DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT

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Contributing Members
Good Meat, Inc.
Polsinelli PC
AR Toxicology
Burdock Group Consultants

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1. FIRM AND CONTACT PERSON

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.
2000 Folsom Street
San Francisco, CA 94110
Tel: 858-349-8338
Fax: 415-682-6990

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.

Date

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2. EXECUTIVE SUMMARY

GOOD Meat, Inc. ("Good Meat") is a subsidiary of Eat Just, Inc. ("Eat Just"). Good Meat has developed cultured chicken cells intended to be used as a human food ingredient when mixed with other safe and suitable food ingredients. The final chicken product resembles a conventional chicken product (*e.g.*, chicken bites and boneless chicken breasts). This dossier describes the safety of the cell line, cell culturing conditions including media constituents, manufacturing processes utilized by Good Meat, and the overall safety of the bulk chicken cell ingredient, termed "cultured chicken". JOINN Biologics US Inc (CA, USA) currently produces the chicken cells in a dedicated manufacturing suite.

Good Meat has characterized the cell banking process utilized in the production of cultured chicken by detailing the cell line origin, properties of the cell line, methods and characterization tools used to create the Master Cell Banks (MCB) and Master Working Cell Banks (MWCB). In addition, criteria for cell bank release are discussed. These criteria indicate that cells derived from cell banks have a standard cell viability and proliferation rate, are sterile (not contaminated by viral or bacterial agents) and are pure (match a set identity according to gene expression and PCR sequencing).

The manufacturing process includes expanding cells in flasks followed by cell proliferation in bioreactors, washing and harvesting of cultured chicken cells. These cultured chicken cells are then packaged in food grade packaging and stored at <-20 °C intended for further processing as a chicken ingredient.

All raw material specifications are detailed in this dossier, particularly cell culture media constituents and details of their safe use. Also included is a discussion of the regulatory status of media constituents and potential consumer exposure from cultured chicken consumption. Analytical testing of media constituents identified by the FDA during premarket consultation demonstrates no carryover of unsafe concentrations of media constituents in cultured chicken on a *per* serving basis.

Cells are harvested, washed, and analyzed to meet Good Meat specifications following the final growth cycle. In addition, the final wash is analyzed to ensure minimal carryover of media components in the cultured chicken. Analyses of representative batches demonstrate that cultured chicken consistently meets specifications and is stable for up to 6 months following production.

Good Meat's food safety plan is described in detail, illustrating a series of robust Hazard Analysis and Risk-Based Preventive Controls (HARBPC) utilized throughout the production of cultured chicken.

The intended use and nutritional profile of cultured chicken are detailed. This profile is similar to that of conventional chicken.

The data and information provided in this dossier demonstrate the overall safety of the cell line, cell culturing conditions and raw material inputs, manufacturing process, and final cultured chicken ingredient. Following the review process with FDA, Good Meat intends to seek authorization from the Food Safety Inspection Service of the U.S. Department of Agriculture ("FSIS") to produce cultured chicken meat products from cultured chicken cells. Good Meat will assist FSIS in ensuring that cultured chicken meat products are safe, can bear the USDA mark of inspection, and are properly labeled.

3. INTRODUCTION

Good Meat has developed cultured chicken cells intended to be utilized as a human food ingredient to be mixed with other food ingredients. The produced chicken product resembles a conventional chicken product (*e.g.*, chicken bites and boneless chicken breasts). This dossier describes the safety of the cell line, cell culturing conditions, including media constituents, manufacturing processes utilized, and the overall safety of cultured chicken.

This dossier provides an overview of Good Meat's cultured chicken cell program. It discusses:

- Cell banking methods, including cell source, culture, characterization, and banking conditions.
- The production process - including material inputs, growth conditions, processing methods, and characterization and specifications of the final cultured chicken cells.
- The safety of the product and media components utilizing relevant data from literature, analytical testing, and pre-existing regulation. Discussion of the safety-in-use of raw material inputs is provided alongside relevant labeling information.
- The food safety plan, including GMP and HARBPC, implemented in the production of the cultured chicken is disclosed.

4. CELL BANK

4.1. Cell Origin

Cells utilized in the production of the master cell bank originate from the commercially available chicken cell line UMNSAH/DF1[1], a chicken fibroblast cell line that was deposited at American Type Culture Collection (ATCC, Manassas, Virginia, USA) (Appendix 1), on October 11, 1996, under the terms and conditions of the Budapest Treaty, having the reference number ATCC® CRL12203™.

The cell line was certified by the supplier as negative for Avian Influenza (Type A), Avian Reovirus, Avian Adenoviruses (Groups I-III), Avian Encephalomyelitis Virus, Fowl Pox, Newcastle Disease Virus, Paramyxovirus (type 2), *Mycoplasma*, *Salmonella*, and other infectious agents known to infect poultry stock.

The following is a brief description of the generation of the cell line: "Chicken embryonic primary cells were obtained by removing the embryonic torso of 10-day old embryos, followed by mincing of the tissue and placement of the cells in culture. Clusters of morphologically uniform cells were then selected to generate the immortalized chicken cells. These cells have undergone greater than 400 population doublings and greater than 160 passages. No chromosomal aberrations were found after cytogenetic examination of 100 cells."

The UMNSAH/DF1 cell line was ordered from ATCC and received by Good Meat on February 22, 2018, with the batch number designated as 70006277. The cell line was internally labeled as "C1F" and briefly expanded in adherent conditions for two passages for the generation of parental C1F cell banks.

[1] www.atcc.org/Products/All/CRL-12203.aspx

The product sheet for the cell line includes the following standard disclaimer "This product is intended for laboratory research use only. It is not intended for any animal or human therapeutic use, any human or animal consumption, or any diagnostic use. Any proposed commercial use is prohibited without a license from ATCC". Despite this statement and the original application of this UMNSAH/DF1 cell line for research purposes, the safety and quality characterization performed on the C1F cells cultured by Good Meat and described in follow-up sections confirmed that these cells, when adapted and cultured using the methods proposed by Good Meat and when used as an ingredient for cultured chicken meat products, are safe for human consumption. Moreover, appropriate process controls and an adequate food safety plan to control and monitor any potential hazard introduced during the manufacturing process were implemented. Hence, the ATCC disclaimer is not relevant in this context.

4.2. Properties of C1F Cells

The main characteristics of immortalized chicken cell lines, relative to their parental isolates, are higher expression of genes associated with cell cycle progression and proliferation, downregulation of cell death pathways, and accelerated capacity for molecular transport (Kong *et al.*, 2011).

C1F cells are not recombinant or engineered (*i.e.*, non-GMO) and have not been exposed to any viruses or viral DNA. The culture protocol involves growing cells in low-serum cell culture media using nutrients native to the human body and harvesting them for use when they have grown to an appropriate cell density. Antibiotics are not used in the culture of C1F cells. Further, C1F cells were assayed for adventitious agents as part of the cell bank release testing (further details in section 4.6). The purpose of this testing is to address common public health hazards that have the potential to propagate in cell culture and cultured meat. Specifically, Master Cell Banks (MCBs) and Master Working Cell Banks (MWCBs) used for production were tested for a panel of human and avian viruses and bacteria. This analysis confirms that during the initial culturing process of C1F cells, human pathogenic viruses and bacteria did not contaminate the cell culture.

Critical to the characterization of an immortalized cell line is its tumorigenic potential. The developers of the parental cell line conducted two types of tests to determine if the cells have tumorigenic potential. The developers noted that "immortalized cells are differentiated from transformed cells in that unlike transformed cells, immortalized cells are density dependent and/or growth arrested (*e.g.*, contact inhibited). Transformed cells are capable of growth in soft agar and are usually able to form tumors when injected into laboratory animals." Accordingly, the first of these tests examined the ability of the cells to grow and form colonies in soft agar. The ability of cells to grow on soft agar in this way is commonly seen in tumor cells (Borowicz *et al.*, 2014). The cell line in question could not grow in suspension in soft agar, indicating that it likely did not have tumorigenic potential. To confirm the lack of tumorigenicity, the cell line developers tested the tumorigenic potential of the cells by injecting six adult chickens with four million of the cells. A positive result for tumorigenicity would be formation of tumors in these injected chickens; however, no tumors developed in any of the chickens, and all remained healthy. These data indicate that the starting cells used for cultured chicken do not have tumorigenic potential.

4.3. Cell Culture of C1F Cells

C1F was selected as the cell source for Good Meat cultured chicken due to its avian nature, its spontaneously immortalized phenotype, and its stability in cell culture.

Details of the initial cell culture of C1F cells to form Master Cell Banks are provided in Appendix 10.3.3. In short, culture media supplemented with bovine serum is utilized to expand the parental cells and adapt them to suspension culture conditions. Further, the cells are weaned to grow in lower levels of bovine serum and internally identified as C1F-P1 cells. All serum sources are tested by the suppliers for bovine viruses according to USDA standards. Further, the manufacturers of the bovine serum have certified that the serum is not derived from material at risk of transmitting bovine spongiform encephalopathy (BSE) and that the serum is collected from USDA-approved harvest facilities.

Cell culture follows internal Standard Operating Procedures (SOPs) and is performed by authorized and trained personnel. General procedures for maintaining clean environment and cell culture laboratory safety are implemented to reduce risk of contamination significantly. All internal cell culture procedures are performed by trained staff under aseptic conditions in biosafety cabinets which are used for cell handling, cell passaging,[2] and change of culture media. Cell passaging of C1F cells was performed following standard operating procedures that describe methods to routinely passage mammalian and avian cells, both in adherent and suspension conditions. Briefly, for each sample of C1F suspension cultures, cell suspension was collected under aseptic conditions and transferred to a 24-well culture well plate to visually inspect the cultures, using a phase-contrast microscope (typical micrograph of cell suspension is shown in Figure 1.

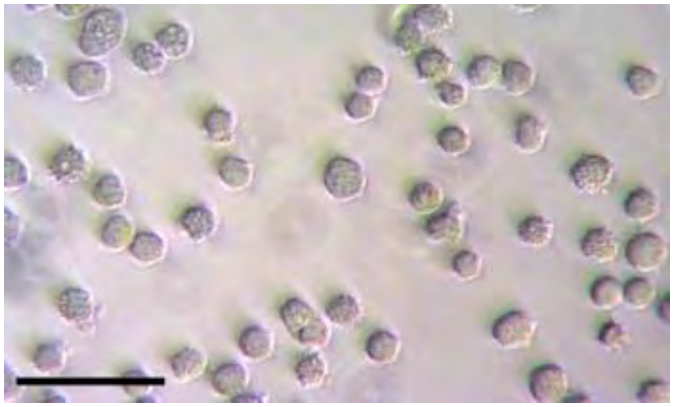


Figure 1. Inverted microscope imaging of C1F cells cultured in suspension. Scale bar represents 50 μm.

C1F cells are passaged every 2 – 3 days of culture in suspension. Determination of proliferation and viability of C1F cells was performed, as was the quantification of viable cell density. To quantify viable cell density, a representative volume of the C1F cell suspension is collected and centrifuged. The supernatant is discarded or used to determine metabolite concentrations. If the culture shows the formation of small cell aggregates, a sample of C1F culture is centrifuged, cell pellet is resuspended in TrypLE enzyme and incubated for few minutes until all chicken cells are single cells, followed by inactivation of enzymatic activity by adding culture media containing serum prior to cell count. The total volume is transferred to sampling cups for cell density and viability quantification using the Vi-Cell XR Cell Viability Analyzer ("Vi-Cell"). Based on viable cell density measured in the Vi-Cell equipment, the volume split ratio for C1F

[2] Cell passaging refers to the process of dividing cells from a given culture into new cultures and fed with fresh media to facilitate further expansion.

cells is determined and typically ranges between 1:3 to 1:5 (*v/v*). For a cell split ratio of 1:3, one-third of the total volume of the original C1F suspension is kept and two-thirds of total volume of fresh culture media are added. Passage number (number of subcultures) and Population Doubling Time (PDT) are recorded for each cell passage to monitor C1F health status and culture progression. After each cell passage, a new measurement of cell density and viability post-split is done following the same protocol previously described. The protocol herein listed applies to both parental C1F cells and C1F-P1 cells adapted to low serum-containing culture media.

When scaling up C1F-P1 cells for cell banking or product development, SOPs for split passaging and cell harvesting of suspension cultures are followed. These SOPs detail the method for split passage of C1F-P1 cells in 2 to 2.5 L of working volume of cell suspension, and the harvesting and washing of the C1F-P1 cells. A complete description of the process workflow and methodologies is found in Section 5 ("Method of Manufacturing").

4.4. Creation of Master Cell Banks (MCB)

Cell banking creates a processed and cryogenically stored collection of cells distributed into containers in a single operation and stored to ensure uniformity and stability of content. A Master Cell Bank (MCB) is a bank of cells from which all subsequent Master Working Cell Banks (MWCBs) used for production will be derived (see Section 4.5).

The C1F-P1 chicken cell line was cultured and expanded as described in Section 4.3 to produce the MCB and MWCB. Each MCB and MWCB preparation from C1F cells in suspension had its own Batch Record document and number, which ultimately provided the cell bank's designated code. Cryopreservation and thawing of cells are performed according to SOPs and GMP chain of custody documentation during retrieval (Appendix 10.3.3). Same protocols apply for the same cell line cultured in low serum (C1F-P1).

C1F-P1 cells are well adapted to suspension growth and can grow in culture media with low serum concentration. This cell line has had a consistent doubling time and viable cell density when evaluated at >200 passages.

4.5. Creation of Master Working Cell Banks (MWCB)

MWCB is generated through the expansion of cells derived from the MCB. Cells from MWCB are used in the production of cells for the manufacturing of commercial products, following cGMP-compliant procedures (CBER, 1993). Four vials of cells were retrieved from the C1F-P1 MCB to establish C1F-P1 MWCB. The cells were thawed according to internal SOP: CA-SOP040. The protocol for establishing the C1F-P1 MWCBs is described in Appendix: 10.3.4. Briefly, C1F-P1 MCB cryovials were removed from the liquid nitrogen storage and immediately thawed. C1F-P1 cell suspension from each vial was transferred into conical tubes containing culture media in a laminar flow hood, and the diluted C1F-P1 cell suspension was pooled together and centrifuged. The supernatant was aseptically aspirated without disturbing the cell pellet. C1F-P1 cells were then resuspended in culture media and transferred to a spin culture flask.

C1F-P1 cells were cultured under agitation for 11 days during which five steps of scale-up occurred. Scale-up steps involved culturing the chicken cells in a humidified incubator, with progressively increasing working volume and cells sub-cultured every 3 days with a split ratio of 1:3 (*v/v*) as described in Section 4.3. Following these scale-ups, C1F-P1 cells were harvested for creation of MWCB.

C1F-P1 cells in the final expansion culture were collected, centrifuged, and resuspended in a lower volume of culture media. The concentrated C1F-P1 cells were sampled and counted as described previously. The cells then went through another centrifugation cycle and were resuspended in cryopreservation media. Cells were then transferred to vials and stored in a vapor phase liquid nitrogen storage system. Vial content and banked storage position were recorded in a controlled database, and GMP chain of custody documentation (vial identity confirmation) was utilized to ensure the appropriate vial(s) are retrieved from the MWCB for cell bank release testing and cultured chicken production.

4.6. Cell Bank Release Testing

The purpose of this section is to describe the procedures for evaluation of quality attributes: cell viability, identity, purity, safety, and stability of cell banks, following internal SOP CA-SOP005 "Cell Bank Release Testing for Chicken C1F Cells". The tests described in this dossier are for cGMP cell banks to be used for commercial purposes. Tests for identity and purity must be performed once for each MCB. When the MCB and MWCB have passed all appropriate tests, the remaining vials in the MCB and MWCB (respectively) can be considered validated since the cells originated from a common pool and were handled under identical conditions

Table 1 lists the quality attributes for C1F-P1 MCB and C1F-P1 MWCB used for commercial production, their specification limit, and whether the assays were performed internally or by a contract laboratory. The attributes are listed by category.

Table 1. Characterization of Quality Attributes for GMP C1F-P1 MCB and C1F-P1 MWCB release for commercial purposes.

Quality Attribute	Assay	Specification	Performed at Good Meat	Performed at third party laboratory
Cell Viability	CA-SOP057 Trypan Blue Exclusion Method	Cell Viability >70% after thawing Cell Viability >80% after cell passage	X	
Cell Proliferation	CA-SOP057 Trypan Blue Exclusion Method	Doubling time comparable to historical data	X	
Safety	Sterility	Negative		X
Safety	*Mycoplasma*	Negative		X
Safety	Viral and bacterial contamination (Adventitious Agents)	Negative		X
Purity/Identity	CA-SOP024 Gene expression	Expression of FSP-1	X	
Purity/Identity	CA-SOP013 Species Identification: PCR and sequencing	Absence of cross-species contamination	X	X

Descriptions for all Cell Banking Release Assays are presented in Appendix: 10.3.5.

4.7. Cell Bank Stability

The stability of the generated C1F-P1 cell banks is evaluated following the internal SOP CA-SOP008 "Cell Bank Stability Testing". This testing is only conducted on GMP cell banks to be used for commercial purposes. Tests for cell bank stability are performed at 6, 12, 18 and 24 months post-creation of each MCB, followed by annual tests after that. Each MWCB used for commercial purposes must be tested for stability at 6, 12, 18, 24 months post-creation of each MWCB.

When a culture from the MCB or MWCB has passed all appropriate evaluations for stability, the stability of the remaining vials in the MCB or MWCB, respectively, can be considered validated since the cells originated from a common pool and were handled under identical conditions. Conversely, if there is a significant decrease in cell proliferation and cell viability or the cell bank does not meet the specified criteria for stability, periodicity of assessment will be increased.

Further details regarding cell bank stability and the testing and parameters evaluated are in Appendix: 10.3.6.

5. METHOD OF MANUFACTURE

5.1. Manufacturing Process

Cultured chicken production is currently performed at JOINN Biologics US, located at 2600 Hilltop Dr., Richmond, CA 94806, USA. Food safety plan information for this cell culture facility is presented in Section 7.

5.1.1. Cell Growth

Figure 2 provides an overview of the manufacturing process of cultured chicken, initiated from qualified C1F-P1 cell banks and scaled up to bioreactor cultures. Avian cells from a qualified cell bank are first thawed and cultured in a seed expansion step. After the final shake flask expansion step, the culture is transferred to a wave bag for further expansion. Upon completion of expansion, the entire contents of the wave bag are transferred to a 200L bioreactor, for incubation, followed by a 1000L production bioreactor for final incubation. Details of each cell growth step are in Appendix: 10.4.1.

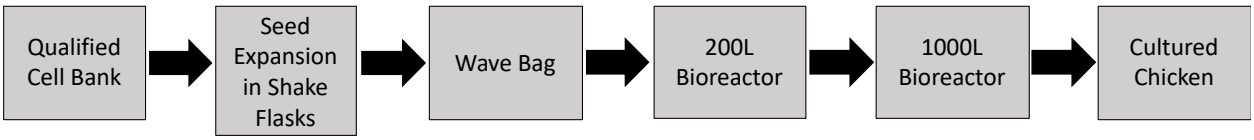


Figure 2. Overview of the manufacturing process for Good Meat cultured chicken.

5.1.2. Cultured Chicken Production

The workflow for harvest and storage of chicken cells is depicted in **Figure 3**. Following cell growth (Section 5.1.1), the cell culture broth is concentrated using centrifugation methods and subsequently washed. Concentrated and washed chicken cells are then packaged and stored at <-20 °C. The wash solution is analyzed to ensure wash efficacy (Section 5.4). Details of each production step are described in Appendix: 10.4.2.

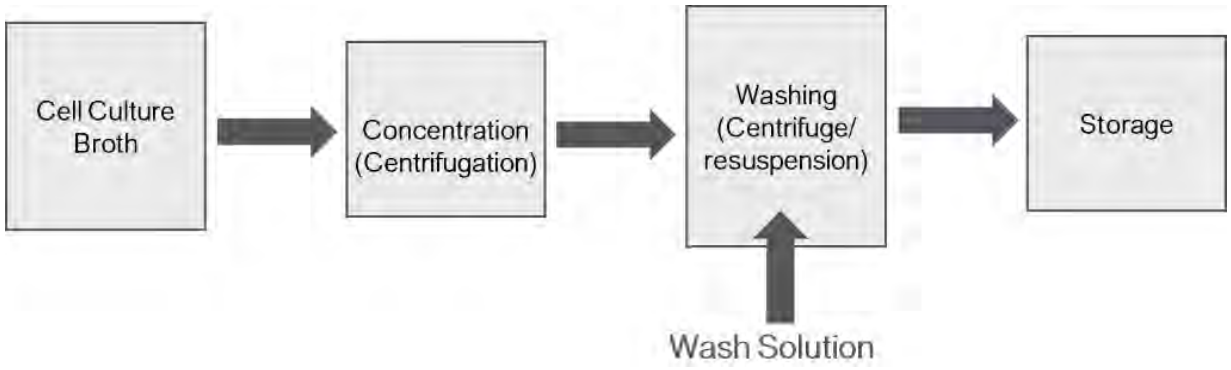


Figure 3. Overview of the harvest process for Good Meat cultured chicken production.

5.2. Raw Material Specifications

In this section, Good Meat identifies each component of the media used in seed and production of cultured chicken. Some of the components used in cell culture media may not have an explicit regulatory status; in such instances, data were assembled to demonstrate the safe use of these materials in the manufacturing process.

5.2.1. Growth media

The media components used in the seed and production cell cultures consist of a basal media, comprised of amino acids, vitamins, inorganic salts, and other components, which is then supplemented with fetal bovine serum. Where a regulation either restricts the use of the constituent, or there is tangential regulation of a similar substance, a discussion of the safety-in-use of each substance is provided. Table 2 lists the media components under this category. For the other media constituents, pertinent regulation is in place to assure safety of exposure. This discussion will relay exposure estimates from use in culture and compare to toxicological and/or existing use levels. A full breakdown of media components is available upon request.

Table 2. Media components with non-covered regulation for the manufacturing process of cultured chicken.

Category	Component	Use or Purpose	Grade
Vitamins	Folic Acid	Nutrient	USP/EP
Inorganic Salts	Ferric nitrate ($Fe(NO_3)_3$-$9H_2O$)	Nutrient	ACS
Other Components	Hypoxanthine Na	Nutrient	GIBCO*
	Lipoic acid	Nutrient	GIBCO*
	Putrescine 2HCl	Nutrient	GIBCO*
	Sodium pyruvate	Nutrient	USP
	Thymidine	Nutrient	USP
	Pluronic F-68	Anti-clumping agent	USP
Serum	Fetal Bovine Serum (FBS)	Nutrient/ Growth factor	USP/EP

ACS: A chemical grade of highest purity and meets or exceeds purity standards set by American Chemical Society.
EP: Chemicals manufactured under current Good Manufacturing Practices meets European Pharmacopeia.
USP: A chemical grade of sufficient purity to meet or exceed requirements of the U.S. Pharmacopeia; acceptable for food, drug, or medicinal use.
GIBCO*: Gibco grade is as *per* internal qualification standards established at GIBCO company for the media components that do not have a grade certified by the supplier. Gibco media products are manufactured according to quality system as outlined under ISO 9001:2015 and utilized in bioprocessing for cellular culture applications.

5.2.2. Media Constituents Covered by Regulation

Each media constituent with relevant existing regulation is denoted in Appendix: 10.4.3. In addition, where relevant, a discussion of the grade specifications is provided.

5.2.3. Media Constituents Not Explicitly Covered by Regulation

Each media constituent is discussed below in terms of its regulatory status, its toxicity and safety profile *per* relevant literature, and its estimated dietary exposure. A safety evaluation is provided based on analytical testing of residue levels present in final Good Meat cultured chicken batches.

5.2.3.1. Folic acid

In the manufacturing process of C1F-P1 cultured chicken, folic acid is part of the basal media and is a component of the seed and production cell culture media.

Folate and folic acid are water-soluble, nutritionally essential B vitamins. Folate occurs naturally in many different foods, while folic acid is the synthetic form of this vitamin that is internationally added to food. The naturally occurring food forms of folate include tetrahydrofolates (THF) and dihydrofolate, which can exist with reduced glutamate groups (*i.e.*, mono- and poly-glutamates) (NIH, 2021). The form of folic acid that exists with a fully oxidized monoglutamate group is used in food fortification and dietary supplements, as it is considered more chemically stable (EFSA, 2014). Natural sources of folate include dark green vegetables, fruits, beans, beef liver, and legumes, while fortified foods typically consist of fortified grain products; however, cooking and processing foods can significantly decrease the amounts of available folate (IOM, 1998; NIH, 2021). Folic acid has been added to cold cereals, flour, breads, pasta, bakery items, cookies, and crackers, as required by federal law since 1998 and is available in a wide range of supplements that provide up to 800 µg folate/day (NIH, 2020).

In humans, an exogenous source of folate is required for nucleoprotein synthesis and the maintenance of normal erythropoiesis. Folic acid is the precursor of tetrahydrofolic acid, which is involved as a cofactor for transformylation reactions in the biosynthesis of purines and thymidylates of nucleic acids (DNA and RNA) and the metabolism of amino acids (IOM, 1998; NIH, 2021).

Folic acid, the most oxidized and stable form of folate, is an essential water-soluble vitamin (vitamin B9) added to virtually all cell culture media formulations. Folic acid must be reduced and methylated to become the metabolically active form (folate) found in tissues. Folate acts as a cofactor for enzymes involved in DNA and RNA biosynthesis and is also involved in the supply of methyl groups to the so-called methylation cycle, which uses methionine and makes homocysteine (Schnellbaecher et al, 2019). Folic acid plays an important role in genomic stability, and deficiencies have been reported to induce chromosomal breaks in human genes. Deficiency in folic acid can also lead to an elevated rate of DNA damage and altered DNA methylation, which in the context of *in vitro* cell culture can lead to changes in cell phenotype, gene expression and cell line stability (Fenech, 2001).

As we explore regulatory status, recommended dietary allowance, safety evaluation and dietary exposure, it is important to properly distinguish total folate and folic acid. Total folate is an umbrella term used to represent the different forms of the vitamin B. Food folate is the form that occurs naturally in food sources. Folic acid is the form of vitamin found in fortified foods and dietary supplements. The term dietary folate is used to represent food folate and folic acid in

fortified foods together. Total folate encompasses all dietary and supplemental exposure to folate and folic acid.

5.2.3.1.1. Regulatory Status

Folic acid is a regulated food additive under 21 CFR § 172.345 as nutrient supplement not to exceed the amounts specified in the regulation. In 1996, the FDA issued regulations requiring that enriched grains (enriched flour, bread, rolls and buns, farina, corn grits, cornmeal, rice, and noodle, but not whole-grain products) be fortified with folic acid. Folic acid is regulated as a special dietary and nutritional additive under 21 CFR § 172.345 and is permitted to be added at 400 µg *per* serving to breakfast cereals, to infant formula at 4 µg *per* 100 kcal of infant formula, 1 mg/1 lb. of corn grits, to foods represented as meal-replacement products at 400 µg/serving if the food is a meal replacement that is represented for use once/day, or 200 µg/serving if the food is a meal-replacement that is represented for use more than once/day. Folic acid can be added to medical food at levels not to exceed the amount necessary to meet the distinctive nutritional requirements of the disease or condition for which the food is formulated, and for food for special dietary use not to exceed the amount necessary to meet the special dietary needs for which the food is formulated.

Folic acid must be present in the culture media to produce the final cultured chicken product. Based on the exposure assessment described below, folic acid is not detected in cultured chicken (<0.1 µg/g). Total dietary folate was found at approximately 1/4th the daily recommended intake (NIH, 2021), and approximately 1/10th the recommended upper daily intake level (UL; 1,000 µg/day) for folate discussed in the final folate rule (Federal Register, 1996). As such, it is highly unlikely that an individual consuming cultured chicken would be over-exposed to folic acid. Thus, folic acid under this specified use and detection level may be considered safe and suitable.

5.2.3.1.2. Relevant Literature

5.2.3.1.2.1. Absorption, Distribution, Metabolism and Excretion (ADME)

Oral bioavailability of naturally occurring folate differs from that administered as supplemental folic acid. The oral bioavailability of food folate is reported as approximately 50%, while the bioavailability of folic acid in supplements or fortified foods is approximately 85% to 100%, depending on whether it is consumed in or with food (Field and Stover, 2018; NIH, 2021). Following consumption, naturally occurring conjugated food folates are hydrolyzed to the monoglutamate folic acid form by folylpoly-γ-glutamate carboxypeptidase in the gut prior to absorption by active transport across the intestinal mucosa of the duodenum and upper part of the jejunum (Blom and Smulders, 2011; NIH, 2021). The monoglutamate is then reduced by dihydrofolate reductase before entering the blood stream as 5-methyl-tetrahydrofolate (5-methyl-THF), which is subsequently taken up throughout the body by carrier- or receptor-mediated transport (Blom and Smulders, 2011). In contrast, supplemental synthetic folic acids are absorbed rapidly from the small intestine, primarily from the ileum primarily by passive diffusion. After folate ingestion, plasma concentration increases and is maintained at an elevated concentration for up to approximately four hours followed by a rapid decrease (EFSA, 2014). Folates which are not bound to specific and non-specific binding proteins are subjected to catabolism by oxidative cleavage at the C9–N10 bond, generating p-aminobenzoylglutamates which in turn are acetylated in the liver before excretion (EFSA, 2014).

Folate is filtered through the kidney glomerulus but most of it is reabsorbed in the proximal tubule with the assistance of folate-binding proteins and specific transporters (EFSA, 2014). As a

result, most of the folate in the urine is in the form of breakdown products, with only 1 – 2 % of the excreted amount being active folate.

5.2.3.1.2.2.Recommended Dietary Allowance (RDA)

The Institute of Medicine (IOM, 1998) established estimated average requirement (EAR), adequate intake (AI), and recommended dietary allowance (RDA) values for folate (as dietary folate equivalents or DFE), which range from 120 to 300 µg/day in children aged 1 to 13 years of age, 320 to 400 µg/day in males and non-pregnant/non-lactating females (aged 14 to >70 years of age), and 450 to 600 µg/day for pregnant and lactating women (<18 to 50 years of age). Women seeking to become pregnant or that are pregnant are recommended to consume at least 400 µg/day of folic acid from fortified foods and/or supplements, in addition to food folate, to help reduce the incidence of neural tube defects (NTDs) in the developing fetus (IOM, 1998). For women with previous history of NTD-pregnancy or those with diabetes, intake recommendations increase to 5,000 µg folic acid/day (SACN, 2017). FDA currently accepts and recommends these values.[3]

Tolerable upper intake levels (ULs) for folic acid from fortified foods and supplements, based on the potential for masking adverse cognitive effects of vitamin B12–deficiency (IOM, 1998), are presented in Table 3. These were derived based on case reports and observational studies in elderly populations and adjusted according to body weights for adolescents and younger age categories (IOM, 1998).

Table 3. Folic Acid Tolerable Intake Levels (ULs) for Different Human Age Categories (IOM, 1998)

Age Category	**Recommended Dietary Allowance (RDA)**	**Limit (for folic acid from fortified foods and supplements)**
0-12 months	Not Applicable	Not possible to establish for supplemental folate
1 to 3 years	150 µg DFE/day	300 µg/day
4 to 8 years	200 µg DFE/day	400 µg/day
9 to 13 years	300 µg DFE/day	600 µg/day
14 to 18 years	400 µg DFE/day	800 µg/day
19 and older (adults)	400 µg DFE/day	1,000 µg/day
14 to 18 years (during pregnancy or lactation)	500 µg DFE/day	800 µg/day
19 years and older (during pregnancy or lactation)	500 µg DFE/day	1,000 µg/day

5.2.3.1.2.3.Safety Evaluation

Several other agencies have reviewed the safety and requirements of folate/folic acid, including the European Commission Scientific Committee on Food (SCF, 2000), the Expert Group on Vitamins and Minerals (EVM, 2003), the German Federal Institute for Risk Assessment (BfR, 2018), the United Kingdom Scientific Advisory Committee on Nutrition (SACN, 2017), the Norwegian Scientific Committee for Food Safety (VKM, 2015), and the European Food Safety Authority (EFSA, 2014). The EVM (2003) and SCF (2000) both derived UL intakes for folic acid of 1,000 µg/day for adults and 800 µg/day for adolescents aged 15 to 17 years, based on a similar

[3]https://www.fda.gov/food/new-nutrition-facts-label/folate-and-folic-acid-nutrition-and-supplement-facts-labels (last accessed July 20, 2021)

concern for potential masking effects of vitamin B12-deficiency. In contrast, the BfR (2018) derived a recommended daily intake limit of 200 µg/day for folic acid from supplements in adults, including adolescents, which was calculated by dividing the adolescent UL of 800 µg/day (SCF, 2000) by 2 to separately delineate limits for folic acid intake from supplements and fortified foods (400 µg/day for each category) and applying a 2-fold safety factor due to uncertainties in intake levels of folic acid supplements. The BfR also raised concerns about potential for adverse effects from folic acid intake at or near the existing ULs derived by the SCF. The BfR still recommended that women of childbearing age and pregnant women consume 400 µg/day of supplemental folic acid for the prevention of NTDs.

As noted in several recent reviews (NTP, 2015; Boyles *et al.*, 2016; SACN, 2017; Field and Stover, 2018; Maruvada *et al.*, 2020) the potential health impacts of high folic acid intakes associated with various adverse health conditions have been critically assessed. These reviews indicated that although further research may be warranted for some outcomes, there is currently insufficient evidence to establish a causal relationship between high folic acid intakes (at or above the UL) and the following endpoints: cancer (prostate, colorectal, breast, overall cancer risk), diabetes-related disorders, long-term effects of systemic unmetabolized folic acid, hypersensitivity-related outcomes, and thyroid disease. The published data related to these health outcomes were reported to be limited, inconsistent, or otherwise insufficient to assess the potential relationship. Furthermore, some health endpoints have been associated with improvements with folic acid supplementation, such as certain cancers, cardiovascular disease, and depression, and as such, further research is needed to elucidate how folic acid has beneficial properties in some cases and possible negative effects in others.

5.2.3.1.3. Dietary Exposure

5.2.3.1.3.1. Dietary Sources

Folic acid is also known as Vitamin B9 making it part of the B-complex of vitamins. Dietary sources of folic acid include dark green leafy vegetables (spinach, broccoli, and dandelion greens), dried beans, peas, legumes, dairy products, poultry, meat, eggs, seafood, citrus fruits and juices (NIH, 2021). Folate is the active form that is found in food and in the human body. The folate content of various plant foods is presented in Table 4, with the highest folate content being in beans at an upper range of 525 µg/100g.

Table 4. Folate content in various plant foods (adapted from Robinson *et al.,* 2015)

Food Source	**Folate content (µg/100g)**
White rice	6 - 9
Sweet potato	11
Onions	10 - 19
Tomato	8 – 30
Potato	11 – 37
Banana	13 – 30
Carrot	16 – 19
Corn (yellow)	19
Orange	18 – 30
Cassava	27
Peas (green)	25 – 65
Strawberry	13 - 96
Snap beans	37
Wheat	38

Lettuce (fresh)	38 – 43
Corn (sweet, white or yellow)	46
Rye (grain)	60 – 78
Wild rice	95
Broccoli	63 – 114
Spinach	100 – 194
Peanut	110 – 240
Lentils	151 – 479
Beans (navy, pinto, great northern)	143 - 525

Intake recommendations for folate and other nutrients are provided in the Dietary Reference Intakes (DRIs) developed by an expert committee of the Food and Nutrition Board (FNB) at the National Academies of Sciences, Engineering, and Medicine.[4] The FNB developed dietary folate equivalents (DFE) to reflect the higher bioavailability of folic acid than that of food folate. At least 85% of folic acid is estimated to be bioavailable when taken with food, whereas only about 50% of folate naturally present in food is bioavailable. Based on these values, the FNB defined DFE as follows:

- 1 µg DFE = 1 µg food folate
- 1 µg DFE = 0.6 µg folic acid from fortified foods or dietary supplements consumed with foods
- 1 µg DFE = 0.5 µg folic acid from dietary supplements taken on an empty stomach

The National Institute of Health (NIH) has released a Folate Fact Sheet for Health Professionals with the folate content of various foods listed. These values are reported in Table 5 and demonstrate that a 3 ounce (85 g) roasted chicken breast contains 3 µg folate *per* serving.

Table 5. Folate content of various foods, from the Folate Fact Sheet for Health Professionals (NIH, 2021)

Food	**Micrograms (µg) DFE *per* serving**	**Percent DV***
Beef liver, braised, 3 ounces	215	54
Spinach, boiled, ½ cup	131	33
Black-eyed peas (cowpeas), boiled, ½ cup	105	26
Breakfast cereals, fortified with 25% of the DV	100	25
Rice, white, medium-grain, cooked, ½ cup	90	22
Asparagus, boiled, 4 spears	89	22
Brussels sprouts, frozen, boiled, ½ cup	78	20
Spaghetti, cooked, enriched, ½ cup	74	19
Lettuce, romaine, shredded, 1 cup	64	16
Avocado, raw, sliced, ½ cup	59	15
Spinach, raw, 1 cup	58	15
Broccoli, chopped, frozen, cooked, ½ cup	52	13
Mustard greens, chopped, frozen, boiled, ½ cup	52	13
Bread, white, 1 slice	50	13

[4] Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes: Th9iamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Washin8gton, DC: National Academy Press; 1998. Accessible at: https://ods.od.nih.gov/factsheets/Folate-HealthProfessio7nal/

Green peas, frozen, boiled, ½ cup	47	12
Kidney beans, canned, ½ cup	46	12
Wheat germ, 2 tablespoons	40	10
Tomato juice, canned, ¾ cup	36	9
Crab, Dungeness, 3 ounces	36	9
Orange juice, ¾ cup	35	9
Turnip greens, frozen, boiled, ½ cup	32	8
Peanuts, dry roasted, 1 ounce	27	7
Orange, fresh, 1 small	29	7
Papaya, raw, cubed, ½ cup	27	7
Banana, 1 medium	24	6
Yeast, baker's, ¼ teaspoon	23	6
Egg, whole, hard-boiled, 1 large	22	6
Cantaloupe, raw, cubed, ½ cup	17	4
Vegetarian baked beans, canned, ½ cup	15	4
Fish, halibut, cooked, 3 ounces	12	3
Milk, 1% fat, 1 cup	12	3
Ground beef, 85% lean, cooked, 3 ounces	7	2
Chicken breast, roasted, 3 ounces	3	1

*DV = Daily Value. The FDA developed DVs to help consumers compare the nutrient contents of foods and dietary supplements within the context of a total diet. The DV for folate is 400 µg DFE for adults and children aged 4 years and older [11], where µg DFE = µg naturally occurring folate + (1.7 x µg folic acid). The labels must list folate content in µg DFE *per* serving and if folic acid is added to the product, they must also list the amount of folic acid in µg in parentheses. The FDA does not require food labels to list folate content unless folic acid has been added to the food. Foods providing 20% or more of the DV are considered to be high sources of a nutrient, but foods providing lower percentages of the DV also contribute to a healthful diet.

Table 6 describes dietary folate and folic acid content in various food products which are further categorized as excellent, good, moderate, and poor sources of folate. This table is generated using data from the Nutrition Coordinating Center of the University of Minnesota, Minneapolis (Food Database version 5A: Nutrient Database version 20, 1991). Dietary folate equivalent values for fortified foods were derived from the study by Suitor and Bailey in 2000 using files provided by the US Department of Agriculture (USDA) (S.E. Gebhardt, Nutrient Data Laboratory, Agricultural Research Service, June 1998).

