In Vitro Culture of Bovine Fibroblasts using Select Serum-Free Media Supplemented with *Chlorella vulgaris* Extract

Manuscript type: Article, New Results

Shortened title: Culture of fibroblasts in select serum-free media with algae extract

Authors

Galileo Defendi-Cho, Timothy M. Gould*

Affiliation

Colorado College, Department of Chemistry and Biochemistry, Colorado Springs, CO 80903.

*Correspondence to: tgould@coloradocollege.edu

Keywords

Serum replacement, serum-free media, algae extract, bovine fibroblast, cell cultured meat

Competing Interests

There are no competing interests.

Abstract

Standard cell culture practices require addition of animal-derived serum to culture media to achieve adequate cell growth. Typically, 5-10% by volume of fetal bovine serum (FBS) is used, which accounts for a vast majority of the cost of media while also imposing environmental and ethical concerns associated with the use of animal serum. Here we tested the efficacy of culturing cells by replacing serum in the media with algae extract and select additives. Using LC-MS, we compared molecular signatures of FBS to *Chlorella* algae extracts and identified NAD(H)/NADP(H) as common and relatively abundant features in their characteristic profiles. Bovine fibroblasts, cultured in serum-free media supplemented with *C. vulgaris* extract and just two growth factors plus insulin, showed significant growth with enhanced viability compared to control cells cultured without serum, albeit still lower than that of controls cultured with 10% FBS. Moreover, *C. vulgaris* extract enhanced cell viability beyond that of cells cultured with the two growth factors and insulin alone. These results suggest that key components in serum which are essential for cell growth may also be present in *C. vulgaris* extract, demonstrating that it may be used at least as a partial alternative to serum for cell culture applications.

Introduction

In 2013 it was estimated that raising livestock contributed roughly 14% to total greenhouse gas emissions, more than half of which was attributable to meat production (Gerber et al., 2013). Cell cultured meat has the potential to offer substantial environmental and social advantages over traditional agriculture practices using livestock (Ladak and Anthis, 2021; Tuomisto and Teixeira De Mattos, 2011), yet analysis on the economics of cultured versus conventional meat production shows high cost barriers, particularly those associated with the nutrients required (Humbird, 2021). Cell culture media is commonly supplemented with fetal bovine serum (FBS) because animal serum promotes cell growth (Penttinen and Saxén, 1959). Based on current commercial prices, the cost of culture-grade FBS is estimated at around \$1.20 per mL and that of other media components at \$0.04 per mL; thus, for typical complete growth media containing 10% FBS, most of its total cost is attributed to FBS, by roughly three-fold. Therefore, low cost and environmentally favorable alternatives to serum are attractive for cell culture applications, particularly for the cultured meat industry.

Because animal-derived serums like FBS are very complex, including thousands of different molecules that can be highly varied in their relative abundance due to many dynamic physiological factors, it is unlikely that synthetic approaches to serum alternatives will prove cost effective if technically feasible. Researchers have studied the culture of cells in serum-free conditions for many decades with varying degrees of success, yet most cell culture practices continue using FBS. Following studies which identified key components necessary to culture cells in the absence of serum (Chen et al., 2011; Murakami and Masui, 1980), there are now chemically-defined serum-free media formulations that are commercially available (e.g., E8™).

However, the cost of these serum-free formulations is still quite high, primarily due to the protein growth factors required. Such formulations were developed mainly for the purpose of consistently culturing human induced pluripotent stem cells (iPSCs); they were not originally intended for cultured meat applications although this does typically involve culturing stem cells. Nevertheless, determining the minimal components and concentrations at which they are needed to achieve adequate and consistent cell growth remains an important undertaking for the cultured meat industry (Specht, 2020).

