification procedures suggested here are examples of numerous possibilities that companies can adopt.

Each FBO should consider routine and particularities to establish the best method and its frequency for each CCP, which can include observations, auditing (internal and external), calibration, sampling and testing, and record review. Verification should be conducted by a third person responsible for monitoring and implementing corrective actions (FAO, 1998), and also tackle the HACCP plan to ensure the whole system is controlled.

3.7. Documentation and record-keeping (Principle 7)

Documentation and record-keeping should be appropriate to the nature of the operation and sufficient to assist the FBO in verifying whether the CCPs and the HACCP plan are under control (FAO, 1998). As for principles 4, 5 and 6, the record-keeping developed here comprises an example among numerous possibilities. Each FBO should consider its reality to establish the best record keeping system.

Table 4 summarizes the HACCP plan developed for the target product, covering all the above principles.

CHAPTER 3

The results

Cultivated burger: Mosa Meat

1. Flow Diagram

Bioreactors: UPSIDE Foods

1.1. Process Flow Diagram

Figure 1 illustrates the process flow diagram for producing cultivated meat burger and describes each step to complete the 24-step process, from the donor animal selection to product storage and distribution, including cell isolation, cell banking, upstream, downstream, and product processing.

Broadly speaking, the bioprocess includes a sequential set of unit operations separated into 'upstream' and 'downstream' steps. Upstream processing include cell expansion, inoculum production and bovine muscle cell biomass production in batches in 500L stirred-tank (STR) bioreactors in parallel. Appendix 01 details the seed train expansion designed in this modeled process. Downstream processing covers unit operations such as harvesting and centrifugation, involved in the obtention of the concentrate cell intermediary product (bovine muscle cell biomass used to formulate the target-product.

CCP steps were labeled in the flow diagram with a number followed by the type of hazard—biological (B), chemical (C) and physical (P). Moreover, we analyzed all the inputs to determine critical materials among raw materials, ingredients, processing aids and packaging (Table A4, Appendix 04).

Figure 1. Flow diagram of cultivated meat burger - part one

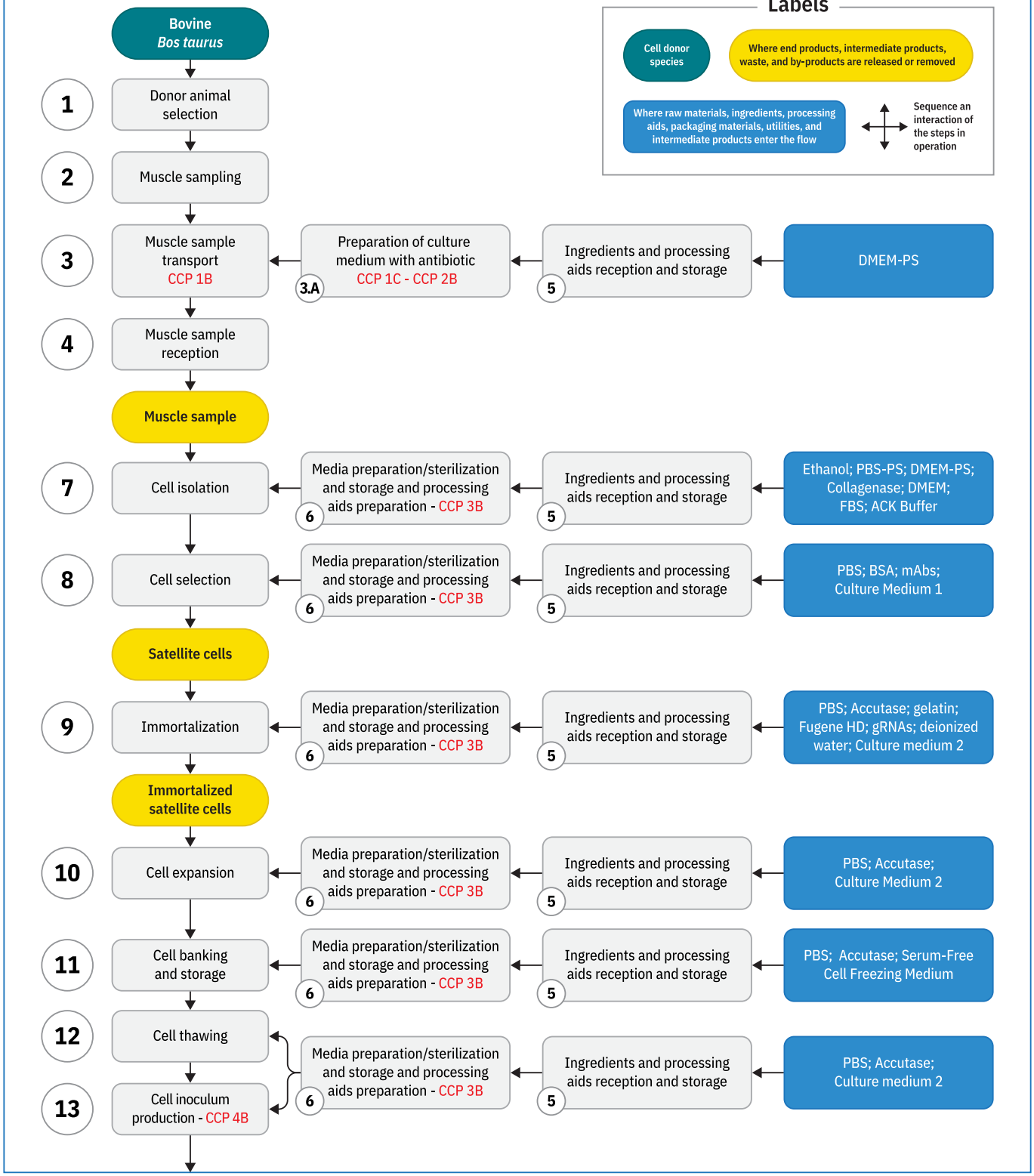
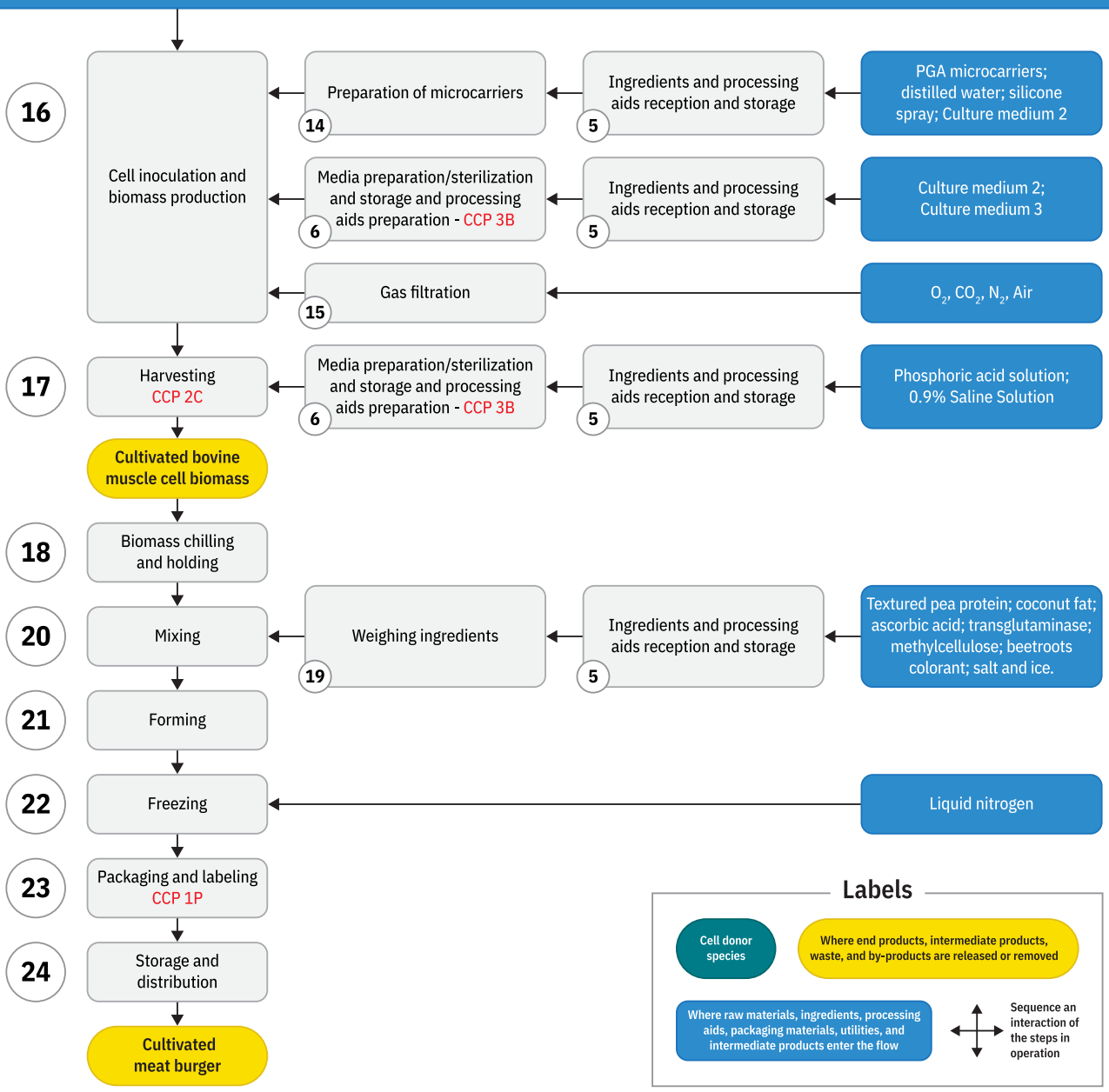


Figure 1. Flow diagram of cultivated meat burger - part two



1.2. Description of steps

This section offers a detailed description of each step of the cultivated meat burger production process, including all inputs, equipment and activities used.

1 - Donor animal selection

A two-year old, male bovine (*Bos taurus*) live animal was chosen as donor animal for the muscle sample. Veterinary inspection, which considers clinical parameters, vaccines, and disease history, including administration history of veterinary drugs such as antibiotics, was performed to attest to health conditions in the field.

2 - Muscle sampling

After the semimembranosus muscle region is washed, shaved, and cleaned with ethanol, the animal receives local lidocaine-based anesthesia and muscle sampling is performed via incision using a sterile metallic tube. Muscle sampling is performed in the field (open environment) at room temperature.