Table 6. Food sources of Folic acid and Total Folate categorized as Excelled, good, moderate and poor sources (adapted from Suitor and Bailey, 2000)

Food Source	**Serving Weight (g)**	**Folic Acid content (µg/serving)**	**Total Folate (µg DFE/serving)**
Excellent sources of folate (100 – 200 µg DFE/serving)			
Breads and Cereals			
Cereals, ready-to-eat, fortified to 25% DV	30 – 58	100	170
Macaroni, enriched, cooked	120 – 140	80 – 90	140 – 160
Noodles, enriched, cooked	160	90	160
Rice, white, enriched, processed, cooked	175	95	170
Spaghetti, enriched, cooked	140	90	160

Tortilla, flour, enriched, soft	70	80	140
Vegetables			
Asparagus, cooked	75	0	100
Okra, cooked	92	0	135
Spinach, raw	56	0	110
Spinach, cooked	95	0	100
Legumes			
Beans, black, cooked	86	0	130
Beans, kidney, cooked	91	0	115
Beans, navy, cooked	91	0	125
Beans, pinto, cooked	86	0	145
Clack-eyed peas, cooked	83	0	105
Chickpeas, cooked	82	0	140
Lentils, cooked	99	0	180
Meats			
Beef liver	85	0	185
Food Source	**Serving Weight (g)**	**Folic Acid content (µg/serving)**	**Total Folate (µg DFE/serving)**
Good sources of folate (50 – 100 µg DFE/serving)			
Breads and Cereals			
Bagel, enriched	57	30	70
Grits, enriched, cooked	121	40	70
Toaster pastry	55	40	70
Wheat germ, toasted	14	0	50
Fruits			
Orange juice, ready-to-drink	249	0	80
Strawberries, fresh	151	0	80
Vegetables			
Avocado	87	0	55
Broccoli, cooked	92	0	50
Brussels sprouts, cooked	78	0	80
Corn	123	0	55
Mustard greens, cooked	75	0	90
Tomato juice	243	0	50
Turnip greens, cooked	75	0	85
Food Source	**Serving Weight (g)**	**Folic Acid content (µg/serving)**	**Total Folate (µg DFE/serving)**
Moderate sources of folate (25 – 49 µg DFE/serving)			
Breads and Cereals			
Breads, rolls, biscuits, muffins, English muffin (half), enriched	28	15	25 – 40
Crackers, saltines, melba, enriched	14	15	25
Pretzels, hard, enriched	14	15	30
Fruits			
Cantaloupe	231	0	40
Grapefruit juice, ready-to-drink	247	0	25
Grapes	160	0	40
Orange	131	0	40
Vegetables			

Broccoli, raw	36	0	30
Cauliflower, cooked	90	0	35
Cauliflower, raw	50	0	25
Lettuce, iceberg	55	0	30
Lettuce, romaine	30	0	40
Potato, Idaho, baked, flesh, skin	156	0	25
Meat and substitutes			
Egg	50	0	25
Meats and fish, breaded or batter-fried with enriched flour	100	15 – 20	25 – 50
Peanut butter	32	0	25
Peanuts, dry roasted	28	0	40
Soups with enriched noodles (no beans), ready-to-eat	180	10	20
Food Source	**Serving Weight (g)**	**Folic Acid content (µg/serving)**	**Total Folate (µg DFE/serving)**
Poor sources of folate (<25 µg DFE/serving)			
Breads and cereals			
Bread, whole wheat	28	0	15
Crackers, wheat	15	5	10
Oatmeal, cooked, not fortified	234	0	10
Other			
Popcorn	12	0	0
Puffed wheat or rice, not fortified	15	0	15
Shredded wheat, plain, not fortified	30	0	15
Fruits			
Apple, with skin	138	0	5
Apple juice	248	0	0
Applesauce	122	0	0
Banana	114	0	20
Blueberries, fresh	73	0	5
Grapefruit	146	0	15
Peaches, canned, juice pack	122	0	5
Pears, canned, juice pack	122	0	5
Pineapple, canned, juice pack	123	0	5
Raisins, uncooked	78	0	5
Tomato, raw	62	0	10
Vegetables			
Beans, green, string, cooked	68	0	5
Cabbage, green, cooked	75	0	15
Carrots, cooked	78	0	10
Carrots, raw, sliced	71	0	10
Celery, raw	71	0	20
Coleslaw	60	0	20
Corn, cooked	82	0	20

Cucumber, raw	52	0	5
Kale, boiled, drained, chopped	65	0	5
Pepper, green, raw	50	0	10
Pepper, red, raw	50	0	10
Potatoes, French fries, frozen	50	0	5
Potatoes, mashed	123	0	10
Squash, butternut, baked, cubed	102	0	10
Squash, yellow summer, sliced, boiled, drained	90	0	15
Milk products			
Cheese, hard, American	28	0	5
Milk, fluid	244	0	10 – 15
Meat, fish, and poultry			
All except organ meats and products prepared with enriched cereal grains (e.g., breaded, batter dipped, and meatloaf)	90	0	5 – 15

5.2.3.1.3.2.History of Consumption

In 2010, Bailey and others conducted a nationwide study to determine folate and folic acid intake from foods and dietary supplements in the US population from 2003 – 2006 (Bailey et al., 2010). Design of this study was based on the National Health and Nutrition Examination Survey (NHANES) cross-sectional survey that samples noninstitutionalized, civilian US residents with the use of a complex, stratified, multistage, probability cluster sampling design. All data were collected by the National Center for Health Statistics of the Centers for Disease Control and Prevention. Briefly, participants were asked to complete 3 components: an in-person household interview, a health examination in a mobile examination center (MEC) ≈3 weeks later, and a phone interview to collect additional dietary data.

The use of dietary supplements was reported by 53.4% of NHANES 2003–2006 participants, and 34.5% reported the use of supplements that contained folic acid. Non-Hispanic whites (39%) reported a higher prevalence of use of folic acid dietary supplements than did non-Hispanic blacks (19%) and Mexican Americans (18%). Use and mean contribution from dietary supplements that contained folic acid was highest for 51–70-year-olds, with 47% of men and 53% of women in this age group reporting use. In this age group, the mean (±SE) contribution of folic acid from supplements was 436 ± 21.4 μg, and 5% were above the UL from dietary supplements alone when the sexes were examined together. In general, dietary folate intakes were relatively stable for women across age groups (Table 7).

In contrast, total folate intakes (diet plus supplements) were higher in those aged >51 years. Among men, no differences were noted in age groups for dietary folate intakes. Men >51 years of age had significantly higher total folate intakes compared with the reference group. Among both sexes, non-Hispanic whites had higher total folate intakes than did non-Hispanic blacks and Mexican Americans. Across all age and racial-ethnic groups, men had higher mean dietary and

total folate intakes than did women (between-sex differences not statistically compared). However, women had a higher percentage than men of total intake contributed by dietary supplements.

Table 7. Folic Acid, Dietary Folate and Total Folate intake stratified by sex, age group, and race-ethnicity in the United States, 2003-2006 (adopted from Bailey et al., 2010)

	n	Folic Acid (µg)	Above UL (%)	Total Intake (µg)	Above UL (%)
Women					
14 – 18 y	1250	201 ± 7.5	0.0 ± 0.0	248 ± 13.1	0.4 ± 0.2
19 – 30 y	914	165 ± 5.6	0.0 ± 0.0	274 ± 9.5	2.2 ± 0.6
31 – 50 y	1350	154 ± 6.7	0.3 ± 0.2	297 ± 16.1	2.7 ± 0.7
51 – 70 y	1251	148 ± 5.7	0.0 ± 0.0	407 ± 22.2	5.2 ± 0.8
≥ 71 y	787	156 ± 6.0	0.0 ± 0.0	358 ± 15.8	3.2 ± 0.8
Non-Hispanic white	2540	162 ± 4.8	0.1 ± 0.01	359 ± 14.0	3.8 ± 0.5
Non-Hispanic black	1406	136 ± 4.9	0.0 ± 0.0	220 ± 10.2	1.4 ± 0.2
Mexican American	1200	152 ± 4.3	0.0 ± 0.0	232 ± 8.6	1.3 ± 0.3
Men					
14 – 18 y	1351	280 ± 9.5	1.2 ± 0.5	322 ± 12.4	2.3 ± 0.7
19 – 30 y	1097	245 ± 9.0	0.5 ± 0.3	317 ± 16.1	2.1 ± 0.9
31 – 50 y	1439	217 ± 5.6	0.0 ± 0.0	341 ± 12.8	2.1 ± 0.4
51 – 70 y	1215	197 ± 7.6	0.5 ± 0.2	406 ± 21.5	4.8 ± 0.8
≥ 71 y	808	200 ± 5.2	0.0 ± 0.0	421 ± 15.5	5.0 ± 0.7
Non-Hispanic white	2707	233 ± 4.7	0.4 ± 0.1	389 ± 12.7	3.9 ± 0.4
Non-Hispanic black	1479	185 ± 5.9	0.2 ± 0.1	261 ± 8.4	1.3 ± 0.5
Mexican American	1341	190 ± 5.7	0.3 ± 0.15	248 ± 11.3	1.3 ± 0.3

Different sample sizes were available for the age groups and racial-ethnic groups by sex because the "all other" racial category was included in the age group analysis. Dietary folic acid represents folic acid from fortified foods. Total folic acid represents folic acid from fortified foods combined with folic acid from dietary supplements. The Tolerable Upper Intake Level (UL) is 800 µg synthetic folic acid for individuals aged 14–18 y and 1000 µg for individuals aged ≥19 y.

Mean ± SE (all such values). SEs were calculated with the use of Fay's modified balanced repeated replication technique.

The relative SE is >40%; this estimate is unreliable.

Significantly different from the referent age group (19–30 y) or the referent racial group (non-Hispanic whites), $P \leq 0.003$ (Bonferroni-adjusted).

The relative SE is >30% and <40%.

Table 7. Dietary Folate and Total Folate intake compared with the estimated average requirement (EAR) stratified by sex, age group, and race-ethnicity in the United States, 2003 – 2006 (adopted from Bailey et al., 2010)

	n	Dietary Folate (DFE)	Below EAR (%)	Total Folate (DFE)	Below EAR (%)
Women					
14 – 18 y	1250	496 ± 14	22.4 ± 2.7	577 ± 24	19.0 ± 2.6
19 – 30 y	914	460 ± 12	21.8 ± 2.3	645 ± 17	16.9 ± 2.4
31 – 50 y	1350	470 ± 12	22.9 ± 2.1	714 ± 29	14.6 ± 1.7
51 – 70 y	1251	460 ± 11	22.8 ± 2.5	900 ± 39	12.7 ± 1.9
≥ 71 y	787	454 ± 10	23.7 ± 2.3	797 ± 26	14.4 ± 1.9

Non-Hispanic white	2540	476 ± 9.0	22.2 ± 1.4	811 ± 25	13.0 ± 1.1
Non-Hispanic black	1406	402 ± 9.4	28.7 ± 2.9	544 ± 18	23.2 ± 2.5
Mexican American	1200	457 ± 7.9	20.2 ± 2.8	593 ± 15	12.6 ± 1.2
Men					
14 – 18 y	1351	674 ± 19	9.5 ± 2.0	745 ± 24	9.2 ± 2.0
19 – 30 y	1097	652 ± 16	5.9 ± 1.7	774 ± 28	5.2 ± 1.6
31 – 50 y	1439	633 ± 11	4.1 ± 0.8	843 ± 23	3.6 ± 0.8
51 – 70 y	1215	583 ± 14	8.2 ± 1.5	938 ± 37	6.3 ± 1.3
≥ 71 y	808	558 ± 8.2	9.3 ± 1.4	935 ± 24	5.5 ± 1.1
Non-Hispanic white	2707	644 ± 8.9	5.3 ± 0.8	909 ± 23	4.3 ± 0.7
Non-Hispanic black	1479	522 ± 13	12.7 ± 2.4	651 ± 156	10.6 ± 2.1
Mexican American	1341	570 ± 12	7.8 ± 1.7	670 ± 22	7.3 ± 1.6

Different sample sizes were available for the age groups and racial-ethnic groups by sex because the "all other" racial category was included in the age group analysis. Dietary folate represents the combination of food folate and folic acid in fortified foods. Total folate encompasses dietary folate and folic acid from dietary supplements combined. Both dietary and total folate are in the DFE metric.

The EAR for individuals aged 14–18 y is 330 DFE and is 320 DFE for individuals aged ≥19 y.

Mean ± SE (all such values). SEs were calculated with the use of Fay's modified balanced repeated replication technique.

Significantly different from the referent age group (19–30 y) or the referent racial group (non-Hispanic whites), $P \leq 0.003$ (Bonferroni-adjusted)

5.2.3.1.3.3.Dietary Folate levels in conventional chicken

The USDA National Nutritional Database for Standard Reference (SR) is the major source of food composition data in the United States and provides the foundation for most food composition databases in the public and private sectors. Dietary folate content of various food products is listed in the USDA National Nutrient Database for Standard Reference Release 28 published on January 14, 2016[5]. This database was used to establish folic acid, total folate, and dietary folate content in various raw and cooked chicken products (Table 8). From this database, raw chicken breast (skinless and boneless) is most closely related to GOOD Meat cultured chicken, which is intended as raw ingredient in various finished products like cultured chicken nuggets, breasts, and tenders. Therefore, raw chicken breast (skinless and boneless) with FDC ID: 171077, is selected as the standard reference for folic acid and total folate content in chicken as an ingredient.

[5] U.S. Department of Agriculture, National Nutrient Database for standard reference release 28, accessible at: https://ods.od.nih.gov/pubs/usdandb/Folate-Food.pdf#search=%22folic%20acid%22

Table 8. Folic Acid, Total Folate and Dietary Folate content in chicken products

Chicken Sample	Folic Acid (µg/100g)	Total Folate (µg/100g)	Folate, DFE (µg/100g)	USDA Reference
Raw Chicken				
Raw Chicken (Ground)	0	1	1	FDC ID: 171116[6]
Raw Chicken Leg (Meat and skin)	0	4	4	FDC ID: 172378[7]
Raw Chicken Breast (Skinless and boneless)	0	9	9	FDC ID: 171077[8]
Raw Chicken Breast (Meat and skin)	0	4	4	FDC ID: 171474[9]
Raw Chicken Thigh (Meat and skin)	0	3	3	FDC ID: 172385[10]
Raw Chicken Drumstick and Thigh (Meat and skin)	0	2	2	FDC ID: 172855[11]
Cooked Chicken				
Chicken Breast (Cooked and roasted)	0	4	4	FDC ID: 171477[12]
Chicken Thigh (Cooked and roasted)	0	5	5	FDC ID: 172388[13]
Roasted Chicken (Meat and skin)	0	5	5	FDC ID: 173635[14]

[6] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 171116, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/171116/nutrients

[7] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 172378, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/172378/nutrients

[8] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 171077, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/171077/nutrients

[9] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 171474, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/171474/nutrients

[10] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 172385, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/172385/nutrients

[11] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 172855, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/172855/nutrients

[12] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 171477, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/171477/nutrients

[13] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 172388, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/172388/nutrients

[14] U.S. Department of Agriculture, FoodData Central Nutritional database, FDC ID: 171116, accessible at: https://fdc.nal.usda.gov/fdc-app.html#/food-details/173635/nutrients

5.2.3.1.4. Folic Acid levels in Cultured Chicken and Estimated Consumer Exposure

To establish estimated consumer exposure to folic acid in cultured chicken, four (4) representative batches of cultured chicken were evaluated by AOAC 2011.06 UHPLC-MS method. Calculations for dietary exposure are based on RACC values stated in 9 CFR § 317.312. All four (4) representative batches were found with folic acid below the limit of detection (<0.1 µg/g). Assuming the maximum content of folic acid in cultured chicken to be the LOD 0.1 µg/g, the estimated maximum folic acid content in cultured chicken was calculated to be <8.5 µg/serving/person for ready-to-serve portions based on a RACC value of 85 g chicken *per* serving. The estimated maximum folic acid content was calculated to be <11 µg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum folic acid content was calculated to be <0.1 µg/g in cultured chicken, which is consistent with the maximum folic acid levels in conventional chicken (USDA National Nutritional Database for Standard Reference; Table 8).

Table 9. Estimated consumer exposure values of Folic Acid from representative batches (RB) of cultured chicken.

Representative Batches (RB)	**Folic Acid amount *per* gram of cultured chicken (µg/g)**	**Folic Acid amount *per* 100 g of cultured chicken (µg/100g)**	**Estimated intake of Folic Acid in ready-to-serve food (RACC 85g) (µg/serving/person)**	**Estimated intake of Folic Acid in ready-to-cook food (RACC 114g)[15] (µg/serving/person)**
RB-1	<0.1	<10	<8.5	<11
RB-2	<0.1	<10	<8.5	<11
RB-3	<0.1	<10	<8.5	<11
RB-4	<0.1	<10	<8.5	<11
Average	**<0.1**	**<10**	**<8.5**	**<11**
Maximum	**<0.1**	**<10**	**<8.5**	**<11**

The values reported in Table 9 confirm that folic acid was found below the detection limit and that residual folic acid from the culture media was either completely washed off or is present at negligible levels that do not represent a safety concern for human consumption.

Reported daily limits for consumption of folic acid from fortified foods and supplements range from 300 µg/day to 1,000 µg/day (Table 3). Assuming a worst-case scenario of folic acid content in cultured chicken and assuming an exclusive source of folic acid derived from this food product in the human diet, it would require a daily consumption of >3Kg/day to >10 Kg/day of cultured chicken cells, to surpass the reported daily limits of folic acid from fortified foods and supplements, depending on the age group of the consumers.

5.2.3.1.5. Total Folate and Dietary Folate Levels in Cultured Chicken

To establish estimated consumer exposure to total folate in cultured chicken, three (3) representative batches of cultured chicken were evaluated by validated method AOAC 944.12 and AACC 86-47.01 (Table 10). Dietary Folate Equivalent concentrations were calculated using results from Total Folate (AOAC 944.12 and AACC 86-47.01) and Folic Acid by UHPLC-MS (AOAC 2011.06), further discussed in Table 9 shown above.

[15] 9 CFR §381.412

Based on the total folate and folic acid results, maximum Dietary Folate Equivalent levels were found at 91 µg per 100 g of cultured chicken.

Table 10. Estimated consumer exposure values of total folate from representative batches (RB) of cultured chicken.

Representative Batches (RB)	**Total folate amount *per* 100 g of cultured chicken (µg/100 g)**	**Folic Acid amount *per* 100 g of cultured chicken (µg/100 g)**	**Dietary Folate Amount per 100 g of cultured chicken (µg/100 g)**
RB-1	91	<10	81-91
RB-2	91	<10	81-91
RB-3	60	<10	50-60
Maximum	**91**	**<10**	**91**

Table 10. Estimated consumer exposure values of total folate from representative batches (RB) of cultured chicken.

Representative Batches (RB)	**Dietary folate amount *per* gram of cultured chicken (µg/g)**	**Dietary folate amount *per* 100 g of cultured chicken (µg/100g)**	**Estimated intake of Dietary Folate in ready-to-serve food (RACC 85g) (µg/serving/person)**	**Estimated intake of Dietary Folate in ready-to-cook food (RACC 114g) (µg/serving/person)**
RB-1	0.91	91	77	104
RB-2	0.91	91	77	104
RB-3	0.60	60	50	67
Average	**0.81**	**81**	**68**	**92**
Maximum	**0.91**	**91**	**77**	**104**

The estimated consumer exposure values of dietary folate from Good Meat cultured chicken were calculated based on RACC values stated in 9 CFR § 317.312. The estimated maximum dietary folate content in Good Meat cultured chicken was calculated to be 77 µg DFE/serving/person for ready-to-serve portions based on a RACC value of 85 g *per* serving. The estimated maximum dietary folate content was calculated to be 104 µg DFE/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum dietary folate content was calculated to be 0.91 µg DFE/g (equivalent to 91 µg DFE/100g), which is approximately 10X the average dietary folate equivalents (DFE) content in raw chicken breast[16] (9 µg DFE/100g). This places cultured chicken in the food group as an excellent source of folate (0.1-0.2 mg DFE/serving), as Table 6 indicates. Similar levels were found for different types of legumes and vegetables, specifically spinach, beans, and asparagus. However, this level is below the recommended daily intake of folate (400 µg/day for adults and children 4 years and older). Similarly, the NTP report (2015) did not conclude that adverse effects could be solely attributed to high folic acid intake. The FNB of the Institute of Medicine (IOM) did not establish an upper limit for folate from food because "…high intakes of folate from food sources have not been reported to cause adverse effects."[17]

Furthermore, Good Meat cultured chicken will be used as ingredient in manufacturing of various cultured chicken finished products (bites, boneless breasts, tenders, etc) ranging from 60%

[16] U.S. Department of Agriculture, FoodData Central; Chicken, broiler or dryers, breast, skinless, boneless, meat only, raw nutrient values. https://fdc.nal.usda.gov/fdc-app.html#/food-details/171077/nutrients (last accessed January 10, 2022).

[17] Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes: Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Washington, DC: National Academy Press; 1998.

to 75% (w/w) [18] of overall formulation, therefore contributing 55 – 70 µg DFE/100g towards finished product. There is a long history of consumer consumption of various foods with similar levels of dietary folate (as shown in Table 6), such that exposure to folate does not pose a safety risk. Examples of food categories identified as excellent or good sources of Dietary Folate at levels above or comparable to cultured chicken include, but are not limited to bread and cereals, vegetables, legumes, fruits, and meats (food liver).

5.2.3.1.6. Conclusion

To summarize the key points in this discussion:

1) Folic acid is required for growth of cultured cells, just as folate is required for cells within an intact plant or animal. However, folic acid added to the cell culture medium is not detected in the harvested chicken cells (when using validated analytical methods).
2) The total amount of folate (as expressed as DFE) is lower than many foods safely consumed in the United States (Table 6). Cultured chicken products have a DFE range of 55 – 70 µg DFE/100g. Current serving size of the cultured chicken meat is approximately 50 grams or 28-35 µg DFE/serving.
3) Although we present a comparison with traditional chicken, this should not be a benchmark for evaluating the safety of this new protein source. Our cultivated chicken is safe as evidence by the fact that approximately 40% of the foods listed in Table 6 have higher folate per serving than cultured chicken.
4) It is also important to point out that differentiating natural sources (e.g., spinach) of folate from cultivated meat is erroneous. Plants are similar to chicken cells in that folate is required for metabolic processes of cells. There are a wide host of reasons for different levels of folate found in different organisms (plants and animals), including genetics, soil conditions and climate. Folate is stored in cellular tissues (whether those tissues are in an intact plant or animal or grown in culture) and utilized when needed. Folate is an essential component of all living things, regardless of whether they are cultivated in a well-controlled bioreactor or in a less controlled farm field.

5.2.3.2. Ferric nitrate ($Fe(NO_3)_3$-$9H_2O$)

In the manufacturing process of C1F-P1 cultured chicken, ferric nitrate is part of the basal media and a component of the seed and production cell culture media.

5.2.3.2.1. Regulatory Status

Ferric nitrate does not have a regulatory reference; however, in aqueous solution it dissociates into ferric and nitrate ions.[19] Iron (ferric ion) is a direct food substance affirmed as generally recognized as safe *per* 21 CFR §184.1375 with no use limitation other than current good manufacturing practice. Another regulation tangentially similar to ferric nitrate is ferric phosphate, an affirmed GRAS nutrient supplement with no limitation other than cGMP (21 CFR § 184.1301). However, it should be noted that dissociation of ferric phosphate does not occur readily in aqueous solution but does occur in acidic solution.[20] Nitrates are regulated as a food additive under 21 CFR

[18] w/w=weight/weight
[19] https://pubchem.ncbi.nlm.nih.gov/compound/25251#section=Solubility (last accessed July 20, 2021).
[20] https://pubchem.ncbi.nlm.nih.gov/compound/24861#section=Experimental-Properties (last accessed July 20, 2021).

§ 170.60 and 21 CFR § 172.170 (which allows nitrates to be added in curing premixes for meat not to exceed 500 parts *per* million in the finished product). Similar substances containing nitrates exist in regulation such as sodium nitrate and potassium nitrate (21 CFR § 181.33; 181.34; 172.160). As a meat preservative, these substances are added at a level of approximately 100 – 150 mg/kg (Karwowska and Kononiuk, 2020), which is approximately 5 – 7.5 times more than that found in cultured chicken (see Exposure Assessment below). However, the utility of nitrate in cultured chicken is not that of a preservative, but rather a source of nitrogen for cell culture.

While nitrate as a dissociated component of ferric nitrate is not intended as a preservative, the nitrate levels in cultured chicken are less than levels commonly utilized for preservation of meat. Similarly, iron levels in cultured chicken are less than levels commonly utilized for fortification such as those levels to fortify breakfast cereal (18 mg/serving).[21] As such, the regulation regarding nitrate and similar salts, as well as iron as a direct food additive is relevant to the overall safety of ferric nitrate as a media constituent in the culture of C1F-P1 cells.

While the use of ferric nitrate for culture of cultured chicken is not explicitly stated in regulation, it is necessary to produce the final cultured chicken product. Nitrate is used as a source for nitrogen for cell culture while iron aids in electron transport and cellular respiration, proliferation, and regulation of gene expression (Boldt, 1999). Presence of iron in cell culture media is especially relevant in formulations that use a low concentration of bovine serum as iron deficiency can increase oxidative stress and favor genomic instability, compromising cell viability and proliferation of cultivated chicken cells. Based on the exposure assessment below, neither iron nor nitrates are incorporated into cultured chicken at an unsafe level. It is highly unlikely that an individual consuming cultured chicken would be overexposed to either iron or nitrates.

5.2.3.2.2. Dietary Exposure to Fe^{3+}

The ferric ion is an iron atom that has lost three electrons to form Fe^{3+}. Iron is a mineral that is naturally present in many foods, added to some food products, and available as a dietary supplement. Iron is also an essential component of hemoglobin, an erythrocytic (red blood cell) protein. FDA has established the Recommended Dietary Intake (RDI) value of iron to be 18 mg *per* day for adults and children over 4 years of age, 11 mg *per* day for infants through 1 year of age, 7 mg *per* day for children of age 1 through 3 years and 27 mg *per* day for pregnant women (21 CFR § 101.9). According to the NIH, "the average daily iron intake from foods and supplements is 13.7 – 15.1 mg/day in children aged 2 – 11 years, 16.3 mg/day in children and teens aged 12 – 19 years, and 19.3 – 20.5 mg/day in men and 17.0 – 18.9 mg/day in women older than 19. The median dietary iron intake in pregnant women is 14.7 mg/day." [22]

To establish estimated consumer exposure values to iron in cultured chicken, four (4) representative batches of cultured chicken were evaluated for iron mineral by the ICP-MS method (Table 11).

Table 11. Estimated consumer exposure values of Iron from representative batches (RB) of cultured chicken

Representative batches (RB)	**Iron amount per gram of cultured chicken (µg/g)**	**Iron amount *per* 100 g of cultured chicken (µg/100g)**	**Estimated intake of Iron in ready-to-serve food (RACC 85g) (µg/serving/person)**	**Estimated intake of Iron in ready-to-cook food (RACC 114g) (µg/serving/person)**

[21] https://ods.od.nih.gov/factsheets/Iron-%20HealthProfessional/ (last accessed July 20, 2021).
[22] https://ods.od.nih.gov/factsheets/Iron-%20HealthProfessional/ (last accessed July 20, 2021).

RB-1	< 0.50 (LOQ)	50	43	57
RB-2	< 0.50 (LOQ)	50	43	57
RB-3	1.10	110	94	125
RB-4	1.10	110	94	125
Average	0.8	80	68	91
Maximum	1.10	110	94	125

Calculations for dietary exposure are based on RACC values (9 CFR § 317.312). Estimated average and maximum iron intake was calculated as 68 µg/serving/person and 94 µg/serving/person, respectively, for ready-to-serve portions based on RACC value of 85 g chicken *per* serving. The estimated average and maximum iron intake from cultured chicken was calculated at 91 µg/serving/person and 125 µg/serving/person, respectively, for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Estimated average and maximum iron content in 100 g serving was calculated at 80 µg/100g and 110 µg/100g, respectively, which is lower than the average nutritional iron content defined by U.S. Department of Agriculture for conventional chicken at 370 µg/100g.[23]

The iron content in cultured chicken is approximately 1/3rd that of conventional chicken (0.37 mg/100g) and approximately 1/450th that of the UL for adults. This is based on the US Institute of Medicine Panel on Micronutrients, which states that the "Tolerable Upper Intake Level (UL) for adults is 45 mg/day of iron, a level based on gastrointestinal distress as an adverse effect" (IOM, 2001). As the maximum daily iron intake from foods and supplements is much lower than Recommended Daily Intake values, the daily intake from either conventional chicken or cultured chicken would not push iron intake to or above the UL level. Therefore, there is little concern regarding overexposure to iron from consumption of cultured chicken.

5.2.3.2.3. Dietary Exposure to Nitrates

Nitrates occur naturally in vegetables and are added to cured and processed meats. Following ingestion, nitrate is converted in the body to nitrite and stored and circulated in the blood (Jones, 2014). In addition, body stores of nitrate and nitrite may also be increased exogenously through the diet, mainly through the consumption of green leafy vegetables such as lettuce, celery, cress, and beetroot, which typically contain over 250 mg nitrate *per* 100 g fresh vegetable weight (Bryan, 2006; Hord *et al.*, 2009; Lundberg *et al.*, 2009). Nitrate levels in fresh vegetables were analyzed by ion-exchange chromatography which showed tah tsai (also known as mustard spinach) and spinach contain over 450 mg nitrate *per* 100 g fresh weight (Shimada and Ko, 2004). The concentration of nitrates in vegetables (Table 12) and meats (Table 13) are listed below.

[23] U.S. Department of Agriculture, FoodData Central; Chicken, broiler or dryers, breast, skinless, boneless, meat only, raw nutrient values. https://fdc.nal.usda.gov/fdc-app.html#/food-details/171077/nutrients (last accessed January 10, 2022).

Table 12. Mean concentration of nitrate in vegetables as adapted from HSU *et al.* (2009)

Vegetables	**Nitrate concentration (mg/kg)**
English spinach	4849.6 ± 573.6
Bok choy	1841.1 ± 84.0
Choy sum	1376.9 ± 56.0
Chinese cabbage	236.2 ± 27.4
Gai choy	1642.3 ± 126.0
Iceberg lettuce	48.0 ± 30.2

Table 13. Mean concentration of nitrates in meat (Hsu *et al.,* 2009; Refai and Sebaei, 2020)

Meat	**Nitrate concentration (mg/kg)**
Poultry meat (fresh)	13.0 ± 9.9
Poultry meat (minced)	58.5 ± 69.7
Poultry meat (strips)	27.2 ± 33.8
Poultry meat (luncheon)	20.6 ± 24.6
Beef meat (fresh)	20.4 ± 19.6
Beef meat (canned)	108.6 ± 131.6
Beef meat (basturma)	98.8 ± 15.9
Beef meat (luncheon)	25.4 ± 24.7
Hot dog	69.9 ± 11.3
Ham	19.0 ± 8.1
Salami	142.5 ± 36.3
Bacon	23.3 ± 8.2
Frankfurt	54.9 ± 8.7
Minced Beef	18.7 ± 6.2
Beef medallion	38.5 ± 14.9

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1995 established an ADI for nitrates as 3.7 mg/kg bw/day, which is equivalent to 222 mg nitrate *per* day for a 60 kg adult (JECFA, 1995).

To establish estimated consumer exposure to nitrates in cultured chicken, three (3) representative batches of cultured chicken were evaluated for nitrate ion *via* the EPA 353.2 colorimetry method (Table 14). The calculations for dietary exposure are based on RACC values (9 CFR § 317.312). The estimated maximum nitrate content was calculated to be 1.7 mg/serving/person for ready-to-serve portions based on a RACC value of 85 g chicken *per* serving. The estimated maximum nitrate content was calculated to be 2.28 mg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum nitrate content was calculated to be 20 µg/g (equivalent to 20 mg/kg), which is consistent with average nitrate content in fresh poultry meat (13.0 ± 9.9 mg/kg) (Refai and Sebaei, 2020). Nitrate levels in cultured chicken are also below the ADI value of 222 mg *per* day established by JECFA.

Table 14. Estimated consumer exposure values of Nitrate ion (NO^{3-}) from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Nitrate amount *per* gram of cultured chicken (µg/g)**	**Nitrate amount *per* 100g of cultured chicken (gm/100g)**	**Estimated intake of Nitrate in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Nitrate in ready-to-cook food (RACC) 114g) (mg/serving/person)**
RB-1	< 20	< 2	< 1.7	< 2.28

RB-2	< 20	< 2	< 1.7	< 2.28
RB-3	20	2	1.7	2.28
Maximum	20	2	1.7	2.28

5.2.3.2.4. Conclusion

Multiple batches of cultured chicken were analyzed for iron and nitrate ions. While the iron nutrient content was higher than the average nutritional iron content in conventional chicken, nitrate levels were consistent with the average nitrate content in fresh poultry meat. However, as indicated, iron nutrient content is not a concern based on recommended daily intake values and the tolerable upper intake level for iron. This testing demonstrates no carryover of unsafe concentrations of ferric nitrate in cultured chicken.

5.2.3.3. Hypoxanthine Sodium (Hypoxanthine Na)

In the manufacturing process of cultured chicken, hypoxanthine sodium is a component of the seed and production cell culture media. Hypoxanthine is a purine derivative. Purines naturally occur as nitrogenous bases of nucleotides.

5.2.3.3.1. Regulatory Status

Hypoxanthine Na dissociates into sodium and hypoxanthine in solution.[24] According to 21 CFR § 73.1329, hypoxanthine purine is allowed as a component of the guanine color additive used in coloring for externally applied drugs, provided that hypoxanthine is not more than 25% of overall composition.

5.2.3.3.2. Relevant Literature

Wilson and Wilson (1962) investigated the digestion, absorption, and metabolism of the purine ribonucleotides adenosine monophosphate and guanosine monophosphate in adult rat or hamster intestinal tissues. As part of these experiments, they also investigated hypoxanthine as a metabolite of purine ribonucleotide metabolism. The results of this study indicate that, generally, purine nucleosides and free bases absorbed from the intestinal lumen are subjected to degradation as well as utilization in the intestinal mucosa. The authors reported that the catabolism of hypoxanthine occurs through oxidation to xanthine, and xanthine to uric acid *via* the enzyme xanthine oxidase. There is a very high activity of xanthine oxidase in the intestinal mucosa, which can convert most of the absorbed purines into uric acid (Auscher *et al.*, 1980). Salati *et al.* (1984) found that hypoxanthine was metabolized in the rat intestine (primarily to uric acid) within 15 minutes of oral administration. As a result, absorbed purines enter the liver as uric acid and are thus not available for utilization by the body for incorporation into tissue nucleic acids.

As indicated, hypoxanthine is metabolized to form uric acid. At physiologic pH, uric acid is a weak acid, and exists primarily as urate, the salt of uric acid. As urate concentration increases in blood, uric acid crystal formation increases. The normal reference interval of uric acid in human blood is 1.5 to 6.0 mg/dL in women and 2.5 to 7.0 mg/dL in men. Normally, most daily uric acid disposal occurs *via* the kidneys (Maiuolo *et al.*, 2016). The solubility of uric acid in water is low, and in humans, the average concentration of uric acid in blood is close to the solubility limit (6.8 mg/dL). When the level of uric acid is higher than 6.8 mg/dL, crystals of uric acid form as

[24] https://pubchem.ncbi.nlm.nih.gov/compound/Hypoxanthine#section=Solubility (last accessed July 20, 2021).

monosodium urate (MSU). Humans cannot oxidize uric acid to the more soluble compound allantoin due to the lack of uricase enzyme (Jin *et al.*, 2012). This commonly leads to gout. Therefore, for the management of hyperuricemia and gout, the recommended daily intake of dietary purines should be less than 400 mg/day (Kaneko *et al.*, 2014).

5.2.3.3.3. Dietary Exposure

Additionally, hypoxanthine is a naturally occurring purine that is present in a variety of foods. The purine content in food groups was reported by Wu *et al.* (2019), and further reported values for hypoxanthine in a variety of food items (Table 15).

Table 15. Hypoxanthine content in various food groups (Wu *et al.,* 2019)

Food Items	**Hypoxanthine content (mg/100 g wet weight)**		
	Mean	**Minimum**	**Maximum**
Beef organ products	35.2	0	96.6
Beef (other than organs)	62.2	36.7	87.2
Cereal grains and pasta	2.5	0	31.9
Dairy and eggs	0.2	0	1.5
Fish and Shellfish	68.7	0	512
Fruits and fruit juices	0.3	0.1	0.5
Lamb organ products	37	20	54
Lamb (other than organs)	78.3	65.3	100.8
Legumes	12.7	0	32.9
Pork organ products	54.4	34	71
Pork (other than organs)	65.5	43.6	90.4
Poultry organ products	40.1	0	71
Poultry (other than organs)	80.6	22.2	131
Sausages and luncheon meats	47	15	92.1
Soups and sauces	112	0.5	657
Vegetables	4.1	0	73.3

This data indicates that mean hypoxanthine content (mg/100g) is highest in the soups and sauces group (112 mg); and poultry products group (80.6 mg). Mean hypoxanthine is lowest in plant-based foods, dairy, and eggs (0 – 12.7 mg).

Other data indicates that the endogenous hypoxanthine levels in traditional, uncooked chicken ranges from approximately 300 – 1115 mg/kg (Sarwar *et al.*, 1985), and 353 mg/kg (Scarborough *et al.*, 1993). Scarborough *et al.* (1993) also reported that the hypoxanthine level in cooked chicken is approximately 121 mg/kg.

To establish estimated consumer exposure to hypoxanthine in cultured chicken, four (4) representative batches of cultured chicken were evaluated for hypoxanthine *via* a Colorimetric Xanthine/Hypoxanthine Assay (Sigma Aldrich MAK186) (Table 16).

Table 16. Estimated consumer exposure values of Hypoxanthine from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Hypoxanthine amount *per* gram of cultured chicken (µg/g)**	**Hypoxanthine amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of Hypoxanthine in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Hypoxanthine in ready-to-cook food (RACC 114g) (mg/serving/person)**

RB – 1	337.25	33.725	28.67	38.45
RB – 2	727.70	72.77	61.85	82.96
RB – 3	496.80	49.68	42.23	56.64
RB – 4	312.85	31.29	26.59	35.66
Average	**468.65**	**46.87**	**39.84**	**53.43**
Maximum	**727.70**	**72.77**	**61.85**	**82.96**

Calculations for dietary exposure are based on RACC values as stated in 9 CFR § 317.312. Estimated average and maximum hypoxanthine content from cultured chicken was calculated to be 39.84 mg/serving/person and 61.85 mg/serving/person respectively for ready-to-serve portions based on a RACC value of 85 g *per* serving. The estimated average and maximum hypoxanthine content was calculated to be 53.43 mg/serving/person and 82.96 mg/serving/person respectively for ready-to-cook cultured chicken portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated average and maximum hypoxanthine content in cultured chicken was calculated to be 468.65 µg/g (equivalent to 46.87 mg/100g) and 727.7 µg/g (equivalent to 72.77 mg/100g), respectively. Maximum hypoxanthine content in cultured chicken is below the average hypoxanthine content in poultry products (80.6 mg/100g) (Wu *et al.*, 2019). Accordingly, considering the worst case scenario, cultured chicken does not pose a risk for individuals that may be monitoring their purine intake due to gout or hyperuricemia, as the recommended daily intake of purines in this population is advised to be less than 400 mg/day (Kaneko *et al.*, 2014), which is 10X above the average level present in cultured chicken.