We were intrigued by a recent study in which the authors reported growth effects of algae extract on mammalian cells (Ng et al., 2020). Ng and colleagues report that extract derived from *Chlorella vulgaris* promoted growth of Chinese hamster ovary (CHO) and mesenchymal stem cells (MSC) under certain conditions, some of which did not contain any serum. In another study, researchers reported high levels of glucose and amino acids present in various microalgae extracts, with particularly high levels of aspartate and glutamate detected in extract from *C. vulgaris* (Okamoto et al., 2020); these authors proceeded to show that they could use such algae extracts as a substitute to culture cells in media deficient in glucose and amino acids. Here we tested the efficacy of culturing cells by replacing serum in the media with algae extract and found that we could achieve appreciable growth and viability of bovine fibroblasts using select serum-free media supplemented with *C. vulgaris* extract (CVE). We characterized molecular features of algae extracts, compared them to that of FBS, and found evidence suggesting that NAD(H)/NADP(H) are common and relatively abundant components. This study establishes how algae extract may be used to culture cells in serum-free conditions while providing further insight into essential components required for cell growth.

Materials and Methods

Chemicals and Reagents

Liquid chlorella growth factor (SKU 504) and *Chlorella vulgaris* extract capsules (SKU 583) were purchased from BioPure® Healing Products (Woodinville, WA). Concentrated living *Chlorella vulgaris* (item # 152068) and Alga-Gro® freshwater medium (item # 153752) were purchased from Carolina Biological Supply Company (Burlington, NC). Pierce™ universal nuclease for cell lysis was purchased from Thermo Fisher Scientific Corporation (Waltham, MA). Recombinant human fibroblast growth factor (FGF-2, catalog # 100-18B) and transforming growth factor beta (TGF-β, catalog # 100-21) were purchased from PeproTech, Inc. (Cranbury, NJ). Bovine insulin, sodium L-ascorbate and selenium dioxide were from Sigma-Aldrich. Cell titer 96® aqueous one solution cell proliferation (MTS) assay (catalog # G3580) was purchased from Promega Corporation (Madison, WI).

Fractionation of Algae Extracts

Algae extracts termed *Chlorella vulgaris* extract (CVE) and chlorella growth factor (CGF) were derived from two different strains, *Chlorella vulgaris* (CVE) and *Chlorella pyrenoidosa* (CGF). CVE was obtained from capsules in dry powder form and dissolved directly in serum-free DMEM. CGF stock solution was a liquid from which all solutions were made on a percent volume basis by diluting CGF directly in serum-free DMEM. For fractionation experiments, crude solutions of 10 g/L CVE and 10% CGF were first prepared in serum-free DMEM. Crude CVE and CGF were then either sterile-filtered through 0.22 μm nylon filters or centrifuged at 10,000 x g for 10 minutes in a benchtop microcentrifuge. After centrifugation, supernatants were removed, and the pellets were resuspended directly in serum-free DMEM.

LC-MS

Sample separation was performed by liquid chromatography (LC) using a UPLC BEH C18 column (2.1 x 50 mm, 1.7 μ m) fitted to an Acquity UPLC system with PDA detector (Waters). Chromatographic profiles were acquired at wavelengths 200-400 nm. Injection volume was 7.5 μ L after equilibration, and the column temperature was held at 30°C. Samples were eluted from the column using a gradient mobile phase that consisted of phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile). The linear gradient elution procedure was as follows: (step 1) 100% A from 0-3 minutes, (step 2) 100% to 60% A from 3-20 minutes, (step 3) 60% to 0% A from 20-21 minutes, (step 4) 0% A from 21-24 minutes, (step 5) 0% to 100% A from 24-25 minutes. The column flow rate was set at 0.2 mL/min from 0-21 minutes, increased to 0.6 mL/min from 21-24 minutes, and then returned to the initial rate from 24-25 minutes. Electrospray ionization mass spectrometry (ESI-MS) was performed using an Acquity QDa detector (Waters). Capillary and cone voltages were 0.8 kV and 10 V, respectively. MS measurements were collected in positive ion mode with a mass scan range from 100-1200 m/z .