3 - Muscle sample transport

The sample (muscle tissue) is then transferred to a sterile tube with Dulbecco's Modified Eagle Medium (DMEM) and Penicillin-Streptomycin (PS) and stored at 4°C inside a thermal box. Muscle samples are transported to the laboratory within two hours.

3.A - Preparation of culture medium for sample transport

Under aseptic conditions (biological safety cabinet), previously sterile-filtered liquid DMEM culture medium are mixed with sterile Penicillin-Streptomycin (PS) to a final concentration of 1%. The media is placed in sterile flasks and stored in a cold chamber (2-8°C) until use.

4 - Muscle sample reception

Upon reception at the industry, the thermal box and plastic bag protecting the muscle sample are externally decontaminated with 70% Ethanol. Temperature and preservation conditions of the sample are verified (visual inspection of the sample and internal temperature control of the box).

5 - Ingredients and processing aids reception and storage

All ingredients and processing aids (described in Table A3.1 and A3.3) are purchased from suppliers and received by the FBO. All materials are checked to ensure adequate shipping conditions (temperature), packing integrity, expiration date, and sterility (when required). Processing aids and ingredients are kept under the supplier's recommended storage conditions until use. Information regarding batch, brand, and expiration date of the ingredients and processing aids received are recorded.

6 - Media preparation/sterilization and storage and processing aids preparation

Media ingredients (from Culture Medium 1, 2, and 3, described in Table A3.2) are dosed and mixed with ultrapure water in a single-use mixing vessel. The desired pH (7.2-7.4) is adjusted using phosphoric acid (H_3PO_4; 1M) or sodium hydroxide (NaOH; 1M) basic solutions, previously weighed and diluted in water. DMEM is purchased fractionated and added as a powder directly into the mixing vessel. A piping connected to filtration units removes the media from the mixer, where the media is then sterilized through a filtration process (0.2 or 0.1 µm). The sterilized media is placed in sterile bags and stored in a cold chamber (2-8°C) until use.

Processing aids that are sterile and that will have direct contact with the product must be handled (mixed, diluted, aliquoted, etc.) in a clean room, using sterile materials inside the biological safety cabinet.

7 - Cell isolation

Before opening, the thermal box and sample tube are cleaned with 70% ethanol. The tube is then placed under sterile conditions, and the muscle sample is transferred to an appropriate new sterile tube. Next, the tissue is washed with 70%

Ethanol and Phosphate buffered saline (PBS)-PS, dissociated (scalpel), digested with collagenase in DMEM-PS at 37°C (CO_2 incubator) for 1.5 h, and mixed (vortex) for 10 minutes. After digestion, 20% Fetal Bovine Serum (FBS) in DMEM is added to the sample and mixed with a pipette. Muscle fragments are centrifuged at 80 x*g* for 3 minutes, and the supernatant collected. The precipitated debris is again triturated with a 20-gauge needle in PBS and centrifuged at 80 x*g* for 3 minutes. The supernatant is collected, mixed, and centrifuged at 1,000 x*g* for 5 minutes. The cells are washed twice with PBS, followed by DMEM with 20% FBS. Next, the cells are filtered (100 µm and 40 µm cell strainers), centrifuged at 1,000 g for 5 minutes at 4°C and incubated with Ammonium-Chloride-Potassium Lysing Buffer (ACK Buffer) for 5 minutes on ice. Finally, the cells are washed twice with PBS.

8 - Cell selection

Cells are centrifuged in a sterile tube, and the pellet is reconstituted with buffer (1% BSA[2] in PBS) plus antibodies (conjugated mAbs). Then, the cell suspension is incubated in ice. Subsequently, labeled cells are washed with PBS, reconstituted in Culture medium 1 and selected by cell sorting in a flow cytometry equipment located under a laminar flow module. The selected cell suspension of satellite cells is transferred to culture flasks and cultivated in a CO_2 incubator for two passages in Culture medium 1.

9 - Immortalization

After removal of the culture flasks containing the satellite cells from the CO_2 incubator and placement under sterile conditions, the cells are washed with PBS, detached from flasks with Accutase, and resuspended in Culture medium 2. The cells are then transfected using CRISPR-Cas9, guide of ribonucleic acid (gRNA), and selected by first isolating individual clonal populations from the parental pool of cells subjected to one transfection cycle using 0.1% gelatin-coated wells. Plasmid DNA construct delivery is mediated by non-liposomal DNA complex forming transfection reagent (Fugene HD). gRNAs targeting the 5'region of the *Bos taurus* CDKN2A gene encoding p16 are designed for expression from a transfected plasmid. Plasmids are diluted in sterile deionized water before use. After immortalization, immortalized satellite cells are placed in Culture medium 2 and cultivated in a CO_2 incubator.

10 - Cell Expansion

The immortalized satellite cells are submitted to serial subculture until a sufficient number of cells is reached. For the subculture procedure, the immortalized satellite cells are washed (with PBS), detached from flasks (Accutase), washed with new Culture medium 2, centrifuged, replated in culture medium 2 and cultivated in T-flasks (CO_2 incubator).

11 - Cell banking and storage

When a sufficient cell number is reached, the subculturing steps are repeated to prepare a master cell bank (MCB) and a working cell bank (WCB). The cells are resuspended in a cryopreservation medium (Serum-Free Cell Freezing Medium), dispensed into individual cryovials, and frozen for at least 4 hours at -80°C (Ultra-freezer) and then permanently at -150°C. The WCB is derived from one or more cell vials from the MCB. MCB and WCB must be stored in ultra-freezers at -150°C (which are placed in a room with controlled temperature

[2] Bovine Serum Albumin

between 20 and -25ºC), under the same conditions in two or more separate locations.

12 - Cell thawing

One or more WCB vials are removed from the ultra-freezers and thawed rapidly by immersion in a bead bath at 37ºC. Vials are placed under sterile conditions (biological safety cabinet), the cells are washed with PBS, centrifuged using a laboratory centrifuge, and resuspended in a Culture medium 2.

13 - Cell inoculum production

Cells are cultured and expanded in Culture medium 2 at 37ºC using monolayer culture systems (T-flasks, Cell Factories, or Cell Stacks) until the number of cells required to inoculate the bioreactor is reached.

14 - Preparation of microcarriers

Under sterile conditions (biological safety cabinet), dry sterile Polygalacturonic Acid (PGA) microcarriers are placed inside a sterile glass bottle siliconized with a food-grade silicone spray. After adding distilled water and gently swirling the suspension, it is transferred to sterile plastic bags and stored for up to 1 week at 4°C before use.

The microcarrier suspension is placed in a sterile Schott bottle to settle and the water is aspirated with a pipette. A small volume of Culture medium 2 is then added, and the liquid is aspirated again to remove all water from the suspension. Lasty, the desired volume of Culture medium 2 is added, and the suspension is transferred to a sterile bag. Culture mediums 2 and 3 and microcarriers suspension stored in bags are transported to the bioreactor area and transferred to the bioreactor using aseptic connections and peristaltic pumps.

15 - Gas filtration

Gases—N_2, O_2, CO_2, and synthetic air, which is a mixture of Nitrogen (80%) and oxygen (20%)—must undergo a filtration process (0.2 µm polytetrafluoroethylene - PTFE - hydrophobic membranes, single-use or sterilized in place) before entering into the bioreactors at a determined rate. The bioreactors are fed a mixture of the four gases (proportions will vary throughout the culture) to maintain the dissolved oxygen (DO) concentration during the proliferation and differentiation stages.

16 - Cell inoculation and biomass production

Before inoculation in the bioreactor (previously sterilized), cells are washed (with PBS), detached from flasks (Accutase), rewashed (Culture medium 2), centrifuged, resuspended in Culture medium 2 and placed inside sterile bags. After overnight equilibration, the cell suspension is inoculated in the bioreactor using sterile connections. Cells adhered to the PGA microcarriers are kept inside the stirred-tank bioreactor with Culture medium 2 under controlled temperature (37ºC), pH (7.2-7.4), and dissolved oxygen (DO) concentration. The pH is controlled by adding CO_2/basic solution (Sodium bicarbonate buffered medium) or, alternatively, by adding an acid and base solution. DO concentration is maintained at the desired setpoint by controlling the impeller speed (agitation) and the gas flow using the 4-gas mixture (N_2, O_2, CO_2, and synthetic air). During biomass production, partial medium exchange (50%) can be conducted by adding fresh Culture medium 2. This biomass production step is held until the desired number of cells is reached. Then, a full medium exchange (100%) to Culture medium 3 is performed to start skeletal muscle differentiation.

17 - Harvesting

Harvesting requires the agitation/aeration to be turned off so that the cells settle and spent Culture medium 3 is removed for the washing procedure with sterile 0.9% Saline Solution. Agitation is then turned on, pH of the saline solution is reduced to pH 5.5-5.8 using phosphoric acid solution (H_3PO_4), and the bioreactor temperature is reduced to 15°C. The solution is mixed until equilibrium of pH and temperature. Subsequently, the cell biomass is de-watered by centrifugation (Disk-stack centrifuge). Spent saline solution is a waste product removed during centrifugation.

18 - Biomass chilling and holding

The de-watered cell biomass is transferred to a tank and chilled to ≤5°C. Once chilled, the cell biomass is kept in the holding tank, automatically weighted, and transferred to the mixing tank.

19 - Weighing of ingredients

The ingredients for the cultivated meat burger (as described in Table A3.1) are placed in a clean room for weighing on a calibrated scale. Food handlers must be well-trained and follow procedures for correctly weighing ingredients.

20 - Mixing

The de-watered cell biomass is mixed with textured pea protein, coconut fat, ascorbic acid, transglutaminase, methylcellulose, beetroot colorant, salt, and ice in an appropriate mixing tank for 10 to 15 minutes until a homogeneous mass with fine binding is obtained. Mass temperature must be kept ≤5°C to allow proper forming while avoiding fat separation and microbiological growth. This step is conducted under non-aseptic conditions at a room temperature ≤10°C.

21 - Forming

The mass obtained is sent to automatic burger formatters for proper size and thickness.

22 - Freezing

Quick freezing is performed by feeding the burger patties through a liquid nitrogen tunnel, reaching a temperature of ≤–18°C. The burger patties are then separated individually by paraffin paper.

23 - Packaging and labeling

Frozen cultivated meat burgers are packed in a paper box, interleaved paraffin paper in a room at ≤10°C temperature. The paper boxes pass through a metal, magnetic, or x-ray detector.