It is important to note that the data provided on hypoxanthine content in conventional chicken samples are from multiple published studies, all of which used an HPLC method. For the cultured chicken samples, hypoxanthine data was produced by a colorimetric assay in a 96-well plate format from a commercially available kit. While different methods, both have the sensitivity to accurately measure the hypoxanthine content in conventional chicken samples or cultured chicken samples. The HPLC data for conventional chicken samples is not from validated methods, whereas the cultured chicken data comes from a method that has been validated. However, the conventional chicken data is published in peer-reviewed journals, which provides an appropriate comparison to the cultured chicken samples.

5.2.3.3.4. Conclusion

Multiple batches of cultured chicken were analyzed for hypoxanthine. Hypoxanthine content is lower than the average hypoxanthine content in conventional chicken, such that exposure to hypoxanthine does not pose a safety risk, even to those individuals monitoring their daily purine intake. Further, this testing demonstrates no carryover of unsafe concentrations of hypoxanthine in cultured chicken.

5.2.3.4. Lipoic acid

In the manufacturing process of cultured chicken, lipoic acid is part of the basal media and a component of the seed and production cell culture media.

Lipoic acid is a necessary cofactor for mitochondrial α-ketoacid dehydrogenases, and thus serves a critical role in mitochondrial energy metabolism. Lipoic acid is synthesized by plants and animals, including humans (Carreau, 1979; Reed, 2001), but may also be absorbed intact from dietary sources, and transiently accumulates in many tissues (Wollin and Jones, 2003).

Lipoic acid contains two thiol (sulfur) groups, which may be oxidized or reduced; dihydrolipoic acid is the reduced form of lipoic acid (Figure 4) (Higdon et., 2002).

Figure 4. Chemical structures of Lipoic Acid (Higdon *et al.*, 2002)

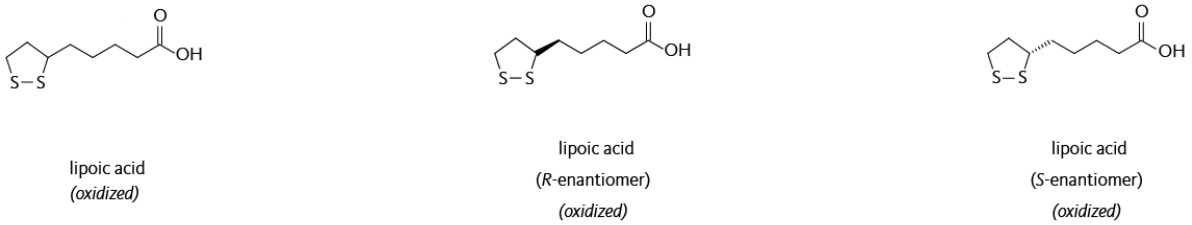


Figure 4. Chemical structures of Lipoic Acid (Higdon *et al.*, 2002)

Lipoic acid also contains an asymmetric carbon, which means that lipoic acid can exist as one of two possible optical isomers, or enantiomers: *R*-lipoic acid and *S*-lipoic acid. Only the *R*-enantiomer is endogenously synthesized and covalently bound to protein. *R*-lipoic acid occurs naturally in food, and free (unbound) lipoic acid dietary supplements may contain either *R*-lipoic acid or a 50:50 (racemic) mixture of *R*-lipoic acid and *S*-lipoic acid. *R*-lipoic acid is the isomer that is synthesized by plants and animals and functions as a cofactor for mitochondrial enzymes in its protein-bound form. Direct comparisons of the bioavailability of the oral racemic mixture and *R*-lipoic acid supplements have not been published; however, following the ingestion of *R*,*S*-lipoic acid, peak plasma concentrations of *R*-lipoic acid were found to be 40% – 50% higher than *S*-lipoic acid, suggesting better absorption of *R*-lipoic acid. Both isomers were nonetheless rapidly metabolized and eliminated (Gleiter *et al.*, 1996; Hermann *et al.*, 1996; Breithaupt-Grögler *et al.*, 1999). Based on our review, published human studies have solely used *R*,*S*-lipoic acid (racemic mixture), and not strictly *R*- or *S*-lipoic acid. This racemic mixture is commonly referred to as *alpha*-lipoic acid, or ALA, which is the subject of the toxicokinetic and safety evaluation below.

5.2.3.4.1. Regulatory Status

Currently, there is no relevant regulation related to lipoic acid.

5.2.3.4.2. Relevant Literature

While food sources of ALA (as R-(+)) are reported to be absorbed as lipoyllysine and bound in plasma, supplemental ALA (as racemic mixture or as R-(+)) is readily absorbed and is present in the circulation in a free form. The free form ALA in circulation is thought to originate as a result of the endogenous production of ALA (EFSA, 2021). Apart from its free form and lipoyllysine, ALA also circulates systemically, weakly bound to proteins *via* hydrogen bonds (Khan *et al.*, 2015).

In humans, maximum plasma concentrations (T_{max}) of ALA following ingestion are generally reached within 1 hour, although the formulation (solid *vs*. liquid) and presence of food may delay absorption, resulting in a mean T_{max} of up to 2.5 hours (EFSA, 2021). Following

absorption, ALA is rapidly cleared from circulation (mean plasma half-life of ~30 minutes). ALA is taken up by cells throughout the body and reduced to dihydrolipoic acid and metabolised *via* β-oxidation. The main metabolite found in plasma is 4,6-bis(methylthio)hexanoic acid, which is excreted in the urine (EFSA, 2021). Overall, the ALA pharmacokinetic parameters support a linear and proportional dose-dependent response over an oral dose range of 50 – 600 mg (EFSA, 2021).

Available data support that ALA has low acute toxicity when administered by gavage to rats (LD_{50} > 2,000 mg/kg body weight) (Cremer *et al.*, 2006a). Feeding studies in male and female rats administered up to approximately 60 mg/kg bw/day for 4 weeks or 2 years, reported no adverse effects and was concluded to be the NOAEL in both studies (Cremer *et al.*, 2006a, 2006b). In the 4-week study, the highest dose tested (121 mg ALA/kg body weight/day) resulted in slight alterations in liver enzymes and some histopathological effects in the liver and mammary gland (Cremer *et al.*, 2006a). At a dose of 180 mg/kg bw/day in the 2-year study, a significant reduction in feed intake was noted in both sexes, which was associated with a concomitant decrease in body weight. The decreased bodyweight was associated with some significant absolute weights of certain organs; however, no gross or histopathological changes were associated with these findings and were therefore not considered to be toxicologically significant (Cremer *et al.*, 2006b).

ALA was not mutagenic in bacterial reverse mutation assays *in vitro* and did not elicit genotoxicity in either an *in vivo* micronucleus or chromosome aberration tests in mice (Cremer *et al.*, 2006a; Tripathi *et al.*, 2020).

ALA has been evaluated in numerous clinical studies conducted to assess the impact on various health conditions including psychiatric and neurological disorders (de Sousa *et al.*, 2019), effects on lipid profiles (Mousavi *et al.*, 2019), symptomatic diabetic polyneuropathy (McIlduff and Rutkove, 2011; Han *et al.*, 2012; Nguyen and Takemoto, 2018), inflammatory biomarkers (Akbari *et al.*, 2018; Vajdi *et al.*, 2021) and glycemic indices (Mahmoudi-Nezhad *et al.*, 2021). General reviews conducted by Salehi *et al.* (2019) and Moura *et al.* (2015), also evaluated additional effects including reduction in miscarriages in pregnant women.

Oral doses in those studies reviewed ranged between 200 and 2,400 mg/day (most were between 300 and 600 mg/day) for durations ranging from 2 weeks up to 4 years (generally between 1 to 12 months) (Nguyen and Gupta, 2021). ALA was found to be well-tolerated at doses of up to 600 mg/day, although the incidence of adverse events was not reported in all studies (McIlduff and Rutkove, 2011). Higher single doses of 1,200 and 1,800 mg/day were in some cases associated with an increased incidence of adverse events including nausea, vomiting, and headaches, as well as vertigo. However, doses of ALA divided throughout the day (600 mg, 3 times daily) were reported to be well-tolerated in a 5-week study (McIlduff and Rutkove, 2011).

Recently, an association of ALA intake and incidence of insulin autoimmune syndrome (IAS) has been investigated by Health Canada (2016), the European Food Safety Authority Panel on Nutrition, Novel Foods and Food Allergens (EFSA, 2021), and other authoritative bodies as summarized in the EFSA opinion, following consumption of supplemental (dietary supplements) or medicinal ALA. IAS is an autoimmune disease characterized by spontaneous hypoglycemic episodes due to high titers of insulin autoantibodies. There is a genetic predisposition to increased risk of developing the disease due to the presence of Human Leukocyte Antigen HLA-DR4, with Asian populations having a higher frequency of the allele (up to 12% of the population) *vs.* <6.0% in European populations (generally between 0.1% to 3.9%) (EFSA, 2021). Based on the EFSA Panel's review of the scientific literature, 49 cases of IAS were linked to ALA intake. The doses, when reported, varied between 200 and 800 mg/day, while the time to onset in the investigated

case reports ranged from 1 week to 4 months (7 to 120 days). There was no obvious association between dose and time to onset. Among 27 cases in which symptoms of adverse effects were reported, 12 included subjects losing consciousness or going into hypoglycemic coma. Additional symptoms reported consisted mostly of sweating, tremors, dizziness, fatigue, weakness, confusion, hunger, and palpitations. In 44 cases this occurred without concomitant intake of other substances that have been reported to be a potential trigger of IAS. The reported times to onset of IAS are compatible with the emergence of an autoimmune disease and resolved upon withdrawal of ALA within several weeks to months.

EFSA concluded that "there is an association between the consumption of ALA and an increased risk of development of IAS in individuals with certain polymorphisms in the HLA region" (EFSA, 2021). No threshold dose for induction of IAS could be established due to a lack of sufficient data. Health Canada (2016) arrived at a similar conclusion that ALA may be associated very rarely with IAS in genetically predisposed individuals.

ALA-associated IAS is a very rare occurrence given the widespread use of ALA as a dietary supplement and the extensive clinical research conducted with this compound and as such would not be a significant safety concern for the majority of consumers. It also resolved in all cases following cessation of intake of ALA within weeks or months.

5.2.3.4.3. Dietary Exposure

Typical dietary sources of lipoic acid are muscle meats, heart, kidney, and liver, and to a lesser degree, fruits and vegetables (Akiba *et al.*, 1998; Packer *et al.*, 2001; Wollin and Jones, 2003). The approximate lipoic acid content in meat and various foods is shown in Table 17 and Table 18, respectively. Lipoic acid is also widely used in dietary supplements and cosmetics due to its antioxidant properties (Wollin and Jones, 2003).

Table 17. Lipoic acid content in commercial quality meat (adapted from Mattulate and Baltes, 1992)

Meat source	**Lipoic Acid content (mg/kg)**			
	Liver	**Kidney**	**Heart**	**Muscle**
Bovine	0.6 – 1.1	0.9 – 1.3	0.7 – 1.0	0.2 – 0.4
Calf	0.3 – 0.5	0.5 – 0.7	0.5 – 0.7	0.07 – 0.15
Lamb	0.7 – 0.8	0.5 – 0.7	0.5 – 0.7	0.2 – 0.4
Pig	0.6 – 0.8	0.4 – 0.7	1.1 – 1.6	0.15 – 0.3

Table 18. Lipoic acid content in various foods (adapted from Durrani *et al.,* 2010)

Food	**Lipoic Acid (mg/kg)**
Fresh chicken egg yolk	0.5 – 0.9
Boiled chicken egg yolk	< 0.2
Fresh chicken egg white	< 0.3
Boiled chicken egg white	< 0.1
Dried chicken egg powder	1.3
Mayonnaise	0.5
Fresh potatoes	1.5 – 4.2
Canned peas	0.5 - 1

Some studies reported that dietary supplementation of ALA in poultry can influence growth performance indicators, immunological, biochemical characteristics, lipid metabolism, and

oxidative stress as well as increase antioxidant potential and storability of poultry meat and meat products (El-Senousey *et al.*, 2013; Sohaib *et al.*, 2017). Khan *et al.* (2015) conducted a study investigating the effects of ALA on various poultry production parameters. As part of the study design, lipoic acid content was measured in the thigh meat of broilers following dietary feeding of basal feed as compared to feed supplemented with ALA. Table 19 demonstrates that without supplementation of ALA in the diet, broiler thigh meat contains approximately 22.17 mg/kg lipoic acid. With supplementation in the diet, the maximum mean lipoic acid content in chicken thigh meat was observed to be 69.40 mg/k (Khan *et al.*, 2015).

Table 19. Lipoic acid content in chicken boiler thigh meat, adapted from Khan *et al.* (2015)

Basal feed duration	**Lipoic Acid in chicken thigh meat (mg/kg)**
Day 0	25.8 $\pm$ 1.2
Day 30	22.1 $\pm$ 1.4
Day 60	18.6 $\pm$ 0.9
Mean	22.2 $\pm$ 1.5

To establish estimated consumer exposure to lipoic acid from cultured chicken, four (4) representative batches of cultured chicken were evaluated for lipoic acid *via* USP38-NF35[25] (Table 20). Results of this analysis demonstrate that lipoic acid content is below the limit of quantification (LOQ) of 20 ppm in all representative batches analyzed. Thus, cultured chicken contains no residual lipoic acid above the LOQ. Assuming the level of lipoic acid is at the LOQ in cultured chicken, the estimated maximum lipoic acid content was calculated to be 1.7 mg/serving/person for ready-to-serve portions of cultured chicken based on a RACC value of 85 g chicken *per* serving. The estimated maximum lipoic acid content was calculated to be 2.28 mg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum lipoic acid content was calculated to be 20 µg/g (equivalent to 2.0 mg/100g, or 20 mg/kg).

Table 20. Estimated consumer exposure values of Lipoic Acid from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Lipoic acid amount *per* gram of cultured chicken (µg/g)**	**Lipoic acid amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of Lipoic acid in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Lipoic acid in ready-to-cook food (RACC 114g) (mg/serving/person)**
RB-1	< LOQ	< LOQ	< LOQ	< LOQ
RB-2	< LOQ	< LOQ	< LOQ	< LOQ
RB-3	< LOQ	< LOQ	< LOQ	< LOQ
RB-4	< LOQ	< LOQ	< LOQ	< LOQ
Maximum	**<20**	**<2.0**	**<1.7**	**<2.28**

LOQ = Limit of Quantification (20 ppm)

Based on the mean lipoic acid content in chicken broiler thigh meat reported in literature (22.17 mg/kg), assuming the level of lipoic acid in cultured chicken is at the LOQ, the lipoic acid content would approximately be equivalent in both conventional chicken and Good Meat cultured chicken.

By utilizing the dose in literature that exhibited no adverse effects (NOAEL), an ADI can be established. Utilizing 60 mg/kg bw/day as the NOAEL (Cremer *et al.*, 2006a, 2006b) and

[25] USP 38- NF 35, pp 6128 - 6130, The United States Pharmacopeial Convention, Inc. Rockville, MD. (2015)

utilizing safety factors to account for interspecies and intraspecies toxicodynamic and toxicokinetic differences (a 100-fold safety factor), an ADI of 0.6 mg/kg bw/day for humans may be stated. This value is approximately equivalent to 36 mg/day for an adult weighing 60 kg. The level of lipoic acid in cultured chicken (<2.28 mg/serving) is far below this value, such that dietary exposure to lipoic from cultured chicken does not pose a safety risk.

5.2.3.4.4. Conclusion

Multiple batches of cultured chicken were analyzed for lipoic acid. Lipoic acid is present below the LOD in cultured chicken. Even after assuming the level of lipoic acid in cultured chicken is equivalent to the LOQ, the anticipated exposure is far below the calculated ADI from literature, such that exposure to lipoic acid does not pose a safety risk. Further, this testing demonstrates no carryover of unsafe concentrations of lipoic acid in cultured chicken.

5.2.3.5. Putrescine 2HCl (1,4 Butanediamine 2HCl)

In the manufacturing process of cultured chicken, Putrescine 2HCl (1,4 Butanediamine 2HCl) is part of the basal media and a component of the seed and production cell culture media.

Putrescine and other polyamines are involved in various biological processes, such as cell proliferation and differentiation, and also have antioxidant properties that have important implications on human health, intestinal maturation, and development of the immune system (Muñoz-Esparza *et al.*, 2019). They are found in all living cells, including microorganisms, plants, and animals.

5.2.3.5.1. Regulatory Status

1,4-Butanediamine is discussed as a manufacturing component of nylon resins in the FDA indirect food contact substance list, found at 21 CFR § 177.1500. There is no other relevant regulation related to putrescine.

5.2.3.5.2. Relevant Literature

5.2.3.5.2.1. Absorption, Distribution, Metabolism, and Excretion (ADME)

In the *de novo* synthesis of polyamines, putrescine is converted to spermidine *via* spermidine synthase; subsequently, spermidine is transformed into spermine *via* spermine synthase (Muñoz-Esparza *et al.*, 2019). The interconversion of polyamines is a cyclic process that controls their turnover and regulates intracellular homeostasis.

Polyamines are absorbed in the duodenum and in the first portion of the jejunum by various mechanisms, including transcellular (through passive diffusion and transporters) and paracellular pathways (Larqué *et al.*, 2007). Polyamines are partly metabolized in the intestinal wall before reaching the blood circulation, and those that pass into the circulation are distributed throughout the organism and captured by the tissues, where they can undergo interconversion reactions. The highest concentrations of polyamines are found in the intestine, thymus, and liver. Polyamines arrive in the intestinal lumen in millimolar concentrations and disappear from the lumen rapidly and completely. However, their content in systemic circulation hardly exceeds 10 – 20 µM. Despite this apparent paradox of high supply/low utilization of luminal polyamines, it has been repeatedly shown that luminal polyamines are indeed absorbed in sufficient amounts and utilized for growth support throughout the body. In addition, they are involved in normal, adaptive, and neoplastic epithelial cell proliferation in the gut (Milovic, 2001).

5.2.3.5.2.2. Safety Studies

Experiments conducted in rats administered radiolabeled putrescine report that polyamines are readily taken up from the gut lumen, most likely by passive diffusion. Evidence of this absorption is supported by *in vitro* assay data (Bardócz *et al.*, 1995; Milovic *et al.*, 2001; EFSA, 2011). Extensive metabolism was determined following absorption, with more than 80% of the orally administered putrescine converted to other polyamines and non-polyamine metabolites, such as succinate (Bardócz *et al.*, 1995, 1998). In humans, ingestion of putrescine resulted in only 20% being recovered in blood, similarly indicating both rapid and extensive metabolism, likely taking place in the intestinal wall and/or liver (Rauscher-Gabernig *et al.*, 2012). Ingested putrescine is metabolized by diamine oxidase *via* oxidative deamination and is primarily excreted in the urine as monoacetyl-putrescine.

Oral administration of closely related surrogate compounds, ethylenediamine (2-carbon diamine) and 1,6-hexamethylene diamine (6-carbon diamine), to rats and humans corroborates that water-soluble polyamines (like putrescine) are rapidly (< 24 hours) excreted primarily in the urine (Yang and Tallant, 1982; Brorson *et al.*, 1990).

In an unpublished acute oral study in Wister rats (5/sex/group), the median lethal dose for putrescine was determined to be 740 mg/kg body weight (95% confidence limits: 650 – 850 mg/kg bw) (ECHA, 2014a). Likewise, a study conducted by Til *et al.* (1997) reported that the "approximate" median lethal dose (LD_{50}) following gavage administration of putrescine to 4 rats, was 2,000 mg/kg body weight, based on the occurrence of 2 deaths at this dose, and death in all 4 rats administered 5,000 mg/kg body weight. The authors reported that most animals showed symptoms of sluggishness and piloerection following administration of numerous polyamines, while animals that died following putrescine administration showed signs of tremor. The same investigators conducted a subchronic study in which weanling Wistar rats (10/sex/group) were administered diets containing 0, 200, 2000, or 5000 ppm putrescine for 4 weeks.[26] There was no mortality in any of the groups and the only significant finding was a slight but statistically significant reduction in body weights in females of the highest dose group. As such the NOAEL was concluded to be 2000 ppm, which was reported to be equivalent to 180 mg/kg bw/day. No significant differences were reported in relation to food intake (except high dose males), food efficiency, water intake, blood pressure, hematology, clinical chemistry (except slight but statistically significant increase in plasma alanine aminotransferase in high dose females vs. controls), or organ weights (except increased brain weight in high dose females), or macro- and micro-histopathology evaluations, which included amongst other organs/tissues, the liver, kidney, heart, and reproductive organs. Furthermore, a 90-day study briefly summarized by (Rauscher-Gabernig *et al.*, 2012), following administration of putrescine to rats at dose levels of up to 56 mg/kg body weight/day, reported no adverse effects.

Several in vitro mutagenicity and genotoxicity studies were summarized in the REACH registration dossier for putrescine (ECHA, 2014a), including a bacterial reverse mutation assay conducted in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100, and *Saccharomyces cerevisiae* D4 (up to 5 µL/plate), a DNA damage and repair assay in *Escherichia coli* indicator strains WP2uvrA, W3110/pol A+, and p3478/pol A- (up to 5 µL/plate), and a gene mutation test in mouse lymphoma L5178Y cells (up to ~700 to 825µg/mL depending on test

[26] Stated as 5 to 6 weeks.

conditions). All assays included the presence and absence of exogenous metabolic activation. Putrescine did not elicit a mutagenic or genotoxic response in any of the assays.

5.2.3.5.2.3. Dietary Exposure

Putrescine is one of the simplest polyamines, plays a role in cell division during cell culture, and has antioxidant properties. EFSA conducted an exposure assessment for putrescine in 2011; however, as putrescine is overly abundant in foods of plant origin, no maximum level of exposure to putrescine was proposed. Food categories that present consumers with the highest putrescine exposure (on a daily intake assessment) are fermented vegetables (4.9 – 164.7 mg putrescine/day), vegetables (13.4 – 93.6 mg putrescine/day), fermented sausages (14.5 – 83.6 mg putrescine/day), meat products (20.9 – 36.9 mg putrescine/day) and cheese (14.3 – 35.3 putrescine mg/day) (EFSA, 2011). Putrescine can accumulate at high concentrations in dairy fermented products such as cheese (up to 1560 mg/kg) and in fish and fish products (up to 337 mg/kg). Putrescine has also been detected in alcoholic beverages, like red wine and beer (EFSA, 2011), as well as breast milk and infant formula, and various other fruits, vegetables, nuts, seeds, and cereals (Muñoz-Esparza *et al.*, 2019). The background cumulative 95th percentile one-day intake of putrescine from all food sources, calculated across several European countries ranged between 35 and 138 mg/day (EFSA, 2011).

Humans consume putrescine from the background diet in a range of foods, particularly in certain fermented foods. No reports were identified that would indicate any adverse effects following the consumption of these foods, and a dose-response relationship delineating potentially toxic effects has not been established (EFSA, 2011). As mentioned above, polyamines can be found in all types of foods in a wide range of concentrations. Putrescine is present in breast milk and first formula and virtually in all foods of plant origin, being particularly abundant in citrus fruits and vegetables (Handa *et al.*, 2018). Table 21 lists putrescine concentrations in different food samples, such as grapefruits, oranges, peas, potatoes, among others.

Table 21. Putrescine content in various food samples (adapted from Ali *et al.,* 2011)

Foods	**Putrescine (mg/kg or mg/mL)**				**Total Polyamines**
	Mean	**SD**	**Min**	**Max**	**Mean**
Dairy products					
Milk 0.5% fat	1.2	0.2	0.9	1.5	2.20
Milk 1.5% fat	0.6	0.2	0.41	0.86	2.14
Milk 3% fat	0.36	0.1	0.26	0.6	0.57
Milk 4.5% fat	0.15	0.02	0.12	0.2	0.45
Yogurt	0.1	0.08	0.1	0.13	0.25
Sour milk	0.62	0.23	0.5	0.88	1.90
Swedish hard cheese 28% fat	1.63	0.05	1.58	1.62	3.40
Swedish hard cheese 31% fat	1.67	0.3	1.5	2.1	6.37
Danish hard mature cheese	52.3	5	66.1	73.8	56.10
Swedish hard cheese 28% fat	4.8	1	3.8	6.2	21.80
Swedish hard cheese 17% fat	4.4	0.5	3.9	5	20.10
Meat and Fish products					
Beef (raw, lean)	5.7	-	5.5	5.9	61.1

Beef (ground)	10.1	14.3	0.8	38.5	42.9
Pork (raw, lean)	3.0	-	2.9	3.1	6.9
Pork (raw, chops)	0.2	0.3	0	0.7	32.3
Sausage	14.2	-	13.8	14.5	45.3
Pork (ham, smoked)	4.1	-	4.0	4.3	-
Chicken (raw)	2.86	-	2.8	2.9	71.4
Chicken (grilled)	2.0	0.5	1.3	2.7	63.7
Cod Fish (raw)	1.4	0.9	0.5	3.1	2.6
Salmon (raw)	2.7	1.0	1.6	4.6	5.0
Chicken eggs (boiled)	0.3	-	0.3	0.4	0.5
Fruits and vegetables					
Potato (fresh)	9.7	2.1	5.8	12.8	23.9
Potato (cooked)	8.5	2.3	5.6	12.4	21.6
Cauliflower (fresh)	5.3	2.1	3.3	8.9	39.7
Cauliflower (cooked)	4.0	1.2	2.6	5.9	36.5
Broccoli (fresh)	6.4	2.9	2.5	3.4	57.6
Broccoli (cooked)	5.6	2.9	2.5	8.9	40.0
Spinach	12.9	23.7	2.5	119	22.4
Cucumber	6.9	1.4	5.5	8.7	15.5
Carrot	1.5	0.7	0.7	2.7	11.9
Tomato (concentrated)	25.9	8.2	7.9	41.1	36.4
Onion	6.4	-	5.5	7.2	14.2
Garlic	2.3	-	0.7	6.1	19.2
Ginger	2.6	-	0.6	3.7	6.7
Lettuce	5.6	1.3	4.5	7.3	15.5
Celeriac	6.1	-	3.7	7.7	32.8
Maize	50.7	-	18.3	85.4	73.3
Parsley	8.7	-	4.0	13.0	15.3
Asparagus	2.9	-	2.0	3.8	13.2
Pumpkin	6.6	-	3.2	10.8	44.3
Apple	1.0	-	0.4	1.7	3.5
Pear	24	-	23.6	24.2	-
Orange	137	11.3	119	153	141.3
Mandarin	122	44.2	67.3	200	124.7
Banana	12.3	-	11.2	13.8	18.7
Strawberry	1.0	-	0.8	1.2	3.4
Cherry	1.6	-	0.4	4.3	4.0
Wheat (whole grain)	3.4	0.5	2.5	4.0	22.8
Rice	0.2	-	0.2	0.3	1.2
Breakfast cereals	2.1	-	2.0	2.2	32.7
Bread (white)	1.7	-	1.5	1.8	10.4
Red kidney beans	0.4	-	0.3	0.4	44.2

Green peas (frozen)	46.3	27.0	11.7	107	96.7
Green peas (cooked)	4.9	-	4.3	5.4	18.2
Coffee	10.3	1.0	9.1	16.3	20.7
Orange Juice	85.0	11.4	76.6	100	87.5

Table 22 provides the amount of putrescine in the top 12 food sources on a mg/portion basis.

Table 22. Putrescine content for top 12 food sources (adapted from Ali *et al.*, 2011)

Food Item	**Portions in grams**	**Putrescine (mg/portion)**
Grapefruit juice, fresh	200	19.6
Orange juice	200	17
Sauerkraut	80	14.6
Orange	110	14
Crab, conserved	75	9.2
Maize	100	5.1
Peas, green	100	4.6
Pear	100	3
Soybean, cooked	190	1.7
Potato, cooked	150	1.68
Paprika, green	30	1.64
Soy sauce	18	1.6

To establish estimated consumer exposure to putrescine in cultured chicken, four (4) representative batches of cultured chicken were evaluated for total polyamine content using Fluorometric Total Polyamine Assay – Sigma Aldrich MAK349 (Table 23). Calculations for dietary exposure are based on RACC values (9 CFR § 317.312). The estimated average and maximum total polyamine content was calculated at 1.31 mg/serving/person and 1.91 mg/serving/person, respectively, for ready-to-serve portions based on a RACC value of 85 g chicken *per* serving. The estimated average and maximum total polyamine content from cultured chicken was calculated at 1.76 mg/serving/person and 2.57 mg/serving/person, respectively, for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated average and maximum total polyamine content was calculated to be 15.4 µg/g (equivalent to 1.54 mg/100g) and 22.5 µg/g (equivalent to 2.25 mg/100g), respectively. Although the total polyamine content in cultured chicken is higher than the putrescine content (0.29 mg/100g) in conventional raw chicken – Table 21, it is significantly lower (about five times lower than average and 3 times lower than maximum) than total polyamine content in conventional chicken (7.14 mg/100g) (Ali *et al.*, 2011). Even assuming that total polyamines content in cultured chicken is putrescine, this level is modest in comparison to dietary exposure from other sources (33.7 – 156 mg/100g). Additionally, utilizing the dose that exhibited no adverse effects (NOAEL) in literature, an ADI can be established. Utilizing 180 mg/kg bw/day as the NOAEL (Til *et al.*, 1997) and utilizing safety factors to account for interspecies and intraspecies toxicodynamic and toxicokinetic differences (a 100-fold safety factor), an ADI of 1.8 mg/kg bw/day for humans may be stated. This value is approximately equivalent to 108 mg/day for an adult weighing 60 kg. Considering the worst-case scenario, the maximum level of putrescine in cultured chicken is far below this value, such that dietary exposure to putrescine from cultured chicken does not pose a safety risk.

Table 23. Estimated consumer exposure values of Total Polyamines from representative batches (RB) of cultured chicken.

Representative batches (RB)	Total Polyamines amount *per* gram of cultured chicken (µg/g)	Total Polyamines amount *per* 100 g of cultured chicken (mg/100g)	Estimated intake of Total Polyamines in ready-to-serve food (RACC 85g) (mg/serving/person)	Estimated intake of Total Polyamines in ready-to-cook food (RACC 114g) (mg/serving/person)
RB – 1	9.1	0.91	0.77	1.04
RB – 2	15.0	1.50	1.28	1.71
RB – 3	22.5	2.25	1.91	2.57
RB – 4	15.1	1.51	1.28	1.72
Average	**15.4**	**1.54**	**1.31**	**1.76**
Maximum	**22.5**	**2.25**	**1.91**	**2.57**

Polyamine values in conventional chicken published by Ali *et al*. 2011 are from HPLC methods, either using UV or fluorescence detection. The method used here to analyze polyamines in cultured chicken samples utilizes a commercially available 96-well plate fluorescence assay. While not identical methods, both utilize the same method of detection and have similar sensitivities as demonstrated by comparable LODs (0.1 ppm for individual amines by HPLC methods, 0.01 ppm for total polyamines by 96-well plate assay). The HPLC data for conventional chicken samples is not from a validated method, whereas the cultured chicken data comes from a validated method. However, the conventional chicken data are published in peer-reviewed journals, which provide an appropriate comparison to the cultured chicken samples.

5.2.3.5.3. Conclusion

In summary, putrescine is a compound produced endogenously in all mammals and plants and is consumed in the background diet from a wide range of foods, including cheese, fermented sausages, fish sauces, fermented vegetables, red wine and beer and various fruits, vegetables, nuts, seeds, and cereals (EFSA, 2011; Rauscher-Gabernig *et al.*, 2012; Muñoz-Esparza *et al.*, 2019). Exposure from the background diet can be as high as 138 mg/day, which is considerably greater than would be present within cultured chicken as an impurity from the growth media. The body appears to have a strong capacity to metabolize ingested putrescine *via* the enzyme diamine oxidase, which is present at high levels throughout the intestinal tract. Studies with intestinal cells from both rats and humans support the extensive metabolism of ingested putrescine. Feeding studies in rats indicate that intake of up to 180 mg/kg body weight/day does not have any serious adverse effects. Multiple batches of cultured chicken were analyzed for total polyamines, and total polyamine content was determined to be at a level of exposure that does not pose a safety risk.

5.2.3.6. Sodium pyruvate

In the manufacturing process of cultured chicken, sodium pyruvate is a component of the seed and production cell culture media. Sodium pyruvate dissociates into sodium and pyruvate in aqueous solution.[27]

5.2.3.6.1. Regulatory Status

Pyruvic acid is a FEMA-recognized GRAS flavoring agent with no safety concern according to JECFA (the threshold for human intake as a flavoring agent was established as 1.8

[27] https://www.chemicalbook.com/ChemicalProductProperty_EN_CB4252757.htm (last accessed January 8, 2022).

mg/day based on structural class) (JECFA, 2001). Pyruvic acid (as well as isoamyl pyruvate and ethyl pyruvate) is among the substances approved as a flavoring substance by FDA (21 CFR § 172.515) for use in the minimum quantity required to produce the intended effect and otherwise in accordance with cGMP. While pyruvic acid as a dissociated component of sodium pyruvate is not intended as a flavoring agent, ultimately, its levels in cultured chicken are similar (1.04 – 1.42 mg/serving based on RACC values, see below). As such, the regulation regarding pyruvic acid and similar salts is relevant to the overall safety of sodium pyruvate as a media constituent in the culture of C1F-P1 cells.

5.2.3.6.2. Relevant Literature

Pyruvate (the ionic form of pyruvic acid) is a breakdown product of normal body metabolism and is the 3-carbon intermediate product of the glycolysis pathway (Koh-Banerjee *et al.*, 2005). Pyruvic acid supplies energy to cells though the citric acid cycle in the presence of oxygen by aerobic respiration. Hence, pyruvate is present in every living cell. Although there is a lack of consensus on its mechanism of action, some authors have suggested that orally consumed pyruvate induces weight loss *via* increased fat metabolism (Kalman *et al.*, 1999).

In 2009, EFSA reviewed the safety of calcium pyruvate and magnesium pyruvate as added for nutritional purposes to food supplements (EFSA, 2009). While sodium pyruvate was not included as part of this evaluation, the Panel noted that it is likely that calcium and magnesium are readily absorbed from orally ingested soluble organic salts, such that it is expected the salts would dissociate to their respective ions. Therefore, this evaluation is relevant to sodium pyruvate, as it is also expected to dissociate into its respective ions. The Panel stated that no UL has been established for pyruvate. Based on the proposed use of calcium pyruvate and magnesium pyruvate, the maximum exposure to pyruvate *per* day would be 3.4 g/day. Therefore, the Panel concluded that the intake of pyruvate "from the corresponding sources is not of safety concern" (EFSA, 2009).

5.2.3.6.3. Dietary Exposure

Pyruvate occurs naturally in plant tissues as a part of metabolism intermediates, and as such high levels of pyruvate can be found in onion, leek, shallot, garlic, red potato, carrot, bell pepper, and broccoli (Table 24) (Yoo and Pike, 2001).

Table 24. Pyruvic acid concentration in food items (adapted from Yoo and Pike, 2001).

Food Item	**Pyruvic Acid concentration ($\mu mol\ mL^{-1}$) ± SD**
Onion (Chilean)	4.48 ± 0.46
Onion (red)	9.26 ± 1.52
Onion (yellow)	7.33 ± 1.00
Onion (white)	9.27 ± 1.01
Leek	9.98 ± 0.54
Shallot	11.82 ± 0.59
Garlic	66.24 ± 3.31
Red potato	0.09 ± 0.01
Carrot	0.10 ± 0.03
Bell Pepper	0.24 ± 0.05
Broccoli	1.31 ± 0.27

While pyruvate content of traditional chicken meat has not been determined, Belo *et al.* (1976) determined the unfasted blood concentration of pyruvate in chickens to be 222 nmoles/mL

(approximately equivalent to 19.5 µg/g of blood), after conducting a study of blood metabolites and glucose metabolism in fed and fasted chickens. While not a substitute for tissue levels, blood levels provide some context as to anticipated levels of pyruvate in tissues, though either may be influenced by the metabolic state of the organism (active *vs.* resting state).

Pyruvate has also been represented as a supplement that may assist in weight loss and/or increased stamina during exercise. For this reason, various clinical trials have administered high doses of pyruvate (5 – 6 g/day) to human subjects for periods ranging from 4 – 6 weeks (Kalman *et al.*, 1999; Koh-Banerjee *et al.*, 2005). The results of these studies indicate that pyruvate doses of this magnitude and duration are generally well-tolerated.

To establish estimated consumer exposure to pyruvate in cultured chicken, four (4) representative batches of cultured chicken were evaluated for pyruvate *via* a Colorimetric Pyruvate Assay (Sigma Aldrich MAK071) (Table 25). Calculations for dietary exposure are based on RACC values as stated in 9 CFR § 317.312. Estimated average and maximum pyruvate content was calculated at 1.04 mg/serving/person and 1.59 mg/serving/person, respectively, for ready-to-serve portions of cultured chicken based on a RACC value of 85 g chicken *per* serving. The estimated average and maximum pyruvate content was calculated to be 1.39 mg/serving/person and 2.13 mg/serving/person, respectively, for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated average and maximum pyruvate content was calculated to be 12.2 µg/g (equivalent to 1.22 mg/100g) and 18.7 µg/g (equivalent to 1.87 mg/100g), respectively, which is slightly less than the average pyruvate content in poultry blood (1.95 mg/100g) (Belo *et al.*, 1976). Considering the worst-case scenario, the maximum pyruvate content in cultured chicken is similar to the threshold for human intake as a flavoring agent (1.8 mg/day), and according to EFSA's evaluation of other pyruvate salts, an exposure of up to 3.4 g pyruvate/day did not pose a safety concern.

Table 25. Estimated consumer exposure values of Pyruvate from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Pyruvate amount *per* gram of cultured chicken (µg/g**	**Pyruvate amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of Pyruvate in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Pyruvate in ready-to-cook food (RACC 114g) (mg/serving/person)**
RB – 1	11.05	1.11	0.94	1.26
RB – 2	9.45	0.95	0.80	1.08
RB – 3	18.7	1.87	1.59	2.13
RB – 4	9.6	0.96	0.82	1.09
Average	**12.2**	**1.22**	**1.04**	**1.39**
Maximum	**18.7**	**1.87**	**1.59**	**2.13**

5.2.3.6.4. Conclusion

Multiple batches of cultured chicken were analyzed for pyruvate. Maximum pyruvate content in cultured chicken is lower than the average pyruvate content in conventional chicken blood; is similar to the level used for flavoring (1.8 mg) and is far below the level stated by EFSA as posing no safety concern (3.4 g), such that exposure to pyruvate does not pose a safety risk. Further, this testing demonstrates that there is no carryover of unsafe concentrations of pyruvate in cultured chicken.

5.2.3.7. Thymidine

Thymidine is a pyrimidine nucleoside composed of the pyrimidine base, thymine attached to the sugar deoxyribose. In the manufacturing process of cultured chicken, thymidine is a component of the seed and production cell culture media.