Cell Culture

The EBTr (NBL-4) cell line (bovine fibroblasts derived from embryonic tracheal tissue) was purchased from ATCC (CCL-44^{TM}). HEK-293 (human embryonic kidney) cells were a gift from Dr. Jörg Goronzy. Both cell types were routinely cultured in an incubator at 37°C with 5% CO₂ in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (Thermo Fisher Scientific, cat no. 15140148); serum-free DMEM included antibiotics but no FBS. Passaging was routinely done as follows: cells were first washed with 1X phosphate-buffered saline (PBS), then placed in an incubator

with 0.25% Trypsin-EDTA solution (Gibco) for approximately 2 minutes immediately after which complete DMEM was added to quench trypsin activity; cells were collected and centrifuged at 300 x *g* for 5 minutes, and then the cell pellet was resuspended in fresh media. Both cell types were passaged every 3-4 days at a 1:3 (EBTr) or 1:10 (HEK-293) passage ratio. Excess cells were stored in liquid nitrogen at $1-2 \times 10^6$ cells/mL in freezing media containing 90:10% FBS:DMSO. The number of passages for experiments was limited to less than twenty.

Viability (MTS) Assays

EBTr and HEK-293 cells were first harvested, counted, and resuspended at a density of 2.5×10^5 cells/mL in serum-free DMEM. Cells were then seeded in clear, flat-bottom 96-well plates to achieve a target density of approximately 2×10^4 cells (80 μ L) per well. Seeded cells were incubated for 2-4 hours to allow sufficient time for cells to adhere to plates prior to experiments. For all viability experiments/assays, each condition corresponded to a single column in a 96-well plate. Test media conditions were achieved by delivering 20 μ L of 5X test solutions (prepared in serum-free DMEM) to the appropriate column wells, bringing total well volumes to 100 μ L. Columns 1 and 12 as well as the top and bottom rows of all 96-well plates were treated as mock wells and excluded from data analysis to control for plate edge effects (Burt et al., 1979). Immediately after adding 5X test solutions to plates, cells were returned to the incubator for the experiment duration (typically 3 days or approximately 72 hours) after which 20 μ L of MTS reagent was added to each plate well. Plates were then incubated for another 4 hours. (For the experiment shown in Figure 4c, only 10 μ L of MTS reagent was added to plate wells because there was not enough reagent to add 20 μ L to all 96 wells.) End-point absorbance measurements were taken with a plate reader at 490 nm (BioTek).

Cell Counting and Imaging

Cells were harvested, pelleted, and resuspended in serum-free DMEM. Two replicate 50 μ L samples of cell suspensions were each diluted 1:1 in trypan blue dye, from which 10 μ L was sampled and pipetted onto a hemocytometer (improved Neubauer ruling pattern). Live and dead/blue (non-intact) cell counts were performed in duplicate (i.e., one count per sample). Brightfield images were obtained using an inverted tissue culture microscope (Hund Wetzlar) fitted with a fixed microscope adapter connected to a 5.1-megapixel digital camera (AmScope). Original grayscale image tiles were merged into a single multi-panel image representing each experiment. Multi-panel images were processed with Fiji/ImageJ2 open-source software (NIH). Image processing involved pseudo flat field correction to balance uneven illumination across images, Fast Fourier Transform (FFT) bandpass filtering to reduce image scan lines, and brightness/contrast adjustments.

Data Analyses and Statistics

LC-MS datasets, including both matrix LC and MS data from 3 replicate injections of the same sample type, were first averaged and normalized; mean data matrices were then visualized using R software (v4.1.0) with viridis and ggplot2 packages. Principal component analysis (PCA) on averaged MS data was performed in R using the prcomp function. Potential parent species were identified in a mass spectral database (MassBank.eu) by searching the m/z value and average relative intensity of peaks shared between samples. All other data were analyzed and/or visualized using Prism software (v9.2.0, GraphPad), in which t -tests with Welch's correction were used to determine statistical significance between experimental groups.

Results

Molecular features of FBS and algae extracts show both distinct and common components.