24 - Storage and distribution

The product must be stored and distributed at ≤-18°C.

Cultivated salmon: Wildtype

2. HACCP Plan for Cultivated Meat Production

This section presents the HACCP plan for producing cultivated meat burger from cultivated bovine muscle cell biomass (an intermediary bioproduct used as an ingredient), as an example of a cultivated meat-based product intended to be commercialized in restaurants.

Here we detail the hazards more likely to be associated with raw materials, ingredients and processing steps, as well as their control measures. We developed it by gathering data from the scientific literature on the safety of conventional meat products and conditions employed in pharmaceuticals, bioprocesses, cell culture, and biological products. Tables 1 and 2 give a full description of the target product and its composition. Table 3 summarizes the results of the Hazard Analysis (principle 1), and Table 4 presents the HACCP worksheet to meet principles 2 to 7.

Since we used a batch process as reference, differences may exist in terms of hazards and control measures if a FBO employs a continuous process. Similarly, facility, equipment, formulation, and processing differences can directly affect hazard analysis. Thus, the potential hazards identified in this HACCP plan may not be the only hazards associated with all cultivated meat and cultivated meat-based products. For any food product, the safety assessment should always be product-specific.

Nonetheless, the information presented here might contribute to developing safe cultivated meat-based products and support FBOs in meeting future regulatory requirements related to pre-requisite programs and HACCP.

2.1. Assumptions and considerations for the HACCP plan

As technology for cultivated meat production is still under development, we adopted some assumptions to guide the HACCP plan execution and interpretation, as detailed below. Each assumption may be directly linked to one or more steps of the cultivated bovine biomass and cultivated meat burger production processes.

- If unlike the modeled process, which considers the use of cells collected directly from a donor animal, the cells are purchased from a third-party cell bank, a letter of guarantee (LOG) and good manufacturing practices (GMP) certificate should be provided;
- The modeled process considers the use of ingredients and processing aids purchased from qualified external suppliers;
- The modeled process considers that all equipment, such as bioreactors and filters (for gas and media sterilization), were cleaned-in-place (CIP)/sterilized-in-place (SIP) using food grade cleaning products, except for the single-use mixer (for media preparation);
- For the approach used here, labeling was deemed a key control measure for specific hazards identified. But since labeling regulations may vary among countries, in concrete cases, FBOs should comply with the country's regulations/guidelines, and if needed, rework the hazard analysis.
- Different FBOs could employ many different processes to produce cultivated meat. If any element or aspect of a concrete process differ from those considered here, significant hazards may be modified leading to changes in the process flow diagram and the hazard analysis.
- Since the conditions, parameters, specifications, standards, and critical limits employed were based on the available scientific data, use of updated references and validated critical limits may imply the need to revise the plan.

2.2. Product Description

Table 1. Product Description Form

1. Product name	Cultivated bovine muscle cell biomass	Cultivated meat burger
2. Product definition	An aggregate of microcarriers and bovine skeletal muscle cells produced in bioreactors	A patty consisting of cultivated bovine muscle cells biomass and added ingredients, shaped and subjected to an adequate technological process
3. Important characteristics of the final products, such as, pH, water activity (aw), etc.	pH=5.5-5.8 (desirable) aw=0.98-0.99	pH=5.5-5.8 (desirable based on beef meat). aw=0.98-0.99.
4. Instruction for use and/or consumption	Ingredients for the process of obtaining cultivated meat products	Heat treatment (grilled, roasted, fried, cooked, etc.)
5. Packaging characteristics	Single-use or reusable (by sanitizing) plastic buckets/bowls	Plastic packaging for frozen products
6. Expiration date	To be defined (additional research is needed)	At least 90 days under freezing (based on conventional meat)
7. Where the product will be sold	Business-to-business	Restaurants
8. Storage	Between 0 to 4°C	Frozen at -18°C
9. Information included in the label	• Product name; • Ingredient list; • Batch; • Expiration date; • Storage instruction.	• Product name; • Ingredient list; • Batch; • Expiration date; • Storage instruction; • Allergen declaration; • Preparation/instruction for use.

2.3. Composition

Table 2. Product Composition Form (Raw Materials, Ingredients, Additives, etc.)		
Product name	Cultivated bovine muscle cell biomass	Cultivated meat burger
Raw material	Bovine semimembranosus muscle	Cultivated bovine muscle cell biomass
Ingredients	Cultivated bovine muscle cells	Textured pea protein
		Coconut fat
		Ascorbic acid
		Transglutaminase
		Methylcellulose
		Beetroots colorant
		Salt
		Water/ice/deionized water
	Polygalacturonic Acid Sodium Salt (PGA) microcarrier	
Facility Gases	O_2, CO_2, N_2, synthetic air (mixture of nitrogen (80%) and oxygen (20%)	
Packaging material	Single-use bioprocess bags	Paraffin paper
	Cell culture flasks (Polystyrene)	Carton packs
	Plastic buckets/drums (LLDPE)	Plastic material (LLDPE)

2.4. Hazard Analysis

Below we present an overview and considerations about the physical, chemical, and biological hazards analysis conducted. Details on the steps in which these hazards were identified and their respective control measures are shown in the Hazard Analysis Worksheet (Table 3).

2.4.1. Biological hazards

Our analysis identified the following biological hazards: foodborne pathogens that could be introduced from the donor animal (bovine), ingredients, and processing aids or entered by cross-contamination due to improper storage, handling, or sanitation. Given the current lack of public scientific data on whether and what foodborne pathogens can grow or survive in cultivated meat, the experts agreed to use a conservative approach contemplating the analysis of groups of the major pathogens recognized as meat contaminants. On that basis, this HACCP plan addressed the following potential biological hazards: *Brucella abortus*, *Mycobacterium sp.*, prion - Bovine Spongiform Encephalopathy (BSE), *Toxoplasma gondii;* Shiga-toxigenic *Escherichia coli* (STEC), *Salmonella, Cryptosporidium parvum, Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus*. In conventional ways of meat processing, most of these hazards can be inactivated by heat treatments (cooking, frying, grilling).

In the cultivated meat burguer production, the initial phases of the process, which includes a sampling procedure conducted in an open field can

be problematic in terms of biological contamination of the sampled tissue. Thus, it is essential to follow GAPs and conduct sampling using sterile material and adding antibiotics at the proper concentration during sample transportation. Once the cells undergo cultivation under controlled conditions, pathogens are not expected to propagate in the cell culture and go undetected until the end process (FAO; WHO, 2023a; Treich, 2021).

Those hazards can also be avoided or controlled by aseptic handling of cells and inputs, monitoring the cultures to verify signs of contamination, ensuring the materials' quality by adopting a supplier qualification program and implementing programs such as GHP and GCCP. For the process evaluated here, ensuring the adequate application of the method to sterilize media and inspect cultures for signs of contamination before inoculation in the bioreactors are specific control measures to prevent contamination by foodborne pathogens. Additional testing to detect microbiological contaminants in cells and sterility testing of the culture medium are also recommended to avoid biological contamination in cell cultures (Geraghty *et al*., 2014).

Salmonella, Shiga-toxigenic *E. coli* (STEC), and *L. monocytogenes* can be found in the farm environment (feces, manure, soil, feeding silage, etc.), and can also colonize meat processing facilities and equipment (Roberts, 2005; ICMSF, 2011; Singh; Thippareddi, 2019; Matle; Mbatha; Madoroba, 2020). These pathogens have been isolated from raw meat or linked to a foodborne outbreak caused by meat and meat products (RASFF, 2022a; 2022b; 2022c; FSIS, 2022a; 2022b; 2022c, 2022d, CDC, 2019a, 2019b, 2022).

Staphylococcus aureus can be found in cattle's fur, hide, or skin as part of the microbiota (Roberts, 2005), or introduced into the manufacturing plant by cross-contamination or handling, reaching the final product (Roberts, 2005; Singh; Thippareddi, 2019; Fang; Chen; Kuo, 1999). Staphylococcal foodborne disease (SFD) is one of the most common worldwide, resulting from food contamination by preformed *S. aureus* enterotoxins (Kadariya; Smith; Thapaliya, 2014).

Bacillus cereus was considered a potential biological hazard here due to some plant-based ingredients used across the process and the likelihood of biofilm formation (Ellouze *et al*., 2021; Akamatsu *et al*., 2019; Majed *et al*., 2016; Lin; Briandet; Kóvacs, 2022). Moreover, this foodborne pathogen has already been isolated from meat and meat products (Tewari; Singh; Singh, 2015).

Cultivated meat burger processing applies recombinant proteins as processing aids at different stages of production. Recombinant proteins are produced using microorganisms as host systems; in the present study, all recombinant proteins used are produced using Escherichia coli, which has a history of safe use in food production and approval for food additives production in Europe (Kallscheuer, 2018) and GRAS approval in the US (FDA, GRN 000897). Except for strains known to be pathogenic, E. coli is considered a Class 1 Agent under the National Institutes of Health (NHI) guidelines, which covers all non-human organisms or animal pathogens. Its use as a cell factory is well-established and has been commercially exploited by various industries. The strain E. coli K-12, for example, is non-pathogenic, non-toxigenic, and not likely to pose a risk to human or animal health, plants, or other microorganisms, and has been utilized for decades, often in industrial settings with high volumes and cell densities. In cases where

recombinant proteins are produced using recipient strains that lack a history of safe use, their safety must be first established.

2.4.2. Chemical hazards

Chemical hazards in conventional food production generally include natural toxins (mycotoxins, animal toxins, and phytotoxins), pesticides, veterinary drugs, environmental pollutants, heavy metals, allergens and additives in inadequate levels. These toxic chemicals may contaminate food at different stages of the production chain (Arisseto-Bragotto; Feltes; Block, 2017), from production (including operations carried out in crop, livestock, and veterinary medicine) to manufacture, processing, preparation, treatment, packaging, transport, or holding of such food, or as a result of environmental contamination (CAC, 2016).

Analysis of chemical hazards consulted publications from recognized entities, like the Codex Alimentarius Commission (CAC), the United Nation's Joint Food and Agriculture Organization (FAO), the World Health Organization (WHO) Expert Committee on Food Additives (JECFA) and the International Agency for Research on Cancer (IARC/WHO). Carcinogenicity of each listed chemical was verified by consulting the IARC database (IARC Monographs[3][4])(IARC, [2023]). For allergens, we consulted the US FDA database which reinforces the need to control cross-contact and labeling of nine major food allergens (soybeans, sesame, milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, and wheat)[5]. Severity of the chemical hazard identified was scored using Acceptable Daily Intake (ADI), and its probability depended on the amount consumed and on the amount of contaminant present in the animal production chain as residues or in the culture media and reagents used in the cultivated meat production.