5.2.3.7.1. Regulatory Status

Currently, there is no relevant regulation related specifically to thymidine. However, several pyrimidines are listed as FEMA recognized GRAS flavoring agents or adjuvants in FDA's Substances Added to Food Database.[28]

5.2.3.7.2. Relevant Literature

Thymidine is a natural nucleoside and is a precursor to DNA synthesis. Mechanistically, thymidine in the cell is phosphorylated by thymidine kinase and is incorporated into DNA synthesis as a nucleotide. As a constituent of DNA, thymidine pairs with adenine in the DNA double helix. Alternately, thymidine can undergo catabolism into thiamine and a pentose sugar, which may be utilized in nucleotide synthesis and glycolysis, respectively (Scarborough *et al.*, 1993).

During the normal course of food intake, nucleic acids, from either intact or degraded DNA, first undergo digestion by pepsin in the stomach (Liu *et al.*, 2015), followed by pancreatic nuclease cleavage into nucleotides, which are then cleaved into nucleosides and phosphoric acid by alkaline phosphatase and nucleotidases found on the luminal surfaces of mucosal cells (Jonas *et al.,* 2001). Nucleosides are then available for absorption intact or can be further cleaved by nucleosidases to produce sugars and purine and pyrimidine bases (ECHA, 2014b). Transport of nucleosides into the enterocyte occurs *via* both facilitated diffusion and a specific Na^+-dependent carrier-mediated mechanism (He *et al.*, 1994; Jonas *et al.*, 2001). Once absorbed, the nucleosides and bases are rapidly degraded within the enterocyte, and catabolic products are utilized in DNA/RNA production or excreted in the urine and feces. Systemic metabolism is mainly *via* thymidine kinase leading to the formation of β-aminoisobutyrate, ultimately resulting in the formation of water and CO_2 (ECHA, 2014b). Based upon information obtained from animal studies, only 2% to 5% of nucleotides consumed within the diet are incorporated into tissue pools (Jonas *et al.*, 2001), which indicates that most of the ingested nucleotides and nucleosides within the various foods are extensively metabolized and excreted.

The main pathway of thymidine biosynthesis occurs by conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) (which then undergoes additional phosphorylation to thymidine triphosphate (dTTP) before incorporation into DNA) (Selman and Kafatos, 1974). Thymidine can be readily phosphorylated to dTMP by thymidine kinase, an enzyme that is part of the salvage pathway for nucleic acid biosynthesis. If thymidine is administered in very large quantities, the ensuing high level of dTTP exerts end-product feedback inhibition on a number of the *de novo* and salvage pathway enzymes (*e.g.*, ribonucleotide reductase, deoxycytidylate deaminase, thymidine kinase) leading to DNA synthesis (Martin *et al.*, 1980).

As summarized in the REACH registration dossier for thymidine (ECHA, 2014b), the toxicity of thymidine was evaluated in acute and repeat-dose oral studies in rodents. The acute oral

[28] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances (last accessed January 8, 2022).

medium lethal dose (LD_{50}) in female Wistar rats was greater than 2,000 mg/kg body weight, while the NOAEL following gavage administration of thymidine to male and female Sprague Dawley rats (10 animals/sex/dose) for six months was 1,000 mg/kg body weight/day, the highest dose tested. No treatment-related adverse effects were noted in relation to mortality, clinical observations, body weight, food consumption, ophthalmology, clinical pathology, including hematology, coagulation, and chemistry, or macroscopic/microscopic pathology between the treated and control animals.

In a combined rat fertility and embryo-fetal development study, male and female Sprague Dawley rats (25 animals/sex/group) were administered thymidine by gavage at doses of 0, 100, 500, or 1,000 mg/kg body weight/day, for 28 days prior to mating for males, and from 15 days prior to mating until gestation Day 17 for females (ECHA, 2014b). Male fertility was evaluated based on mating and impregnation rate; sperm count, mobility, and morphology; and microscopic examinations of testes and epididymides. Female fertility was evaluated based on estrous cycling, mating and pregnancy rate, number of corpora lutea, number and distribution of implantation sites, early resorption, and microscopic examination of ovaries. Fetotoxicity and teratologic potential were evaluated based on mid and late resorptions; the numbers of live and dead fetuses; external sex; body weight; and gross external alterations and microscopic internal alterations, such as major malformations, minor external visceral and skeletal anomalies, and common skeletal variants. At the conclusion of the study, there were no test article-related effects observed in male or female fertility. The pregnancy rate was 76% in rats dosed at 500 mg/kg body weight/day and 72% in rats dosed at 1,000 mg/kg/day. Although these rates were slightly lower than that of the control group (92%), these remained within the historical control range of the testing facility. It was concluded that the NOEAL for male and female fertility, and fetal developmental effects was 1,000 mg/kg body weight/day, the highest dose tested (ECHA, 2014b).

The mutation potential of thymidine was evaluated in a bacterial reverse mutation assay in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and *Escherichia coli* strain WP2 using the plate incorporation and preincubation methods at concentrations of up to 5,000 µg/plate, with and without exogenous metabolic activation (ECHA, 2014b). It was reported that thymidine did not elicit a positive mutagenic response in any of the strains or concentrations, with and without metabolic activation and was therefore concluded to be non-mutagenic under the conditions of the test.

An *in vitro* cytogenicity/chromosome aberration study in Chinese hamster lung fibroblasts (V79) was conducted at concentrations of up to 5000 µg thymidine/mL with and without metabolic activation (ECHA, 2014b). Thymidine did not elicit structural chromosome aberrations under any of the conditions of the assay and was concluded to be not clastogenic.

In Chinese hamster ovaries (CHO), thymidine evaluated at concentrations of up to 5000 µg/mL with and without metabolic activation tested over a 5-hour period did not induce increases in mutation frequency compared to the background; however, when evaluated over 20 hours without metabolic activation, the mutant frequency of the cells showed statistically significant increases in mutation frequency at concentrations of 3500 to 5000 µg/mL (ECHA, 2014b). These increases were considered biologically significant and dose dependent. In the presence of metabolic activation, no increase in mutation frequency was observed over 20 hours.

In addition, genotoxicity studies on the structural analog L-thymidine, were summarized, which indicated that in an Ames test and two chromosome aberration tests (one using Chinese hamster ovary cells and one using human peripheral blood lymphocytes) no mutagenic or

clastogenic responses were observed. In an *in vivo* micronucleus test in male and female CD-1 mice administered L-thymidine at single gavage doses of 0, 500, 1000, or 2000 mg/kg bw, no evidence of chromosome damage was observed (ECHA, 2014b).

Although a positive mutagenic response was observed with thymidine in CHO cells without metabolic activation when evaluated after 20 hours, all the other results within the same study were negative. In conjunction with the negative results in the Ames assay, chromosome aberration assay in V79 cells evaluating thymidine, and the negative results in the *in vitro* assays and micronucleus test in mice with L-thymidine, thymidine was concluded to be non-genotoxic (ECHA, 2014b).

No specific safety studies in humans were identified in the literature for nucleotides or nucleosides; however, supplemental nucleotides have been evaluated generally for use in infant formula and various health conditions, as well as exercise performance (Carver, 1999; Dancey *et al.*, 2006; Hess and Greenberg, 2012). These studies provide limited evidence that exogenous nucleotides may have health-supportive properties under certain conditions.

It has been reported that high dietary intakes of DNA/RNA could be associated with increased urinary uric acid levels due to elevated purine catabolism, which can be a risk factor for developing gout (Hess and Greenberg, 2012); however, this would not apply to catabolism of pyrimidines like thymidine, as these do not produce uric acid.

5.2.3.7.3. Dietary Exposure

In healthy humans, because thymidine is produced endogenously, exogenous nucleoside/nucleotide intake within the diet is not considered necessary; however, with certain health conditions, supplemental nucleotide intake has been shown to potentially provide some supportive benefits with regards to gastrointestinal and immune issues and has been investigated in both adults and infants (Carver, 1999; Gil, 2002; Hawkes *et al.*, 2005; Dancey *et al.*, 2006). Nucleotides have also been evaluated for supporting exercise performance in healthy adults (Hess and Greenberg, 2012). As noted by Hawkes *et al.* (2005), human milk contains higher amounts of non-protein nitrogen as nucleotides, nucleosides, and nucleic acids compared with bovine milk, and as a result, the addition of nucleotides to infant formula has been investigated in relation to improving neonatal growth and intestinal tract maturation (Singhal *et al.*, 2010; Hess and Greenberg, 2012). It is reported that concentrations of nucleotides in human milk range from 0.4 to 7 mg/dL (Carver, 1999), which indicates that infants may be exposed to exogenous levels of thymidine.

While literature regarding the thymidine content of various foods has not been studied, the level of thymine, the defining constituent of thymidine can be utilized for comparison. The content of thymine in numerous foods, especially carbohydrate-rich ones, has been investigated by Lassek and Montag (1990). As shown in Table 26, broccoli has a high level of thymine content, at 493 mg/kg. The thymine content in meat, however, is shown to be much lower, at <3 mg/kg (Scarborough *et al.*, 1993) (Table 27). In a similar study, Sarwar *et al.* (1985) determined the thymine content of various poultry products on a mg/100mg total nitrogen basis. Calculating for thymine content on a mg/kg tissue basis, the mean thymine content was determined to be <1 mg/kg tissue.

Table 26. Thymine content in various fruits and vegetables (adapted from Lassek and Montag, 1990)

Food Group	**Thymine (mg/kg)**
Broccoli	493
Brussel sprouts	127
Green cabbage	177
Cauliflower	340
Young spinach	260
Avocado	81
Potato	149
Oyster mushrooms	213
Pea soup	144
Kidney beans	126
Chickpeas	182
Wheat	73

Table 27. Thymine content in meat, adapted from a study by Scarborough *et al.* (1993)

Meat Source	**Thymine (mg/kg)**
Raw chicken	< 3
Cooked chicken	< 3
Raw turkey	< 3
Chicken breast	< 3
Chicken leg	< 3
Turkey breast	< 3
Turkey leg	< 3
Turkey neck	< 3

To establish estimated consumer exposure to thymidine in cultured chicken, four (4) representative batches of cultured chicken were evaluated for thymidine *via* HPLC-UV (Table 28). In 3 of the representative batches (RB-1, RB-2 and RB-3), thymidine was below the limit of quantification (LOQ) of 3.13 µg/mL. In representative batch RB-4, thymidine content was 3.96 µg/mL which is slightly above the limit of quantification (LOQ). Thus, cultured chicken may contain extremely low residual thymidine content. The estimated maximum thymidine content was calculated to be 0.34 mg/serving/person for ready-to-serve portions of cultured chicken based on a RACC value of 85 g chicken *per* serving. The estimated maximum thymidine content was calculated to be 0.38 mg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum thymidine content was calculated to be 3.96 µg/g (equivalent to 0.396 mg/100g, or 3.96 mg/kg). Calculations for dietary exposure are based on RACC values as stated in 9 CFR § 317.312.

Table 28. Estimated consumer exposure values of Thymidine from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Thymidine amount *per* gram of cultured chicken (µg/g)**	**Thymidine amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of Thymidine in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Thymidine in ready-to-cook food (RACC 114g) (mg/serving/person)**
RB – 1	< 3.13 (LOQ)	< 0.31	< 0.27	< 0.36
RB – 2	< 3.13 (LOQ)	< 0.31	< 0.27	< 0.36
RB – 3	< 3.13 (LOQ)	< 0.31	< 0.27	< 0.36
RB – 4	3.96	0.40	0.34	0.38
Maximum	**3.96**	**0.40**	**0.34**	**0.38**

LOQ = Limit of Quantification (3.13 µg/mL)

Additionally, utilizing the dose that exhibited no adverse effects (NOAEL) in literature, an ADI can be established. Using 1000 mg/kg bw/day as the NOAEL (ECHA, 2014b) and safety factors to account for interspecies and intraspecies toxicodynamic and toxicokinetic differences (a 100-fold safety factor), an ADI of 10 mg/kg bw/day for humans may be stated. This value is approximately equivalent to 600 mg/day for an adult weighing 60 kg. The maximum level of thymidine in cultured chicken is far below this value, such that dietary exposure to thymidine from cultured chicken does not pose a safety risk.

The data provided here on thymidine content in cultured chicken samples uses a reversed-phase HPLC method similar to that used by Scarborough *et al.* (1993) to measure thymine content in conventional chicken samples. In both methods, thymine and thymidine content is determined using standard curves of peak area versus concentration of analytical standards. The sensitivity of the methods is similar, with an LOQ of 3.13 ppm for the thymidine method used for cultured chicken cells and an LOQ of 3 ppm for thymine in the method by Scarborough *et al.* (1993). The HPLC data for conventional chicken samples is not from a validated method, whereas the cultured chicken data comes from a validated method. However, the conventional chicken data are published in a peer-reviewed journal, which provides an appropriate comparison to the cultured chicken samples.

5.2.3.7.4. Conclusion

Multiple batches of cultured chicken were analyzed for thymidine, and the estimated maximum thymidine content was calculated to be 3.96 µg/g, respectively. Given the degradation pathway for thymidine, it can be assumed that all of the thymidine is degraded into thymine. Therefore, assuming that maximum total thymine content in cultured chicken is equivalent to total thymidine content 3.96 mg/kg, which is approximately equivalent to the level found in sources of poultry meat (< 3 mg/kg). Additionally, the maximum potential level of thymidine in cultured chicken is far below the calculated ADI from literature, such that thymidine's use as a media constituent does not pose a safety risk at the limits of detection. Further, this testing demonstrates that there is no carryover of unsafe concentrations of thymidine in cultured chicken.

5.2.3.8. Pluronic F-68 (Poloxamer 188)

In the manufacturing process of cultured chicken, Pluronic F-68 is a component of the seed and production cell culture media. Pluronic F-68 is a surfactant that functions as anti-clumping agent in cell culture.

Pluronic® F-68 (also known as "Poloxamer 188"[29]) is a non-ionic, polyoxyethlyene-polyoxypropylene block polymer (average molecular weight: 7.68 to 8.83 kDa) used in the media to produce cultured chicken as a surfactant aiming to: (i) control shear forces in suspension cultures; (ii) reduce cell attachment to glass, and (iii) reduce foaming in the culture. It is also used for different functions and in products including cosmetics, as a pharmaceutical excipient, an investigative therapeutic agent for sickle cell disease (Orringer *et al.*, 2001; Casella *et al.*, 2021) and cancer therapy, as a stool softener (EMC, 2020), and has been investigated for its wound-healing and cleansing properties (Percival *et al.*, 2018). Poloxamers have been shown to modify drug efflux transport protein activity, thereby enhancing the effectiveness of anticancer drugs in

[29] CAS#'s associated with Pluronic F-68 include: 106392-12-5; 691397-13-4; 9003-11-6; for clarity, Poloxamer 188 and Pluronic F-68 are used interchangeably in this dossier.

multidrug-resistant cancer cells and to increase the bioavailability of orally administered pharmaceutical drugs (CIR Expert Panel, 2008).

Poloxamers are synthesized at high temperature and pressure from propylene glycol, to which propylene oxide is added followed by ethylene oxide in the presence of an alkaline catalyst (CIR Expert Panel, 2008). Commercially available Poloxamer 188 has been reported to contain impurities that include low-molecular-weight substances such as aldehydes and formic/acetic acids, as well as 1,4-dioxane, and residual ethylene oxide and propylene oxide (CIR Expert Panel, 2008). The United States Pharmacopeia (USP) (2004) established the maximum limits of ethylene oxide, propylene oxide, and 1,4-dioxane in poloxamers at 1, 5, and 5 ppm, respectively.

5.2.3.8.1. Regulatory Status

Poloxamer 188 is listed at 21 CFR § 310.545(a)(12)(iii) as a stool softener. Poloxamer 188 is also listed as an inactive ingredient[30] in 23 approved drug products administered *via* intramuscular, intravenous, ophthalmic, oral, periodontal, and topical routes. For those approved drug products, poloxamer 188 has varying maximum daily exposure values ranging from 6 – 200 mg for orally administered drugs. Although certain molecular weight poloxamers are stated as permitted direct and indirect food additives *per* the FDA Substances Added to Food Database,[31] poloxamer 188 is not specifically stated.

5.2.3.8.2. Relevant Literature

No toxicokinetic studies were identified following oral administration of Pluronic F-68; however, data from intravenous studies in several animal species, as well as humans, indicates that it is rapidly excreted in the urine with limited metabolism (CIR Expert Panel, 2008).

In humans administered Pluronic F-68 at infusion doses of 10 to 90 mg/kg bw/hour for 24 to 72 hours, 72 to 94% of the administered dose was eliminated in the urine. Although the mechanism of clearance is not established, it was hypothesized by the investigators to be *via* glomerular filtration based on the lack of electrical charge and macromolecular structure of the Poloxamer 188 (Jewell *et al.*, 1997). However, there may be some restriction in filtration due to its molecular size. Pluronic F-68 displayed no apparent infusion rate dependence in its pharmacokinetic properties, such as clearance, elimination, and apparent volume of distribution.

Grindel *et al.* (2002) investigated the distribution, metabolism, and excretion of Pluronic F-68 in rats, dogs, and humans. They reported that 75 to 95% of the intravenous dose was excreted in the urine (~90% in humans), primarily as unchanged polymer, with <5% excreted in the feces. Upon fractionation of urine and/or plasma, a single metabolite, with a molecular of 16,000 Da and a block copolymer structure, was identified at low concentrations in all species, which overall supported the conclusion that less than 5% of the administered dose was metabolized. Pluronic F-68 was primarily distributed in extracellular water with little to no uptake by red blood cells. The tissues with the highest concentration of Pluronic F-68 were the kidneys, lymph nodes, liver, spleen, and urinary bladder with the gastrointestinal tract. Metabolism was limited (< 5%), and the clearance from the body was slow and almost entirely by renal excretion. The 48-hour infusion doses of Pluronic F-68 were cleared in all species by approximately 1 week after the cessation of dose administration. The distribution, metabolism, and excretion pattern of poloxamer 188 was

[30] https://www.accessdata.fda.gov/scripts/cder/iig/index.cfm?event=BasicSearch.page (last accessed July 20, 2021).
[31] https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&id=ETHYLENEOXIDEPROPYLENEOXIDECOPOLYMER (last accessed July 20, 2021)

similar to patterns expected for other nonionic block copolymers with physical and chemical characteristics similar to poloxamer 188.

Several studies reported by the Cosmetic Ingredient Review (CIR) Expert Panel (2008) investigated the pharmacokinetics of Pluronic F-68 administered *via* intravenous perfusion. In rats, pharmacokinetic parameters were found to be dose dependent, reaching steady state within 46 hours, while in dogs a dose-dependent steady state was reached at 7 Days, with a mean plasma clearance between 49.4 and 87.9 mL/hour. In humans, the plasma concentration of Pluronic F-68 administered intravenously reached a maximum at 1 hour. Pluronic F-68 distribution within the extracellular fluid with minimal tissue uptake was further confirmed by Gibbs and Hagemann (2004); however, Pluronic F-68 was detectable at low concentrations primarily in the liver, lung, and kidney tissues of dogs following intravenous administration. It was also found to be transported in the plasma, with about 26% attached to the albumin fraction while 74% circulated free.

Oral toxicity studies conducted in rats and dogs indicate that Pluronic F-68 is of low oral toxicity with no specific toxicity identified. These studies are described below. Despite a lack of reproductive and developmental toxicity studies, the available data suggested that there would be little or no exposure to Pluronic F-68 of sex organs or the developing fetus during reproduction (CIR Expert Panel, 2008).

In an acute oral toxicity study, Pluronic F-68 dissolved in water or corn oil (not specified) was administered as a 50% (*w/v*) solution at a dose range of 2 to 15 g/kg bw (CIR Expert Panel, 2008). The LD_{50} was determined to be 5.5 g/kg bw. No adverse effects were reported in animals at the lowest doses (not specified), while rats exhibited mild sedation at increasing doses, which increased in severity with time. Severe respiratory depression was observed in animals that died, in addition to pulmonary edema with rales. Necropsy revealed marked engorgement of the lungs, stomach distention, and massive vascular dilation of the intestines.

In a study summarized by the CIR Expert Panel (Comai and Sullivan, 1980, as cited by CIR Expert Panel, 2008) female Charles River CD strain rats were divided into groups of 10 animals and fed either a control high-fat diet, or a diet with the same caloric density with added Pluronic F-68 (3% or approximately 1.5 g/kg body weight/day), which was substituted for the cellulose component of the diet. Animals were maintained on the diets for 42 days. Fecal-fat elimination and dietary fat absorption were determined throughout the study. At the end of the study, rats were killed and blood samples, and liver and adipose tissues, were taken. Pluronic F-68 caused no significant effect on feed intake, fecal-fat elimination, or dietary fat absorption compared to control rats and there were no significant differences in liver weights.

In a 6-month feeding study conducted in groups of 45 rats, animals were administered diets containing Pluronic F-68 at 0%, 3%, or 5% by weight in food (approximately equivalent to 0, 1.5, and 2.5 g/kg bw/day[32]) (Leaf, 1967, as cited by CIR Expert Panel, 2008). During the test period, 2 and 14 animals died in the 3% and 5% groups, respectively. Mortality was attributed to a combination of infection and inanition. Animals killed throughout the period for pathological examination showed no overt signs of adverse effects. The same authors also conducted a study in 12 dogs administered capsules of Pluronic F-68 at doses of 0, 0.05, or 0.1 g/kg before feeding (daily dose not reported). No differences were observed between the test and control dogs. Results

[32] Assuming average body weight of 400 g (PAFA Conversion Table, 1993).

of blood and urine analysis of test animals were comparable to those of control dogs and gross and microscopic examinations were unremarkable.

In a two-year feeding study summarized by the CIR Expert Panel (Leaf, 1967, as cited by CIR Expert Panel, 2008) rats were administered Pluronic F-68 in the diet at concentrations of 0%, 3%, 5%, and 7.5% (approximately 1.5, 2.5, and 3.75 g/kg bw/day, respectively[33]). No adverse effects were observed in any of the animals, with the exception of continuous moderate diarrhea in the two highest dose groups. A small decrease in growth was reported in the 7.5% dosage group, but no pathological effects were observed in any of the rats. The mortality rate of the control animals was higher than that of the test groups.

No studies specifically evaluating the oral toxicity of Pluronic F-68 in humans were identified; however, it has been investigated as an intravenous therapeutic agent for various health conditions and was reported to be safe and well-tolerated in both adults and children (Gibbs and Hagemann, 2004). Side effects have been reported to be generally mild and transient without renal function impairment; however, modest but statistically significant increases in alanine aminotransferase and direct bilirubin levels were reported in one trial (Orringer *et al.*, 2001), which returned to baseline levels at the 35-day follow up. In a second trial (Casella *et al.*, 2021), adverse events were more frequent in the group receiving poloxamer 188 *vs*. placebo, including incidence of hyperbilirubinemia (12.7% vs 5.2%).

5.2.3.8.3. Dietary Exposure

To establish estimated consumer exposure to Pluronic F-68 in cultured chicken, four (4) representative batches of cultured chicken were evaluated for Pluronic F-68 *via* a Colorimetric Assay (CA-SOP029) (Table 29).

Table 29. Estimated consumer exposure values of Pluronic F-68 from representative batches (RB) of cultured chicken.

Representative batches (RB)	**Pluronic F-68 amount *per* gram of cultured chicken (µg/g)**	**Pluronic F-68 amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of Pluronic F-68 in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of Pluronic F-68 in ready-to-cook food (RACC 114g) (mg/serving/person)**
RB – 1	<LOD	<LOD	<LOD	<LOD
RB – 2	<LOD	<LOD	<LOD	<LOD
RB – 3	<LOD	<LOD	<LOD	<LOD
RB – 4	<LOD	<LOD	<LOD	<LOD
Maximum	**<100**	**<10**	**<8.5**	**<11.4**

LOD = Limit of Detection (0.01%)

In all representative batches tested, Pluronic F-68 was below the limit of detection (LOD) of 0.01% (equivalent to 100 ppm, or 100 mg/kg cultured chicken). Thus, cultured chicken contains no residual Pluronic F-68 above the LOD. Assuming the level of Pluronic F-68 is at the LOD in cultured chicken, the estimated maximum Pluronic F-68 content was calculated to be 8.5 mg/serving/person for ready-to-serve portions of cultured chicken based on a RACC value of 85 g chicken *per* serving. The estimated maximum Pluronic F-68 content was calculated to be 11.4 mg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum Pluronic F-68 content was calculated to be 100 µg/g (equivalent to 10 mg/100g).

[33] Assuming average body weight of 400 g (PAFA Conversion Table, 1993).

The available toxicological and safety data reported in rats, dogs, and humans indicates that Pluronic F-68 is not anticipated to be associated with any adverse effects following oral exposure at moderate to high levels of exposure. No specific toxic effects were noted in the long-term feeding study in rats and dogs, except for diarrhea, which was only evident at high doses. Utilizing the dose that exhibited no adverse effects (NOAEL), an ADI can be established. Using 1500 mg/kg bw/day as the NOAEL (CIR Expert Panel, 2008) and safety factors to account for interspecies and intraspecies toxicodynamic and toxicokinetic differences (a 100-fold safety factor), an ADI of 15 mg/kg bw/day for humans may be stated. This value is approximately equivalent to 900 mg/day for an adult weighing 60 kg. As indicated, the maximum Pluronic F-68 intake from consumption of cultured chicken was calculated to be 11.4 mg/serving, which is equivalent to 0.190 mg/kg bw/day for a 60 kg adult. Therefore, the level of Pluronic F-68 in cultured chicken is far below ($1/75^{th}$) that of the calculated ADI, such that the levels of Pluronic F-68 do not pose a safety risk.

5.2.3.8.4. Conclusion

Multiple batches of cultured chicken were analyzed for Pluronic F-68. Pluronic F-68 content in cultured chicken is below the LOD, which equates to a maximum approximate content of 11.4 mg/serving. This value is far below the calculated ADI from the NOAEL reported in the literature, such that there is no safety risk posed by utilizing Pluronic F-68 as a media constituent. Further, this testing demonstrates no carryover of unsafe concentrations of Pluronic F-68 in cultured chicken.

5.2.3.9. Bovine serum

In the manufacturing process of cultured chicken, Fetal bovine serum (FBS) is a component of the seed and production cell culture media. Bovine serum albumin (BSA) is the major component of FBS, representing 50 – 60% of the total serum proteins (Boone *et al.*, 1971). BSA was selected as the reference component for quantification of residual bovine serum in cultured chicken because it is one of the most prevalent proteins present in the mixture with qualified analytical assays for determination of concentration.

5.2.3.9.1. Regulatory Status

Serum is a biological product obtained from the blood of animals and has been approved in vaccine production by FDA as a nutrient for cell culture.

5.2.3.9.2. Dietary Exposure

Serum is an inherent part of blood-based food products such as blood sausages. Albumin is a family of globular proteins most common of which are serum albumins which are present in abundance in beef, pork and chicken meat and dairy products including milk, cheese, yogurt, and whey protein. BSA represents the most abundant (50 – 60%) protein present in the plasma fraction of bovine blood (Dàvila *et al.*, 2007).

Plasma contains about 7.9% protein, consisting principally of immunoglobulins (4.2%), albumins (3.3%) and fibrinogen (0.4%) (Howell and Lawrie, 1984). Blood plasma is a versatile product, which is used in food industry as a binder in meat products, egg replacer in bakery, in protein enriched pasta, fat replacers, or even polyphosphate or caseinates substitute (Ofori and Hsieh, 2012). In many countries, whole blood and its separated RBCs are consumed as food or in combination with meat and other ingredients to prepare some meals such as in blood sausages, blood puddings, blood curd, bread, biscuits, and cakes (Toldrà *et al.*, 2019).

Plasma can be incorporated in meat products to take advantage of its gelling and thickening properties. It can also be used as an egg replacer in the bakery industry because it has foaming and gelling properties (Jayathilakan *et al.*, 2012). Frozen blood plasma is also used in cooked hams and hot dogs to improve their texture and color characteristics (Autio *et al.*, 1984). Cofrades *et al.* (2000) used plasma proteins as fat substitutes in fermented sausages and concluded that plasma is a good source of soluble proteins which could be useful in meat industries to replace fat. The authors further concluded that plasma-derived proteins such as immunoglobulins, fibrinogen, and serum albumin may be added to food and feed ingredients because of their gelation and emulsification properties. Serum albumin has been used as a substitute for egg albumin and utilized as sausage casing. Other common types of albumin proteins are abundantly present in egg and dairy products (Toldrà *et al.*, 2019). Table 30 demonstrates the wide variety of products containing blood plasma proteins.

Table 30. Blood plasma proteins used in food products, adapted from Toldra *et al.* (2019)

Blood Fraction	Function	Food Product
White animal blood	Protein source and color	Spanish blood sausage
Bovine hemoglobin	Iron fortification	Cookies
Bovine globin and plasma	Fat replacer	Ham pate
Animal plasma	Fat replacer	Bologna (fermented) sausage
Porcine TGase, fibrinogen, thrombin	Binder	Restructured meat products
Porcine plasma	Protease inhibitor	Surimi
Bovine plasma	Egg white replacer	Cakes
Processed bovine plasma	Emulsifier, stabilizer	Minced meat

BSA is also present in beef and cow's milk (Goldman *et al.*, 1963b, 1963a; Martelli *et al.*, 2002), contributing to the long history of consumer exposure. BSA's content in milk is provided in Table 31 and Table 32, adapted from a study by Poutrel *et al.* (1983). The mean concentration was 0.193 mg/mL in cow's milk. The density of milk is approximately 1.03 g/mL (Walstra *et al.*, 2006). Therefore, the overall mean of BSA concentration in milk is 0.187 mg/g, or 187.38 mg/kg.

Table 31. Milk bovine serum albumin concentration related to number of lactations (Poutrel *et al.,* 1983)

Lactation number	No. of samples	Milk bovine serum albumin concentration		
		Mean (mg/mL)	Standard deviation (mg/mL)	Range (mg/mL)
1	120	0.182	0.073	0.04 – 0.5
2	95	0.193	0.06	0.62 – 0.370
3	67	0.199	0.098	0.043 – 0.5
4	56	0.210	0.125	0.048 – 0.77
5, 6, 7	38	0.192	0.094	0.055 – 0.415

Table 32. Milk bovine serum albumin concentration related to stage of lactations (Poutrel *et al.,* 1983)

Days of lactation	No. of samples	Milk bovine serum albumin concentration		
		Mean (mg/mL)	Standard deviation (mg/mL)	Range (mg/mL)
30	126	0.173	0.094	0.040 – 0.3
150	125	0.188	0.093	0.053 – 0.77
270	125	0.224	0.094	0.062 – 0.585

All FBS sources used for culture of cultured chicken were tested for bovine viruses, following the procedures described in U.S. Code of Federal Regulations 9 CFR § 113.53(c) (Requirements by USDA for ingredients of animal origin used for production of biologics). Further, suppliers of FBS certify that serum is derived from healthy bovine from USDA-approved harvest facilities, and that bovine have passed ante- and post-mortem inspection and were found free of contagious diseases.

To establish estimated consumer exposure to FBS in cultured chicken, four (4) representative batches of cultured chicken were evaluated for BSA *via* enzyme-linked immunosorbent assay (ELISA) (Table 33). BSA was the selected reference component for quantification of residual bovine serum in cultured chicken due to being one of the most prevalent proteins present in the mixture with qualified analytical assays for determination of concentration. As reference, one of the FBS lots used in manufacturing contained 34 g/L of total protein and BSA accounted for 65% of total protein, with 22 g/L. Calculations for dietary exposure are based on RACC values (9 CFR § 317.312). The estimated average and maximum BSA content was calculated to be 1.33 mg/serving/person and 3.61 mg/serving/person, respectively, for ready-to-serve portions of cultured chicken, based on a RACC value of 85 g chicken *per* serving. The estimated average and maximum of BSA content was calculated to be 1.79 mg/serving/person and 4.84 mg/serving/person, respectively, for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated average and maximum BSA content was calculated to be 15.67 µg/g (equivalent to 1.57 mg/100g) and 42.46 µg/g (equivalent to 4.25 mg/100g), respectively, which is far lower than the average BSA content in cow milk (187.38 mg/kg, or 18.74 mg/100g) (Poutrel *et al.*, 1983).

Table 33. Estimated consumer exposure values of Bovine serum albumin from representative batches (RB) of cultured chicken.

Representative batches (RB)	**BSA amount *per* gram of cultured chicken (µg/g)**	**BSA amount *per* 100 g of cultured chicken (mg/100g)**	**Estimated intake of BSA in ready-to-serve food (RACC 85g) (mg/serving/person)**	**Estimated intake of BSA in ready-to-cook food (RACC 114g) (mg/serving/person)**
RB – 1	42.46	4.25	3.61	4.84
RB – 2	11.97	1.20	1.02	1.36
RB – 3	5.27	0.53	0.45	0.60
RB – 4	3.0	0.30	0.26	0.34
Average	**15.67**	**1.57**	**1.33**	**1.79**
Maximum	**42.46**	**4.25**	**3.61**	**4.84**

FBS is also a source of proteins, electrolytes, lipids, carbohydrates, hormones, enzymes, among other constituents present in low concentrations. Considering the initial low levels of these components in FBS, the low concentration of FBS used in the culture media and similar consumption and washing out ratio observed for BSA during culture and downstream concentration and washing post-harvest, the residual content of these other components is negligible and do not represent a safety concern for human consumption. Growth factors are also heat labile molecules and will go through reduction of their activity during cell culture at physiological temperatures. Moreover, the freezing step for storage and the cooking methods will inevitably affect the stability of any residual bioactive factor present in cultured chicken. In the remote possibility of residual growth factors preserving high activity levels during these multiple

temperature shifts, they would lose stability during their passage through the digestive system, especially as they go through the low pH observed in the stomach (pH 1-2.5).

5.2.3.9.3. Conclusion

Multiple batches of cultured chicken were analyzed for BSA. The BSA content in cultured chicken is higher than the average content in conventional chicken as a result of milk consumption; the average levels present in cultured chicken are approximately 10% and maximum levels are approximately 20% of the levels found in milk and do not pose a safety risk. This further demonstrates that overall bovine serum residual content in cultured chicken is low such that it does not raise any food safety concerns since there is no carryover of unsafe concentrations of bovine serum in cultured chicken.

5.2.4. Cell growth in bioreactor

During growth in the bioreactor additional components are used beyond those listed in section 5.2.1 for nutrient supplementation and pH control. These components are listed below.

5.2.4.1. D-Glucose (Dextrose)

In the manufacturing process of cultured chicken, D-Glucose may be supplemented to the basal media and is a component of the seed and production cell culture media. D-Glucose is affirmed as GRAS under 21 CFR § 184.1857 as food ingredient with no limitation other than cGMP.

5.2.4.2. Sodium hydroxide

In the manufacturing process of cultured chicken, Sodium hydroxide may be added to the basal media or the culture broth for pH control in the bioreactors. Sodium Hydroxide is affirmed as GRAS under 21 CFR § 184.1763 as pH control agent with no limitation other than cGMP.

5.2.4.3. Hydrochloric acid

In the manufacturing process of cultured chicken, hydrochloric acid may be added to the basal media or the culture broth for pH control in the bioreactors. Hydrochloric acid is GRAS under 21 CFR § 182.1057 as pH neutralizing agent when used in accordance with cGMP.

5.2.5. Extractables and Leachables from Single-Use Disposable Systems

Single-use disposable systems are used for the seed expansion and cell growth in the cultured chicken manufacturing process. The disposal systems with long contact time with the culture media include shake flasks, Wave Bags, and media hold bags. The vendors and the materials of construction of the single use systems used in the manufacturing process are summarized in the Table 34. These systems are commonly used in bioprocessing applications for commercial production of biopharmaceuticals. The extractables and leachable profiles of these systems are extensively validated by the vendors and the detailed guides provided by these vendors are reviewed at Good Meat and available upon request.

Table 34. Extractables and leachable information for single-use systems.

Single use system	Vendor*	Material
Erlenmeyer flasks (125 – 5000 mL)	Corning Inc.	Polycarbonate
Wave Bags (20 – 50 L)	GE Health Care Life Sciences	EVA (Ethylene vinyl acetate)
		LDPE (low density polyethylene)

	Sartorius Ag	Flexsafe® (multilayer polyethylene)
Flexel Media bags	Sartorius Ag	Ultra-low-density polyethylene (ULDPE)
Media Storage Bags	Optimum Processing, Inc	Ultra-low-density polyethylene (ULDPE)
UniFuge Single Use Module	Pneumatic Scale Angelus	Polycarbonate
		Polyurethane
		Silicone
		Bioprene
		C-Flex
		Polypropylene

*Example of provider.

Currently, single use disposable sterile components are used in the bioreactor for surfaces that come in direct contact with cultured chicken cells. These components are compliant with USP<88> biological reactivity standard, USP<87> cytotoxicity standard and USP<661> physiochemical standards for plastics. Further, as part of supply chain preventive controls, Good Meat verifies the certificate of sterility and endotoxins for every batch received from suppliers.

The storage bags for harvested material comply with 21 CFR § 177 subpart (b), and thus are authorized for direct food contact. Furthermore, the supplier has provided a letter of continued guarantee for adherence to applicable guidelines, and defect action levels for natural or unavoidable defects, action levels for poisonous or deleterious substances, including those applicable to contamination with aflatoxin, natural toxins, pests, undesirable microorganisms, and extraneous material. These storage bags are certified by suppliers to be free of Bisphenol-A (BPA), phthalates, and other volatile impurities ensuring the levels of extractable and leachable from these storage bags do not pose a food safety risk through contact with cultured cells.

5.3. Washing Step

5.3.1. Sodium chloride

In the manufacturing process of cultured chicken, sodium chloride solution is used to wash cells to remove carry over components. Sodium chloride is GRAS under 21 CFR § 182.1(a).

5.4. Chemical Analysis of the Final Wash Solution

To further corroborate the minimal presence of media components in cultured chicken, Good Meat performed chemical analysis of the media components in the second wash solution (Table 35). All the measured media components are retained at low concentrations in the wash solution (except sodium, as 0.45% NaCl is used as the wash solution), indicating minimal carryover of media components from the growth/spent media in the cultured chicken.

Table 35. Concentration of media components present in second wash solution of a representative production batch of cultured chicken.