Given their potential growth effects, we sought to compare algae extracts (CVE and CGF, each of which was derived from a different *Chlorella* strain) to each other and FBS using LC-MS. Due to limited scope and the anticipated molecular complexity of these samples, our aim was not to look for specific compounds but rather to gain a qualitative profile for each sample type. Figure 1a shows LC chromatograms of FBS compared to CVE and CGF samples. Large broad peaks around 260 nm for species that eluted off the column within the first few minutes were observed in both CVE and CGF but not FBS samples; these large peaks did not correspond to any appreciable signal(s) detected by the mass spectrometer at such early elution times (Figure 1b). All samples showed both substantial absorbance (between 200-250 nm) and mass signals at retention times from approximately 18-21 minutes; the relative magnitude of this cluster of signals was comparable for FBS and CVE samples, whereas that of CGF samples was lower. Figure 1c shows the results of a principal component analysis (PCA) comparing total ion chromatograms (TICs) of all FBS, CVE and CGF samples (i.e., replicates averaged in Figure 1b). Replicate injections of the same sample type clustered together and separated in PC1 but not PC2 values; moreover, CVE and FBS samples were more similar in PC4 values than CGF samples. We also visualized how signals at each m/z value and retention time compared across samples (Figure 1d). Each sample type showed a common pattern with a cluster of relatively high signal peaks corresponding to m/z values ranging from roughly 800-1200 and retention times from about 15-21 minutes. Both CVE and CGF samples also shared signal features at lower m/z values (mainly over the 400-800 range) and retention times from about 5-15 minutes; this

broad cluster of signals was less apparent if not absent in FBS samples. We further examined the most striking MS feature found in all samples, i.e., the cluster of signals at m/z values from about 800-1200 and retention times from 15-21 minutes. Figure 1e highlights five relatively abundant and shared peaks within this cluster, noting their m/z whole integer value, relative abundance, and corresponding potential parent ion identity(s). The top candidate identified by a mass spectral database search (MassBank.eu) for the most abundant shared peak (no. 3 in Figure 1e) was nicotinamide adenine dinucleotide (NAD^+) and/or NAD phosphate (NADP^+).

Algae extracts alone in the absence of FBS slightly improve cell viability but not growth.

We initially set out to test if algae extracts have growth effects on cells cultured in the absence of any FBS, using MTS assays as an initial readout and proxy for potential cell growth. Figure 2 shows the relative viability of HEK-293 and EBTr cells cultured over a 10,000-fold range of concentrations of CVE or CGF. Media supplemented with CVE and CGF alone significantly increased HEK-293 and EBTr cell viability compared to serum-free controls; such effects were more pronounced for CVE than CGF and for relatively high concentrations of CVE. However, increases in relative cell viability due to CVE supplementation alone did not exceed beyond roughly 25% in any condition; moreover, and importantly, upon examining CVE-treated cells under the microscope immediately prior to performing MTS assays, we did not observe any convincing level of growth or cell doubling(s).

Next, we decided to test similar conditions to the experiment described in Figure 2 using MTS assays to assess viability but adding 5% FBS to our test conditions (Figure S1). For HEK-293 cells, 5% FBS alone provided about 90% viability compared to that of 10% FBS controls after 3 days. The addition of 0.01-1 g/L CVE very slightly increased viability (compared to 5% FBS

controls), yet such viability decreased with 10 g/L CVE. Compared to 5% FBS controls, the viability of EBTr cells cultured with 5% FBS plus 0.01-1 g/L CVE showed a similar pattern as that of HEK-293 cells (i.e., not markedly different viabilities compared to 5% FBS controls).

However, 10 g/L CVE with 5% FBS increased viability in EBTr cells relative to 5% FBS controls.

Given the partial but limited growth shown in Figure 2, and the slight decrease in viability at higher concentrations of CVE for HEK-293 cells shown in Figure S1, we wondered if there may be components in the CVE which were acting to inhibit cell growth. We decided to separate the different components of CVE through filtration, centrifugation, and treatment with nuclease, using MTS assays to compare head-to-head the extract in its crude, filtered, supernatant, and pellet forms, each with and without nuclease treatment. Figure 3 shows the relative viability of EBTr and HEK-293 cells cultured with these different CVE fractions. No obvious differences were seen between crude, filtered and supernatant forms of extract, with viabilities of such fractions all falling near the range of 15-20%. The viability of cells treated with the resuspended pellet was notably lower than that of all other fractions, falling to nearly 0%, similar to that of no serum controls. Further, CVE fractions treated with nuclease showed mostly reduced viability compared to the corresponding fractions not treated with nuclease. Figure S2 shows the LC-MS characterization of the supernatant and pellet forms of CVE used in the experiment described in Figure 3. UV absorbance data shows sharp peaks around 20 minutes in the 200-250 nm range for both the pellet and the supernatant. The supernatant also shows a pair of broader peaks at around 200 and 260 nm that appear to have eluted within the first minute, neither of which was present in the pellet form. TICs of mass spectrometry data for m/z values between 400 and 1200 for these two forms of extract show similar