Accordingly, the potential chemical hazards identified were veterinary drugs, antibiotics (PS), growth factors (IGF-1, FGF, EGF), chemical substances from the packaging material and substances capable of eliciting allergic responses, albumins (HSA and BSA) and pea protein isolate (Taylor *et al.*, 2021). In the present plan, pea protein is the only input intended to be present in the final product. Veterinary drugs could be unintentionally introduced during production, while all other chemicals are intentionally introduced but are not intended to be present in the final product. Control measures for these chemical hazards include source control (supplier qualification program), adopting GMP, and labeling control (allergens). For our proposed process, controlling the antibiotic formulation and applying a validated washing procedure before harvesting the cell biomass are specific controls to reduce the concentration of chemical hazards in the end product.

Moreover, the gene editing technique (CRISPR-Cas 9) to which satellite cells are subjected for immortalization could result in knock-out expressions of protein p16, extending the cell's replicative capacity to allow pilot production of the biomass. Previous publications have shown that similar methods could affect the levels of endogenous bioactive substances produced by cells, resulting in the expression of novel substances with potential allergenic or hazardous properties for consumers (FAO; WHO, 2023a, Ong

[3] https://monographs.iarc.who.int/wp-content/uploads/2019/07/Classifications_by_cancer_site.pdf
[4] https://monographs.iarc.who.int/list-of-classifications
[5] https://www.fda.gov/food/food-labeling-nutrition/food-allergies

et al., 2021, Soice ; Johnston, 2021). We thus considered them as chemical hazards in the hazard analysis (Table 3). Expression of novel substances could be controlled by inspecting the cultures to identify signs of culture disruption, such as altered growth and viability (FAO; WHO, 2023a). Moreover, testing control measures could be applied to verify that the editing technique resulted in no further changes to the genome (e.g., off-target effects) and to directly identify new protein expression. Besides foods derived from cell culture, gene editing techniques have been applied to plants, animals and microorganisms for agrifood use already commercialized. Thus, the same potential unintended effects may also occur and have been managed in these products (FAO, 2023).

Of the 49 inputs used to produce the cultivated meat burger, 19 contain hazardous substances in their composition and were considered critical materials (Table A4). Of these, most are inputs commonly used for biomedical research purposes and, in general, have not been used in conventional food production. Although these inputs may be hazards in the cultivated meat burger production, due to the lack of information supporting an evidence-based safety assessment, some of them were not listed in Table 3 (see an indication in Table A3.3) but commented detailed in the following text.

All the processing aids intentionally introduced in the processing are not intended to be present in the final product. Nonetheless, those identified as critical materials contain in their composition substances that could lead to adverse events (e.g., sodium azide, phenylalanine, putrescine, etc.) or directly modulate cell function (e.g., growth factors and small molecules). Considering the cultivated meat burger production process, critical materials such as antibiotics, antibodies and immortalization reagents added in low concentration and applied up to the cell banking step are unlikely to remain in the end product after multiple washes, medium fluid exchanges and increases in cell volume (see seed train details in Appendix 01).

On the other hand, residues from inputs added in the final steps, notably prior to harvesting, such as HSA, growth factors, p38 inhibitor and MEM, could reach the final product. For the proposed process, formulation control and application of a validated washing procedure before cell biomass harvesting are specific measures needed to eliminate chemical hazards in the final product. In a concrete production process potential adverse effects (allergenic, mutagenic, carcinogenic, toxic) from processing aids and their maximum residue limits must be determined. If this information is unknown, alternatives must be sought for their use in production. Regarding growth factors and other recombinant proteins, we must determine their equivalence with the native protein, its presence and acceptable levels in the conventional counterparts, and the presence of the microorganisms used to express the recombinant protein in the end product (WHO, 2003; Swartz *et al.*, 2023).

2.4.3. Physical hazards

Although the physical hazards considered were mostly those known for conventional ground meat and burger processing, we considered some particular characteristics of the cultivated meat processing and its ingredients and processing aids.

According to FAO (1998), the main physical hazards to be considered in a HACCP plan are metal, stones, glass, and plastics, as foreign materials have been responsible for the recall of meat and meat products (FSIS, 2019, 2022e). Examples of control

measures for physical hazards include source control (supplier qualification program), processing control (magnets, metal detectors, sifter screens, de-stoners), and environmental control (GMP, including employee training program and equipment maintenance program).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps

(1) Step	(2) Identify potential hazards introduced, controlled or enhanced at this step P= Physical C= Chemical B= Biological		(3) Does this potential hazard need to be addressed in the HACCP plan? Yes*	 No**	(4) Justify your decision for column 3	(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?
1. Donor animal selection	P	None				
	C	Veterinary drug	x		Residues from veterinary drugs used for herd treatment can potentially reach the final product if the withdrawal period and/or the critical limits are not properly respected. Those residues can be a health hazard or elicit an allergic reaction when handled or consumed.	Follow the withdrawal period established for each drug. Veterinary inspection (require vet drug residue analysis reports). Follow relevant good practices[1].
	B	Foodborne pathogens (*Brucella abortus*, *Mycobacterium sp.*, prion – Bovine Spongiform Encephalopathy (BSE), *Toxoplasma gondii;* Shiga-toxigenic *Escherichia coli* (STEC), *Salmonella, Cryptosporidium parvum*)	x		Foodborne pathogens can be present in tissues such as the muscle and/or blood of the donor animal and can survive and/or grow, potentially reaching the final product and causing illnesses in consumers.	Animal health report from the donor. Health inspection of the animal prior to sampling. Follow relevant good practices[1].
2. Muscle sampling	P	None				
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; Staphylococcus aureus*)	x		Food borne pathogens can be present in the environment, animal skin or hair and contaminate the tissue during sampling, potentially reaching the final product causing illnesses in consumers	Trichotomy, cleaning and antisepsis of the animal's semimembranosus muscle region prior to sampling. Health inspection of the sampled tissue for signs of infection. Use of sterile material for tissue sampling.
3. Muscle sample transport	P	None				
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; S. aureus)*	x		Failure to properly cool the material can result in foodborne pathogen growth and toxin formation, potentially reaching the final product causing illnesses in consumers	Control sample storage temperature (4°C) and time of transportation (2h). Use of sterile culture medium to maintain sample viability during transport. Add antibiotic in proper concentration. Follow relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps

(1) Step	(2) Identify potential hazards introduced, controlled or enhanced at this step P= Physical C= Chemical B= Biological		(3) Does this potential hazard need to be addressed in the HACCP plan? Yes*	 No**	(4) Justify your decision for column 3	(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?
3.A – Preparation of culture medium for sample transport	**P**	None				
	C	Penicillin-Streptomycin-(PS)	x		Failure in preparing antibiotic solutions can potentially result in concentrations above the critical limit set for these substances in the final product and become a health hazard or elicit an allergic reaction when handled or consumed	Apply a validated washing procedure to remove PS or reduce their concentration. Food handling training. Follow other relevant good practices[1].
	B	Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; S. aureus)*	x		Failure in preparing and/or sterilizing the culture medium can result in contamination, survival or growth of foodborne pathogens, potentially reaching the final product causing illnesses in the consumers	Aseptic handling of cell culture inputs. Follow other relevant good practices[1].
4. Muscle Sample Reception	**P**	None				
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; S. aureus*)		x	Failure in sample reception can lead to failure in identifying samples previously contaminated by foodborne pathogens, potentially reaching the final product causing illnesses in the consumers	External decontamination of the thermal box and the plastic bag. Checking sample temperature and preservation conditions. Visual inspection of the material (turbidity, color, viscosity. Follow relevant good practices[1].
5. Ingredients and processing aids reception and storage	**P**	Foreign materials (plastic, metal, insect fragments, stones)		x	Fragments of foreign materials can come from ingredients or processing aids and potentially reach the final product, causing health issues/injury to consumers	Adoption of a supplier qualification program. Follow other relevant good practices[1].
	C	Allergens – Pea protein Packaging material		x x	Pea protein will be present in the final product and could elicit allergic reaction when handled or consumed. Toxic substances can potentially migrate from the packaging material to the final product, causing health issues to consumers.	Food allergen labeling. Adoption of an allergen control program. Adoption of a supplier qualification program.
	B	Fetal Bovine Serum (FBS) (Príons, *L. monocytogenes, S. aureus, Bacillus cereus,* STEC, *Salmonella, B. abortus*)		x	Failures in good practices can result in foodborne pathogen growth and toxin formation, potentially reaching the final product causing illnesses in the consumers.	Control storage temperature (≤-10°C). Adoption of a supplier qualification program. Follow other relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?** **Yes***	**No****	**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
6. Media preparation/ sterilization and storage and Processing aids preparation	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials can potentially reach the final product, causing health issues/injury in the consumers.	Adoption of an equipment maintenance program. Follow other relevant good practices[1].
	C	None				
	B	Foodborne pathogens (Príons, *L. monocytogenes, S. aureus, Bacillus cereus,* STEC, *Salmonella*, *B. abortus*)	x		Failure in preparing and/or sterilizing the culture medium can result in contamination, survival or growth of foodborne pathogens, potentially reaching the final productcausing illnesses in the consumers.	Apply a validated sterilization procedure. Control media flow rate and pressure of filters during sterilization. Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
7. Cell isolation	**P**	None				
	C	None				
	B	Foodborne pathogens (Príons, *S. aureus, L. monocytogenes,* STEC, *Salmonella, B. abortus,*)		x	Failures in good practices can result in contamination and growth of foodborne pathogens, potentially reaching the final product causing illnesses in the consumers.	Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
8. Cell selection	**P**	None				
	C	Bovine Serum Albumin (BSA)		x	Residues of albumin can be carried to the final product and elicit allergic reaction when handled or consumed	Food allergen labeling. Apply a validated washing procedure to remove albumin residues or reduce their concentration. Adoption of an allergen control program.
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failures in good practices or during flow cytometry can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product.	Cell sorter cleaning. Aseptic handling of cells and cell culture inputs. Follow relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?**		**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
			Yes*	**No****		
9. Immortalization	**P**	None				
	C	Novel allergenic or hazardous substances generated by unintended effects of immortalization		x	Failures in the immortalization process can potentially lead to the expression of hazardous or allergenic substances. These substances can persist in the cell culture and reach the final product, becoming a health hazard or eliciting an allergic reaction when handled or consumed.	Regular inspection of the cultures examining cell morphology and signs of culture disruption (e.g., altered growth and viability).
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
10. Cell expansion	**P**	None				
	C	None				
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Regular visual inspection of the cultures using microscope. Cell morphology examination: signs of deterioration (e.g., granularity, detachment and vacuolation) and signs of contamination (e.g., medium turbidity, color, viscosity). Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
11. Cell banking and storage	**P**	None				
	C	None				
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failure to properly cool the material can result in foodborne pathogen growth and toxin formation, causing illnesses in the consumers of the final product	Control storage temperature (≤-80ºC). Correctly label cryovials and check for leakage. Quarantine the cells until their origin has been authenticated and are shown to be free of microorganisms. Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?**		**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
			Yes*	**No****		
12. Cell thawing	**P**	None				
	C	None				
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
13. Cell inoculum production	**P**	None				
	C	None				
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)	x		Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Regular visual inspection of the cultures using microscope. Cell morphology examination: signs of deterioration (e.g., granularity, detachment and vacuolation) and signs of contamination (e.g., medium turbidity, color, viscosity). Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].
14. Preparation of microcarriers	**P**	None				
	C	None				
	B	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella*)		x	Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Control storage temperature (4°C). Aseptic handling of microcarriers and inputs. Follow other relevant good practices[1].
15. Gas filtration	**P**	None				
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes, B. cereus*)		x	Failures in gas filtration can result in survival or growth of foodborne pathogens, causing illnesses in the consumers of the final product	Adoption of a filter maintenance program.