Category	**Component**	**Concentration in second wash solution**
Free Amino Acids	Glycine	<0.01% (w/w)
	Alanine	<0.01% (w/w)
	Arginine	<0.01% (w/w)
	Aspartic acid	<0.01% (w/w)
	Glutamic acid	<0.01% (w/w)
	Histidine	<0.01% (w/w)

	Isoleucine	<0.01% (w/w)
	Leucine	<0.01% (w/w)
	Lysine	<0.01% (w/w)
	Methionine	<0.01% (w/w)
	Phenylalanine	<0.01% (w/w)
	Serine	<0.01% (w/w)
	Threonine	<0.01% (w/w)
	Tyrosine	<0.01% (w/w)
	Valine	<0.01% (w/w)
Vitamins	Biotin	< 0.05 ppm
	Pantothenate	2.0 ppm
	Niacin	2.0 ppm
	Riboflavin	<0.2 ppm
	Thiamine	<0.2 ppm
	Vitamin B12	0.047 ppm
Minerals/ Metals	Calcium	8 ppm
	Copper	0.01 ppm
	Iron	<0.5 ppm
	Magnesium	7.46 ppm
	Phosphorous	46 ppm
	Potassium	68.4 ppm
	Sodium	1670 ppm
	Titanium	0.06 ppm
	Zinc	0.3 ppm
Fats/Lipids	Fats by fatty acid profile	0.4 ppm
Others	Folic Acid	Section 5.2.3.1
	Ferric Nitrate	Section 5.2.3.2
	Hypoxanthine	Section 5.2.3.3
	Lipoic Acid	Section 5.2.3.4
	Putrescine	Section 5.2.3.5
	Pyruvate	Section 5.2.3.6
	Thymidine	Section 5.2.3.7
	Pluronic F-68	Section 5.2.3.8
	Bovine Serum	Section 5.2.3.9

Table 35 confirms the minimal carryover of media components in the cultured chicken after the downstream washing steps. By washing, the effective reduction of the media component carryover in the cultured chicken is at least 25-fold. Except for glucose, glutamine, and sodium, the carryover of the media components is empirically estimated to be very low, at < 10 ppm based on the 25-fold dilution at the end of washing. Glucose and glutamine are consumed as carbon/nitrogen sources during cell culture. Also, all the media components are generally recognized as safe or do not pose a safety risk at measured levels, as elaborated in Section 5.2.

Out of the media components stated in Table 35, Good Meat characterized Pluronic F-68 and bovine albumin concentrations in second wash solution for multiple production batches to determine the efficiency of cell washing. The initial concentration of the Pluronic F-68 in the growth media is 0.1% *w/v* (1000 mg/L). Data on bovine albumin concentration in the second wash solution was also gathered consistently for production runs to ascertain minimal carryover of serum in the cultured chicken. Bovine albumin concentration in the growth media is 412.5 mg/L on average.

5.4.1. Measurement of Pluronic F-68 as Indicator of Washing Efficiency

Pluronic F-68 in the wash solution is measured using a colorimetric cobalt thiocyanate method. Pluronic F-68 in the sample forms a complex with cobalt thiocyanate that sediments upon centrifugation. The precipitate is dissolved in acetone, and the color intensity is correlated to the Pluronic F-68 concentration in the linear range for quantification.

Ascertaining washing efficiency by tracking Pluronic F-68 in wash solution may/may not be compromised depending on the accumulation of Pluronic F-68 within the cell. To elucidate this further, Good Meat measured the amount of Pluronic F-68 in (i) growth media (ii) spent media (iii) C1F cell lysate and (iv) second wash solution.

Based on the results (summarized in the Table 36 and Table 37), it is confirmed that negligible amounts are accumulated within the cell (< 0.01% in the cell lysate) and most of the Pluronic F-68 in growth media is retained in the spent media. As described before, by washing the spent media 25-fold, the Pluronic F-68 concentration in the second wash solution is measured to be below the LOD (<0.01%), indicating Pluronic F-68 concentration can be used as an indicator of washing efficiency. The consistency of washing efficiency is demonstrated by below the limit of detection levels from six representative batches as summarized Table 37, below.

Table 36. Pluronic F-68 level by cobalt thiocyanate assay in a representative production batch of cultured chicken.

Sample Type	**Pluronic F-68 concentration (% w/v)**
Growth media	0.10 ± 0.0023
Spent media	0.10 ± 0.015
Cell lysate	<0.01
Second wash solution	<0.01

w/v = weight/volume

Table 37. Chemical analysis (Pluronic F-68) of the final wash solution from 1000 L harvests of cultured chicken (*N*=6 representative batches (RB)).

Parameter	**Basis Method**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Pluronic F-68 Concentration (%)	Cobalt thiocyanate method	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

LOD for Pluronic F-68 = 0.01%

5.4.2. Bovine Serum Albumin

The results of this analysis are presented below. Bovine albumin concentration in the growth media is 412.5 mg/L on average, and that the concentration in the final wash solution is less than 1/100th the concentration in media (Table 38) indicating minimal carryover of serum in the cultured chicken.

Table 38. Chemical analysis (bovine albumin) of the final wash solution from 1000 L harvests of cultured chicken (*N*=6 representative batches (RB)).

Parameter	**Basis Method**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Bovine Albumin Concentration (mg/L)	ELISA	< 0.31	1.40	0.60	< 0.31	< 0.31	0.35

LOD = 0.31 mg/L.

5.5. Good Meat Cultured Chicken Analysis

The data reported in this dossier are based on at least three harvests from 1000 L bioreactors. This section will detail the various characterization methods performed on cultured chicken to ensure it meets Good Meat specifications.

5.5.1. Characterization of Cell Line Stability

Characterization of cell line stability of chicken cells used for manufacturing of cultured chicken was performed by (i) cytogenetic analysis and (ii) RNA sequencing.

5.5.1.1. Cytogenetic Analysis of Parental and Cultured Chicken Cells

Cytogenetic analysis is used as one method for the characterization of cell line stability during manufacturing. A cell line is considered stable if modal chromosome number does not change significantly and there is no accumulation of cytogenetic aberrations (chromatid-type, chromosome-type, and severely damaged) during the process (Chang and Delany, 2004). If such characteristics are not stable, then it should be demonstrated that the instability does not adversely impact manufacturing or product consistency. Results from cytogenetic analysis on both parental and cultured chicken cells indicate that a majority of cells in the parental and production cell bank contained between 68 – 78 chromosomes (Note: the normal number of chicken chromosomes is 78) (Mendonça *et al.*, 2016).

Cytogenetic analysis was performed on parental chicken cell bank and end of production chicken cell bank: (1) parental cell bank established from purchased UMNSAH/DF1 (ATCC) cells; (ii) end of production chicken cell bank from one representative cultured chicken harvest. A sterile volume of culture from end of production run was collected pre-harvest. Chicken cells were pooled and banked following internal CA-SOP035 “Cryopreservation and LN2 Storage of Research Cell Banks”. Cryovials from both samples were shipped to Charles River to perform cytogenetic analysis using AGL-CCP.2 method. Testing was performed at Applied Genetics Laboratories, Inc (Melbourne, FL 32901, U.S.A).

The chicken origin of both samples was confirmed. The chromosome count for cells from parental cell bank ranged from 62 to 156 chromosomes *per* metaphase, with a modal chromosome number of 76 (Note: 76% of the cells contained between 72 and 78 chromosomes). The polyploid frequency was 14%. No chromosome aberrations were found in the 100 cells analyzed. The mitotic index was 3.1%, based on 1000 cells counted.

The chromosome count for cells from the end of the production cell bank ranged from 63 to 415 chromosomes *per* metaphase, with a modal chromosome number of 72 (Note: 67% of the cells contained between 68 and 74 chromosomes). The polyploid frequency was 12%. No chromosome aberrations were found in the 100 cells analyzed. The mitotic index was 2.7%, based on 1000 cells counted.

For most species, chromosomes can be distinguished relatively easily by either classical (*e.g.*, G-banding) means or molecular cytogenetics. Birds are a notable exception because they present a high number of chromosomes and many cytologically indistinguishable micro-chromosomes (Masabanda *et al.*, 2004). Therefore, to further confirm those cytogenetic changes did not negatively impact manufacturing, product consistency, and safety, RNAseq of C1F cells during culture adaptation and manufacturing was performed.

5.5.1.2. RNAseq of Chicken Cells along the Culture Adaptation Process and Manufacturing Steps

RNA sequencing analysis of chicken cells used to produce cultured chicken was performed by comparing the parental cells used as a starting point for culture adaptation towards manufacturing purposes with the chicken cells harvested from bioreactors at the end of production. Altogether, this comparison evaluates the drift in transcriptomic signature during the culture adaptation process to suspension cultures in low serum and during manufacturing. Results from this analysis indicate that the functional gene changes that occur during culture are mostly related to the culture of C1F cells in anchorage-independent conditions and in culture media with reduced serum content. There is no indication of significantly altered gene expression from parental to cultured cells that would raise concerns regarding the food safety of cultured chicken.

Functional annotation analysis showed that most of the different expressed genes between parental cells and end-of-production chicken cells associated with enriched pathways were grouped to Extracellular Matrix (ECM) organization, cytoskeleton, and cell adhesion (44% of 88 genes in the dataset). Some extracellular molecules (like integrins, collagen, fibronectin) are downregulated in cells collected at the end of production (grown in suspension) compared to those in attachment (parental cells). Serum weaning between parental and end-of-production C1F-P1 cells can also justify these differences in expression of genes encoding for adhesion and ECM interaction proteins, as overall protein content provided in culture media was reduced during serum weaning.

Overall, the data collected from cytogenetic and transcriptomic analysis supports the stability of C1F-P1 cells throughout culture adaptation and manufacturing of cultured chicken.

5.5.2. Characterization of Purity and Identity of Good Meat cultured chicken

Cell line purity is evaluated for the parental chicken cell banks by performing a species identification assay and phenotype confirmation. For the characterization of end-of-production chicken cells, the same protocols were used.

5.5.2.1. Species Identification Assay

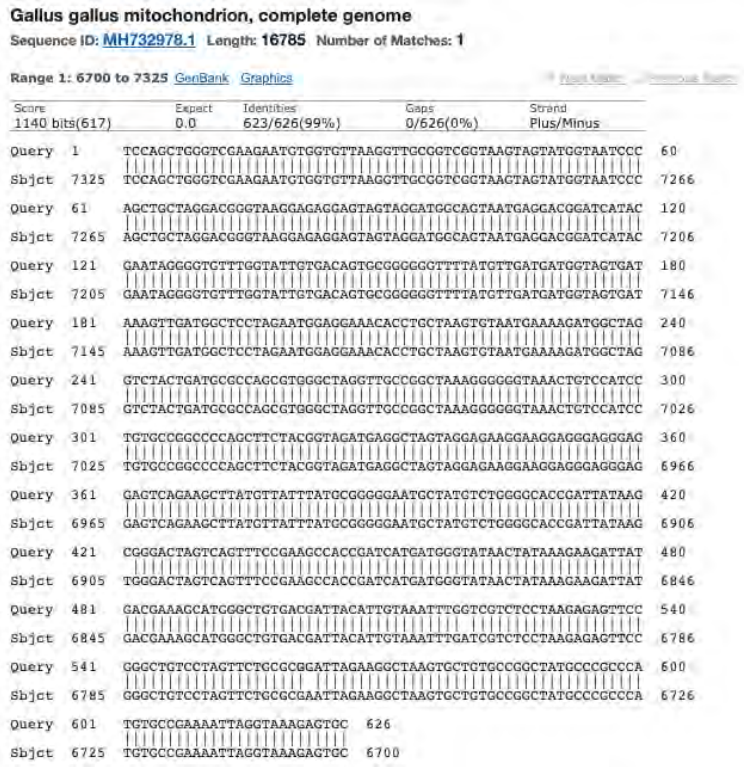
Cultured chicken from independent 1000 L production runs was analyzed using conventional PCR following an internal CA-SOP013 “Avian Species Identification by PCR Sequencing” (See Appendix: 10.3.1).

Cultured chicken from independent 1000 L production runs was analyzed using conventional PCR following an internal CA-SOP013 “Avian Species Identification by PCR Sequencing”. Pellets from production runs of cultured chicken were collected aseptically. DNA was extracted, and the PCR reaction was performed. Afterward, the amplicons were run on agarose gels to assure amplicon purity and size. The DNA fragment was amplified by PCR reaction with the expected size.

After confirming the successful reaction, amplicons were purified, and the samples were shipped for DNA Sanger sequencing at Quintara Biosciences. Sequence alignment between the genotyped amplicon and the published chicken consensus sequence (from National Center for Biotechnology Information) was performed using the online software tool “Align Sequences Nucleotide BLAST” available at blast.ncbi.nlm.nih.gov (Figure 5).

The high level of homology between the amplified product and the public genomic databases of chicken confirms the identity of cultured chicken as chicken, specifically *Gallus gallus*.

Representative Batch 1_ M13F



Gallus gallus mitochondrion, complete genome

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Range 1: 6700 to 7325 GenBank Graphics

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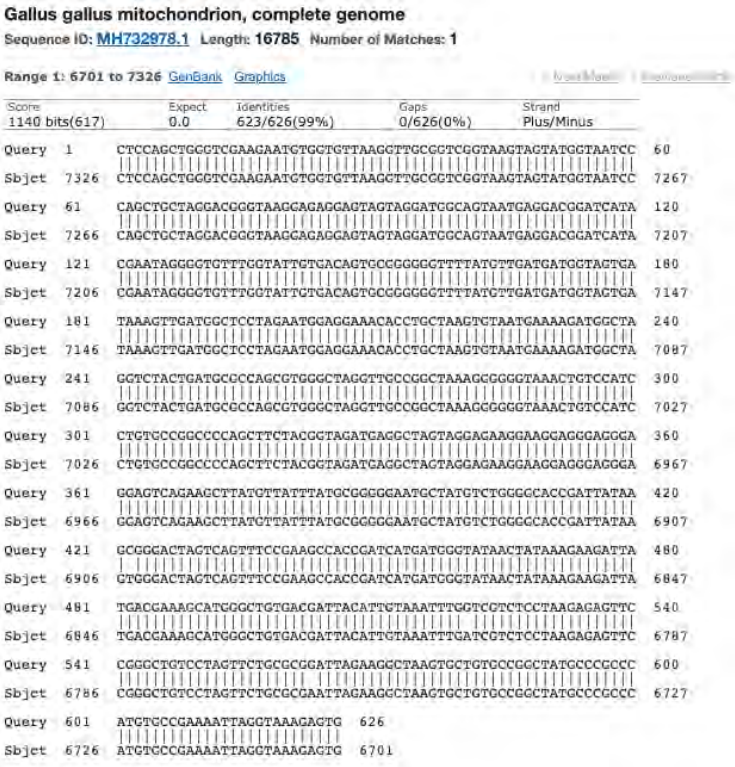
Representative Batch 2_ M13F



Gallus gallus mitochondrion, complete genome

Sequence ID: MH732978.1 Length: 16785 Number of Matches: 1

Range 1: 6701 to 7326 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1140 bits(617)	0.0	623/626(99%)	0/626(0%)	Plus/Minus