footprints. Both show similarly the highest intensity spectra between 15 and 20 minutes, with relatively smaller spectra between 0 and 15 minutes. The spectra of the pellet form show slightly greater relative signals in the 0-6 minute range. Notably, the large UV peaks in both sample types correspond to increased signals in their respective mass spectra at around 20-21 minutes, however the large UV signal eluting early and only in the supernatant did not correspond to any appreciably large signal(s) in its mass spectra.

Select serum-free media supplemented with *C. vulgaris* extract enhances cell viability.

After not seeing many promising results from algae extract alone, we decided to move our research in a different direction, focusing on growth factors, which have previously been used to successfully culture stem cells in an “Essential 8” (E8™) serum-free medium (Chen et al., 2011). Figure 4a shows percent viability of both EBTr and HEK-293 cells treated with different concentrations of basic fibroblast growth factor (FGF) and transforming growth factor β 1 (TGF). Neither growth factor alone improved viability beyond 5-10% relative to serum-free controls, yet viability increased to around 15% for EBTr cells treated with a combination of 10 ng/mL FGF and 10 ng/mL TGF. Thus, we decided to use a combination of FGF and TGF for our next experiment in which we tested these growth factors together with bovine insulin, vitamin C (in the form of L-ascorbate), and selenium (in the form of selenium dioxide), all of which are listed as ingredients of the E8™ media. Figure 4b shows insulin as a critical factor in real cell growth, in conjunction with growth factors; treatments with both insulin and growth factors improved EBTr cell viability to around 35% relative to controls cultured with FBS. (Note that since we used bovine insulin, these effects were only apparent for bovine EBTr and not human HEK-293 cells.) Despite their inclusion in E8™ media, adding L-ascorbate, selenium, or both,

slightly reduced EBTr cell viability compared to that of cells cultured with growth factors and insulin alone. We decided next to bring algae extracts back into our experiments, combining them with insulin and growth factors and/or L-ascorbate and selenium. Figure 4c shows perhaps the most promising results thus far; EBTr cell viability reached approximately 75% (relative to FBS controls) when treated with a combination of CVE, growth factors and insulin. On the other hand, the viability of cells treated with CGF, growth factors and insulin was actually lower than that of cells treated with growth factors and insulin alone. Once again, the addition of L-ascorbate and selenium had little if any beneficial effect on cell viability.

Select serum-free media supplemented with *C. vulgaris* extract promotes cell growth and expansion.

After seeing promising viability results (i.e., Figure 4c), we decided the next step was to test if EBTr cells would expand when treated with insulin, growth factors and CVE in a cell count experiment. Figure 5a shows cell counts of CVE and CGF with supplements (i.e., growth factors, insulin, L-ascorbate, and selenium) compared to controls with and without serum (10% FBS). The number of EBTr cells increased in cultures with CVE or CGF plus supplements by day 2, showing definitive growth, yet still not as much growth as control cells cultured with 10% FBS. By day 4, the number of cells cultured with CGF plus supplements fell to a level near that of no serum controls, but cells cultured with CVE plus supplements continued growth to around half that of controls cultured with 10% FBS. On day 8, the number of cells cultured with CVE plus supplements was comparable to that of controls cultured with 10% FBS, the latter of which had decreased since day 4. Figure 5b shows the corresponding cell death percentages for the experiment described in Figure 5a. Cell death percentages were largely in the 10-15% range,