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?** **Yes***	**No****	**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
16. Cell inoculation and Biomass production	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials originating from equipment, cell culture plastics, packaging materials, can reach the final product, causing health issues or injuries to consumers	Adoption of an equipment maintenance program. Follow other relevant good practices[1].
	C	Human Serum Albumin (HSA) – allergen		x	Residues of albumin can be carried to the final product, becoming a health hazard or eliciting an allergic reaction when handled or consumed.	Food allergen labelling. Adoption of an allergen control program.
		Fibroblast Growth Factor-Basic (FGF), Insulin-like Growth Factor-1 (IGF-1), EGF (Epidermal Growth Factor)	x		Residues of these substances can potentially reach the final product, becoming a health hazard or eliciting an allergic reaction when handled or consumed.	Formulation control – ensure the use of the minimal levels for effective action.
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes)*	x		Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Monitor signs of contamination (e.g., pH, high consumption of alkaline solution for Ph maintenance, turbidity, color). Aseptic handling of cells and inputs. Follow other relevant good practices[1].
17. Harvesting	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Adoption of an equipment maintenance program. Follow other relevant good practices[1].
	C	Fibroblast Growth Factor-Basic (FGF), Insulin-like Growth Factor-1 (IGF-1), EGF (Epidermal Growth Factor)	x		Residues of these substances can potentially reach the final product, becoming a health hazard or eliciting an allergic reaction when handled or consumed	Apply a validated washing procedure to remove residues of chemical compounds. Adoption of a residual testing program.
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes)*	x		Failures in good practices can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Control biomass temperature and Ph prior to harvesting. Aseptic handling of cells and cell culture inputs. Follow other relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?** **Yes***	**No****	**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
18. Biomass chilling and holding	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Adoption of an equipment maintenance program. Follow other relevant good practices[1].
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes)*	x		Failures in GHP can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Follow relevant good practices[1]
19. Weighing of ingredients (burger formulation)	**P**	Foreign materials (insect fragments, stones, plastic, metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Salt sifting. Adoption of a supplier qualification program. Follow relevant good practices[1].
	C	Allergens – Pea protein cross contact		x	Residues of pea protein can be carried to the final product and could elicit allergic reaction when handled or consumed	Food allergen labeling. Adoption of an allergen control program. Follow other relevant good practices[1].
	B	None				
20. Mixing	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Adaptation of an equipment maintenance program. Follow other relevant good practices[1]
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes, S. aureus)*		x	Failures in GHP can result in contamination and growth of foodborne pathogens, causing illnesses in the consumers of the final product	Follow relevant good practices[1]
21. Forming	**P**	Foreign materials (plastic and metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Adoption of an equipment maintenance program. Follow other relevant good practices[1].
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes, S. aureus)*	x		Failures in GHP and in room temperature control can result in contamination and/or growth of foodborne pathogens and toxin production, causing illnesses in the consumers of the final product	Temperature control of the processing room (≤10°C). Follow relevant good practices[1].

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

Table 3. Hazard Analysis Worksheet – (Principle 1) Physical, Chemical and Biological Hazards related to process steps						
(1) Step	**(2) Identify potential hazards introduced, controlled or enhanced at this step** **P= Physical** **C= Chemical** **B= Biological**		**(3) Does this potential hazard need to be addressed in the HACCP plan?** **Yes***	**No****	**(4) Justify your decision for column 3**	**(5) What measure(s) can beapplied to prevent or eliminate the hazard or reduce it to an acceptable level?**
22. Freezing	**P**	Foreign material (plastic, metal)		x	Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Adoption of an equipment maintenance program. Follow relevant good practices[1].
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes*, *S. aureus*)	x		Failure to properly cool the product can result in foodborne pathogen growth and toxin formation, causing illnesses in the consumers	Temperature control of the products (≤-18ºC)
23. Packaging and labeling	**P**	Foreign material (plastic, metal)	x		Fragments of foreign materials can reach the final product, causing health issues/injury to consumers	Inspect the packaged product (x-ray, metal detector, magnetics)
	C	Allergens		x	Failure to declare the presence of allergens on the label of the final product can cause an allergic reaction in consumers	Food allergen labeling
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes*, *S. aureus*)	x		Failures in package integrity and GHP can result in contamination and growth of foodborne pathogens and toxin production, causing illnesses in the consumers of the final product	Temperature control of the processing room (≤10ºC). Visual inspection of package integrity. Food labeling (Consumer instructions – preparation/use instructions, storage conditions)
24. Storage and distribution	**P**	None				
	C	None				
	B	Foodborne pathogens (STEC, *Salmonella*, *L. monocytogenes*, *S. aureus*)		x	Failures in GHP and storage temperature can result in contamination and/or growth of foodborne pathogens and toxin production, causing illnesses in the consumers of the final product	Temperature control (≤-18ºC) of the product. Follow relevant good practices[1].

To answer the question at column 3 as:

*** Yes**: The study team determined that if the potential hazard is not adequately controlled, consumption is likely to result in an unacceptable health risk. Hence, the potential hazard was addressed in the HACCP plan.
****No:** The study team determined that the potential hazard risk is low and good practices can adequately control it. Hence, the potential hazard was not addressed in the HACCP plan.

[1] Good practices may include good agricultural practices (GAP); good manufacturing practices (GMPs); good hygiene practices (GHPs); and good cell culture practice (GCCP).

2.5 HACCP Worksheet

Table 4. HACCP Worksheet (Principles 2 to 7)

Critical Control Points	Significant Hazards		Critical limits	Monitoring				Corrective actions	Verification activities	Records
				What	How	When (frequency)	Who			
CCP 1B **Step 3. Muscle sample transport**	**B**	Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; S. aureus)*	Stored at ≤4°C for up to 2h	Transport time and temperature	Data collection using data loggers	Continuous (real time)	Designee	Determine and eliminate the cause of deviation. Muscle sample segregation for evaluation. Retrain employee.	Instrument calibration (data loggers). Record review. Intermediate checks of sensors and measurement devices Microbiological analysis of the muscle sample.	Data loggers' calibration certificate. Time and temperature profile sheet(s). Microbiological analysis report(s).
CCP 1C **Step 3A Preparation of culture medium for sample transport**	**C**	Penicillin-Streptomycin (PS) (overdose inappropriate concentration of antibiotic	Concentration in the final product: Penicillin < 50* µg/kg. Streptomycin < 600 µg/kg.	Weighing of antibiotics and volume added to the culture medium	Observe the employee preparing the antibiotic solution. Record in the proper form.	At each medium batch preparation	Designee	Determine and eliminate the cause of deviation. Segregate for evaluation. Retrain the employee.	Record review. Observation of the weighing procedure. Analysis of the final product to verify the antibiotic concentrations	Weighing/ volume sheet. Employee training certificate. Reports of antibiotic analysis in the final product.
CCP 2B **Step 3A Preparation of culture medium for sample transport**	**B**	Growth of Foodborne pathogens (STEC, *Salmonella, L. monocytogenes; S. aureus)*	Concentration in the culture medium (per liter): Penicillin. 100U/Ml. Streptomycin 100µg/Ml.	Weighing of antibiotics and volume added to the culture medium	Observe the employee measure the components and mix the solution. Record in the proper form	At each medium batch preparation	Designee	Determine and eliminate the cause of deviation. Segregate for evaluation. Retrain the employee.	Record review. Observation of the preparation procedure. Intermediate checks of sensors and measurement devices. Microbiological analysis of the culture medium.	Pipette calibration certificate. Weighing/ volume sheet. Employee training certificate. Culture medium microbiological analysis reports.
CCP 3B **Step 6. Media preparation/ sterilization and storage and Processing aids preparation**	**B**	Foodborne pathogens (*S. aureus, B. cereus*, STEC, *Salmonella, B. abortus, L. monocytogenes*)	Filter integrity Flow rate and pressure limits established after validation by FBO	Filter integrity Flow rate and pressure of the filter system	Visual inspection of filteremometer/ Manometer	At each batch preparation Continuous (real time)	Designee	Determine and eliminate the cause of deviation. Segregate for evaluation. Retrain the employee	Microbiological analysis of the culture medium and processing aids. Intermediate checks of flow rate and pressure sensors and measurement devices. Supervision of filter integrity systems. Instrument calibration (flow rate and pressure measurement devices).	Culture medium and processing aids microbiological analysis reports. Flow rate and pressure measurement devices calibration certificate. Filter integrity systems sheet. Employee training certificate.