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Query  361   GGAGTCAGAAGCTTATGTTATTTATGCGGGGGAATGCTATGTCTGGGGCACCGATTATAA  420
Sbjct  6966  GGAGTCAGAAGCTTATGTTATTTATGCGGGGGAATGCTATGTCTGGGGCACCGATTATAA  6907
Query  421   GCGGGACTAGTCAGTTTCCGAAGCCACCGATCATGATGGGTATAACTATAAAGAAGATTA  480
Sbjct  6906  GTGGGACTAGTCAGTTTCCGAAGCCACCGATCATGATGGGTATAACTATAAAGAAGATTA  6847
Query  481   TGACGAAAGCATGGGCTGTGACGATTACATTGTAAATTTGGTCGTCTCCTAAGAGAGTTC  540
Sbjct  6846  TGACGAAAGCATGGGCTGTGACGATTACATTGTAAATTTGATCGTCTCCTAAGAGAGTTC  6787
Query  541   CGGCCTGTCCTAGTTCTGCGCGGATTAGAAGGCTAAGTGCTGTGCCCGCTATGCCCCGCCC  600
Sbjct  6786  CGGGCTGTCCTAGTTCTGCGCGAATTAGAAGGCTAAGTGCTGTGCCGGCTATGCCCGCCC  6727
Query  601   ATGTGCCGAAAATTAGGTAAAGAGTG  626
Sbjct  6726  ATGTGCCGAAAATTAGGTAAAGAGTG  6701
```

Representative Batch 3_ M13F

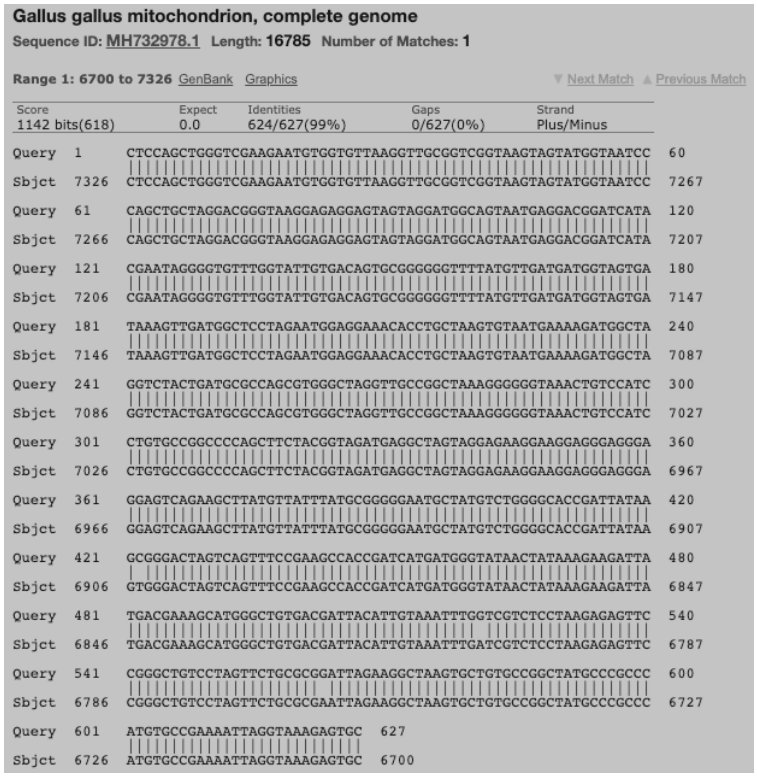

Figure 5. Alignment for forward primer on amplicon from cultured chicken cells from three independent harvests to published chicken mitochondrial genome (100% alignment).

5.5.3. Microbiological Analysis

The safety and purity of cultured chicken production are confirmed by microbiological analysis for every production batch. Sterility of culture, mycoplasma testing, and identification of adventitious viral and microbial agents was validated for six representative production batches as additional characterization data points for the proposed process.

5.5.3.1. Sterility of Culture and Mycoplasma

C1F-P1 culture media supernatants from independent 1000 L production runs were incubated for 14 days in TSB and FTM broth media to evaluate bacterial and fungal growth (Table 39). Samples were analyzed at Charles River Research Animal Diagnostic Services (MA 01887, USA) and data are shown in Table 39.

Table 39. Results from the sterility testing of independent 1000 L cultured chicken representative batches (RB)

Sterility pre-harvest	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
FTM broth	Negative	Negative	Negative	Negative	Negative	Negative	Negative
TSB broth	Negative	Negative	Negative	Negative	Negative	Negative	Negative

The 1000 L production runs passed sterility testing as all independent samples showed the absence of microorganism growth in both TSB and FTM broths.

5.5.3.1.1. Mycoplasma Testing

C1F-P1 media supernatants from 1000 L production runs were tested for *Mycoplasma*. Table 40 shows the results.

Table 40. *Mycoplasma* detection on independent 1000 L cultured chicken representative batches (RB)

Mycoplasma	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Mycoplasma Genus PCR	Negative	Negative	Negative	Negative	Negative	Negative	Negative

All tested 1000 L production runs were considered approved for the absence of *Mycoplasma*.

5.5.3.1.2. Viral Assessment

C1F-P1 cells harvested from 1000 L production runs were also tested for the same panel of human and avian viruses used to test our internal chicken MCB and MWCB used for manufacturing purposes (Table 41).

Table 41. Infectious disease PCR on independent 1000 L cultured chicken representative batches (RB1 to RB6). Cultured were sampled under sterile conditions, immediately pre-harvest #.

	HUMAN ESSENTIAL CLEAR PANEL					
	RB1	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Adeno-associated virus	-	-	-	-	-	-
BK virus	-	-	-	-	-	-
Epstein-Barr virus	-	-	-	-	-	-
Hepatitis A virus	-	-	-	-	-	-
Hepatitis B virus	-	-	-	-	-	-
Hepatitis C virus	-	-	-	-	-	-
Herpes Simplex 1 PCR	-	-	-	-	-	-
Herpes Simplex 2 PCR	-	-	-	-	-	-
Herpesvirus type 6	-	-	-	-	-	-
Herpesvirus type 7	-	-	-	-	-	-
Herpesvirus type 8	-	-	-	-	-	-
HIV-1	-	-	-	-	-	-
HIV-2	-	-	-	-	-	-
HPV-16	-	-	-	-	-	-
HPV-18	-	-	-	-	-	-
Human cytomegalovirus	-	-	-	-	-	-
Human Foamy virus	-	-	-	-	-	-
Human T-lymphotropic virus	-	-	-	-	-	-
John Cunningham virus	-	-	-	-	-	-
Parvovirus B19	-	-	-	-	-	-
Mycoplasma Genus PCR	-	-	-	-	-	-
Mycoplasma pulmonis PCR	-	-	-	-	-	-
	AVIAN VIRUS AND BACTERIA PANEL					
	RB1	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
REV PCR	-	-	-	-	-	-
AEV PCR	-	-	-	-	-	-
ALVA PCR	-	-	-	-	-	-
ALVB PCR	-	-	-	-	-	-
ALVJ PCR	-	-	-	-	-	-
FAV1 PCR	-	-	-	-	-	-
FAV3 PCR	-	-	-	-	-	-
CAV PCR	-	-	-	-	-	-
ARV PCR	-	-	-	-	-	-

Avian *S. pullorum* PCR	-	-	-	-	-	-
Avian *Mycoplasma Genus* PCR	-	-	-	-	-	-

Negative (absence of virus/bacteria) is noted with (-); Equivocal (inconsistent amplification detected by real-time PCR) is noted with (+/-); Positive (presence of virus/bacteria) is noted with (+); Inconclusive (failure of control result) is noted with (I).

5.5.3.1.3. Microbial Analysis

The European Food Safety Authority (EFSA) conducted a review to provide scientific opinion on the public health hazards from poultry meat (EFSA, 2012). This review identified *Campylobacter spp*. and *Salmonella spp*. as most relevant bacteria to cause a biological hazard. A decision tree developed by EFSA was used for risk ranking poultry meat-borne biological hazards. Hazards that are introduced and/or for which the risk to public health relates to growth that occurs during processing steps after carcass chilling were not considered. The risk ranking was based on the following criteria: (I) the magnitude of the human health impact; (II) the severity of the disease in humans; (III) the proportion of human cases that can be attributable to the handling, preparation and/or consumption of poultry meat; and (IV) the occurrence (prevalence) of the identified hazards in poultry flocks and carcasses. The risk ranking did not consider the different poultry species separately.

Based on the risk ranking, the hazards were classified as follows:

i. *Campylobacter spp.* and *Salmonella spp*. were considered high public health relevance for poultry meat inspection.

ii. ESBL/AmpC gene carrying *E. coli* bacteria were considered medium to high risk.

The intestinal tract of domestic and wild animals and birds appears to be the primary reservoir of *Campylobacter jejuni* (Munroe *et al.*, 1983; Bryan and Doyle, 1995). Consumption of food and water contaminated with untreated animal or human waste accounts for 70% of *Campylobacter*-related illnesses each year (FDA BAM, 2000). *Campylobacter jejuni* colonizes primarily the lower gastrointestinal tract of chicks, principally the ceca, large intestine and cloaca (Beery *et al.*, 1988). Absence of intestinal tract and other digestive organs in cultured chicken minimizes the risk of *Campylobacter* species.

A study of factors responsible for the introduction and spread of *C. jejuni* in poultry production revealed that the environment in and near rearing houses, transfer from farm to slaughterhouse, scalding, and defeathering was the most likely source for young chickens (Kazwala *et al.*, 1990). Cultured chicken is harvested from bioreactors after manufacturing under sterile conditions and does not pose a risk of *Campylobacter* contamination from the environment.

FDA BAM chapter 7 (2000) states that *C. jejuni* is a thermophilic species and does not multiply at refrigeration temperatures in airtight packaging and their numbers decrease 2 logs upon freezing at -20°C. Cultured chicken is stored at temperature below -20°C which reduces the risk of *Campylobacter* growth. Based on this risk assessment and absence of gastrointestinal organs in cellular culturing, the probability of *Campylobacter* growth in cultured chicken is negligible. Therefore, no food safety concerns are raised by not testing for *Campylobacter*. Furthermore, Table 42 presents four representative batches of cultured chicken tested for the presence of *Campylobacter,* and all results were negative/25g sample.

Table 42. *Campylobacter* ssp. Testing for four (4) representative batches of cultured chicken

Representative batches (RB)	Method	Specification	Result	Average
RB-1	AOAC-RI 051201	Negative/25g	Negative/25g	Negative/25g
RB-2	AOAC-RI 051201	Negative/25g	Negative/25g	Negative/25g
RB-3	AOAC-RI 051201	Negative/25g	Negative/25g	Negative/25g
RN-4	AOAC-RI 051201	Negative/25g	Negative/25g	Negative/25g

Cultured chicken was also analyzed for the presence of various microbial contaminants described in Table 43. The method for each of these analyses is provided in Appendix: 10.4.4.1.

Table 43. Microbiological analyses for representative 1000 L production batches of cultured chicken (RB1 to RB6).

Representative Batch (RB) Microbiological analysis	Specs	RB1	RB2	RB3	RB4	RB5	RB6
Aerobic plate count (cfu/g)	**<10,000**	<10	<10	<10	<10	<10	<10
Yeast (cfu/g)	**<100**	<10	<10	<10	<10	<10	<10
Mold (cfu/g)	**<100**	<10	<10	<10	<10	<10	<10
Coliforms (MPN/g)	**<24**	<3	<3	<3	<3	<3	<3
E. coli (MPN/g)	**<3**	<3	<3	<3	<3	<3	<3
Enterococcus (cfu/g)	**<10**	<10	<10	<10	<10	<10	<10
Salmonella (per 25g)	**Neg/25g**	Neg/25g	Neg/25g	Neg/25g	Neg/25g	Neg/25g	Neg/25g

Overall, the microbiological results highlight the clean profile of the harvested cultured chicken material, emphasizing the aseptic conditions applied during *in vitro* expansion of C1F-P1 cells in the production of the cultured chicken. Moreover, the manufacturing process has been demonstrated to be robust, as harvests have consistently low counts of microorganisms, even with the absence of antibiotics in the culture media.

It should be noted that the specifications detailed above are those that every batch of cultured chicken must meet. Further breakdown of analytical methods is presented Table 44. The frequency of tests in Table 44 is for every harvest, samples are taken from post-harvested cells for release testing. Testing occurs according to GLP at Silliker Salida laboratory, which is accredited to ISO/IEC 17025 standard.

Table 44. Microbiological specifications and analysis of cultured chicken.

Microbiological Analysis	Basis Method	Specification
Aerobic plate count	FDA BAM – Chapter 3/AOAC 966.23	< 10,000 cfu/g
Coliforms	FDA BAM – Chapter 4/AOAC 966.24	< 24 MPN/g
E. coli	FDA BAM – Chapter 4/AOAC 966.24	< 3 MPN/g
Enterococcus	CMMEF 4th ed. – Chapter 10	< 10 cfu/g
Salmonella	FDA BAM – Chapter 5/ AOAC-RI100201	Negative/25g

Yeast	FDA BAM – Chapter 18	< 100 cfu/g
Mold	FDA BAM – Chapter 18	< 100 cfu/g

5.5.4. Chemical Analysis of Cultured Chicken

5.5.4.1. Proximate Analysis

Table 45 summarizes the results for proximate and heavy metals analysis for cultured chicken harvested from six representative batches. The methods for each parameter are available in Appendix: 10.4.4.2.

Table 45. Chemical and biological analyses for representative 1000 L production batches of cultured chicken (RB1 to RB6).

Representative batch (RB)	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Proximate Analysis							
Moisture (%)	85 - 95	88.73	89.91	90.54	89.87	88.77	89.07
Protein (%)	5 – 10	9.75	7.88	7.75	8.19	8.88	8.12
Fat (%)	0.5 – 2.0	1.55	1.20	1.66	1.38	1.20	1.18
Ash (%)	0.0 – 2.0	0.84	1.00	0.78	0.71	0.95	0.86
Carbohydrate (%)	0.0 – 2.0	< 0.1	< 0.1	< 0.1	< 0.1	0.2	0.77
Heavy Metals							
Arsenic (ppm)	< 0.10	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Lead (ppm)	< 0.20	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Mercury (ppm)	< 0.05	< 0.005	< 0.005	< 0.0005	< 0.005	< 0.005	< 0.005
Cadmium (ppm)	< 0.02	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.001
Chromium (ppm)	< 0.20	0.02	0.01	0.01	0.02	0.01	0.02

ppm = parts *per* million; specs = specifications

5.5.4.2. Amino Acid Analysis

Complete amino acid analysis of cultured chicken is performed *via* the USDA MSS2 (1993) method. Table 46 contains the results for the amino acid analysis of cultured chicken from a representative 1000 L production batch. Cultured chicken offers a balanced amino acid compositional profile comparable to conventional chicken (while overall amino acid content is reduced, the proportion of amino acids to one another is relatively the same). Glutamic acid, aspartic acid, leucine, and lysine are ranked as the top four amino acids at the highest concentration in both cultured chicken and conventional chicken.

Table 46. Complete amino acid analysis of a representative production batch of cultured chicken. Data are listed as amino acid content *per* 100 g of product. Values for conventional chicken are listed in USDA databases.

Amino Acids Analysis (USDA MSS2)	**Cultured Chicken, raw, g**	**Conventional ground chicken (SR LEGACY, 171116), Moisture 73.2%, g**
Aspartic Acid	0.88	1.62
Threonine	0.42	0.73
Serine	0.45	0.73
Glutamic Acid	1.12	2.61
Glycine	0.37	0.84
Alanine	0.48	0.99

Valine	0.46	0.83
Methionine	0.22	0.45
Isoleucine	0.39	0.79
Leucine	0.75	1.36
Tyrosine	0.35	0.60
Phenylalanine	0.40	0.68
Lysine	0.66	1.51
Histidine	0.20	0.53
Arginine	0.63	1.13
Proline	0.37	0.66
Hydroxyproline	< 0.01	0.09
Cysteine	0.09	0.19
Tryptophan	0.11	0.14

5.6. Stability of Cultured Chicken

The stability of cultured chicken was assessed by its proximate composition and oxidative state. Samples from representative batches at Good Meat were packaged and frozen at less than or equal to -20°C. A new package was opened at each test period, thawed, and tested for proximate composition including moisture content, protein content, fat content, ash content, carbohydrate content, and oxidative state by measuring peroxide values (Table 47).

Table 47. Stability analysis of cultured chicken.

Parameter	**Spec**	**0 Month**	**1 Month**	**2 Months**	**3 Months**	**4 Months**	**5 Months**	**6 Months**
Moisture content	85-95	91.9%	92.4%	92.5%	91.1%	91.1%	91.7%	92%
Protein content	5-10	6.7%	5.5%	5.4%	6.4%	6.4%	6.1%	5.9%
Fat content	0.5-2.0	1.4%	0.8%	0.9%	0.9%	0.7%	0.9%	0.8%
Ash content	0.0-2.0	0.6%	0.5%	0.6%	0.6%	0.8%	0.6%	0.7%
Carbohydrate content	0.0-2.0	< 0.1%	0.8%	0.7%	0.9%	0.9%	0.7%	0.6%
Peroxide value	<0.25[1\]	Not Tested	<0.02 meq/Kg	< 0.02 meq/Kg	< 0.02 meq/Kg	< 0.02 meq/Kg	< 0.02 meq/Kg	< 0.02 meq/Kg

The stability of cultured chicken was validated in stored conditions at less than or equal to -20°C for 6 months as no changes were observed on proximate analysis and oxidative state of the frozen cells.

6. INTENDED USE OF GOOD MEAT CULTURED CHICKEN IN FOOD

6.1. Current Regulatory Status and History of Use

Cultured chicken has not been sold in the United States. Good Meat seeks authorization from FDA to use cultured chicken cells to be subsequently processed into poultry products that bear the USDA mark of inspection. The requested authorization is the demonstration that the biological materials exiting the culture process are safe and non-adulterated within the meaning of the Federal Food, Drug, and Cosmetic Act.

Good Meat modeled the cell culture process it implemented for the development of cultured chicken after biopharmaceutical processes that employ animal cell culture. Good Meat has implemented safety and quality controls on the creation of cGMP cell banks for production and the process of manufacturing. Cultured chicken is composed of C1F-P1 cells, which are not exposed to environmental pathogens or antibiotics during manufacture. Thorough analysis was performed on cultured chicken to demonstrate the quality, safety, and identity of cultured chicken as one of the main ingredients of poultry products, such as chicken bites, strips, and other processed formats.

Chicken meat produced through conventional means has been deemed safe for consumption once cooked to an internal temperature of 165°F; hence, the same is expected for cultured chicken.

6.2. Good Meat Cultured Chicken

6.2.1. Intended Use in Cultured Chicken Products

Cultured chicken is intended to be used as a raw material in a finished poultry product, for example, as an alternative for traditional chicken meat in various applications. Good Meat seeks to launch in the U.S. Good Meat cultured chicken products, such as chicken bites, nuggets, breasts, strips, sausages, and patties, among others, containing cultured chicken combined with other safe and suitable ingredients.

6.2.2. Nutritional Comparison of Good Meat Cultured Chicken to Conventional Chicken

The nutritional profile of cultured chicken is similar to that of conventional chicken when 100 grams of dry cultured chicken cells is compared to dry raw chicken meat (USDA, 2012). Table 48 lists ash, total protein, fat, and carbohydrates levels in cultured chicken compared to conventional chicken.

Of note, the high ash content in cultured chicken is due to residual salt, primarily from the 0.45% NaCl washes used to prepare the material and from the culture media used to grow the chicken cells. This was also confirmed by the sodium levels in dry cultured chicken (1.7%). When ash is removed from the analysis (as in Column 4 of 48), protein, fat, and carbohydrate levels are consistent between cultured chicken and conventional chicken.

Table 48. Nutritional analysis of cultured chicken in comparison with conventional boneless chicken breast. Nutritional values are presented as percentage. Analytes qualified using the nutrition analytical method from Silliker (Crete, IL, USA).

Nutritional package	Chicken breast, dry, raw (USDA, 2012)	Good Meat cultured chicken, dry, raw	Good Meat cultured chicken, dry, raw *
Ash	0	7.6	0
Carbohydrates	0	0	0
Protein	87.1	79.4	86.0
Total Fat	8.2	9.2	10.0

*normalized to 0% ash

Table 49 lists the percent saturated, monounsaturated, and polyunsaturated fats in cultured chicken compared to conventional boneless chicken breast. Fat values are presented as a *percent* of specific fat relative to total fat in the sample.

Table 49. Percent saturated, monounsaturated and polyunsaturated fats of Good Meat cultured chicken compared to conventional boneless chicken breast. Fat values are presented as % of total fat. Analytes quantified using the nutrition analytical method from Silliker (Crete, IL, USA).

Nutritional package	**Chicken breast, dry, raw (USDA, 2012) (% of total fat)**	**Good Meat cultured chicken, dry, raw (% of total fat)**
Fat – Saturated	26.1	31.5
Fat – Monosaturated	34.1	50.0
Fat – Polyunsaturated	17.5	12.0
Calories (*per* 100g)	49	112

Cultured chicken has a lower overall caloric value than conventional chicken breast when considering wet analysis, as moisture content is higher in cultured chicken. Monounsaturated fats (commonly referred to as the healthy type of fat) represent the type of fat in higher percentage in both conventional and cultured chicken (34.1% and 50%, respectively), followed by saturated fats and polyunsaturated fats. In summary, cultured chicken preserves the fat distribution observed in conventional chicken.

Analysis also revealed an average cholesterol content of 122 mg *per* 100 g of cultured chicken when following the AOAC Official Method 994.10 for cholesterol determination. This value is consistent with the cholesterol content reported by the USDA for cooked chicken breast (111 to 123 mg *per* 100 g).[34]

Although there are small differences in the ash content, both types of chicken are high in protein and low in fat and represent highly nutritious food sources.

6.2.3. Microbiological Safety of Cultured Chicken

Cultured chicken shares the nutritional benefits of regular chicken (high protein, low saturated fat) but significantly lessens the potential for microbiological contamination issues prevalent among commercially farmed chicken.

While sharing the nutritional benefits of conventional chicken, because the cultured chicken product is grown, processed, and packaged under clean conditions and without the possibility of avian fecal contamination, its microbiological food safety profile is likely to be high.

Table 50 shows a typical microbiological analysis of Good Meat cultured chicken in comparison with conventional chicken (USDA, 2012).

Table 50. Microbiological analysis for Good Meat cultured chicken.

Microbiological Analysis	**Good meat cultured chicken**			**Conventional Chicken[1]**	
	Number of Samples	**Mean value**	**Positive samples (%)**	**Mean value**	**Positive samples (%)**
Aerobic plate count (cfu/g)[2]	>30	<10	0	556,018,333	98.8
Coliforms (MPN/g)[3]	>30	<3	0	2,544	88.5
E. Coli (MPN/g)	>30	<3	0	701	62.3
Campylobacter (cfu/ml)[4]	4	Negative	0	10.41	21.39

[34] https://fdc.nal.usda.gov/fdc-app.html#/food-details/331960/nutrients (last accessed July 20, 2021).

Salmonella (/25g)	>30	Negative/25g	0	0.82	26.3

[1] The Nationwide Microbiological Baseline Data Collection Program: Raw Chicken Parts Survey (USDA, 2012).
[2] CFU/g: Colony Forming Unit *per* g of material.
[3] MPN/g: Most probable number *per* g of material.
[4] *Campylobacter* measurements are in CFU/ml; LOD = 1 CFU/ml. These are total positives; this is the addition of 335 (13.4%) quantifiable samples plus 199 (7.9%) positives detected by qualitative test.

6.2.4. Presence of Residual Hormones in Cultured Chicken

Hormonal substances (anabolic agents) used in food animals fall into two groups: (1) those that are naturally occurring substances in animals (and are either extracted from animals or manufactured using recombinant DNA or some other technology) and (2) those that are produced synthetically and do not occur naturally in animals (xenobiotic compounds). As cultured chicken is produced with bovine serum in the culture media, residual concentrations of synthetic hormones that could have been administered to the cattle from which the serum was derived were quantified.

Safety concerns concerning hormone residues in meat are typically associated with cattle. When natural hormones are considered, claims of zero-tolerance residue levels in food are not meaningful, since these compounds occur in detectable and highly variable concentrations in body fluids and the tissues of all animals, regardless of hormone treatment or not. The hormones of endogenous origin comprise the "classical" steroid sex hormones, such as oestradiol-17β, testosterone, and progesterone. These natural hormones have low bioavailability when administered orally, owing to rapid conjugation and metabolic transformation in the liver. JECFA (2000) concluded that the amount of exogenous oestradiol-17β, progesterone, and testosterone ingested *via* meat from treated cattle would be incapable of exerting any hormonal effects in human beings because bioavailability is very low in the case of orally administered oestradiol-17β, progesterone, and testosterone. For this reason, JECFA recommended that establishing Maximum Residue Limits (MRLs) is unnecessary because exogenous estradiol is structurally identical to that produced endogenously in human beings, showing great variation in levels according to age and sex. MRLs were also considered unnecessary for progesterone and testosterone as the estimated amount of daily intake *via* food consumption (0.069 – 0.231 μg/day and 0.0093 – 0.108 μg/day, respectively) is negligible comparing to the levels of daily production in human beings (150 – 750 μg/day and 30 – 6900 μg/day, respectively) (Paris *et al.*, 2006).

Other growth hormones commonly tested in meat products comprise a category of synthetic xenobiotic growth promoters, including Zeranol, Melengesterol Acetate, and Trenbolone Acetate. These anabolic agents require a somewhat different approach to the evaluation of their safety in comparison to natural sex hormones, with approved MRLs of 2, 1, and 2 μg/kg in cattle muscle, respectively.

Approved feeding doses of Melengestrol Acetate are in the range of daily 0.25 to 0.50 mg *per* head, and residues of this hormone have been consistently below the sensitivity levels of the methods used for quantification (lower than 5 ppb in muscle), whether or not the compound was withdrawn 48 h before slaughter (Lauderdale *et al.*, 1977).

Therefore, Good Meat conducted tested cultured chicken to confirm that no residual hormones are present. To evaluate residual hormones in end-of-production chicken C1F-P1 cells, Good Meat performed the analysis for synthetic hormones at a third-party company (Merieux Nutrisciences). Results from a representative batch of cultured chicken are provided in Table 51. CFIA CVDR-M-3016.11 is the test method for Gestagens performed by BRN Silliker JR

laboratories in British Columbia accredited by Canadian Food Inspection Agency's center for Veterinary Drug Residues. CFIA CVDR-M-3019.17 is the method for Zeranol and related substances performed by BRN Silliker JR laboratories in British Columbia accredited by Canadian Food Inspection Agency's center for Veterinary Drug Residues.

As expected, cultured chicken is extremely low or absent of synthetic xenobiotic hormones, as the results from chicken cells were lower than detection thresholds (Table 51).

Table 51. Synthetic hormone testing of a representative batch of Good Meat cultured chicken produced at 1000 L scale.

Hormone	**Method Reference**	**Good Meat cultured Chicken**
GESTAGENS		
Megestrol Acetate	CFIA CVDR-M-3016.11	<0.005 ppm
Melengesterol Acetate	CFIA CVDR-M-3016.11	<0.005 ppm
Chlormadinone Acetate	CFIA CVDR-M-3016.11	<0.005 ppm
ZERANOL/DES/STILLBENES		
Dienestrol	CFIA CVDR-M-3019.17	<0.0003 ppm
Diethylstilbestrol	CFIA CVDR-M-3019.17	<0.0003 ppm
Hexestrol	CFIA CVDR-M-3019.17	<0.0003 ppm
Taleranol	CFIA CVDR-M-3019.17	<0.0003 ppm
Zearalanone	CFIA CVDR-M-3019.17	<0.0003 ppm
Zeranol	CFIA CVDR-M-3019.17	<0.0003 ppm

CFIA = Canadian Food Inspection Agency; ppm = parts *per* million.

Sex hormones were found to be below detection limits (0.002 ppm) for representative batches of cultured chicken (Table 52). MP 0337 rev2 2010 is the test method for analysis of multiresidual hormone performed by CHL-Silliker Lab in Resana Italy. This method is internally validated test method.

Table 52. Multi-residual hormone testing in a representative batch of Good Meat cultured chicken produced at 1000 L scale.

Hormone	**Method Reference**	**Good Meat cultured chicken**
MULTIRESIDUAL HORMONES		
17-*beta*-Oestradiol	MP 0337 rev 2 2010	< 0.002 ppm
17-*alpha*-Methyltestosterone	MP 0337 rev 2 2010	< 0.002 ppm
17-*alpha*-Ethynyloestradiol	MP 0337 rev 2 2010	< 0.002 ppm
19-Nortestosterone	MP 0337 rev 2 2010	< 0.002 ppm
Androstenedione	MP 0337 rev 2 2010	< 0.002 ppm
Stanozolol	MP 0337 rev 2 2010	< 0.002 ppm
Epitestosterone	MP 0337 rev 2 2010	< 0.002 ppm
Esestrol	MP 0337 rev 2 2010	< 0.002 ppm
Medroxyprogesterone	MP 0337 rev 2 2010	< 0.002 ppm
Medroxyprogesterone Acetate	MP 0337 rev 2 2010	< 0.002 ppm
Progesterone	MP 0337 rev 2 2010	< 0.002 ppm
Testosterone	MP 0337 rev 2 2010	< 0.002 ppm
17-*beta*-Trenbolone	MP 0337 rev 2 2010	< 0.002 ppm
17-*beta*-Trenbolone Acetate	MP 0337 rev 2 2010	< 0.002 ppm
Alpha-Zearalanol	MP 0337 rev 2 2010	< 0.002 ppm
Androstadienedione	MP 0337 rev 2 2010	< 0.002 ppm
Norandrostenedione	MP 0337 rev 2 2010	< 0.002 ppm
17-*alpha*-Boldenone	MP 0337 rev 2 2010	< 0.002 ppm
17-*beta*-Boldenone	MP 0337 rev 2 2010	< 0.002 ppm

Equilin	MP 0337 rev 2 2010	< 0.002 ppm

ppm = parts *per* million

6.2.5. Absence of Tumorigenic Potential

The starting C1F cells used by Good Meat do not have tumorigenic potential; nevertheless, it may theoretically be possible that a few cells might develop tumorigenic potential during the many generations of cell culture as they are grown up from an initial starter culture to the large volume of cells harvested to prepare food products, just as it is possible that a food animal might develop a microscopic tumor, not detectable by visual inspection. RNA sequencing analysis of end-of-production chicken C1F-P1 cells harvested from bioreactors did not reveal an increased tumorigenic potential relative to the parental cell source. Even if such an occurrence were to happen, cells would not present a health risk to consumers as described below.

The cells harvested at JOINN Biologics are frozen for shipment without cryopreservation agents to locations where the finished meat food products are prepared. This step alone virtually kills all cells. While mammalian and avian cells can withstand being frozen if they are suspended in a cryoprotectant such as dimethylsulfoxide (DMSO), during the freezing process without that protectant ice crystals form, which ruptures cell membranes leading to cell death (Dumont *et al.*, 2006).

In a USDA-approved facility, the preparer of the food products partially cooks the products after defrosting the chicken cells before they are stored, again frozen. This cooking and freezing process represents a second kill step. At the restaurant before service, the partially cooked food products are again cooked, a third kill-step that would be lethal to any remaining cells, including any theoretical tumorigenic cells, in the food products before they are served.

In the remote possibility that a few cells with tumorigenic potential survived the freezing and repeated cooking processes, they would then be killed by passing through the digestive system after being eaten, likely by the very low pH in the stomach (pH 1 – 2.5), or by the subsequent action of the various digestive enzymes that would destroy the cell membrane and kill the cells.

Following this logic, cells from Good Meat's cultured chicken products do not present a risk of tumorigenicity to consumers due to the complete safety package presented for both cell banks and the method of manufacturing, in addition to the layers of defense provided by product processing and food digestion.

6.2.6. Allergy Issues

In evaluating potential risks of allergy to cultured chicken cells, it is important to consider current risks of allergy to this widely consumed protein source. This evaluation was accomplished by performing a literature search using the NCBI PubMed database; then by reviewing the primary allergen database, www.AllergenOnline.org from the Food Allergy Research and Resource Program at the University of Nebraska, and the Allergen Nomenclature database at www.allergen.org.

Allergic reactions and the proteins that cause the reactions are specific to the individual. The dominant form of food from chickens that cause allergies are from the egg, and they are ovomucoid (Gal d 1) and ovalbumin (Gal d 2), which are the most abundant proteins in egg white and the most abundant and potent elicitors of allergy for people allergic to egg, which can be life-threatening (Järvinen *et al.*, 2007; Dhanapala *et al.*, 2015). The less important allergens in egg white include ovotransferrin (Gal d 3) and lysozyme (Gal d 4) and in egg yolk chicken serum

albumin [Gal d 5 and rarely YGP42 C-terminal fragment (Gal d 6)] as described by Dhanapala *et al.* (2015).

The prevalence of allergic reactions to chicken meat is low and is presumed to be similar to that of red meat allergy according to one publication (Hemmer *et al.*, 2016), and to range from 0.6% to 5% in food allergic subjects (a small fraction of the total population) according to another (Besler *et al.*, 2001).

Consumption of chicken meat rarely causes food allergy. When it does, primarily mild reactions occur, associated with people allergic to fish due to IgE binding to the enolase or aldolase enzymes in muscle or to parvalbumin (Kuehn *et al.*, 2016) (Table 53). Myosin (Gal d 7) has also been called an allergen, but it does not appear to be an important allergen from the consumption of chicken meat (Hemmer *et al.*, 2016). A recently discovered delayed food reaction has been described for red meats in people who have been sensitized by tick bites (Commins *et al.*, 2009). The carbohydrate responsible for this reaction, *alpha*-gal, does not appear to be present in chicken meat (Wilson and Platts-Mills, 2018).

Table 53. Allergens from representative mammalian and avian meat sources that have been reported to bind IgE antibodies but have not been proven to elicit allergic reactions (Wilson and Platts-Mills, 2018).

Source	Allergen Name	Biochemical Name
Bovine	Bos d 6	Serum Albumin
	Bos d 7	Immunoglobulin
	a-Gal	Gal-a 1,3Gal-b1,4GlcNAcR
Chicken	Gal d 5	Serum Albumin
	Gal d 7	Myosin light chain kinase
	Gal d 8	a-parvalbumin
	Gal d 9	b-enolase
	Gal d 10	Aldolase

The mRNA for enolase, aldolase and parvalbumin proteins do not show upregulation in the end-of-production chicken C1F-P1 cells. Myosin light chain kinase was found downregulated in end-of-production chicken cells (Appendix 10.5). For this reason, there is no expectation for these proteins to be present at a higher level in cultured chicken.

Serum albumin is the only media constituent that may pose an allergenicity concern. Serum albumin is reported to be an important contributor to both mammalian and avian meat allergy. However, the protein denatures after moderate heating and loses IgE binding capacity (Wilson and Platts-Mills, 2018). Individuals with beef meat allergy occasionally show a co-existing allergy to milk. That circumstance is generally due to IgE recognition of serum albumins, which are heat labile. BSA in Good Meat cultured chicken is present in extremely low amounts ranging on average 15 mcg/mL, typically, cow's milk has 150 – 250 mcg of BSA/mL of milk, as mentioned in section 5.2.3.9.2. However, significant allergy to cow's milk is usually due to IgE sensitization to *beta*-lactoglobulin or caseins, which are not present in meat.

While proteins are encoded in the DNA present in all cells of the organism, all genes are not transcribed in all cell types or tissues. The primary allergens from the chicken species are in chicken eggs, and primarily sensitize and cause reactions in young children, although egg allergy can persist into adulthood. It is highly unlikely to have expression of the major egg allergens in fibroblasts that are grown in culture as these allergens are expressed in the oviducts of mature female chickens under control of specific hormones (Stadnicka *et al.*, 2018).

Overall, the incidence of allergic reaction to cultured chicken is likely to be rare and no more common than an allergic reaction to other types of meat, including conventionally produced chicken meat.

6.2.7. Description of Labeling

As the concept of cultured meat products such as cultured chicken is now being introduced globally, several international regulatory agencies are working towards fashioning appropriate regulatory frameworks, including frameworks concerning labeling. As *per* the joint memo issued by FDA and the USDA on March 7, 2019, labeling of the cultured chicken would be regulated by FDA and the finished cultured chicken meat product consisting of a combination of food ingredients and food additives would be regulated by the USDA. Good Meat believes that *cultured chicken* is the most appropriate way to describe the biological material exiting the culture process. Following the culture process, the cultured chicken is then stored at -20°C (or temperatures below) and transported to an USDA-approved facility for further processing into final cultured chicken products. Cultured chicken product labeling will follow USDA regulations and will be submitted through the label submission and approval system (LSAS).

Figure 6 is the proposed label for cultured chicken that Good Meat will be shipping from Richmond (CA, USA). This is a clear label that highlights the storage instruction and labeling of cultured chicken.

Figure 6. Proposed label for Good Meat cultured chicken that will be shipping from Richmond (USA).

6.3. Expert Support

Working with external experts in safety and regulatory compliance, Good Meat has developed a structure that assures the safety of our meat products and provides a structure that is universally applicable for others. By drawing on regulatory and quality expertise in both food and human therapeutic arenas, Good Meat is confident that this process will protect food safety and public health.

A panel of regulatory and safety experts has worked in collaboration with Good Meat as noted below:

Stuart Pape is the Chair, FDA Practice Group at the Polsinelli law firm in Washington, D.C. Stuart Pape has regulatory experience with the U.S. Food and Drug Administration (FDA), U.S. Department of Agriculture (USDA), and similar health and safety regulatory bodies worldwide. Previously, he served in various positions in the Office of the Chief of Counsel at the FDA, including as associate chief counsel for food. He also served as executive assistant to FDA Commissioner Donald Kennedy.

Joseph Rodricks, Ph.D. is a founding Principal of Ramboll, and an internationally recognized expert in toxicology and risk analysis. He has consulted for hundreds of manufacturers, government agencies and for the World Health Organization in the evaluation of health risks associated with human exposure to chemical substances of all types. Joe came to consulting roles after a 15-year career as a scientist at the US Food and Drug Administration (FDA).

Dr. Duncan Turnbull is a board-certified toxicologist with more than 30 years of experience in toxicology, chemical carcinogenesis, mutagenesis and quantitative risk assessment. Duncan is the author or coauthor of more than two dozen peer-reviewed publications in toxicology and risk assessment.

Richard E. Goodman is a Research Professor in the Department of Food Science and Technology, in the Food Allergy Research and Resource Program at the University of Nebraska Lincoln. He was an Allergen Program Manager at Monsanto from 1997-2004. He manages the www.AllergenOnline.org database for risk assessment of Genetically Engineered Organisms and novel food proteins and is Chair of the WHO/IUIS Allergen Nomenclature Sub-Committee since June 2014.

7. FOOD SAFETY PLAN

7.1. Description of Process Controls

Good Meat has developed robust process controls to ensure the safety and quality of cultured chicken. The primary process steps in manufacturing include thawing of cells from a validated cell bank, scale-up of cells in shake flasks and bioreactors, followed by harvest and storage of the cultured cells. A detailed Hazard Analysis (HA) was performed, and Risk-Based Preventative Controls (RBPC) were implemented. The HARBPC plan included in this document summarizes the hazards, risk assessment, preventative measures, confirmation, and corrective actions in case of deviations. In addition, extensive in-process checks and controls of key process parameters are implemented to ensure that the process is well controlled to maintain consistency and reliability. Full documentation of process controls, sanitation and environmental controls, and supply chain controls and their implementation and validation are provided in Attachments A2 – A5.

7.2. Safety Control and Testing

A thorough HARBPC plan was developed for cultured chicken manufacturing process by identifying food safety hazards including biological, chemical and physical along with their corresponding preventative measures and confirmation/corrective actions. The major aspects of the HARBPC plan are summarized below. Food safety hazards in the cultured chicken production process are primarily due to biological/chemical contamination of the cell culture and/or due to sub-standard sanitation of the equipment used.

The preventative measures encompass: (i) use of sterile equipment and supplies for propagating the cell culture (where possible, single use, pre-sterilized consumables are used to alleviate the risk of contamination); (ii) sterile filtration of the growth medium using 0.2-micron filters; (iii) use of aseptic technique for the transfer of culture and the medium from one process step to the other and; (iv) validated sanitation procedures. Broadly, the process steps that utilize these safety controls include the following:

1. Receiving and storage of raw material, media, components & utilities
2. Preparation of growth media

3. Preparation of production media
4. Cultured C1F-P1 cells proliferation through final harvest from the bioreactor
5. Preparation of wash solution
6. Washing of harvested cultured chicken (centrifugation/resuspension)
7. Storage of cultured chicken

Full details of the HARBPC are available as an Attachment 4: Food Safety Plan for C1F-P1 Cultured Chicken Cells.

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The effectiveness of the control measures is confirmed in-process during the cell-growth stages by monitoring the progress of the cell culture for any (a) atypical or stalled growth and (b) visual contamination under microscope. Further validation of control measures is shown by sterility testing of end-of-culture (pre-harvest) samples for the absence of adventitious agents and indicated in Table 54 for a group of six representative manufacturing batches. A culture that tests positive for contamination results in testing of the cultured cells. If reconfirmed, the cultured cells would be rejected and not released for further use.

Table 54. Sterility testing from pre-harvest samples on multiple representative cultured chicken batches (RB1 to RB6).

Sterility pre-harvest	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
FTM broth	Negative	Negative	Negative	Negative	Negative	Negative	Negative
TSB broth	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Mycoplasma	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Mycoplasma genus PCR	Negative	Negative	Negative	Negative	Negative	Negative	Negative

FTM = Fluid Thioglycolate Media; Specs = Specifications; TSB = Trypticase Soy Broth.

The downstream processing procedures, including harvesting and washing of cells followed by packaging and storage, are performed under controlled conditions following current good manufacturing processes (cGMP) procedures. Sanitation of the equipment involved in downstream processing is validated using ATP and APC swabbing. If the ATP swab results are out of limits, the cleaning process is repeated. If the APC swab results are out of limits, the cleaning process is re-evaluated and corrected. The microbiological safety of the cultured chicken is confirmed by the microbiological testing as summarized in Table 55, including six representative manufacturing batches. The batches are released for further use as a food ingredient in cultured chicken products if microbiological specifications are met. A batch is rejected for further use if specifications are not met.

Table 55. Safety release testing on multiple representative cultured chicken batches (RB1 to RB6).

Representative Batch (RB) Microbiological analysis	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Aerobic plate count (cfu/g)	<10,000	<10	<10	<10	<10	<10	<10
Yeast (cfu/g)	<100	<10	<10	<10	<10	<10	<10
Mold (cfu/g)	<100	<10	<10	<10	<10	<10	<10
Coliforms (MPN/g)	<24	<3	<3	<3	<3	<3	<3
E. coli (MPN/g)	<3	<3	<3	<3	<3	<3	<3
Enterococcus (cfu/g)	<10	<10	<10	<10	<10	<10	<10
Salmonella (/25g)	Neg/25g	Neg/25g	Neg/25g	Neg/25g	Neg/25g	Neg/25g	Neg/25g

cfu = colony-forming units; MPN = most probable number; Neg/25g = Negative per 25g; RB = representative batch.

The cell centrifugation (concentration) and washing process were designed to effectively remove the growth media component residuals from cultured chicken. After washing with sodium chloride solution, the wash solution is tested for residual Pluronic F-68. Good Meat has selected Pluronic F-68 as a media component to confirm the washing effectiveness because it is not

consumed by the cells. A result of <100 ppm would represent at least a 90% reduction from the media. If the test result exceeded the limit, an additional washing stage would be applied to ensure sufficient washing. The product would only be released with an acceptable result.

Further, the absence of unwanted toxic heavy metals is ensured to be below the acceptable limits before releasing the batch as indicated in the Table 56.

Table 56. Safety release testing on multiple representative cultured chicken batches (RB1 to RB6).

Heavy metals (cultured chicken)	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Arsenic (ppm)	**<0.10**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lead (ppm)	**<0.20**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Mercury (ppm)	**<0.05**	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Cadmium (ppm)	**<0.02**	<0.001	<0.001	<0.001	<0.001	0.001	0.001
Chromium (ppm)	**<0.20**	0.02	0.01	0.01	0.02	0.01	0.02
Residual media components (wash solution)	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Pluronic F-68 (ppm)	**<100**	<100	<100	<100	<100	<100	<100

ppm = parts *per* million; Specs = specifications. RB = representative batch.

7.3. Quality Control and Testing

Quality control of a product is primarily correlated to consistent process performance achieved through design of a well-controlled process and execution of it using cGMP procedures. While validated sterilization and sanitation programs mostly ensure safety of the product, monitoring and controlling of key process parameters is essential for ensuring consistent process performance. Given the intrinsically slow growth rate of animal cells, in-process sampling and controls can be utilized to assure the success of the process throughout a batch. Written procedures and testing for in-process controls are established and followed. Table 57 summarizes vital process parameters controlled in the manufacturing process of cultured chicken at various stages for consistent performance.

Table 57. Key process parameters in manufacturing process of cultured chicken and the corresponding impacted key performance indicators.

Process step	**Key process parameter (KPP)**	**Key performance indicator (KPI)**
Media preparation	Accurate weighing of components	pH & osmolarity of the media, Supports growth
Growth in shake flasks	Temperature & percent CO_2	Viable cell density, percent viability
Growth in bioreactors	Temperature, Dissolved oxygen, pH	Viable cell density, percent viability
Harvesting	Relative centrifugal force	Pellet formation with limited lysis
Washing	Amount of residual media components	Pluronic F-68 concentrations in the wash solution
Storage	Temperature	Visual absence of any thawing post freezing

The key process parameters are monitored and recorded in the batch records that are approved by Quality Assurance. Equipment sensors indicating and recording key process parameters are put through validated calibration programs to ensure accuracy. The effectiveness of the control of key process parameters for the indicated steps including media preparation and growth in shake flasks and bioreactors is evident in the consistency of the cell growth, indicated by the viable cell density and *percent* of cell viability. If these metrics do not meet a minimum cell density requirement for subsequent transfer or harvest, the process will be aborted and started again from the thawing of cryovials from a validated cell bank.

The washed cells are packaged and stored at < -20ºC until further usage. In addition to calibration of the freezer temperature sensors, the storage temperature is tracked and monitored using alarm systems. If all the key process parameters are monitored and found to be in the acceptable range, the process is "in-control" and the quality of the product (cultured chicken) tested by proximate analysis will be consistent within the predetermined specifications. This is illustrated in Table 58 below, including six representative batches (RB1-RB6).

Table 58. Quality release testing on multiple representative cultured chicken batches (RB1 to RB6)

Proximate Analysis	**Specs**	**RB1**	**RB2**	**RB3**	**RB4**	**RB5**	**RB6**
Moisture (%)	85-95	88.7	89.9	90.5	89.9	88.8	89.1
Protein (%)	5-10	9.8	7.9	7.8	8.2	8.9	8.1
Fat (%)	0.5-2.0	1.6	1.2	1.7	1.4	1.2	1.2
Ash (%)	0.0-2.0	0.8	1.0	0.8	0.7	1.0	0.9
Carbohydrate (%)	0.0-2.0	<0.1	<0.1	<0.1	<0.1	0.2	0.8

RB=representative batch; Specs=specifications.

Any deviations outside the acceptable range for key process parameters are evaluated for any potential risk to the quality of cultured chicken. In addition, a formal change control procedure is in place to evaluate all changes that can potentially affect the quality of the cell culture process and cultured chicken. Any such changes to the process will be reviewed and approved by the production unit and the Quality Assurance department before implementation.

A Certificate of Analysis of key in-process and release testing results from a representative manufacturing batch is provided in Figure 7.

Certificate of Analysis

Product Name	Cultured Chicken (*Gallus gallus*)
Project Code	2927
Batch # or Lot#	21-FP-003-01
Specification	2905-QC-SPE-001.01
Date of Manufacturing (YYY-MM-DD)	2021-04-11
Storage Conditions	< -20°C

Test Item	Test Method	SOP#	Specification	Test Result
Appearance	Visual Examination	2905-QC-SOP-001	Off-White to Pale Yellow	Off-White to Pale Yellow
Moisture Content	AOAC 926.12	QA-0200-4101.20	85 – 95 %	88 %
Protein Content	AOAC 992.23	AS-CC-011.02	5 – 10 %	9 %
Fat Content	AOAC 950.54	QA-0210-4212.22	0.5 – 2.0 %	1.0 %
Ash Content	AOAC 945.46	QA-0225-2001.13	0.0 – 2.0 %	0.9 %
Carbohydrate Content	By difference from the total of moisture, protein, ash, and fat content	Calculation	0.0 – 2.0 %	0.9 %
Arsenic	AOAC 2015.01	M-C043	< 0.10 ppm	< 0.01 ppm
Lead	AOAC 2015.01	M-C043	< 0.20 ppm	< 0.01 ppm
Mercury	AOAC 2015.01	M-C043	< 0.05 ppm	< 0.005 ppm
Cadmium	AOAC 2015.01	M-C043	< 0.02 ppm	0.001 ppm
Chromium	AOAC 2015.01	M-C043	< 0.20 ppm	0.03 ppm
Aerobic Plate Count	AOAC 966.23	QA-0015-0201	< 10,000 CFU/g	< 10 CFU/g
Coliforms	AOAC 966.24	QA-0020-0301	< 24 MPN/g	< 3 MPN/g
E. coli	AOAC 966.24	QA-0025-0302	< 3 MPN/g	< 3 MPN/g
Enterococcus	CMMEF, 4th ed.	QA-0045-0601	< 10 CFU/g	< 10 CFU/g
Salmonella	AOAC-RI100201	QA-0010-0180	Negative/25g	Negative/25g
Yeast	FDA BAM Chapter 18	QA-0035-0501	< 100 CFU/g	< 10 CFU/g
Mold	FDA BAM Chapter 18	QA-0035-0501	< 100 CFU/g	< 10 CFU/g
Albumin	ELISA	101-QCU-034	As reported	0.4 µg/mL
Pluronic	Cobalt Thiocyanate	101-QCU-035	As Reported	< 0.01 %

Figure 7. Typical certification of analysis of key in-process and release testing results and the corresponding specifications for a representative batch of cultured chicken.

7.4. Environmental Monitoring Plan

Environmental monitoring is performed for all processes that are performed outside the bioreactor including C1F-P1 cell expansion, concentration by centrifugation, cell washing, and packaging. These processes take place in a controlled environment inside the cleanroom in which air quality (i.e. the number of airborne particles and microorganisms) is controlled to minimize risk of contamination. The environmental monitoring program ensures maintenance of the required environmental conditions inside the cleanroom. Cleanrooms are further equipped with HEPA filters and differential pressure is controlled to maintain environmental conditions inside the cleanroom. Good Meat has also established environmental controls as part of an environmental monitoring plan (Table 59). The test, method, frequency, and pass criteria are all presented.

Table 59. Environmental monitoring plan.

Site	Test	Method	Frequency	Pass criteria
Biosafety cabinets (BSC)	Active Viable air, non-viable air, Viable surface	USP<1116>	Weekly	ISO Class 5
Biosafety cabinets (BSC)	Passive Viable Air (settle plates), Viable surface (personnel monitoring)	USP<1116>	In-operation monitoring	ISO Class 5
Media preparation room	Active Viable air, non-viable air, Viable surface	USP<1116>	Weekly	ISO Grade D
Inoculation preparation room	Active Viable air, non-viable air, Viable surface	USP<1116>	Weekly	ISO Grade C
Bioreactor Room	Active Viable air, non-viable air, Viable surface	USP<1116>	Weekly	ISO Grade D
Harvest Room	Active Viable air, non-viable air, Viable surface	USP<1116>	Weekly	ISO Grade D
Outlet tubing (cultured cells)	APC swab	AOAC 966.23	Post-harvest	< 100 cfu/swab
Outlet tubing (cultured cells)	*Enterobacteriaceae* Swab	AOAC 2003.01	Post-harvest	< 30 cfu/swab
Outlet tubing (supernatant)	APC Swab	AOAC 966.23	Post-harvest	< 100 cfu/swab
Outlet tubing (supernatant)	*Enterobacteriaceae* Swab	AOAC 2003.01	Post-harvest	< 30 cfu/swab
Sink Drain	*Listeria* Swab	AOAC 2013.10	First and last harvest	Negative/swab
Sink Drain	*Salmonella* Swab	AOAC 2013.01	First and last harvest	Negative/swab

AOAC = Association of Official Analytical Chemists; APC = Aerobic plate count; BSC = biosafety cabinets; cfu = colony-forming unit; hr = hour; USP = United States Pharmacopoeia

8. CONCLUSION

Overall, as described in the above sections, Good Meat has implemented robust process controls program to ensure the safety and quality of the cultured chicken product. Appropriate

product testing is employed to ensure that the cultured chicken product is free of pathogens, contaminants, and any other material (*e.g.*, microbial contaminants, heavy metals) not suitable for human consumption. In addition to validated sanitation and sterilization programs, calibration programs were developed on key equipment and process sensors, critical to assure the quality of food product production. Good Meat extensively characterized the raw material input lacking an appropriate food regulation. Based on the totality of information presented in this dossier, Good Meat concludes that there is reasonable certainty that no harm will result from the consumption of cultured chicken. Accordingly, cultured chicken is safe for its proposed use, such that it should be permitted for sale in the United States after undergoing the appropriate regulatory review by FDA and USDA-FSIS (as previously indicated).

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RESPONSE TO FDA FOR CLARIFICATION OF QUESTIONS RELATING TO GOOD MEAT CULTIVATED CHICKEN

July 8, 2022

Good Meat, Inc.

TABLE OF CONTENTS

INTRODUCTION