slightly increasing over time, with the most death occurring for cells cultured with CGF plus supplements on day 4 jumping to about 40%, corroborating the loss of growth shown for such cultures between day 2 and day 4 in Figure 5a. Figure 5c shows images captured of the experiment described in Figures 5a and 5b. EBTr cells cultured with 10% FBS show sustained growth through day 4 until reaching confluency, at which point growth plateaued between days 4 and 8. Cells cultured with CVE plus supplements also appeared to grow through day 4, reaching a relatively high level of confluency; on day 8 the level of confluency appears about the same as day 4 (perhaps slightly more) with more dead cells in the image. Cells cultured with CGF plus supplements appear to show growth until day 2, though notably less than cells cultured with CVE plus supplements; however, by day 4 most if not all remaining cells appear dead, and by day 8, only dead cells remain in the image. (Note that EBTr cells in wells treated with algae extract appear larger than cells in wells where algae extract was not present.) The no serum control shows some cells with normal morphology on day 2, with many dead cells in the image as well; by days 4 and 8, hardly any cells remain besides a few dead cells.

Given that EBTr cells showed growth and markedly improved viability when cultured with CVE and select supplements, we decided next to attempt to expand EBTr cells from a 1 mL culture (12-well plate) to a 2 mL culture (6-well plate) under these conditions (Figure S3). On day 1, cells cultured with 10% FBS or with CVE plus supplements attached effectively, while the no serum control already shows many dead cells. By day 4, the no serum control well looks nearly identical to that on day 1, with some cells still attached but many dead with no visible growth. By day 4, cells cultured with 10% FBS or CVE plus supplements show obvious growth, nearing confluency. As observed in Figure 5c, cells cultured with CVE plus supplements appear

298 slightly bigger than cells not cultured with CVE, meaning that fewer cells were necessary to
 299 reach similar levels of confluency. After the day 4 pictures were taken, cells were detached and
 300 removed from the 1 mL cultures, then pelleted and resuspended into fresh 2 mL cultures. The
 301 following day, after cells attached to the plate (day 5), the relative amount of cells in the 10%
 302 FBS control well was notably greater than that in the well with CVE plus supplements. By day 8,
 303 cells cultured with 10% FBS reached near-confluency, while cells cultured with CVE plus
 304 supplements remained at nearly 50% confluency. On both days 5 and 8, the no serum control
 305 wells were essentially devoid of live cells, with only a few dead cells remaining.

Discussion

Regarding our characterization of algae extracts compared to FBS, the most striking and obvious feature of the CVE and CGF samples that was not present in FBS samples were large absorbance peaks around 260 nm, which eluted off the column with the aqueous mobile phase within the first couple minutes. Such an absorbance profile is a characteristic property of nucleic acid (Setlow and Boyce, 1960), and not surprising as a relatively abundant component present in crude cellular extracts. Moreover, since no appreciable mass signal(s) at this retention period was/were observed, it supports the notion that the species producing this absorbance spectra are likely nucleic acid species (e.g., DNA/RNA fragments containing at least 4 bases) with m/z values greater than that which could be detected (i.e., >1200). However, because the sensing of foreign nucleic acid often triggers cell death responses (Schlee and Hartmann, 2016), we did not expect that foreign nucleic acid would be beneficial to cell growth/viability, rather on the contrary. Yet our results suggest that this putative nucleic acid at least did not harm cells and may have even been beneficial because nuclease-treated extracts, as well as the insoluble pellet with presumably very little nucleic acid (as evidenced by loss of the characteristic absorbance peak at 260 nm in Figure S2a), both decreased cell viability as compared to cells not treated with nuclease or cells treated with soluble supernatant.

Despite an increase in HEK-293 and EBTr cell viability observed for cells cultured with different concentrations of CVE and GCF alone, when such plates were observed under the microscope, no convincing amount of cell division was visible. When algae extract (CVE) was combined with growth factors, insulin and other additives, a similar marginal increase in cell viability of around 20% was observed in an environment where cells were already dividing.