Table 4. HACCP Worksheet (Principles 2 to 7)

Critical Control Points	Significant Hazards		Critical limits	Monitoring				Corrective actions	Verification activities	Records
				What	How	When (frequency)	Who			
CCP 4B **Step 13.** **Cell inoculum production**	**B**	Foodborne pathogens (*S. aureus, L. monocytogenes,* STEC, *Salmonella)*	Absence of microorganisms No visual changes (turbidity, color, viscosity)	Microbial growth Visual aspect of the cell culture	Rapid sterility test Visual inspection	Each batch	Designee	Determine and eliminate the cause of deviation Segregate for evaluation Retrain the employee	Microbiological analysis/ sterility test of the cell culture. Record review. Supervision of inspection/ sterility test. Intermediate checks of equipment	Microbiological analysis/ sterility test reports. Employee training certificate. Equipment calibration/ maintenance certificate. Inspection/ sterility test sheet.
CCP 2C **Step 17.** **Harvesting**	**C**	Fibroblast Growth Factor-Basic (FGF), Insuline-like Growth Factor 1(IGF-1), Epidermal growth factor (EGF)	Minimum washing cycles to ensure absence of the chemical hazard	Number of washing cycles and conditions	Record in the proper form	Each batch	Designee	Determine and eliminate the cause of deviation. Segregate the product for evaluation. Retrain the employee.	Intermediate checks of washing procedure. Residual testing program (e.g., ELISA test). Recording review.	Washing procedures form. Employee training certificate. Residual testing program reports/ intermediate checks.
CCP 1P **Step 23.** **Packaging and labeling**	**P**	Foreign material (plastic, metal)	≥ 2mm**	Foreign material	Inspection of the packaged product (e.g., x-ray, visual inspection, metal detector, magnet)	Continuous (real time)	Designee	Determine and eliminate the cause of the deviation. Segregate the product for evaluation. Retrain the employee.	Equipment calibration. Record review. Supervision of the inspection. Intermediate checks of inspection (e.g., use of specimens).	Equipment calibration certificate. Inspection sheet. Employee training certificate

*Critical limit suggested based on FAO & WHO (2023b).
**Critical limit suggested based on ANVISA – RDC 623/2022 (Brasil, 2022).

CHAPTER 4

Conclusions and research priorities

Cultivated meat steak-tartare: Mosa Meat

Alternative proteins such as cultivated meat might offer solutions for food security challenges by providing more sustainable products and methods of production, but to effectively deliver cultivated meat to consumers, we must ensure its safety for human consumption. Although food safety authorities worldwide have been confronted with many innovative solutions to cultivated meat production, only two regulatory frameworks (Singapore in late 2020, and the USA in mid-2023) formally processed complete applications for these products. The present study conducted a scientifically-based safety assessment of a hypothetical cultivated meat product to serve as an initial guide for developers working on their products and food safety studies, and contribute to regulators in building and providing clear food safety guidelines for cultivated meat across different regions.

As demonstrated in the present report, HACCP system identified hazards in the cultivated meat burger manufacturing process supporting their safe production. The hazard analysis has indicated most of the hazards identified in the cultivated meat burger are already known and familiar to conventional FBOs. Many potential hazards can occur in producing conventional counterparts of the cultivated meat burger (e.g., foodborne pathogens and veterinary drugs) and other conventional food products (e.g., pea protein). Other potential hazards are unfamiliar to traditional meat processing but may occur in biotechnology-derived foods, such as fermented food and ingredients and foods produced by conventional breeding or genetic engineering (e.g., novel allergenic or hazardous substances). In agreement with previous publications (FAO, 2023; Ong *et al*., 2021), most of the identified hazards can be controlled by existing control measures and relevant good practices.

Due to the nature of the process and the lack of a well-established supply chain, many of the cell culture materials were not previously optimized for food application. Using non-food-grade ingredients and additives is a challenge for both industries and regulators. Establishing appropriate inputs for cultivated meat application is paramount for meeting the lower costs and environmental impacts needs of the final product. However, we highlighted this requirement from the food safety perspective. Besides costs and environmental impact, careful selection of well-characterized and safe inputs, as a safety-by-design strategy, from the early stages of product development contributes to ensure product and process safety while avoiding rework by developers after significant investments in terms of time and resources.

Despite limitations arising from using a modeled flow diagram without in-loco validation, we were able to accurately detail all the manufacturing steps necessary to produce a cultivated meat product and allow the safety assessment to map potential hazards. Conventional meat processing is well-known and relatively well-understood by the general public. Conversely, cultivated meat production is a new and disruptive food operation about which relevant information must be provided to consumers. Thus, the flow diagram developed provides a detailed view of how a cell culture-based production process could work from sampling to final processing, providing transparency to consumers and contributing to an informed decision-making.

In short, this study serves as a starting guide for developers interested in establishing a safety assessment for their products and regulators interested in designing legislation that stimulates innovation and enables the safe development of

cultivated foodstuffs. Discussion sessions held by the team allowed to identify some knowledge gaps. The research priorities described below may serve to support hazard control, control measures, corrective actions, monitoring, and verification procedures, and be essential to implement the HACCP plan and, consequently, to ensure the safety of cultivated meat products:

- Given specific storage conditions, characterize what foodborne pathogens can grow or survive in the final product during storage and distribution;
- Assess the behavior of spoilage microorganisms in the product;
- Understand the role of microbiota in the ingredients used in food processing (e.g., pea protein, coconut fat, beetroot colorant, etc.) and in cultivated meat shelf-life and safety;
- Verify potential cross-contamination during packaging and the growth potential of foodborne pathogens throughout the product's shelf-life;
- Determine the shelf-life of cultivated meat products considering formulation, processing, storage, distribution, and commercialization conditions;
- Identify the limiting factor of shelf-life for cultivated meat products;
- Establish strategies or validate procedures needed to reduce or remove chemical residues from the final product;
- Assess whether cell culture inputs not commonly used in conventional food production bear allergenic/mutagenic/carcinogenic/toxicity potential and identify its acceptable levels in the end product.
- Assess whether *Staphylococcus aureus* toxin can be carried to the final product. Despite the use of sterile microcarriers during its preparation, contamination by this toxin can occur due to inappropriate human manipulation.
- Investigate differences and similarities in transforming muscle into meat between cultivated meat and conventional meat and possible implications to safety and quality.

Finally, we encourage all stakeholders in the cultivated meat field to fill the research gaps raised here using an evidence-based approach and proactive work to advance the technology as a whole.

Cultivated chicken: GOOD Meat

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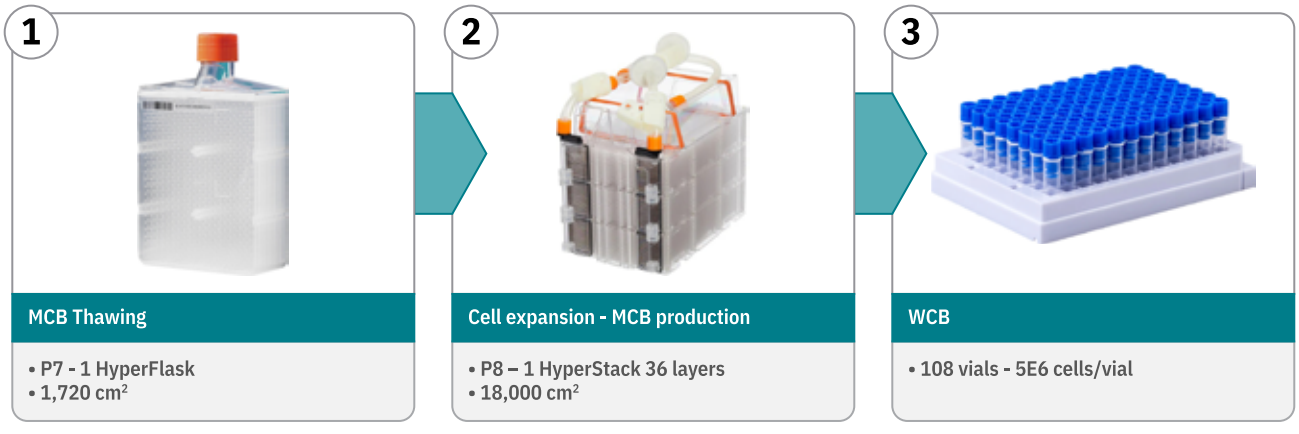
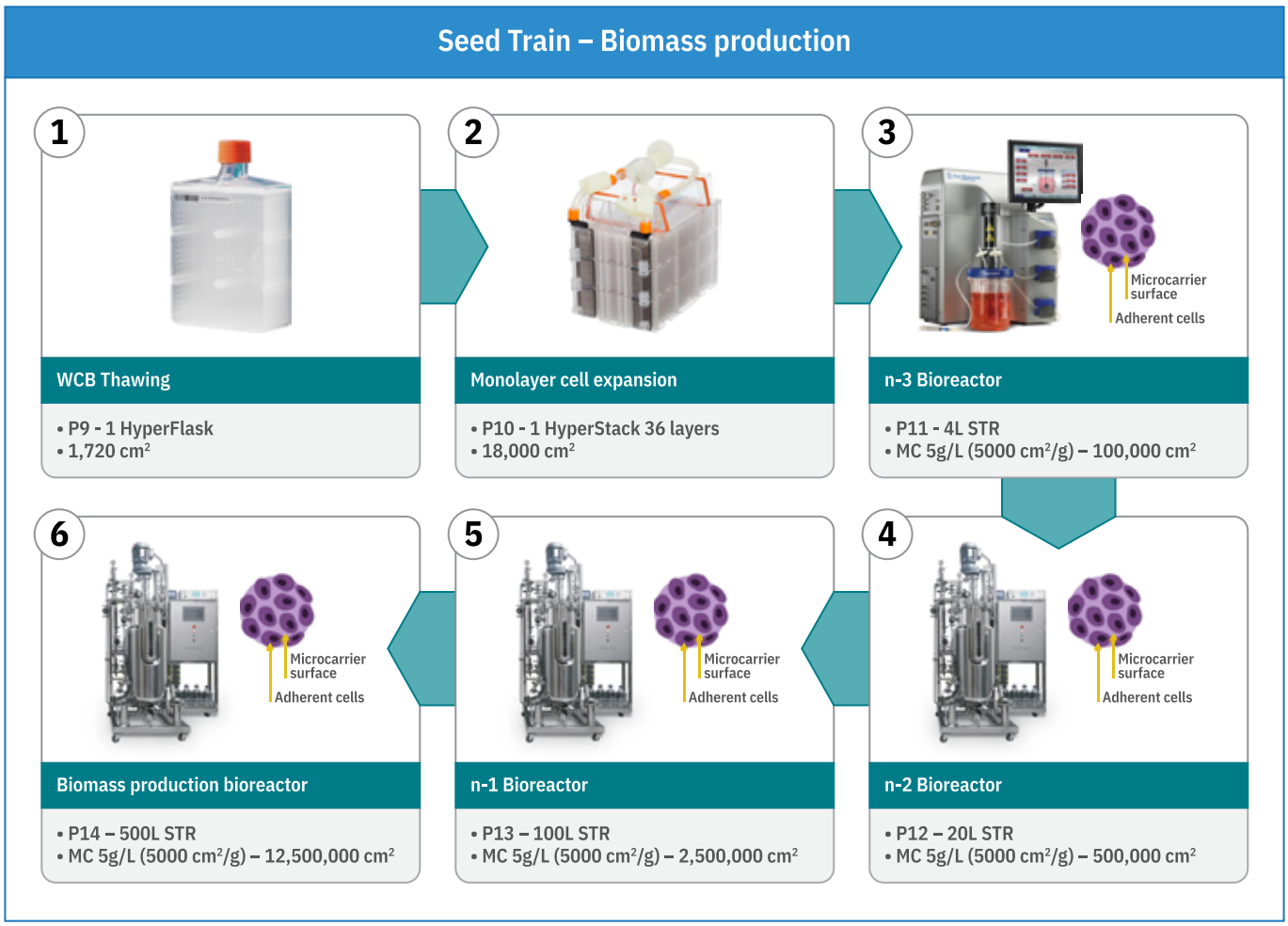
Appendix 01 - Seed train expansion design