The responses reported in this document address the request for additional information received from the FDA on June 29, 2022, after the review of the Final Safety Dossier (CCC 000001) submitted on March 4, 2022. For clarity, FDA requests are included as bold text and our responses follow. We numbered the requests by the FDA for ease of referencing.

1. CELL BANK ESTABLISHMENT

1.1 CELL ORIGIN

1.1.1. Please provide, for addition to the disclosable safety narrative, information about the cell origin as follows:
the statement regarding the patent found in the first paragraph of page 7, Section 10.1 of the confidential supplementary material. Please also provide a copy of Attachment A1.

The statement regarding the patent found in the first paragraph of page 7, Section 10.1 of the confidential supplementary material is below:

> Cells utilized in the production of the master cell bank originate from a commercially available chicken cell line.[1] This cell line, denoted UMNSAH/DF1, was deposited at American Type Culture Collection (ATCC, Manassas, Virginia, USA) on October 11th, 1996 with the reference number ATCC® CRL12203™. The generation of UMNSAH/DF1 cell lines, including isolation, characterization, and use of immortalized cell lines from primary chicken embryo, is described in US Patent 5,672,485 (Attachment A1). The UMNSAH/DF1 cell line was certified by the supplier as negative for Avian Influenza (Type A), Avian Reovirus, Avian Adenoviruses (Groups I-III), Avian Encephalomyelitis Virus, Fowl Pox, Newcastle Disease Virus, Paramyxovirus (type 2), *Mycoplasma, Salmonella,* and other infectious agents known to infect poultry stock.

A copy of Attachment A1 from the Dossier in Support of the Safety of Good Meat Cultured Chicken as a Human Food Ingredient Appendix dated March 4, 2022 is also provided below:

[1] www.atcc.org/Products/All/CRL-12203.aspx (last accessed July 2, 2021).

US005672485A

United States Patent [19]

Foster et al.

[11] Patent Number: 5,672,485

[45] Date of Patent: Sep. 30, 1997

[54] **IMMORTALIZED CELL LINES FOR VIRUS GROWTH**

[75] Inventors: **Douglas N. Foster**; **Linda K. Foster**, both of Roseville, Minn.

[73] Assignee: **Regents of the University of Minnesota**, Minneapolis, Minn.

[21] Appl. No.: **696,200**

[22] Filed: **Aug. 13, 1996**

[51] **Int. Cl.**^{6} **C12Q 1/02**

[52] **U.S. Cl.** **435/40.51**; 435/349; 435/172.3; 435/172.1; 435/235.1

[58] **Field of Search** 424/94.63, 85.2; 455/69.1, 69.3, 240.2, 172.3, 240.1, 320.1, 317.1, 172.1, 6, 91.2, 349, 40.51, 235.1; 800/2

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Primary Examiner—James Ketter
Assistant Examiner—Ire Yucel
Attorney, Agent, or Firm—Mueting, Raasch, Gebhardt & Schwappach, P.A.

[57] **ABSTRACT**

This invention relates to the production and use of immortalized cell lines from primary chicken embryonic fibroblasts. The cells are useful as substrates for virus propagation, recombinant protein expression and recombinant virus production.

9 Claims, No Drawings

the terms and conditions of the Budapest Treaty. In addition, this invention relates to cultures of these cells and to immortalized subclones of the immortalized cell line that support virus replication.

In one aspect of this invention the immortalized cells of this invention contain virus and in another the immortalized cells of this invention contain at least one vector capable of directing expression of recombinant protein in the cells. In one embodiment the cells of this invention express recombinant protein and in another aspect of this invention the vector contained in the cells of this invention encodes at least a portion of a recombinant virus. In another embodiment the vector is a retroviral vector.

In another aspect of this invention a method is disclosed for producing an immortalized cell line from chicken embryonic fibroblasts comprising the steps of: growing primary chicken embryonic fibroblasts in culture; passaging the fibroblasts in culture until they begin cell senescence; concentrating the cells during cell senescence to maintain about 30% to about 60% culture confluence; identifying foci of non-senescent cells; and growing the non-senescent cells for greater than 30 passages.

In yet another aspect of this invention a method is disclosed for growing virus in a cell comprising the steps of: growing a spontaneously immortalized cell line derived from primary chicken embryonic fibroblasts in culture; infecting the cells with virus; allowing the virus to replicate in the cells; and collecting virus that replicated in the cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

At present there are essentially no non-viral, non-viral protein or non-chemically transformed arian cell lines available. Primary cell lines are cumbersome to continually generate for virus stock production and must be separately validated as contaminant free reservoirs for virus growth. This invention discloses the immortalization of chicken embryo fibroblastic (CEF) cells including cells derived from East Lansing Line (ELL-0) chicken embryos.

The term immortalization is used herein to refer to non-rodent cells capable of growing in culture for greater than 30 passages that maintain a doubling time in culture of about 1 to about 2 days and have been in continuous culture for greater than about 6 months. Avian cells are generally considered immortalized after about 20 to about 25 passages in culture. Immortalized cells are differentiated from transformed cells in that unlike transformed cells, immortalized cells are density dependent and/or growth arrested (e.g., contact inhibited). Transformed cells are capable of growth in soft agar and are usually able to form minors when injected into laboratory animals. The cells of this invention are useful as reservoirs for growing virus or for expressing recombinant protein or virus particularly where it is important that the cells do not harbor contaminating virus or viral protein. The cells are also useful for studying the underlying mechanisms of cellular senescence and immortalization.

Chicken Embryo Fibroblastic (CEF) primary cells from 10 day old ELL-0 eggs were obtained by taking the embryonic torso of the 10 day old embryos, mincing the tissue and placing the cells in culture. Fertilized eggs are available Hy-Vac (Adel, Iowa). The eggs and their layers were certified by the supplier as negative for Avian influenza (Type A), Avian reovirus, Avian adenoviruses (Groups I–III), Avian encephalomyelitis virus, Fowl pox, Newcastle disease virus, Paramyxovirus (Type 2), Mycoplasma, Salmonella and other infectious agents known to infect poultry stock. Isolation of primary cells and identification of immortalized cells is provided in Example 1.

The cells were identified because at the time of the discovery of the immortalized line, cell populations were being selected to study the effects of cell senescence. Human and avian cells are known to be some of the most difficult cells to immortalize under tissue culture conditions. Unlike rodent cells, there are no peer-reviewed reports of methods for immortalizing human or chick fibroblasts from normal donors (Smith, et al. *Science* 273:63–67, 1996). In avian fibroblasts, untreated cells typically last only 20–25 passages. That is, by 30 passages primary cultures of these avian cells are dead or dying. As disclosed in this invention, to reach 20 passages, the cells were passed and concentrated (see Example 1) between about passage 12 up to about passage 20 onto smaller plates as needed. Foci of more rapidly growing cells were observed and these loci were isolated using cloning rings (Belleo Glass, Inc. Vineland, N.J.) and expanded in culture.

Senescence is defined herein as cells having population doublings of about 0.5 population doublings or less per day. For this invention, immortalized cells are cells in culture for more than 30 passages, growing at a population doubling rate (as determined by total cell counts and viable cell counts per day using trypan blue exclusion) of about between 0.6 to about 1.2 population doublings per day and preferably between about 0.7 to about 1.0 population doublings per day while exhibiting contact inhibition, density dependence and a normal cell morphology.

The cells obtained from the originally identified loci, as described in Example 1, have undergone greater than 400 (population doublings) and greater than 160 passages. The term foci is used herein to refer to clusters of morphologically uniform cells that can be distinguished from the morphology of the cells around them. These foci of cells can be readily removed and subcloned for further study. The cells of this invention have continued to double every 22–24 hrs. The cells were contact inhibited, reverse transcriptuse negative (see Example 2), density dependent arrested, aneuploid (as observed by chromosome spread analysis under oil emersion microscopy the karyotype was a mixture of diploid/tetraploid karyotypes with some cells displaying an apparent translocation of chromosome 1), and grow to high plating densities of between $1.1–1.9\times10^5$ cells/cm^2. No multinucleated giant cells were observed. The cells have a uniform phenotype. The cells also maintain a characteristic pattern of rapid growth which is important for virus propagation.

The cells were nontransformed as demonstrated by their inability to grow in soft agar assays (see Example 3). In addition, the cells did not produce tumors when injected into the wings of chickens (see Example 4). Exemplary cells of this invention were designated UMNSAH-DF 1 cells and are deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville Md., 20852 as accession number CRL-12203, deposited on Oct. 11, 1996 under the terms and conditions of the Budapest Treaty.

This invention also relates to the immortalized chicken embryonic fibroblast cells of this invention in culture and to subclones of the immortalized cells of this invention. For example, the cells of this invention are identified as spontaneous immortalized cells. The cells are obtained from known virus-free, known chemical contaminant-free layers (hens producing the embryonic tissues that are the source of this invention) and the embryonic tissues used to produce the cells of this invention are also chemical contaminant-free

(i.e, free from treatment by known carcinogens or other agents known to transform rodent cells) and free from known virus. Once the immortalized cells of this invention are in culture, it is possible to further subclone the cells to select for other physiological parameters that may vary in the cell population while still maintaining contact inhibition and susceptibility to virus infection.

Cells were tested for their ability to replicate HVT (Herpesvirus of Turkeys), arian herpesvirus (serotype III), Fowl Pox virus, and reovirus. Cells can be tested for their ability to replicate Circodnavirideae, chicken HSV serotype II for a variety of other viruses and have been tested as a substrate for transfection. The cells were useful for propagating both avian and non-avian viruses. Example 5 details methods for propagating HVT, Fowl Pox virus and reovirus. The cells are useful as a substrate for viral production, and in particular the cells are useful for retrovirus production since the cells and their layers (i.e., their mothers) did not have detectable retrovirus infections. The cells are able to support the replication of Avian Sarcoma Leukemia Virus and Rous Sarcoma Virus.

To produce virus stock, the cells of this invention can be seeded into tissue culture flasks, roller bottles, stir culture, into hollow fiber reactors or other mass culture systems. For roller bottle virus propagation, the cells are seeded at about $2–5\times10^4$ cells/cm^2 of surface area. The multiplicity of infection (ratio of infectious virus particles to cells) to initiate virus stock growth will vary depending on virus strain. Those skilled in the art of virology and skilled in the growth of particular viruses and strains of viruses will be able to maximize virus stock yield through the standard manipulation of the multiplicity of infection, temperature, media variations, and the like, without undue experimentation.

Methods for harvesting the virus after infection to obtain infectious virus stock also varies with virus strain. Enveloped viruses egress into the culture media more slowly than non-enveloped virus. Stocks of virus can be obtained from the culture media alone or from cell lysates pooled with the conditioned media. For lytic viruses (those efficient at lysing a cell during virus egress), harvesting the conditioned culture media (e.g., spent media containing virus) after a gentle centrifugation step to remove cell debris is sufficient. Again, methods for harvesting and saving virus from a wide range of virus strains are well known in the art.

There are a variety of methods, also all known in the art, for quantitating virus growth from a culture of cells. For example, the titer of a virus stock for members of the Herpesvirus family and for a variety of viruses producing foci of cytopathology on a cell monolayer surface are readily quantitated by plaque assay (as plaque forming units/ml of culture fluid or as plaque forming units/dose for vaccine inoculum virus quantitation) or as tissue culture infectious dose-50 ($TCID_{50}$). Rapidly lytic viruses are better quantitated by $TCID_{50}$ as the dose or dilution of virus stock capable of infecting 50% of the cultures in a defined time period. Methods for growing and quantitating virus are known in the art and sources for teaching virus quantification methods are found in Fields, et al. (eds) *Fundamental Virology* 1991, Raven Press, New York or in Mandell, et al. (eds.) *Principles and Practice of Infectious Diseases,* 1985, John Wiley & Sons, New York.

In addition to supporting virus growth, the cells of this invention can be used as packaging lines to produce recombinant virus, including retrovirus. The cells can also be used to produce recombinant proteins, including viral proteins, and the like. Methods for incorporating nucleic acid encoding recombinant protein into a nucleic acid vector under the control of regulatory elements capable of directing expression of a protein in a eukaryotic cell, such as the immortalized cells of this invention, are well known in the art. Expression vectors are replicable nucleic acid fragments that can direct expression of a recombinant protein. Many expression vectors, including retroviral vectors, are available in the art through journal publications and commercial suppliers. Replicable expression vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, enhancer elements, promoter elements, optional signal sequences and transcription termination sequences. The selection or marker genes encode protein that serves to identify a population of transformed or transfected cells. Typical selection genes encode proteins that confer resistance to antibiotics or other toxins, complement auxotrophic deficiencies or supply critical nutrients not available from complex media.

Expression vectors having nucleic acid encoding recombinant protein are transfected into the cells and are used to direct expression of the recombinant protein in the immortalized cells of this invention. The vector preferably can encode any recombinant protein capable of expression in chicken embryonic fibroblast cells, including, but not limited to, virus protein, including reverse transcriptase and/or viral structural protein. Examples of vectors to produce recombinant protein in a cell include retroviral vectors to produce tumor suppressive protein, or viral structural protein such as those disclosed by Givol, et al. *Oncogene* 11(12):2609–2618, 1995, Givol, et al. *Cell Growth & Differentiation* 5(4):419–429, 1994, Federspiel, et al. *Virology* 203(2):211–220, 1994 and Boyer, et al. *Oncogene* 20:457–66, 1993.

The cells of this invention can serve as substrate to express recombinant virus, including, but not limited to recombinant retrovirus. The cells of this invention are suitable to serve as packaging cell lines for genetically engineered virus useful for gene therapy, or the like. Constructs and methods for using a particular cell line as a packaging cell line are known in the art. For example, Boerkoel, et al. (Virology 195(2):669–79, 1993) discloses methods for packaging virus using primary chicken embryonic fibroblasts as the packaging cell line. These same methods can be used to package virus in the immortalized cells of this invention.

Since most avian cell lines and all transformed avian cells as well as virtually all mouse transformed cell lines either contain viral contaminants such as endogenous virus or produce viral protein, they are not suited for the production of human or animal vaccines. The cells cannot be used to produce recombinant protein because the endogenous contaminants can contaminate purified recombinant protein preparations. Advantageously, the cells of this invention provide a suitable alternative to these problems.

The cells of this invention can also serve as a substrate for supporting virus growth from other cells. These other cells include primary cells, or cultured cells that show improved growth or longevity in culture in the presence of other cells or in the presence of extracellular matrix proteins such as collagens, laminins, and the like. In one embodiment, cells are mixed with virus and then mixed with the cells of this invention preferably in a ratio of cells: to cells of this invention of about between 1:5 cells to about 1:20 cells and more preferably in a ratio of about 1:10 (1 cell to about 10 cells of this invention). The mixed cells are then placed into culture. In a second embodiment the cells are mixed with

virus and plated onto the surface of the immortalized cells of this invention are already attached to a tissue culture surface. The cells of this invention serve as a support for the other cells and, without intending to limit the scope of this invention, the cells of this invention can supply growth factors and the like as well as extracellular matrix components, and the like to support the other cells while they are producing virus. Example 6 provides an example of the use of the cells of this invention as a cell substrate.

Particular embodiments of this invention will be discussed in detail and reference has been made to possible variations within the scope of this invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention.

Example 1

Establishment of Spontaneous Chicken Fibroblast Cell Line

Two dozen ELL-0 eggs were ordered from East Lansing USDA poultry stocks. The eggs were incubated in a sterilized isolated incubator for 10 days and were processed for primary cultures. Embryonic tissue was dissociated using a trypsin/EDTA solution and plated in DMEM media (Gibco) containing 10% fetal calf serum (Gibco), 1% antibiotic/antimycotic (Gibco) containing and 2 mM L-glutamine (Gibco). The dissociated cell suspension was collected in a 50 ml centrifuge tube containing 10% ml fetal bovine serum to inactivate the trypsin and centrifuged at 700 × g for 10 minutes.

The cells were resuspended in 10 ml Dulbecco's modified Eagles's medium enriched with 36 μg/ml insulin (Sigma), 1.6 μg/ml transferrin (Sigma, St. Louis, Mo.), 2 mM L-glutamine, 10% fetal calf serum, 1% antibiotic/antimycotic solution and pipetted into a 25 cm^2 coming tissue culture flask and incubated at 40.5° C. in 5% CO_2, 95% air. After 24 hours of incubation, the media was changed. The primary culture contained numerous explants with centers of epithelial-like cells and radiating fibroblasts.

Cultures were allowed to grow to confluency (5 days) and were removed from the plates using a trypsin/EDTA solution (0.05% trypsin and 0.02% ethylene diamine tetra acetic acid (EDTA) in PBS) and replated for second passage. At second passage some of the cells were frozen in a conditioned media containing 50% DMEM media, 12% DMSO and 38% fetal calf serum. These cells were frozen in the vapor phase liquid nitrogen for 24 hours then transferred to the aqueous liquid nitrogen for long term storage.

Cells at second passage (P2) were replated at a seeding density of 2.7×10^4 cells/cm^2. The cells were sub-cultured for several months. The cultured fibroblasts grew rapidly for 8 to 9 passages, then began to slow down with significant cell death. During crises, the cells were passed using an ATV solution (8 gm/l NaCl, 0.4 gm KCl, 1 gm dextrose, 0.58 gm $NaHCO_3$, 0.5 gm trypsin (Difco 1:250), 0.2 gm versene (disodium salt) in 1000 mL). Cells were grown in Dulbecco's modified Eagles's medium enriched with 36 μg/ml insulin (Sigma), 1.6 μg/ml transferrin (Sigma), 2 mM L-glutarnine, 10% fetal calf serum and 1% antibiotic/antimycotic solution. It was noted that the majority of the cells at passage 11 (P11) were dead or dying; however, a small subpopulation of cells appeared to be healthy fibroblasts. The P11 cells remained on the dish for four weeks with refeeding every three days with fresh media. Some cells were frozen and the remaining cells were concentrated into a smaller area and were allowed to grow another two weeks before they were confluent enough for a second subculturing. By P15, the cells were appearing to be more homogeneous in cellular morphology and were growing at a rate of 0.32 population doublings per day. By P20, the population doublings increased to about 0.7 to about 0.8 population doublings per day. At this time the cells appeared to have a very uniform morphology. The cells were denoted UMNSAH/DF #1 and have been in continuous culture for over nineteen months. The cells are currently at passage **160**. Cells were frozen (as above) and thawed from P5. The subcloned cells were expanded and the reproducibility of the method was confirmed through the identification of other clones. Several more subclones were obtained by P 11.

Example 2

Testing cells for virus contaminants

The cells of this invention are tested for viral contaminants using PCR to identify contaminating nucleic acid fragments. There are a wide variety of commercially available test kits for a variety of viruses that can be used to determine whether the cells of this invention contain contaminating virus. Similarly, there are commercially available tests to detect viral antigert (e.g., commercially available ELISA assays and the like), where the antigen is derived from a variety of different viruses. These tests can be used on the cells of this invention using routine experimental techniques to demonstrate that the cultures are free of contaminating virus.

In one series of tests, the cells were tested for reverse transcriptase activity. 1×10^6 cells from rapidly growing cultures were isolated in 4 ml. of media. The media was taken through several freeze thaws at −80° C. to lyse the cells. The media with lysed cells were layered over a 10% glycerol gradient. The gradient was spun for 60 minutes at 40,000 rpm using an SW40 rotor (Beckman Instruments, Palo Alto, Calif.). Virus particles, if present were pelleted. The media was discarded and the pellet was resuspended in 20 μl of Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.).

An eppendorf tube was heated at 41° C. 5 μl of sample was added to 45 μl of reverse transcriptase cocktail containing 45 mM Tris, pH 7.8, 2 mM 2-β mercaptothanol, 2 mM manganous acetate, 0.1% Triton X-100, 10 μM each dATP, dCTP, dGTP (Boehringer Mannheim Biochemical, Indianapolis, Ind.), 2.4 μg polyA (Sigma), 60 ng primer dT 12-19 (Pharmacia), 0.4 μCi/reaction ^3H thymidine triphosphate (15,000 to 28,000 cpm/pmole activity, Amersham).

The reaction was incubated for one hour at 41° C. A negative control included 5 μl of ddH_2O and 45 μl of the cocktail. Two known positive controls were included with the assay. The assay was stopped by adding 1 ml of 10% trichloroacetic acid (TCA, Columbus Chemical Industries, Inc., Columbus, Wis.). The mixture was filtered through a Whatman GF/C glass 0.45 micron pre filter. Several washes were performed using 5% TCA. The filter was transferred to a Beckman Instruments Scintillation Counter using scintillation vials containing 5 mls of scintillation counting fluid. Samples were counted on a 050 to 600 window setting. An increase of threefold counts over the cocktail background (neg. control) was considered positive.

The primary cultures tested negative for reverse transcriptase as did the immortalized cells obtained in this invention. For further information on reverse transcriptase assays see (Crittenden, et al. *Virology* 57:128–138, 1974).

Example 3

Soft Agarose Colony Formations Assay to Assess Tumorigenic Potential of Cells

To test for tumorigenic potential, the cells were tested for growth in soft agar. A soft agarose base was made by mixing

12 ml of a 2% agarose solution (that had been autoclaved and cooled to 56° C.) in 21.6 mls of enriched McCoy's 5A medium [Gibco, 120 mls fetal calf serum (heat inactivated, 5 mls Na pyruvate (2.2% stock), 1 ml L-serine (21 mg/ml stock), 5 mls L-glutamine (200 mM stock), 12.5 mls Hepes (1M stock)], 5.9 mls Asparagine (4.4 mg/ml filtered sterilized stock). Seven mls of warm media/agarose was poured onto a 100 mm^2 tissue culture dish and allowed to solidify at room temperature in a tissue culture hood for 1 hr.

Cells were removed from actively growing cultures (about 40% to about 70% confluent) by trypsinization to achieve a single cell suspension in fresh DMEM media containing 10% fetal calf serum (with L-glutamine and antibioticsantimycotic). Approximately 1×10^6 cells was added to 4.25 ml of DMEM media containing 10% fetal calf serum, 0.75 ml of 1% agarose, and 50 µl 2βmercaptoethanol. Care was needed to be certain that the warm media/agarose was at 42° C. before adding the cells. Quickly, 5 ml of the above cell suspension was overlaid on the agarose plates.

Cells were grown at 37° C. in a 5% CO_2 95% air incubator and observed for 35 days. Duplicate plates were stained with 3 p-nitrophenyl-5-phenyl tetrazolium chlorite (INT stain) and examined at days 0, 5, 10, 15, 20, 30 and 35 for colony formation and growth. All stained colonies greater than 60 µm were considered positive.

All cells tested negative. Further information related to the soft-agar assay is available from Hamburger et al., *Prog. Clin. Biol. Res.*, Cloning of Human Tumor Stem Cells, 48, 43–52 (1980); S. Salmon, *Prog. Clin. Biol. Res.*, Cloning of Human Tumor Stem Cells, 135–151 (1980); and B. Kressner et al., *Prog. Clin. Biol. Res.*, Cloning of Human Tumor Stem Cells, 179–193 (1980).

Example 4

Tumorgenicity of Immortalized cells

Under the guidelines outlined in the University of Minnesota Animal Usage Protocol (protocol #950300-1, March 1995-December 1996) cells were injected into test animals to determine whether or not the cells were tumorigenic.

Actively growing cells were removed from cell culture plates and were injected into six SPAFAS line adult chickens (Hy-Vac, Adel, Iowa). Subcutaneous injections of 4×10^6 cells were introduced into the wing webs of the chickens. The sites of injection were examined weekly for 3.5 months. No tumors were observed at the injection site for any of the transfected cells produced to date with all animals remaining healthy. The experiment demonstrated that the immortalized cells were nontumorigenic.

Example 5

Ability of Cells to Support Virus Growth

The cells were seeded into roller bottles at 5.0×10^5 cells/cm^2. The cells were allowed to attach for 24 hours and a control was harvested for cell counts. Cells were grown for virus infection in DMEM (4.5 g/L glucose), 4% Fetal Bovine Serum, 2 mM L-Glutamine, 50 mg/L Gentamicin. Cells were infected at a multiplicity of infection of 0.0006 HVT virus particles per cell. The roller bottles were watched daily for progression of CPE. The bottles were harvested at 46 hs. post infection when there was approximately 50% CPE. HVT infected cells were frozen in growth medium with 10% DMSO at a concentration of 2.0×10^7 cells/ml. Titers of HVT were quantitated by plaque assay. Virus was serially diluted in growth media and placed onto confluent monolayers of permissive cells. Cultures were incubated for a designated time and the cells were fixed and stained. Plaques on the monolayers were counted and virus liter was expressed as plaque forming units per dose.

These cells were also tested for their ability to support reovirus production. 2.5×10^8 cells were infected with WSS-Reo 1733 strain of Reovirus having a tiler of 8.2 $TCID_{50}$/ml. Cells were infected at a multiplicity of infection of 0.005, 0.001 or 0.0005 infectious virus particles/cell. Infected cells were grown in roller bottles and tested at 48, 64 and 72 hours after infection to demonstrate productive vital growth.

Experiment 6

Use of Transfected Skin Cells as a Cell Substrate

The cells of this invention are useful as a substrate for supporting virus replication of primary cells. In these experiments the immortalized cells are mixed with primary cells. In one study the primary cells are infected and mixed with the immortalized cells and placed in culture and in another study the primary cells are infected and placed onto the immortalized cells where the immortalized cells are already positioned as a lawn in the tissue culture flask. In one example the virus is Egg Drop Syndrome virus and the primary cells are primary chicken embryonic liver cells. In a second example the primary cells are endothelial cells, preferably kidney endothelial cells and the virus is infectious bronchitis virus. The preferred ratio of primary cells to immortalized cells is about 1:5 to about 1:20 and more preferably about 1:10. Virus titers from primary cells growing in the mixed cell population are higher than virus titers from primary cells in culture alone. The immortalized cells allow the primary cells to be used for virus propagation under commercial conditions.

All cited publications are incorporated by reference in their entirety into this text. Although the invention has been described in the context of particular embodiments, it is intended that the scope of coverage of the patent be limited only by reference to the following claims.

What is claimed is:

1. A spontaneously immortalized cell line, derived from primary chicken embryonic fibroblasts, having the characteristics of UMNSAH-DF 1 cell line and deposited with the American Type Culture Collection as Accession #CRL-12203.

2. A culture or immortalized subclone of the immortalized cell line of claim **1** that support virus replication.

3. A cell of the cell line of claim **1** or **2** which contains virus.

4. A cell of the cell line of claim **1** or **2** which contains at least one vector capable of directing expression of recombinant protein in the cells.

5. A cell of the cell line of claim **4** which expreses recombinant protein.

6. A cell of the cell line of claim **4**, wherein the vector encodes at least a portion of a recombinant virus.

7. A cell of the line cell line of claim **4**, wherein the vector is a retroviral vector.

8. A method for producing an immortalized cell line from chicken embryonic fibroblasts comprising the steps of:

growing primary chicken embryonic fibroblasts in culture;

passaging the fibroblasts in culture until they begin cell senescence;

concentrating the cells during cell senescence to maintain about 30% to about 60% culture confluence;

identifying foci of non-senescent cells in the culture;

isolating the non-senescent cells; and

growing the non-senescent cells for greater than 30 passages.

9. A method for growing virus in a cell comprising the steps of:

growing the cells of claim **1** in culture;

infecting the cells with virus;

allowing the virus to replicate in the cells; and

collecting virus that replicated in the cells.

* * * * *

1.1.2 Please provide, for addition to the disclosable safety narrative, information about the cell origin as follows:
the statement regarding cytogenic examination of the cells found in the second paragraph of page 7, Section 10.1.

The statement regarding cytogenic examination of the cells found in the second paragraph of page 7, Section 10.1 is provided below:

"After cytogenetic examination of 100 cells, polyploid rate was determined to be 14%, with a modal chromosome number of 76 and no chromosome aberrations."

1.1.3 Please provide, for addition to the disclosable safety narrative, information about the cell origin as follows:
the material included on pages 9-11.

The material included on pages 9-11 of the Dossier in Support of the Safety of Good Meat Cultured Chicken as a Human Food Ingredient Appendix dated March 4, 2022, is provided below:

[The remainder of this page is blank]

ATCC

Product Sheet

UMNSAH/DF-1 (ATCC® CRL-12203™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: UMNSAH/DF-1 (ATCC® CRL-12203™)

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800 638 6597 or 703 365 2700
Fax: 703 365 2750
Email: Tech@atcc.org

Or contact your local distributor

Description

Organism: *Gallus gallus*, chicken
Strain: East Lansing Line (ELL-0)
Tissue:
embryo
Cell Type: fibroblast spontaneously transformed
Age: 10 days gestation
Morphology: fibroblast
Growth Properties: adherent
Cytogenetic Analysis: Number of cells examined = 59; Modal Chromosome Number = 75 with a range of 65 to 79; Polyploidy Rate = 22%

Batch-Specific Information

Refer to the Certificate of Analysis for batch-specific test results.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37-39°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL complete growth medium and spin at approximately 125 x g for 5 to 7 minutes.
4. Resuspend cell pellet with the recommended complete growth medium (see the specific batch information for the recommended dilution ratio). It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).
5. Incubate the culture at 39°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).
2. **If the cells are still attached,** aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 39°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.
3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at

ATCC

Product Sheet

UMNSAH/DF-1 (ATCC® CRL-12203™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

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Complete Growth Medium

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Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: UMNSAH/DF-1 (ATCC® CRL-12203™)

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor

125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 mL of this medium and add to 25 cm^2 flask. Incubate at 39°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes are given for a 75 cm^2 flask. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other sizes.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 39°C to facilitate dispersal.
4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 39°C.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:10 is recommended
Medium Renewal: Twice per week

Cryopreservation Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Comments

foci of non-senescent cells were identified and grown for greater than 30 passages.
No clonal proliferation was observed in soft agar cultures, indicating that these cells were immortalized but not transformed.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

ATCC Warranty

ATCC® products are warranted for 30 days from the date of shipment, and this warranty is valid only if the product is stored and handled according to the information included on this product information sheet. If the ATCC® product is a living cell or microorganism, ATCC lists the media formulation that has been found to be effective for this product. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this product. If an alternative medium formulation is used, the ATCC warranty for viability is no longer valid.

Disclaimers

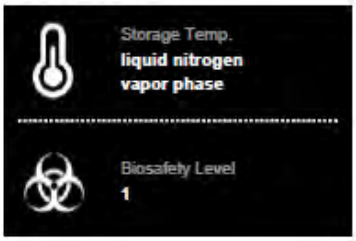
This product is intended for laboratory research purposes only. It is not intended for use in humans.
While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.
This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of materials on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of such materials.
Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this

ATCC

Product Sheet

UMNSAH/DF-1 (ATCC® CRL-12203™)

Please read this FIRST



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Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: UMNSAH/DF-1 (ATCC® CRL-12203™)

product. The MTA is also available on our Web site at www.atcc.org

Additional information on this culture is available on the ATCC web site at www.atcc.org.

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor

1.2 MICROBIAL AND VIRAL TESTING

1.2.1 Please provide, for addition to the disclosable safety narrative, additional discussion of the analysis of serum sources, including the second and third paragraph, as well as Table A4 of page 17-18, Section 10.3.2 of the confidential supplementary material.

Additional discussion of the analysis of serum sources, including the second and third paragraph, as well as Table A4 of page 17-18, Section 10.3.2. of the confidential supplementary material are provided below. Table numbering follows the original Dossier in Support of the Safety of Good Meat Cultured Chicken as a Human Food Ingredient Appendix dated March 4, 2022.

All serum sources used for culture of C1F and C1F-P1 cells were tested for bovine viruses, following the procedures described in U.S. Code of Federal Regulations 9 CFR § section 113.53(c) (Requirements by USDA for ingredients of animal origin used for production of biologics) (Table A4).

Table A 4. Bovine virus testing on serum batch used for culture and production of C1F-P1 cells at Good Meat.

Virus	**Result**
Bluetongue	Negative
Bovine Adenovirus	Negative
Bovine Parvovirus	Negative
Bovine Respiratory Syncytial Virus	Negative
Bovine Viral Diarrhea Virus	Negative
Rabies	Negative
Reovirus	Negative
Cytopathogenic Agents (IBR)	Negative
Hemadsorbing Agents (PI3)	Negative

Additionally, Seradigm certifies that FBS does not contain and are not derived from material of bovine origin at risk of carrying bovine spongiform encephalopathy, such as the skull (including the brain and eyes), tonsils, and spinal cord of bovine animals aged over 12 months.

Additional discussion:

Several controls exist in the selection, testing and processing of bovine serum that further contribute to the safety profile for the intended application.

- **Geography and Source:** Bovine blood is collected from cows sourced in the United States and that have passed ante- and post-mortem inspection. FBS processed in the US, collected exclusively from approved harvest facilities, and performed under the strict guidance of standard operating procedures. As listed in the quoted section above, Seradigm certifies that FBS does not contain and is not derived from material of bovine origin at risk of carrying bovine spongiform encephalopathy (BSE). OIE Resolution No.20, issued in May 2013, upgraded the United States's risk status classification for BSE to "negligible risk' and material from the US is now category A, which is the lowest risk category for BSE.
- **Processing:** FBS is sterile filtered through three consecutive 0.1 µm pore-size filters and heat-inactivated at 56°C for 30 minutes.
- **Testing:** Besides the bovine viruses listed in Table A4, FBS is also tested for sterility, endotoxin and *mycoplasma*.

We satisfy the United States' safety guidelines on the use of bovine serum in cell culture biopharmaceutical manufacturing processes. Moreover, by reducing the serum level to a fraction of what is typically used in animal cell culture, implementing thorough washing of cells post-harvest, the rigorous testing of the FBS, and the long history of safe consumption of bovine materials we are confident in the safety of the cultivated chicken.

1.2.2 Please provide, for addition to the disclosable safety narrative, additional discussion of adventitious agent testing as follows:
the discussion found on pages 22-24, Sections 10.3.5.3-10.3.5.4.2 of the confidential supplementary material.

The discussion of adventitious agent testing found on pages 22-24, Sections 10.3.5.3-10.3.5.4.2 of the confidential supplementary material is provided below. Table numbering follows the original Dossier in Support of the Safety of Good Meat Cultured Chicken as a Human Food Ingredient Appendix dated March 4, 2022.

10.3.5.3. Mycoplasma Detection

Mycoplasmas are the smallest free-living microbes known. They lack a cell wall and unlike other types of bacteria, can be difficult to trace and identify in culture with conventional microscopes. They are naturally

resistant to several common antibiotics, such as penicillin that target cell wall synthesis (Razin et al., 1998).

Mycoplasma species may be found in research laboratories as contaminants in cell culture. Even though they can remain undetected for long periods as *Mycoplasma* and eukaryotic cells co-exist, the presence of *Mycoplasma* can induce changes in cell metabolism and cell growth. Therefore, *Mycoplasma* detection is performed as one of the release testing assays of the MCB and MWCB. Samples were analyzed at Charles River Research Animal Diagnostic Services (MA 01887, USA). Results indicate that MCB and MWCB are absent of *Mycoplasma.*

10.3.5.4. PCR for Adventitious Agents

The European Food Safety Authority (EFSA) conducted a review study to provide scientific opinion on the public health hazards from poultry meat (EFSA, 2012). This review identified Avian influenza virus, Avian leukosis virus (ALV), Hepatitis virus, Newcastle disease virus, Fowl adeno virus (FAV) as viruses most commonly found in poultry. Based on this scientific opinion from EFSA, Good Meat selected the comprehensive Human and Avian adventitious agent panel at Charles River encompassing majority of these viruses. Although the avian microorganisms included in the panel are not transmissible to humans (Avian Reticuloendotheliosis virus, Avian encephalomyelitis virus, Avian Leukosis Virus A, Avian Leukosis Virus B, Avian Leukosis Virus J, Fowl Adeno Virus 1, Fowl Adeno Virus 3, Chicken Anemia Virus, Avian Reovirus) with exception of Avian S. Pullorum (*Salmonella*), Good Meat included this testing inf our analysis to further demonstrate Good cell culture practices and the quality of Good Meat's chicken cell banks throughout R&D and manufacturing.

Furthermore, ATCC deposited cells were certified as negative for Avian Influenza (Type A), Avian Reovirus, Avian Adenoviruses (Groups I-III), Avian Encephalomyelitis Virus, Fowl Pox, Newcastle Disease Virus, Paramyxovirus (type 2), *Mycoplasma*, *Salmonella* and other infectious agents known to infect poultry stock (Section 4.1).

10.3.5.4.1. Description of Method

Unlike biotechnology products used for therapeutic purposes that undergo multiple chromatography steps, in harvesting the cell mass for food purposes, one cannot develop processes for robust removal of viruses.

Thus, it is incumbent on the manufacturer of the cell mass to assure that all cell banks are free of adventitious agents.

Taking into consideration that C1F-P1 cells were internally established from parental UMNSAH/DF1 (ATCC), and these cells were proposed for virus propagation, Good Meat performed a thorough analysis of adventitious human and avian viruses and bacterial agents through an infectious Disease Polymerase Chain Reaction (PCR) performed by a third-party (Charles River Research Animal Diagnostic Services) – Human Essential CLEAR Panel; Avian Virus and Bacteria Panel.

C1F-P1 MCBs and MWCBs are considered valid for viral assessment if a minimum of $0.4x\sqrt{n}$ independent and randomly selected cryovials (from a cell bank of n size) from the tested bank are thawed and their cell pellets provide a negative result for the full panel of adventitious agents listed in Table A6.

10.3.5.4.2. Results

C1F cell pellets from independent vials of the C1F-P1 MCB and MWCB were evaluated for the presence of viral and bacterial adventitious agents. Samples were analyzed at Charles River Research Animal Diagnostic Services (MA 01887, USA). Results are listed in Table A6.

Both C1F-P1 MCB and C1F-P1 MWCB are considered approved for the absence of adventitious avian and human viral and bacterial agents as the independent cell pellets from each cell bank were negative for the entire human and avian panels. As previously mentioned, the absence of bovine viruses in serum used for culture is cleared by the negative viral testing results provided by manufacturers Seradigm.

Table A 6. Infectious disease PCR on independent cultures thawed from C1F-P1 MCB and MWCB.

HUMAN ESSENTIAL CLEAR PANEL		
	MCB	MWCB
Adeno-associated virus	-	-
BK virus	-	-
Epstein-Barr virus	-	-
Hepatitis A virus	-	-
Hepatitis B virus	-	-
Hepatitis C virus	-	-
Herpes Simplex 1 PCR	-	-

Herpes Simplex 2 PCR	-	-
Herpesvirus type 6	-	-
Herpesvirus type 7	-	-
Herpesvirus type 8	-	-
HIV-1	-	-
HIV-2	-	-
HPV-16	-	-
HPV-18	-	-
Human cytomegalovirus	-	-
Human T-lymphotropic virus	-	-
John Cunningham virus	-	-
Parvovirus B19	-	-
Mycoplasma Genus PCR	-	-
Mycoplasma pumonis PCR	-	-
HUMAN ESSENTIAL CLEAR PANEL		
	MCB	MWCB
REV PCR	-	-
AEV PCR	-	-
ALVA PCR	-	-
ALVB PCR	-	-
ALVJ PCR	-	-
FAV1 PCR	-	-
FAV3 PCR	-	-
CAV PCR	-	-
ARV PCR	-	-
Avian S. pullorum PCR	-	-
Avian Mycoplasma Genus PCR	-	-

1.2.3 Please provide, for addition to the disclosable safety narrative, additional discussion of adventitious agent testing as follows:
clarification regarding the frequency of adventitious agent testing for the master working cell bank (MWCB).

Adventitious agent testing of MWCBs is performed as part of Good Meat's cell bank release testing. Master Working Cell Banks (as well as Master Cell Banks) are considered cleared for presence of adventitious agents if a minimum of $0.4x\sqrt{n}$ independent and randomly selected cryovials (from a cell bank of "n" size) from the tested bank are thawed and their cell pellets provide a negative result for the full panel of adventitious agents listed in Table A6 above. These cell banks are sealed and maintained cryopreserved in the vapor phase of liquid nitrogen freezers. This testing for adventitious agents as part of the release process is considered sufficient since there is no known risk for contamination after the banks are prepared, sealed and stored.

1.3 CELL PROLIFERATION AND VIABILITY

1.3.1 Please provide, for addition to the disclosable safety narrative, information about cell proliferation and viability as follows:
the discussion found in paragraphs two through seven on pages 20-21, Section 10.3.5.1 of the confidential supplementary material.

The requested information about cell proliferation and viability found in paragraphs two through seven on pages 20-21, Section 10.3.5.1 is provided below:

> Cell proliferation is broadly defined as a process leading to an increase of cell number, essentially depending on a balance between cell cycle progression/cell division, cell death, and cellular senescence. This process is physiologically important to maintain animal cells in culture and in manufacturing of cultured chicken.
>
> A variety of methods exist to analyze cell proliferation which include "direct" and "indirect" approaches. Direct approaches measure the number of cells actively dividing in a cell population, whereas indirect approaches extrapolate from cell number/viability or metabolic activity to cell proliferation.
>
> Direct approaches use either incorporation of nucleoside analogs during DNA synthesis (such as BrdU), detection of cell cycle-associated proteins (such as Ki-67) with antibodies, or photometric methods using cytoplasmatic proliferation dyes that dilute with each cell division equivalently to daughter cells (such as carboxyfluorescein diacetate succinimidyl ester – CSFE). Indirect methods include cell counting (with and without cell viability stains, such as trypan blue) and metabolic activity assays (such as resazurin).
>
> During the S phase of the cell cycle (genome replication), DNA polymerases incorporate nucleosides (e.g., deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) into new strands of DNA. Direct cell proliferation methods incorporate chemically or radioactively labeled nucleosides into the sample of interest, which are then incorporated into newly synthesized DNA during S phase. Other assays involve antibody staining targeting cell cycle-associated proteins. Additional sample processing involves sample fixation and permeabilization is not practical for an in-process characterization of cell density.

Among the variety of applicable methods, trypan blue exclusion is described as a robust, easy-to-perform, and cost-effective method to assess cell proliferation in an experimental setting. When staining cells with trypan blue, viable cells with intact membranes do not incorporate the dye, whereas dead cells without intact membranes do, which makes it possible to distinguish viable from dead cells. In this text, a cell suspension is mixed with the dye and then visually examined to determine whether cells take up or exclude the dye. A viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm (Strober, 2015). There also several instruments commonly used in the biotechnology space that apply this method for determination of cell density, such as Vi-Cell XR Cell Analyzer (Beckman Coulter) or Bioprofile ® FLEX2 (Nova Biomedical), with automation and high throughput capacity with potential integration for online sampling and real-time cell analysis for improved bioprocess monitoring and control.

Compared to other indirect methods such as metabolic assays, the readout is not disturbed by potential metabolic changes due to different culture conditions (Funk and Musa, 2021). Alternatively, exclusion can also be measured with other light emitting dyes such as propidium iodide. Detailed comparison of estimates of live versus dead cells using trypan blue exclusion and flow cytometry indicate that the two techniques provide very similar results in experienced hands (Strober, 2015). Moreover, rather than trying to identify exactly the percentage of cells actively dividing, an assay like trypan blue will provide reliable information about the total cell biomass by applying a simultaneous particle count and membrane integrity assessment.

1.3.2 Please provide, for addition to the disclosable safety narrative, information about cell proliferation and viability as follows:
clarification on the specification for cell viability. The specification for cell viability presented in Table 1 of the disclosable safety narrative appears to be inconsistent with the second bullet on page 21, Section 10.3.5.1.

The specification for cell viability of C1F-P1 presented in Table 1 of the disclosable safety narrative is the following: (i) cell viability >70% after thawing; (ii) cell viability >80% after cell passage.

The description of cell proliferation and viability used in Section 10.3.5.1 was listed based on actual cell viability values from representative Master Cell Bank release read-outs and

not directly referring to the specifications. The statement for viability on page 21, Section 10.3.5.1 should read as follows:

Cell proliferation and viability for MCB release testing for C1F-P1 cells in culture showed:

- Viability of 95.9±1.6% (higher than the 70% specification) after thawing of vials
- Viability equal or higher than 93% (higher than the 80% specification) for the three passaging cycles
- Average PDT lower than 49 h for the three passaging cycles
- Above mentioned quality attributes are similar to the MCB established for C1F-P1 cells

1.4 CELL STERILITY

1.4.1 Please provide, for addition to the disclosable safety narrative, information about cell sterility, including the discussion found in paragraphs one, four, five, Table A5, and paragraph seven on pages 21-22, Section 10.3.5.2 of the confidential supplementary material.

The requested information about cell sterility including the discussion found in paragraphs one, four, five, Table A5, and paragraph seven on pages 21-22, Section 10.3.5.2. of the confidential supplementary material is found below. For reference, the table number was maintained as the one listed in Section 10.3.5.2. of the confidential supplementary material included in the Appendix Dossier in Support of the Safety of Good Meat Cultured Chicken as Human Food Ingredient dated March 4, 2022.

> Cell sterility assays typically involve the incubation of culture supernatant with complex enriched media routinely used to grow certain pathogenic bacteria and fungi. Moreover, cell cultures at Good Meat are routinely monitored by microscopic inspection to confirm the absence of visual contamination.
>
> TSB is recommended for use in sterility testing for the detection of contamination with low incidence fungi and aerobic bacteria.[2] FTM is used for the evaluation of aerobic, microaerophilic, and anaerobic microorganisms. The FTM formulation is the standard medium recommended by the Food and Drug Administration, National Institute of