These observations suggest that algae extract improves cell viability without stimulating proliferation. The possibility that algae extract enhances the cellular metabolic potential without promoting cell division requires further investigation. Cell viability assays using tetrazolium salts like MTS are often used as a proxy for cell growth, yet what such assays actually measure is the activity of mitochondrial NADH and NADPH-dependent dehydrogenase enzymes which reduce the reagent to a formazan product (Dunigan et al., 1995). In most cases, the more cells there are the more mitochondria are available to reduce the MTS reagent, but it is possible that the algae extracts have a positive effect on such metabolic activity without promoting cell growth. Given that a common and relatively abundant signal feature found in the mass spectra of algae extract samples mapped to NAD⁺/NADP⁺ as the parent species, it is possible that enriched levels of NAD(H)/NADP(H) present in the algae extracts may have caused enhanced activity of the dehydrogenase enzymes in the cells thus explaining the observed increase in viability. Further, it was previously shown that CVE also contains high levels of glutamate (Okamoto et al., 2020), as *Chlorella* algae use NADPH-dependent glutamate dehydrogenase enzymes to control their cell cycle (Talley et al., 1972). Glutamate has been shown to increase oxidation of NADH (Kannurpatti and Joshi, 1999; Maalouf et al., 2007), and increase cancer cell viability and growth (Gelb et al., 2015; Reiman et al., 1981; Takano et al., 2001; Yamaguchi et al., 2020). Moreover, in fibroblasts, glutamate reduced toxicity induced by high levels of cysteine (Bannai and Ishii, 1982), and glutamate dehydrogenase deficiency reduced cell viability (Tatsumi et al., 1989). Therefore, it is possible that enriched levels of glutamate in algae extracts may also be responsible for the increased viability observed.

When examined alone, there were cases in which cells cultured with 5% FBS and algae extract showed viability nearly matching 10% FBS controls. In one case, viability was greater than that of 10% FBS controls, however we are cautious not to overinterpret this data. It is important to note that any gains in cell viability were marginal compared to 5% FBS controls. Often, cells can be cultured in 5% FBS nearly as effectively as 10% FBS (Talbot et al., 2004). Thus, the reason that addition of CVE increased cell viability could also be attributed to the same phenomenon discussed above, i.e., by enhancing metabolism without promoting growth.

Our experiments with components of the E8^{TM} media clearly showed that both insulin, basic-FGF and TGF- β are necessary for cell growth, and that these components are roughly additive in terms of their benefits to cell viability. Early studies examining serum-free culture conditions showed FGF and insulin as key determinants for growth (Mcfarland et al., 1991). Insulin causes cells to import glucose, providing energy and nucleotide building blocks needed for replication. In viability assays, addition of CVE also proved to be roughly additive, with CVE in addition to basic-FGF, TGF- β , insulin, and other additives combined yielding the highest viability reading of any serum-free condition tested. We demonstrated that EBTr cells can grow and expand into a new culture vessel when cultured in serum-free media that included at minimum basic-FGF, TGF- β , insulin and algae extract in the form of CVE, although such growth was not as robust as that of cells cultured with 10% FBS. Recent studies have shown that mitochondrial NADP(H) levels are essential for cell growth (Tran et al., 2021), without which cells cannot make proline required to support nucleotide and protein synthesis. Thus, it is plausible that enriched levels of NAD(H)/NADP(H) in CVE extract may provide a true benefit to cell growth; however, whether the additive increase in viability from CVE translates to any real

benefit in growth rate beyond that from basic-FGF, TGF- β and insulin alone remains unclear. Regardless of whether algae extract is truly beneficial to cell growth, it is important to note that bovine fibroblasts could be cultured with only a subset of the E8^{TM} components present. This may at least suggest some potential economic benefit due to lower cost serum-free formulations requiring fewer components. Since growth factors and insulin directly interact with their respective receptors through a relatively small range of residues across the entire protein, further experiments could test whether smaller peptide fragments mapping to such regions within these proteins remain actionable enough to sustain cell growth. Such substitutes may simplify and further lower the cost of serum-free formulations in which cells can be effectively cultured. Similarly, small molecules that act as agonists on basic-FGF and/or TGF- β receptors could also be tested for the same purpose.