Initial Cell Expansion – MCB production

WCB production



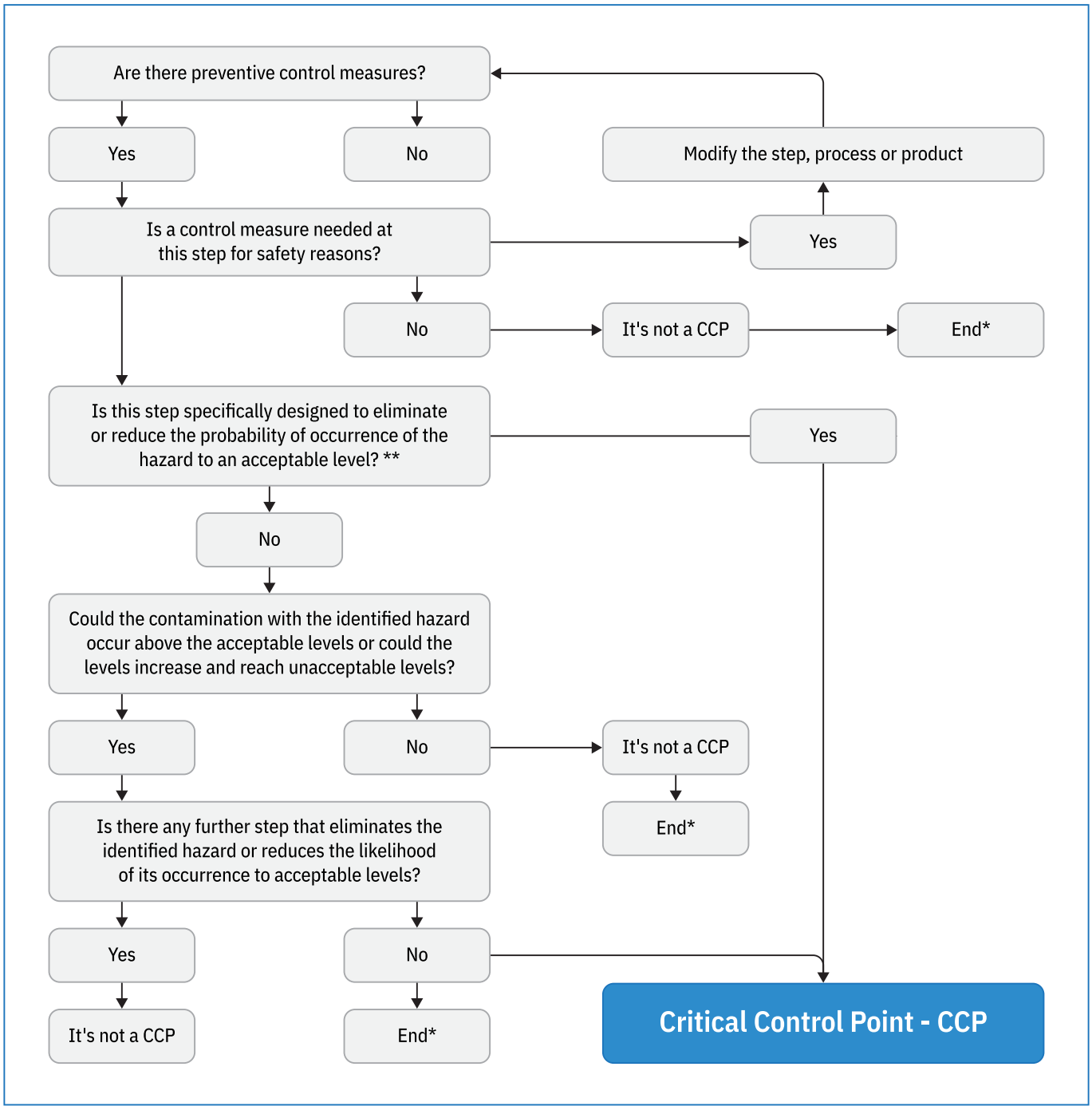


After immortalization, cells need to be expanded into monolayer cultures to produce the master (MCB) and working cell banks (WCB). In this process, conventional culture systems like well plates and T-flasks can be employed in the first passages. When a larger surface area is required, culture systems such as HyperFlasks and HyperStacks are preferable due to smaller footprint, when compared to conventional monolayer systems, and possible operation under closed conditions, being therefore more GMP-compliant. Additional monolayer passages should be employed to obtain MCB/WCB with a higher number of vials/cell density.

Batch production begins with cell thawing from WCB. After monolayer cell expansion in HyperFlasks and HyperStacks (step 10), the cells are inoculated in increasing volume bioreactors until reaching the final bioreactor for biomass production and differentiation. The flow diagram developed considered a 5 times scale-up in the bioreactor seed train, as is usual for microcarriers-based processes. Growth area was estimated by considering a microcarrier concentration of 5g/L (5,000 cm²/g), as in Vebbrugen et al., 2018.

Operating multiple production bioreactors in parallel requires multiple thawing vials and seed train expansion processes. A process with a higher volume biomass production bioreactor (1,000-2,000L) might require an additional seed train step in bioreactor.

Appendix 02 - Decision tree to identify CCPs



Reference: CAC, 2023

Appendix 03 - Ingredients, culture media and processing aids composition

Table A3.1. Burger Patty - Ingredients composition	
Ingredient	**Composition**
Ultrapure Water	Potable water
Deionized water	Potable water
Ice	Ice from potable water
Cell biomass	Cultivated bovine muscle cells Polygalacturonic Acid Sodium Salt (PGA) microcarrier
Pea protein	Pea extract
Coconut fat	Crude Coconut Oil
L-ascorbic acid 2-phosphate	Vitamin C, Antiscorbutic factor L-Threo Ascorbic acid: C6H8O6
Transglutaminase	Glutaminase from *E. coli*
Methylcellulose	Sodium carboxymethyl cellulose
Beetroot Red colorant	Beet plant
Salt	NaCl, Iodine

Table A3.2. Culture media - Formulation	
Culture medium	**Composition**
Culture medium 1 (Cell selection medium)	Dulbecco's Modified Eagle Medium-F12 Penicillin-Streptomycin Chemically-defined FBS replacement Water Phosphoric acid and sodium hydroxide (pH adjustment)
Culture medium 2 (Proliferation medium)	Dulbecco's Modified Eagle Medium-F12 FGF (Fibroblast Growth Factor-Basic) p38 MAP Kinase Inhibitor SB203580 Water Phosphoric acid and sodium hydroxide (pH adjustment)
Culture medium 3 (Differentiation medium)	Dulbecc''s Modified Eagle Medium-F12 EGF IGF-1 Human Serum Albumin L-ascorbic acid 2-phosphate (Vitamin C) MEM amino acids solution $NaHCO_3$ Water Phosphoric acid and sodium hydroxide (pH adjustment)

Table A3.3. Processing Aids - Classification and Composition		
Processing aids	**Classification**	**Composition**
Ethanol	Alcohol	70% ethyl alcohol
Penicillin-Streptomycin (PS)	Antibiotic	Stock solution contains 10,000 units/mL of penicillin 10,000µg/mL of streptomycin in a 10 mM citrate buffer (for pH stability)
Phosphate-buffered saline (PBS)	Buffer solution	1X working concentration contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4
Collagenase	Enzyme	Protease from *Clostridium histolyticum*
Fetal Bovine Serum (FBS)	Serum	Proteins, attachment factors, growth factors, amino acids, trace elements, vitamins, lipids, and hormones.
Ammonium-Chloride-Potassium Lysing (Buffer-ACK)	Buffer solution	Ammonium Chloride; Potassium Bicarbonate; ethylenediaminetetraacetic acid (EDTA)
Bovine Serum Albumin (BSA)	Protein	Cohn Fraction V
Fibroblast Growth Factor-Basic (FGF)	Growth factor	Recombinant protein; 20mM potassium phosphate with 750mM NaCl
Insulin-like Growth Factor-1 (IGF-1)	Growth factor	Recombinant protein; 20mM potassium phosphate with 750mM NaCl
Epidermal growth factor(EGF)	Growth factor	Recombinant protein; 20mM potassium phosphate with 750mM NaCl
*NCAM1-PE-Cy7**	Monoclonal antibody	Recombinant protein, sodium azide, BSA, fluorochrome R-phycoerythrin (PE) coupled to the cyanine dye (Cy7)
*CD29-APC**	Monoclonal antibody	Recombinant protein, sodium azide, BSA, Allophycocyanin (APC)
CD31-FITC*	Monoclonal antibody	Recombinant protein, sodium azide, BSA, Fluorescein isothiocyanate (FITC)
*CD45-FITC**	Monoclonal antibody	Recombinant protein, sodium azide, BSA, Fluorescein isothiocyanate (FITC)
Collagen type 1	Protein	Bovine collagen
Accutase	Enzyme	Invertebrate (crab)-derived enzyme; EDTA (ethylenediaminetetraacetic acid); phenol red.
*Serum-Free Cell Freezing Medium**	Freezing medium	10% DMSO and methylcellulose
Agar	Hydrocolloid	Gum agar, Agar-agar
Calcium chloride	Chemical compost	$CaCl_2$
*Chemically-defined FBS replacement**	Chemical compost	Proteins, attachment factors, growth factors, amino acids, trace elements, vitamins, lipids, and hormones
p38 MAP Kinase Inhibitor SB203580*	ATP-competitive inhibitor	4-(4'-Fluorophenyl)-2-(4'-methylsulfinylphenyl)-5- (4'-pyridyl)-imidazole
Human Serum Albumin (HSA)	Protein	Recombinant protein
*MEM amino acids solution**	Culture media supplement	**Amino Acids:** L-Arginine hydrochloride, L-Cystine, L-Histidine hydrochloride-H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine.
Phosphoric acid	Chemical compost	H_3PO_4
Sodium bicarbonate	Chemical compost	$NaHCO_3$
Gelatin	Gelling agent	Gelatin from bovine skin
Silicone spray	Lubricating spray	Dimethyldichlorosilane
*Fugene HD**	Transfection reagent	Mixture of lipids; 80% ethanol
*Cas9 protein**	Enzyme	Recombinant protein; glycerol