[2] The Official Compendia of Standards. USP-NF. United States Pharmacopeial Convention, Rockville, MD.

Health, the National Formulary, and the U.S. Pharmacopeia for sterility testing of clear fluid biologics and other sterile products.[3]

The C1F-P1 MCBs and MWCBs are considered valid for sterility if a minimum of $0.4x\sqrt{n}$ independent and randomly selected cryovials (from a cell bank of n size) from each bank are thawed and their culture supernatants do not demonstrate bacterial and fungal growth in the referenced culture broth media.

Table A 5. Results from the sterility testing of C1F-P1 MCB and C1F-P1 MWCB.

Sterility Test	**MCB**	**MWCB**
TSB	Negative for growth	Negative for growth
FTM	Negative for growth	Negative for growth

C1F-P1 MCB and C1F-P1 MWCB passed sterility testing as all independent samples showed absence of microorganism growth in both TSB and FTM broths.

Additional discussion:
Sanitation, process and environmental controls all provide additional measures to assure microbial control during manufacture. These controls include equipment cleaning and sterilization, facility sanitization, water quality controls, 0.2 µm filtration of media and cell culture monitoring.

Additionally, routine microbiological testing is conducted on each harvest of cultivated chicken cells to confirm microbial limits are within release criteria. This information is presented in Section 5.5.3.1.3, Microbial Analysis, including Tables 43 and 44.

1.5 CELL PURITY AND IDENTITY

1.5.1 Please provide, for addition to the disclosable safety narrative, information about assessment of cell purity and identity as follows:
the discussion found on pages 24-25, Section 10.3.5.5 of the confidential supplementary material.

The information requested on the assessment of cell purity and identity in the discussion found on pages 24-25, Section 10.3.5.5 of the confidential supplementary material is provided below:

[3] Federal Security Agency, Food and Drug Administration, Compilation of Regulations for Test and Methods of Assay and Certification of Antibiotic Drugs; National Institutes of Health Circular: Culture Media for the Sterility Test, 2nd rev. Feb. 5, 1946.

Cell purity and identity can be determined by a variety of molecular tests. This testing is performed to establish that a C1F-P1 MCB or MWCB stock cell population is phenotypically homogeneous (*e.g.*, pure), or it is not significantly different from the parental C1F cells.

A panel of assays used to verify the identity of the C1F-P1 banked cells includes a species identification assay and a PCR evaluation of fibroblast marker indicative of the phenotype of C1F cells.

DNA was chosen as the target molecule for species identification purposes owing to its higher stability when compared to proteins. Protein-based methods can give satisfactory results in raw meats; nevertheless, they can be significantly less sensitive when applied to thermally processed foods due to protein denaturation and alterations of specific epitopes.

DNA identification with polymerase chain reaction presents a fast, sensitive, and highly specific identification method in complex processed foods. Despite these methods being developed for chicken cells in culture, considerations of thermal degradation for future cooking of the products were taken into account. Thermally treated products can present DNA with high level of degradation. Therefore, the primers for PCR amplification should be designed to target small target DNA sequences of the mitochondrial genome. It is particularly advisable to use sequences from the mitochondrial DNA, which are several-fold more abundant than those of nuclear genome, as well as detecting short sequences to increase the possibility of amplifying fragmented DNA. The intraspecific variability of mitochondrial DNA offers the possibility of species discrimination.

1.5.2 Please provide, for addition to the disclosable safety narrative, information about assessment of cell purity and identity as follows:
the discussion found in the first, second, and third paragraph, as well as the final sentence on page 25, Section 10.3.5.5.1.

The information requested regarding the assessment of cell purity and identity in the discussion found in the first, second, and third paragraph, as well as the final sentence on page 25, Section 10.3.5.5.1 is provided below:

Good Meat independently confirmed species identity of C1F-P1 cells by PCR followed by an external genotype sequencing analysis of the

amplified products from the PCR reaction at Quintara Biosciences (CA 94545, USA).

C1F-P1 cells were also analyzed using conventional PCR following an internal CA-SOP013 "Avian Species Identification by PCR Sequencing" (Appendix 10.3.1). Briefly, PCR amplification is performed using primers designed to amplify highly conserved regions of the mitochondrial genome specific for individual species. The primers are listed in Table A7.

Amplified DNA is then sequenced. Sequences are compared to published avian sequences in national databases to confirm species identity. Since the amplified regions are highly conserved during evolution (therefore, less prone to mutations) and simultaneously are highly specific of each species, this method offers great confidence for reliable identification of species type for the tested cells (Amaral *et al.*, 2015).

C1F-P1 cells are considered validated as chicken cells when the percentage of alignment with the mitochondrial genome is higher than 95%.

1.5.3 Please provide, for addition to the disclosable safety narrative, information about assessment of cell purity and identity as follows:
the discussion found on pages 25-26, Section 10.3.5.5.2 of the confidential supplementary material.

The information requested regarding the assessment of cell purity and identity in the discussion found on pages 25-26, Section 10.3.5.5.2 is provided below. The table and figure numbers refer to the sequence from the original Appendix Dossier in Support of the Safety of Good Meat Cultured Chicken as Human Food Ingredient dated March 4, 2022.

C1F-P1 cell pellets from independent vials of the C1F-P1 MCB was collected aseptically. DNA was extracted, and the PCR reaction was performed using the PCR primer indicated in Table A7. Afterward, the amplicons were run on agarose gels to assure amplicon purity and size. The DNA fragment was amplified by PCR reaction with the expected size. After confirming the successful reaction, amplicons were purified, and the samples were shipped for DNA Sanger sequencing at Quintara Biosciences. Sequence alignment between the genotyped amplicon and the published chicken consensus sequence (from National Center for Biotechnology Information) was performed using the online software tool "Align Sequences Nucleotide BLAST" available at blast.ncbi.nlm.nih.gov.

The high level of homology between the amplified product and the public genomic databases of chicken confirms the identity of C1F-P1 cells as chicken, specifically *Gallus gallus*. C1F-P1 cells from the MCB were 99% aligned to the sequence of the chicken mitochondrion (Sequence ID: MH732978.1) – Figure A3.

Of note, the sequenced C1F amplicons were also aligned against other species' mitochondrial genome available online at National Center for Biotechnology Information. The percentage of alignment of C1F cells with other species was significantly lower: 86% with turkey (NC_010195.2) and Japanese quail (KX712089.1); no significant similarity with bovine (MG736676.1) or with human (NC_012920.1) mitochondrion genomes.

2. CELL CULTURE PROCESS

2.1 CELL GROWTH IN BIOREACTOR

2.1.1 Please provide, for addition to the disclosable safety narrative, additional discussion of the parameters monitored during cell culture, including the first through third paragraph of page 29, Section 10.4.1 of the confidential supplementary material.

Below is the first through third paragraph of page 29, Section 10.4.1 of the confidential supplementary material:

Avian cell culture process control is necessary to achieve a high yield of the desired product. The cell density in a bioreactor depends on the concentration of certain nutrients and end-products of metabolism.

Six carbon sugars like glucose and the amino acid L-glutamine are provided as the two major energy sources for cell health and to support proliferation in culture. The lactate concentration is increased in culture over time because of glycolytic metabolism as glucose is converted into pyruvate and then to lactate through glycolysis. This results in a decrease of pH, which is balanced by the settings pre-set in our bioprocess. Cell growth, metabolic rates and protein expression are all affected by pH, so monitoring and in-process control of pH is necessary to avoid excessive acidification of the culture media and to keep C1F cells in their ideal pH range to support normal metabolism and cell division. Glutamine is converted to glutamate and ammonia, hence monitoring these levels of glutamine and glutamate during culture contribute for a characterization of their normal metabolic state during manufacturing.

Consequently, by monitoring and controlling pH, glutamine, lactate, and glucose concentrations in cell cultivation, Good Meat can monitor the metabolism of C1F-P1 cells and confirm if metabolite production and consumption rates are meeting the typical one observed in the culture process used in manufacturing of cultured chicken.

Additional discussion:
The table below summarizes parameters monitored during cell culture.

In-Process Parameter	**Monitored/Controlled**	**Rationale**
Cell Density	Monitored	- Monitor cell growth - Trend process performance
Cell Viability	Monitored	- Monitor cell health - Trend process performance
Glucose	Monitored & controlled	- Monitor carbon source - Controlled addition to medium - Trend process performance
Glutamine	Monitored & controlled	- Monitor key nutrient source - Controlled addition to medium - Trend process performance
Lactate	Monitored	- Monitor by-product accumulation - Trend process performance
Temperature	Monitored & controlled	- Provide optimal temperature for growth
Gassing	Monitored & controlled	- Dissolved oxygen control - pH control
Agitation	Monitored & controlled	- Homogenous mixing - Gas-liquid mass transfer and dissolved control
Dissolved oxygen	Monitored & controlled	- Provide oxygen for growth - Trend process performance
pH	Monitored & controlled	- Provide optimal pH for growth - Trend process performance

The parameters outlined above are routinely measured in cell culture manufacturing processes because they are the hallmarks of a well-controlled process. Each of these measurements and the resulting data assure that we have a consistent process from batch to batch (i.e., cell growth and cell densities are consistent throughout the process). Most importantly, a consistent process, coupled with release testing, assure that our product is consistent from batch to batch.

3. FOOD SAFETY PLAN

3.1 SAFETY AND CONTROL TESTING

3.1.1 On page 9 of the disclosable safety narrative, you state, "JOINN Biologics US Inc (CA, USA) currently produces the chicken cells in a dedicated manufacturing suite". Please provide an explicit statement affirming that JOINN Biologics US Inc. will be appropriately mitigating food safety risks from potential adventitious agents or other contaminants for your products.

JOINN Biologics US Inc. (CA, USA) will mitigate food safety risks from potential adventitious agents or other contaminants for Good Meat products. This is included in the Food Safety Plan designed to comply with 21 CFR 117, 21 CFR 121 and related regulations. It is included in the formal agreement between JOINN and Good Meat.

3.1.2 On page 80 of the disclosable safety narrative, you state, "Full details of the HARBPC are available as an Attachment 4: Food Safety Plan for C1F-P1 Cultured Chicken Cells". On the same page, you discuss process controls. The section references process, sanitation, and environmental controls, but does not elaborate on anything other than the environmental monitoring program (page 85). Additionally, on page 80 you state, "Full documentation of process controls, sanitation and environmental controls, and supply chain controls and their implementation and validation are provided in Attachments A2-A5." For addition to the disclosable safety narrative, please provide a brief, general summary of the manufacturing controls not directly mentioned in the text (e.g., sanitation controls, supplier controls, environmental monitoring).

A thorough HARBPC plan was developed for cultured chicken manufacturing process by identifying food safety hazards including biological, chemical and physical along with their corresponding preventative measures and confirmation/corrective actions. The major aspects of the HARBPC plan are sanitation controls, supplier controls, process controls and environmental monitoring to ensure the safety of the cultured chicken.

The sanitation controls include monitoring of water quality, equipment cleaning and overall facility sanitation. Reverse osmosis deionized (RODI) water or equivalent is used for all cell culture processing, including sterilized in-place (SIP) and cleaned-in-place (CIP). Water is monitored to assure it meets compendial requirements including bioburden, conductivity and TOC. The bioreactors and storage tanks are CIP and SIP before and after each use. Both SIP and CIP performances are monitored and confirmed to meet acceptance criteria. All other direct contact components are single-use pre-sterilized by gamma radiation and comply to USP biological reactivity and cytotoxicity standard. The manufacturing areas are compliant to ISO Class 8

cleanroom standards. The cleaning in these manufacturing areas is performed before and after operation and weekly in packaging areas.
The supplier controls include vendor qualifications and review of supplier documentation including Certificate of Analysis, Certificate of Sterilization, Certificate of Compliance, Letter of Continued Guarantee (as appropriate) for all incoming materials, components and utilities for their intended purposes. We have established physical, chemical, biological and regulatory compliance specifications for all materials and components. JOINN Biologics performs detailed review of supplier documentation for every batch of material ensuring compliance with established pass criteria before releasing for operational use.

The process preventive controls for food safety include media filtration, monitoring cell culture performance, monitoring cell wash performance and temperature monitoring during storage. Cell culture media is filtered through 0.2 micron filters prior to use. Filters are single use sterile or autoclaved sterilized before use. The cell culture process is monitored by cell counts and viability to monitor growth and assess performance. Cell culture samples are visually inspected for bacterial contamination and cell morphology. During harvest, albumin and pluronic F-68 levels are monitored to assess cell washing efficiency. Finally, temperature monitoring data is reviewed weekly to ensure finished cultured cells are stored at appropriate temperatures.

Environmental monitoring includes testing for active viable air monitoring, non-viable air monitoring and viable surface monitoring on a weekly basis in processing areas and during operation in biosafety cabinets. *Listeria* and *Salmonella* are routinely monitored as sanitation indicator organisms. Further, cultured cell and supernatant outlet tubings are tested for APC and *Enterobacteriaceae* post-harvest to ensure absence of pathogenic microorganisms in the final cultured cells.

All batches of cultured chicken are tested for microbiological safety as summarized in Table 55 of original safety dossier. The batches are only released for further use as a food ingredient in cultured chicken products if microbiological specifications are confirmed to the pass criteria. A batch is rejected for further use if specifications are not met.

[The remainder of this page is blank]

4. POINTS OF CLARIFICATION

4.1.1. For addition to the disclosable safety narrative, please provide a copy of page 8 of the disclosable safety narrative, and page 6 of the confidential supplementary material, that includes the signature and date.

The signed copy of page 8 of the disclosable safety narrative and page 6 of the confidential supplementary material is provided below:

[The remainder of this page is blank]

DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT

1. FIRM AND CONTACT PERSON

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.
2000 Folsom Street
San Francisco, CA 94110
Tel: 858-349-8338
Fax: 415-682-6990

[redacted] 7/1/2022

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.

Date

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DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT - APPENDIX

FIRM AND CONTACT PERSON

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.
2000 Folsom Street
San Francisco, CA 94110
Tel: 858-349-8338
Fax: 415-682-6990

[redacted]

7/1/2022

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.

Date

[The remainder of this page is blank.]

5. CELL BANK ESTABLISHMENT

5.1.1 On page 13, Section 4.4 of the disclosable safety narrative, you state, "Cryopreservation and thawing of cells are performed according to SOPs and GMP chain of custody documentation during retrieval (Appendix Error! Reference source not found.)". Please provide a statement correcting the reference source not found error.

On page 13, Section 4.4. of the disclosable safety narrative, it should state: Cryopreservation and thawing of cells are performed according to SOPs and GMP chain of custody documentation during retrieval (Appendix 10.3.3).

6. CELL CULTURE PROCESS

6.1.1 For addition to the disclosable safety narrative, please provide a statement that you will only use food contact materials which are authorized for their intended use.

Good Meat warrants that all food contact materials used are USP class VI and/or food grade (listed under 21 CFR Parts 175-178, 179.45, and 180) and suitable for their intended use. For any material that is USP class VI and not food grade, the Good Meat FSQA department analyzes all available manufacturing and release data to assure it complies with food-grade standards. The animal cell culture space has been developed for the Biopharmaceutical industry and, as such, some of the materials we rely on to assure a safe, aseptic process may not be classified as "food grade." In each case we have carefully evaluated all materials used in our process and available manufacturing data to assure that the chicken product using such material is safe for human consumption.

6.1.2 Please provide, for addition to the disclosable safety narrative, a statement that all analytical methods used are validated for their intended purpose.

Good Meat Inc. warrants that all analytical methods used are validated for their intended purposes.

6.1.3 Please provide a statement clarifying whether you have made various adjustments to your production process in the course of your discussions with FDA, and identifying the latest version of the process and product that you are presenting for evaluation with regard to any aspect that could affect the properties of the harvested cell material.

Based on discussions with the FDA during pre-market consultation, Good Meat characterized the C1F-P1 cell culture growth requirements for folic acid. This characterization determined that exogenous folic acid provided in the culture medium could be reduced by 90% without impacting culture growth or quality aspects. Folic acid levels in the culture medium have been reduced from 2.65 mg/L to 0.265 mg/L for all steps of the manufacturing process, from vial thaw through the production bioreactor unit operations. Good Meat demonstrated this reduced folic acid process, without any impact in the final product and meeting the release specifications proposed in the original Dossier in Support of the Safety of Good Meat Cultured Chicken as Human Food Ingredient dated March 4, 2022.

7. PRODUCT CHARACTERIZATION

7.1.1 Please provide, for addition to the disclosable safety narrative, the second sentence of the first paragraph of Section 10.4.4.1.3 on page 36 of the confidential supplementary material.

The second sentence of the first paragraph of Section 10.4.4.1.3 on page 36 of the confidential supplementary material is below:

"As outlined in the BAM, Chapter 4, the detection of coliforms is used as a general indicator of sanitary conditions in the food-processing environment while *E. coli* is used to indicate recent fecal contamination or unsanitary processing."

7.1.2 Please provide the citation, for addition to the disclosable safety narrative, the method used for measuring peroxide values, as listed on page 39, Section 10.4.4.2.7 of the confidential supplementary appendix.

The requested citation for the method used for measuring peroxide values as listed on page 39, Section 10.4.4.2.7 of the confidential supplementary appendix is below:

AOCS (2003) Official Method Cd 8-53. American Oil Chemists Society, Champaign, IL.

7.1.3 Please provide the citation, for addition to the disclosable safety narrative, the method used for measuring pluronic F-68, as listed on page 41, Section 10.4.4.2.14 of the confidential supplementary appendix.

The requested citation for the method used for measuring Pluronic F-68 as listed on page 41, Section 10.4.4.2.14 of the confidential supplementary appendix is below:

"Pluronic F-68 concentrations in Good Meat cultured chicken are analyzed by colorimetric assay, utilizing the method developed by Ghebeh et al. ("Development of an Assay for the Measurement of the Surfactant Pluronic F-68 in Mammalian Cell Culture Medium." Analytical Biochemistry, Volume 262, Issue 1, 15 August 1998, Pages 39-44)."

8. FOOD SAFETY PLAN

8.1 PROCESS CONTROLS

8.1.1 On page 80 of the disclosable safety narrative, you state, "The primary process steps in manufacturing include thawing of cells from a validated cell bank, scale-up of cells in shake flasks and bioreactors, followed by harvest and storage of the cultured cells". Establishment of the master cell bank is not included. For addition to the disclosable safety narrative, please clarify if the establishment of the master cell bank is included in the food safety plan.

To this point, the Master Cell Bank and Master Working Cell Banks have been created and managed in a dedicated biological laboratory using aseptic techniques and FSQA approved batch records consistent with FDA guidelines for the biopharmaceutical industry. All banks are prepared using GMPs and under a class 100 biosafety cabinet. Based upon this question, we are moving these programs to a formal Food Safety Plan and supporting Quality System which will comply with the requirements of 21 CFR 117 and related regulations. These activities are currently performed at dedicated Good Meat laboratories with fully tested and released banks being transferred to JOINN for manufacturing.

8.2. SAFETY AND CONTROL TESTING

8.2.1. On page 81 of the disclosable safety narrative, you state, "Sanitation of the equipment involved in downstream processing is validated using ATP and APC swabbing. If the ATP swab results are out of limits, the cleaning process is repeated. If the APC swab results are out of limits, the cleaning process is re-evaluated and corrected". This statement is not included in the Environmental Monitoring Plan in Section 7.4 (page 85). Additionally, Table 55 in this section summarized the results from six representative batches of the final cell cultured product. For addition to the disclosable safety narrative, please comment on the relationship between the sanitation program and the safety of the final cell cultured product.

The equipment involved in downstream processing of cultured chicken that is referred to in the safety narrative is a centrifuge for cell washing after harvest. These centrifuges can be stainless-steel or sterile single-use.

The version of centrifuge that is utilized at JOINN Biologics is sterile-single-use. We are not using stainless-steel centrifuges so the current HARBPC food safety plan does not include pre-operational ATP/APC swab for verification of cleaning. This statement is not relevant to this process.

The sterile-single-use centrifuge contact surfaces are USP class VI, pre-sterilized by gamma radiation; therefore, no CIP or SIP is needed. As part of the food safety preventive control plan, the supplier provides a certificate of sterilization for every batch which is verified by JOINN Biologics at receiving and prior to release for operational use.

The process that we use to cultivate cells uses a closed aseptic environment with aseptic controls to prevent contamination of the culture. Aseptic systems rely upon environmental and sanitation controls to compliment and assure the effectiveness of the process. Sanitation of the bioreactors, including steam sterilization, is core to creating the aseptic process environment. Single use sterile materials maintain and extend the aseptic process. At the final harvest, when the cells are removed from the centrifuge, there is a limited period of exposure to the processing room environment. Environmental monitoring and sanitation programs for the processing areas help to support the aseptic process.

Although the cells and cell culture have limited contact with the processing environment, we understand that sanitation programs play an important role in ensuring that the environment does not contaminate the process or the product. JOINN Biologics has established a robust sanitation program to prevent contamination of cultured cells from the environment, personnel and equipment. Non-conforming results from monitoring

articulated in Table 59 of the submission would require correction and investigation. Product safety and release is determined based upon direct product testing.

[The remainder of this page is blank]

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DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT – PROCESS AMENDMENT

August 4, 2022

FINAL

Good Meat, Inc.

DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT – PROCESS AMENDMENT

1. FIRM AND CONTACT PERSON

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.
2000 Folsom Street
San Francisco, CA 94110
Tel: 858-349-8338
Fax: 415-682-6990

[redacted] (b) (6)

March 2, 2023

Peter Licari
Chief Technology Officer
GOOD Meat, Inc.

Date

2. EXECUTIVE SUMMARY

This dossier describes a change in the composition of the culture medium used for manufacturing of chicken cells utilized by Good Meat, Inc. (Good Meat). Good Meat characterized the C1F-P1 cell culture growth requirements for folic acid. This characterization determined that exogenous folic acid provided in the culture medium could be reduced by 90% without impacting culture growth or quality aspects. Furthermore, Good Meat demonstrated that, using this reduced folic acid process, results in folic acid not being detected in the harvested chicken cells and reduced the total amount of folate in chicken cells while still meeting the release specifications proposed in the original "Dossier in Support of the Safety of GOOD Meat Cultured Chicken as Human Food Ingredient" dated March 4, 2022.

3. FOLIC ACID

Folic acid is part of the basal media and is a component of the seed and production cell culture media in the manufacturing process of C1F-P1 cultured chicken.

Folic acid is an essential water-soluble vitamin added to virtually all cell culture media formulations. Following up on pre-market consultation discussions with FDA, we reviewed the original concentration of folic acid supplied to the culture of C1F-P1 cells and explored reduction of its concentration to lower levels without impacting the manufacturing process and the quality specifications of the harvested chicken product.

Much like we did in the original safety dossier, we distinguish between total folate and folic acid for definition of regulatory status, recommended dietary allowance, safety evaluation and dietary exposure. Total folate is an umbrella term used to represent the different forms of the vitamin B. Food folate is the form that occurs naturally in food sources. Folic acid is the form of vitamin found in fortified foods and dietary supplements. The term dietary folate is used to represent food folate and folic acid in fortified foods together. Total folate encompasses all dietary and supplemental exposure to folate and folic acid. We investigated the impact of reduction of folic acid concentration in the culture medium used for manufacturing of C1F-P1 chicken cells in both folic acid and total folate levels.

3.1. Process Change

The only process change implemented is to reduce the original folic acid concentrations by 90% in all manufacturing steps of the process. Other than reducing folic acid, the same cell banks and method of manufacturing described in Sections 4 and 5, respectively, of the "Dossier in Support of the Safety of GOOD Meat Cultured Chicken as Human Food Ingredient" dated March 4, 2022 are maintained.

3.2. Folic Acid levels in Cultured Chicken and Estimated Consumer Exposure

To establish estimated consumer exposure to folic acid in the new manufacturing process of cultured chicken using lower levels of folic acid in the culture medium, three (3) representative batches of cultured chicken were evaluated by AOAC 2011.06 UHPLC-MS method. Calculations for dietary exposure are based on RACC values stated in 9 CFR § 317.312. All three (3) representative batches were found with folic acid below the limit of detection (<0.1 µg/g). Assuming the maximum content of folic acid in cultured chicken to be the LOD 0.1 µg/g, the estimated maximum folic acid content in cultured chicken was calculated to be <8.5 µg/serving/person for ready-to-serve portions based on a RACC value of 85 g chicken *per* serving. The estimated maximum folic acid content was calculated to be <11.4 µg/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum folic acid content was calculated to be <0.1 µg/g in cultured chicken, which is consistent with the maximum folic acid levels in conventional chicken (USDA National Nutritional Database for Standard Reference: Table 8 of "Dossier in Support of the Safety of GOOD Meat Cultured Chicken as Human Food Ingredient" dated March 4, 2022).

Table 1. Estimated consumer exposure values of Folic Acid from representative batches (RB) of cultured chicken.

Representative Batches (RB)	Folic Acid amount *per* gram of cultured chicken (µg/g)	Folic Acid amount *per* 100 g of cultured chicken (µg/100g)	Estimated intake of Folic Acid in ready-to-serve food (RACC 85g) (µg/serving/person)	Estimated intake of Folic Acid in ready-to-cook food (RACC 114g)[1] (µg/serving/person)
RB-1	<0.1	<10	<8.5	<11.4
RB-2	<0.1	<10	<8.5	<11.4
RB-3	<0.1	<10	<8.5	<11.4
Average	<0.1	<10	<8.5	<11.4
Maximum	<0.1	<10	<8.5	<11.4

The values reported in Table 1 confirm that folic acid was found below the detection limit and that residual folic acid from the culture media was either completely washed off or is present at negligible levels that do not represent a safety concern for human consumption. The original process using higher folic acid concentration in the culture medium for manufacturing of cultured chicken also showed representative batches with folic acid level below the limit of detection.

Reported daily limits for consumption of folic acid from fortified foods and supplements range from 300 µg/day to 1,000 µg/day (Table 3 of "Dossier in Support of the Safety of GOOD Meat Cultured Chicken as Human Food Ingredient" dated March 4, 2022). Assuming a worst-case scenario of folic acid content in cultured chicken and assuming an exclusive source of folic acid derived from this food product in the human diet, it would require a daily consumption of >3Kg/day to >10 Kg/day of cultured chicken cells, to surpass the reported daily limits of folic acid from fortified foods and supplements, depending on the age group of the consumers.

3.3. Total Folate and Dietary Folate Levels in Cultured Chicken

To establish estimated consumer exposure to total folate in cultured chicken, three (3) representative batches of cultured chicken were evaluated by validated method AOAC 944.12 and AACC 86-47.01 (Table 2). Dietary Folate Equivalent concentrations were calculated using results

[1] 9 CFR §381.412

from Total Folate (AOAC 944.12 and AACC 86-47.01) and Folic Acid by UHPLC-MS (AOAC 2011.06), further discussed in Table 1 shown above.

Both folic acid and total folate concentrations in the three (3) representative batches were below LOQ. Considering the worst-case scenario that folic acid and total folate are present at LOQ levels in cultured chicken samples, maximum Dietary Folate Equivalent levels were calculated[2] to be <12.7 DFE µg per 100 g of cultured chicken.

Table 2. Estimated consumer exposure values of total folate from representative batches (RB) of cultured chicken.

Representative Batches (RB)	**Total folate amount per 100 g of cultured chicken (µg/100g)**	**Folic Acid amount *per* 100 g of cultured chicken (µg/100g)**	**Dietary Folate Equivalent Amount per 100 g of cultured chicken (DFE µg/100g)**
RB-1	< 6 (LOQ)	<10 (LOQ)	<12.7
RB-2	< 6 (LOQ)	<10 (LOQ)	<12.7
RB-3	< 6 (LOQ)	<10 (LOQ)	<12.7
Maximum	**< 6 (LOQ)**	**<10 (LOQ)**	**<12.7**

Table 2. Estimated consumer exposure values of total folate from representative batches (RB) of cultured chicken.

Representative Batches (RB)	**Dietary folate amount *per* gram of cultured chicken (DFE µg/g)**	**Dietary folate amount *per* 100g of cultured chicken (DFE µg/100g)**	**Estimated intake of Dietary Folate in ready-to-serve food (RACC 85g) (DFE µg/serving/person)**	**Estimated intake of Dietary Folate in ready-to-cook food (RACC 114g) (DFE µg/serving/person)**
RB-1	<0.127	<12.7	<10.8	<14.5
RB-2	<0.127	<12.7	<10.8	<14.5
RB-3	<0.127	<12.7	<10.8	<14.5
Maximum	**<0.127**	**<12.7**	**<10.8**	**<14.5**

The estimated consumer exposure values of dietary folate from Good Meat cultured chicken were calculated based on RACC values stated in 9 CFR § 317.312. As quantification in cultured chicken batches was below the limit of quantification, the estimated maximum dietary folate content in Good Meat cultured chicken was calculated assuming the worst-case scenario and found to be at 10.8 µg DFE/serving/person for ready-to-serve portions based on a RACC value of 85 g *per* serving. As a comparison, the estimated maximum dietary folate content in Good Meat cultured chicken produced using the original process was calculated to be 77 µg DFE/serving/person for ready-to-serve portions based on a RACC value of 85 g *per* serving, a value more than 7x higher than the one attained with the new proposed process.

Following the same pattern, the estimated maximum dietary folate content was calculated to be 14.5 µg DFE/serving/person for ready-to-cook portions based on a RACC value of 114 g chicken *per* serving. Lastly, the estimated maximum dietary folate content was calculated to be

[2] Dietary Folate Equivalent (DFE) formula as described in U.S. FDA Guidance Document on Converting Units of Measure for Folate, Niacin, and Vitamins A, D, and E on the Nutrition and Supplement Facts Labels. Accessible at: https://www.fda.gov/media/129863/download

Dietary Folate Equivalents = (mcg Total Folates – mcg Folic Acid) + (mcg Folic Acid / 0.6)

0.127 µg DFE/g (equivalent to 12.7 µg DFE/100g), which is similar to the average dietary folate equivalents (DFE) content in raw chicken breast[3] (9 µg DFE/100g).

This reduction of folic acid concentration in the culture media used for manufacturing moved cultured chicken from an excellent source of folate (0.1-0.2 mg DFE/serving) in the original process conditions to a poor source of folate (<25 µg DFE/serving) in the conditions herein described, following the classification indicated in Table 6 of "Dossier in Support of the Safety of GOOD Meat Cultured Chicken as Human Food Ingredient" dated March 4, 2022. Similar levels of folate are described for all meat, fish, and poultry products according to the same table.

Furthermore, Good Meat cultured chicken will be used as ingredient in manufacturing of various cultured chicken finished products (bites, boneless breasts, tenders, etc.) ranging from 60% to 75% (w/w)[4] of overall formulation, therefore contributing with < 10 µg DFE/100g towards finished product. For all the reasons listed above, such exposure to folate does not pose a safety risk for human consumption.

4. Conclusion

To summarize the key points in this discussion:

1) We successfully reduced the original content of folic acid in the culture medium of cultured chicken by 90%.
2) Folic acid added to the cell culture medium is not detected in the harvested chicken cells (when using validated analytical methods).
3) This process change reduced the total amount of folate (expressed as DFE) in cultured chicken (when using validated analytical methods).
4) Cultured chicken products have now < 10 µg DFE/100g. Current serving size of the cultured chicken meat is approximately 50 grams or < 5 µg DFE/serving.
5) Good Meat cultured chicken products are now categorized in poor sources of folate, alongside most meat, fish, and poultry products.
6) This process change successfully reduced total folate in cultured chicken products and significantly reduced its dietary exposure to consumers.

[3] U.S. Department of Agriculture, FoodData Central; Chicken, broiler or dryers, breast, skinless, boneless, meat only, raw nutrient values. https://fdc.nal.usda.gov/fdc-app.html#/food-details/171077/nutrients (last accessed January 10, 2022).

[4] w/w=weight/weight

DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT – RESPONSE DOCUMENT

September 14, 2022

Good Meat, Inc.

1. EXECUTIVE SUMMARY

The responses reported in this document address the request for additional information received from the FDA on September 12, 2022, after the review of the submitted Response to FDA Clarification questions relating to application (CCC 000001) submitted on July 8, 2022. For clarity, FDA requests are included as bold text and our responses follow. We numbered the requests by the FDA for ease of referencing.

2. DESIGNATION OF JULY 8TH, 2022, AMENDMENT AS "CONFIDENTIAL"

2.1. Pages 1-37 of the July 8, 2022, amendment is designated as "confidential". In our June 29, 2022, request for additional information, we stated, "Your response will be appended to the CCC 000001 disclosable safety narrative as an amendment and will subsequently be treated as part of the disclosable safety narrative in the administrative record for CCC 000001 (i.e., we expect to proactively disclose it at the completion of this consultation)". For the administrative record, please provide a statement clarifying whether any of the information contained in the July 8, 2022, amendment is confidential.

The amendment that was submitted on July 8, 2022, is to be considered non-confidential and can be included as part of the CCC 000001 disclosable safety narrative. In addition to this response document, we have included a revised version of the amendment where we have removed "confidential" from the footer; that is the only change to this document.

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3. FOOD SAFETY PLAN

3.1. CELL GROWTH IN BIOREACTOR

3.1.1. On page 28 of the July 8, 2022, amendment, you state, "Cell culture samples are visually inspected for bacterial contamination and cell morphology". Please clarify, for addition to the disclosable safety narrative, what the indicator for bacterial contamination is. Please also provide the resulting steps and/or programs triggered if contamination is observed at this stage.

Cell culture samples from the 1000L bioreactor are visually inspected under a microscope once per day for bacterial contamination, cell morphology and cell growth parameters. Results are recorded in the batch production records. Indicators of contamination can be (including but not limited to):

- Sudden drop in pH or dissolved oxygen of the culture media
- Culture media appearing cloudy, change in color or development of thin biofilms
- Observation of shapes of individual bacteria between the cells (e.g., rod shaped) through microscopic observation

If contamination is determined, then the production run is terminated, and all of the 1000L bioreactor culture is discarded. Samples will be collected for further investigation [Program: 101-MFP-055. Contamination responses for Bioreactor and Inoculum]. The bioreactor undergoes a sterilization process, a cleaning process, and qualification testing for safe use prior to the next production run [Program: 101-MFP-010. Assembly and SIP of components, 101-MFP-011. SIP procedures for Bioreactor]. Quality Assurance personnel will conduct detailed investigation on the incident which includes root cause analysis (RCA) and corrective and preventive actions (CAPA) [Program: 101-QAU-009. Incident, Deviation and CAPA Management].

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3.1.2. On page 28 of the July 8, 2022, amendment, you state, "… temperature monitoring data is reviewed weekly to ensure finished cultured cells are stored at appropriate temperatures". Please explain, for addition to the disclosable safety narrative, the management strategy including corrective actions, employed in an instance where temperature abuse occurs during a given week.

Cultured Chicken cells are stored below -20°C with critical limit of -18°C. The storage freezers have temperature monitoring sensors which monitor and record the internal temperature of the freezers at every 15 minutes interval. If the temperature reading is above -18°C then an alarm will be activated, and email notification will be sent to authorized personnel.

Quality Assurance personnel review the temperature monitoring data routinely on a weekly basis which also includes checking for sensor calibration status, alarm testing and sensor battery status. This is to ensure that temperature monitoring sensors are always active and calibrated to monitor temperature. If in an instance, the temperature is above the critical limit for more than 2 hours, then the product will be evaluated for food safety using FDA's Draft Guidance Document: Hazard Analysis and Risk-Based Preventive Controls for Human Food, Appendix 3: Bacterial Pathogen Growth and Inactivation[1]. Additionally, microbiological and sensory testing of the product can be performed to evaluate suitability for commercial purposes.

Quality Assurance personnel will conduct detailed investigation on the incident which includes root cause analysis (RCA) and corrective and preventive actions (CAPA) [Program: 101-QAU-009. Incident, Deviation and CAPA Management].

Master Cell Banks (MCB) and Master Working Cell Banks (MWCB) are stored in liquid nitrogen freezer (below -175°C) with critical limit of -130°C. The LN2 freezers have temperature monitoring sensors which monitor and record the internal temperature of the freezers at every 15 minutes interval. If the temperature reading is above -130°C then an alarm will be activated, and email notification will be sent to authorized personnel. In the event of equipment or power failure, backup refrigeration is available and MCB/MWCB would be transferred to a backup for emergency cooling. For MCB/MWCB cryovials that would be exposed to temperature abuse, Quality Assurance personnel would conduct a detailed investigation that would include stability evaluation of the cell bank. If those fail to meet our specifications, these MCB/MWCB exposed to temperature abuse would be rejected and replaced by a new MCB/MWCB.

[1] U.S. Food and Drug Administration, Draft Guidance Document for Industry: Hazard Analysis and Risk-Based Preventive Controls for Human Food, Appendix 3: Bacterial Pathogen Growth and Inactivation, Table 3C. Accessible at: https://www.fda.gov/media/99598/download

3.1.3. On page 28 of the July 8, 2022, amendment, you state, "… *Listeria* and *Salmonella* are routinely monitored as sanitation indicator organisms". Please confirm that this refers to the monitoring described in Table 59 (page 85) of your disclosable safety narrative, and also clarify, for addition to the narrative, whether "*Listeria*" refers to *Listeria* spp. or *L. monocytogenes*, specifically.

GOOD Meat, Inc. confirms that statement on page 28 of the July 8, 2022, amendment, "*Listeria* and *Salmonella* are routinely monitored as sanitation indicator organisms" refers to the Environmental Monitoring Plan described in Table 59 (page 85) of the Safety Dossier. The swabs are tested for Genus *Listeria* (*Listeria spp.*) and Genus *Salmonella* (*Salmonella spp.*) using the ELFA technique (Enzyme Linked Fluorescent Assay).

If the indicator organism is detected, the production batch is placed on "Quality Hold" for food safety evaluation. Any in-process material or finished product will be tested for safety pertaining to the incident. Quality Assurance personnel will conduct detailed investigation on the incident which includes root cause analysis (RCA), corrective and preventive actions (CAPA) [Program: 101-QAU-009. Incident, Deviation and CAPA Management].

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3.1.4. The provided information describing your food safety plan did not address control of potential food allergens. Please clarify, for addition to the disclosable safety narrative, whether there is a food allergen plan in place.

GOOD Meat, Inc. has established supply chain preventive controls which includes material evaluation and supplier qualification. Material evaluation includes presence of food allergens, ingredient composition and labelling. Only approved materials from qualified suppliers are authorized to be used for production.

GOOD Meat, Inc. has reviewed and determined that there are no food allergens present in the media components used in the processing of cultured chicken. Cultured chicken processing takes place in a dedicated suite at JOINN Biologics, dedicated to GOOD Meat's cultured chicken production only, so there is no risk of allergen cross-contamination. JOINN Biologics do not have any materials containing food allergens in their warehouse. Furthermore, JOINN Biologics have prepared an allergen control plan [Program: 101-QAU-016. Allergen Management Program] which prohibits storage of any material containing allergens in the building. All materials received at JOINN Biologics are inspected to ensure only approved materials from qualified suppliers are released for production use [Program: 101-SUP-003. Inspection, Quarantine and Disposition of Raw Materials].

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DOSSIER IN SUPPORT OF THE SAFETY OF GOOD MEAT CULTURED CHICKEN AS A HUMAN FOOD INGREDIENT – RESPONSE DOCUMENT

March 2, 2023

Good Meat, Inc.

1. EXECUTIVE SUMMARY

The responses reported in this document address the request for additional information received from the FDA on March 1, 2023, after the review of the submitted Response to FDA Clarification questions relating to application (CCC 000001) submitted on prior. For clarity, FDA requests are included as bold text and our responses follow. We numbered the requests by the FDA for ease of referencing.

2. CELL BANK ESTABLISHMENT

2.1. On page 69 of the disclosable safety narrative, you identify *Campylobacter spp.*, *Salmonella* serovars, and ESBL/ AmpC gene carrying *Escherichia coli* as organisms of medium to high public health relevance for conventional poultry meat. However, in the July 8, 2022, amendment (Table A6, p17-18), it appears that you do not test the master cell bank (MCB) and master working cell bank (MWCB) for these specific microorganisms (with the exception of *S.* Pullorum). Please briefly discuss how you ensure that these microorganisms are not present in the MCB and MWCB in the absence of specific testing for these microorganisms, or otherwise clarify your MCB and MWCB testing process.

Sterility assessment of the Master Cell Banks and Master Working Cell Banks created at Good Meat is done by culture broth testing performed at a third-party company. Two independent tests are performed by a 14-day incubation of cell bank samples in both (i) Trypticase Soy Broth (TSB) and (ii) Fluid Thioglycolate Media (FTM). TSB is recommended for use in sterility testing for the detection of contamination with low incidence fungi and aerobic bacteria.[1] FTM is used for the evaluation of aerobic, microaerophilic, and anaerobic microorganisms. The FTM formulation is the standard medium recommended by the Food and Drug Administration, National Institute of Health, the National Formulary, and the U.S. Pharmacopeia for sterility testing of clear fluid biologics and other sterile products.[2]

The C1F-P1 MCBs and MWCBs are considered valid for sterility if a minimum of $0.4x\sqrt{n}$ independent and randomly selected cryovials (from a cell bank of n size) from each bank are thawed and do not demonstrate bacterial and fungal growth in the referenced culture broth media.

This testing would amplify the organisms of high public health relevancy for conventional poultry meat listed on page 69 of the disclosable safety narrative if they were present, which would lead to the failure of the release testing of those MCBs or MWCBs. For this reason, the current testing ensures that these microorganisms are not present in the C1F MCB and MWCB.

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[1] The Official Compendia of Standards. USP-NF. United States Pharmacopeial Convention, Rockville, MD.

[2] Federal Security Agency, Food and Drug Administration, Compilation of Regulations for Test and Methods of Assay and Certification of Antibiotic Drugs; National Institutes of Health Circular: Culture Media for the Sterility Test, 2nd rev. Feb. 5, 1946.

3. CELL CULTURE PROCESS

3.1. In Table 43 (page 70) of the disclosable safety narrative, you list the specification for aerobic plate count in the harvested cell material as <10,000 CFU/g. This specification is three orders of magnitude larger than the batch analysis data provided in Table 43. Further, as the harvested cell material, as described in the disclosable safety narrative, is a product produced in a controlled system in an aseptic environment, a high aerobic plate count in the final product should not be expected provided the product is produced under cGMP conditions. Therefore, we ask that you please consider lowering this specification to more closely align with the results of batch analysis data or provide a discussion regarding the adequacy of your specification from a food safety perspective.

This limit was set in relation to commercial food safety. Based on your comment and the data we have generated; we will modify the limit to 1,000 CFU/g. Periodically we will review our data and tighten specifications further if warranted.

4. PRODUCT CHARACTERIZATION

4.1. On page 32 of the disclosable safety narrative, you state "Estimated average and maximum iron content in 100 g serving was calculated at 80 µg/100g and 110 µg/100g, respectively, which is lower than the average nutritional iron content defined by U.S. Department of Agriculture for conventional chicken at 370 µg/100g. The iron content in cultured chicken is approximately 1/3rd that of conventional chicken (0.37 mg/100g) and approximately 1/450th that of the UL for adults." However, on page 34 you state "Multiple batches of cultured chicken were analyzed for iron and nitrate ions. While the iron nutrient content was higher than the average nutritional iron content in conventional chicken." Please clarify the discrepancy between these two statements.

The information on Page 32 is accurate while the conclusion statement on Page 34 of the safety dossier is incorrect. The correct statement on Page 34 should be revised as below:

"Multiple batches of cultured chicken were analyzed for iron and nitrate ions. While the iron nutrient content was lower than the average nutritional iron content in conventional chicken, …"

4.2. One page 59 of the disclosable safety narrative, you state "The BSA content in cultured chicken is higher than the average content in conventional chicken as a result of milk consumption." Please clarify this statement.

"As a result of milk consumption" should be removed from the statement on Page 59. The statement should read "The BSA content in cultured chicken is higher than the average content in conventional chicken; …"

5. DOCUMENTATION

5.1. The amendment provided on August 8, 2022, is marked "confidential." Our understanding is that this document may be appended to the publicly disclosable safety narrative. If this is correct, please confirm our understanding.

We agree to have the amendment provided on August 8, 2022, be made nonconfidential.

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