Table A3.3. Processing Aids - Classification and Composition		
Processing aids	**Classification**	**Composition**
*gRNAs**	RNA	Synthetic ribonucleic acid
Synthetic air	Gases	Nitrogen (80%) and Oxygen (20%)
*Dulbecco's Modified Eagle Medium High glucose (DMEM)**	Basal medium	**Amino Acids:** Glycine, L-Arginine hydrochloride, L-Cystine 2HCl, L-Glutamine, L-Histidine hydrochloride-H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Phenylalanine, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine. **Vitamins:** Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, I-Inositol. **Inorganic salts:** Calcium Chloride ($CaCl_2$) (anhyd.), Ferric Nitrate (Fe $(NO_3)_3$"$9H_2O$), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Sodium Chloride (NaCl), Sodium Phosphate monobasic (NaH_2PO_4-H_2O). **Other Components:** D-Glucose (Dextrose)
*Dulbecco's Modified Eagle Medium-F12**	Basal medium	**Amino Acids:** Glycine, L-Alanine, L-Arginine hydrochloride, L-Asparagine-H_2O, L-Aspartic acid, L-Cysteine hydrochloride-H_2O, L-Cystine 2HCl, L-Glutamic Acid, L-Glutamine, L-Histidine hydrochloride-H_2O, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, *L-Phenylalanine**, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine disodium salt dihydrate, L-Valine. **Vitamins:** Biotin, Choline chloride, D-Calcium pantothenate, Folic Acid, Niacinamide, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, Vitamin B12, I-Inositol. **Inorganic Salts:** Calcium Chloride ($CaCl_2$) (anhyd.), Cupric sulfate ($CuSO_4$-$5H_2O$), Ferric Nitrate ($Fe(NO_3)_3$"$9H_2O$), Ferric sulfate ($FeSO_4$-$7H_2O$), Magnesium Chloride (anhydrous), Magnesium Sulfate ($MgSO_4$) (anhyd.), Potassium Chloride (KCl), Sodium Bicarbonate ($NaHCO_3$), Sodium Chloride (NaCl), Sodium Phosphate dibasic (Na2HPO4) anhydrous, Sodium Phosphate monobasic (NaH_2PO_4-H_2O), Zinc sulfate ($ZnSO_4$-$7H_2O$) **Other Components:** D-Glucose (Dextrose), Hypoxanthine Na, Linoleic Acid, Lipoic Acid, *Putrescine 2HCl**, Sodium Pyruvate, Thymidine.

* Inputs not addressed in Table 3. See comments in section 2.4.2 and table A.4 in Appendix 04.

Appendix 04 - Identification of critical material

Table A4. Ingredient or Processing Aids and Packaging Critical Material*				
Raw material ingredient or processing aids and packaging material	**Hazard identified** **P=**Physical **C=**Chemical **B=**Biological	**Question 1** Could the identified hazard occur at levels greater than acceptable or could it increase to undesirable levels? If no. The raw material/ ingredient is not critical. If yes, answer question 02.	**Question 2** Will the process or consumer eliminate or reduce the hazard to an acceptable level? If no. The raw material/ingredient must be considered critical, that is, the process does not guarantee the safety of the product. Modify the product or process. If yes. It's not critical. Repeat question 1 for another raw material/ingredient.	**Critical or Non-critical****
Ultrapure water	None			**Non-critical**
Deionized water	None			**Non-critical**
Ice	None			**Non-critical**
Cell biomass (Bovine muscle cells)	None			**Non-critical**
PGA Microcarriers	None			**Non-critical**
Textured pea protein	**C -** Allergen	Yes	No	**Critical**
Coconut fat	None			**Non-critical**
L-ascorbic acid 2-phosphate	None			**Non-critical**
Transglutaminase	None			**Non-critical**
Methylcellulose	None			**Non-critical**
Beetroots colorant	None			**Non-critical**
Salt	None			**Non-critical**
Ethanol	None			**Non-critical**
Penicillin-Streptomycin (PS)	**C -** Antibiotic residue	Yes	No	**Critical**
Phosphate buffered saline (PBS)	None			**Non-critical**
Collagenase	None			**Non-critical**
Dulbecco's Modified Eagle Medium High glucose (DMEM)	**C** - Presence of L-Phenylalanine and Putrescine 2HCl in the composition	Yes	No	**Critical**

*Source: Mortimore and Wallace (2001).
** For all cases in which a hazardous component was identified in the processing aid, Question 2 was answered as 'No' due to the impossibility of experimentally evaluate the absence of residues in the final product.

Table A4. Ingredient or Processing Aids and Packaging Critical Material*				
Raw material ingredient or processing aids and packaging material	**Hazard identified** **P=**Physical **C=**Chemical **B=**Biological	**Question 1** Could the identified hazard occur at levels greater than acceptable or could it increase to undesirable levels? If no. The raw material/ ingredient is not critical. If yes, answer question 02.	**Question 2** Will the process or consumer eliminate or reduce the hazard to an acceptable level? If no. The raw material/ingredient must be considered critical, that is, the process does not guarantee the safety of the product. Modify the product or process. If yes. It's not critical. Repeat question 1 for another raw material/ingredient.	**Critical or Non-critical****
Fetal Bovine Serum (FBS)	**B -** Foodborne pathogens (Prions, *S. aureus, L. monocytogenes,* STEC, *Salmonella, B. abortus,*)	Yes	No	**Critical**
Ammonium-Chloride-Potassium Lysing (Buffer-ACK)	None			**Non-critical**
Bovine Serum Albumin (BSA)	**C -** Allergen	Yes	No	**Critical**
Fibroblast Growth Factor-Basic (FGF)	**C -** Hormones	Yes	No	**Critical**
Insulin-like Growth Factor-1 (IGF-1)	**C -** Hormones	Yes	No	**Critical**
Epidermal growth factor (EGF)	**C -** Hormones	Yes	No	**Critical**
NCAM1-PE-Cy7 (Conjugated monoclonal Antibodies)	**C -** Presence of sodium azide (NaN_3) in the composition	Yes	No	**Critical**
CD29-APC (Conjugated monoclonal Antibodies)	**C -** Presence of sodium azide (NaN_3) in the composition	Yes	No	**Critical**
CD31-FITC (Conjugated monoclonal Antibodies)	**C -** Presence of sodium azide (NaN_3) and Fluorescein Isothiocyanate Isomer 1 (FITC)	Yes	No	**Critical**
CD45-FITC (Conjugated monoclonal Antibodies)	**C -** Presence of sodium azide (NaN_3) and Fluorescein Isothiocyanate Isomer 1 (FITC)	Yes	No	**Critical**
Collagen type 1	None			**Non-critical**
Accutase	**C -** Presence of Phenol red in the composition	Yes	No	**Critical**
Serum-Free Cell Freezing Medium	**C -** Presence of DMSO in the composition	Yes	No	**Critical**
Ágar	None			**Non-critical**

*Source: Mortimore and Wallace (2001).
** For all cases in which a hazardous component was identified in the processing aid, Question 2 was answered as 'No' due to the impossibility of experimentally evaluate the absence of residues in the final product.

Table A4. Ingredient or Processing Aids and Packaging Critical Material*				
Raw material ingredient or processing aids and packaging material	**Hazard identified** **P=**Physical **C=**Chemical **B=**Biological	**Question 1** Could the identified hazard occur at levels greater than acceptable or could it increase to undesirable levels? If no. The raw material/ ingredient is not critical. If yes, answer question 02.	**Question 2** Will the process or consumer eliminate or reduce the hazard to an acceptable level? If no. The raw material/ingredient must be considered critical, that is, the process does not guarantee the safety of the product. Modify the product or process. If yes. It's not critical. Repeat question 1 for another raw material/ingredient.	**Critical or Non-critical****
Calcium chloride	None			**Non-critical**
Dulbecco's Modified Eagle Medium-F12	**C -** Presence of L-Phenylalanine and putrescine 2HCl in the composition	Yes	No	**Critical**
Chemically-defined FBS replacement	**C -** Hormones and growth factors	Yes	No	**Critical**
p38 MAP Kinase Inhibitor SB203580	**C -** Presence of imidazole in the composition	Yes	No	**Critical**
Human Serum Albumin (HSA)	**C -** Allergen	Yes	No	**Critical**
MEM amino acids solution	**C -** Presence of L-Phenylalanine in the composition	Yes	No	**Critical**
Phosphoric acid	None			**Non-critical**
Sodium bicarbonate	None			**Non-critical**
Gelatin	None			**Non-critical**
Silicone spray	None			**Non-critical**
Fugene HD	None			**Non-critical**
Cas9	None			**Non-critical**
gRNAs	None			**Non-critical**
Synthetic air	None			**Non-critical**
Paraffin paper	None			**Non-critical**
Plastic Bags single use	None			**Non-critical**
Plastic drum (LLDPE - Linear Low Density Polyethylene)	None			**Non-critical**
Grow bottle: Polystyrene	None			**Non-critical**

*Source: Mortimore and Wallace (2001).
** For all cases in which a hazardous component was identified in the processing aid, Question 2 was answered as 'No' due to the impossibility of experimentally evaluate the absence of residues in the final product.

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