Other limitations of our studies include aspects of the growth experiments in which we did not anticipate how differences in cell size could impact the results. In these experiments, it appears from the images as though cells cultured with algae extract were larger in size as compared to those not cultured with algae extract. Notably, increases in cell viability correlate with increased cell size (Dreesen and Fussenegger, 2011), which could at least partially explain this observation since we also found that algae extract typically increased cell viability. However, differences in cell size observed with and without algae extract in the culture make the growth and expansion experiments difficult to interpret. For instance, during the expansion experiment, it appears that more cells were transferred from the 1 mL culture with 10% FBS compared to the culture with CVE plus supplements despite these two groups appearing to have a similar level of confluency on day 4. While both conditions promoted growth, the cells

cultured in 10% FBS not only grow faster but require less space per cell. As a result, in the 2 mL cultures, the cells cultured in 10% FBS had a higher base count from which to start growing and were thus much more confluent by day 8. Furthermore, it is important to note limitations in counting the fraction of dead cells recorded in our cell count experiments. As noted in the methods section, we first removed the old media and washed the cells briefly, which likely removed a large amount of previously dead and floating cells. Thus, the dead cell count more accurately represents the percentage of cells which were nearly dead, or dead, but not yet detached and floating.

Taken together, our studies show that algae extract derived from *Chlorella vulgaris* (CVE) enhances viability of bovine fibroblasts, possibly due at least in part to its enriched levels of NAD(H)/NADP(H) and/or glutamate, and that CVE can be used together with other supplements (basic-FGF, TGF- β , and insulin) to grow cells in the absence of serum. Further studies are needed to determine the extent to which CVE promotes cell growth and whether the supplementary components can be further modified to reduce their cost.

408 **Acknowledgements**

409 This work was supported by the Figge-Bourquin Award and by Student Faculty Collaborative
410 Grants via the Creativity and Innovation and Dean's Offices at Colorado College. The authors
411 thank Rachel Wonciar and Tanya Cervantes in the Department of Chemistry and Biochemistry,
412 and Carrie Moon and Darrell Killian in the Department of Molecular Biology at Colorado
413 College, for their technical assistance. The authors also thank Jörg Goronzy and Wenqiang Cao
414 in the Department of Immunology at the Mayo Clinic (Rochester, MN), for the HEK-293 cells.

415

416 **Author Contributions**

417 G.D.C. and T.M.G. designed the study, performed experiments, analyzed data, and wrote the
418 paper, with both authors contributing equally.

References

- Bannai, S. and Ishii, T. (1982). Transport of Cystine and Cysteine and Cell Growth in Cultured Human Diploid Fibroblasts: Effect of Glutamate and Homocysteate. *Journal of Cellular Physiology* 112, 265–272.
- Burt, S. M., Carter, T. J. N. and Kricka, L. J. (1979). Thermal characteristics of microtitre plates used in immunological assays. *Journal of Immunological Methods* 31, 231-236.
- Chen, G., Gulbranson, D. R., Hou, Z., Bolin, J. M., Ruotti, V., Probasco, M. D., Smuga-Otto, K., Howden, S. E., Diol, N. R., Propson, N. E., et al. (2011). Chemically defined conditions for human iPSC derivation and culture. *Nature Methods* 8, 424–429.
- Dreesen, I. A. J. and Fussenegger, M. (2011). Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. *Biotechnology and Bioengineering* 108, 853–866.
- Dunigan, D., Waters, S. and Owen, T. (1995). Aqueous soluble tetrazolium/formazan MTS as an indicator of NADH- and NADPH-dependent dehydrogenase activity. *Biotechniques* 19, 640–649.
- Gelb, T., Pshenichkin, S., Rodriguez, O. C., Hathaway, H. A., Grajkowska, E., Diraddo, J. O., Wroblewska, B., Yasuda, R. P., Albanese, C., Wolfe, B. B., et al. (2015). Metabotropic glutamate receptor 1 acts as a dependence receptor creating a requirement for glutamate to sustain the viability and growth of human melanomas. *Oncogene* 34, 2711–2720.
- Gerber, P. J., Steinfield, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. and Tempio, G. (2013). Tackling climate change through livestock – a global assessment of

emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO), Rome.

Humbird, D. (2021). Scale-up economics for cultured meat. *Biotechnology and Bioengineering* 118, 3239–3250.

Kannurpatti, S. S. and Joshi, N. B. (1999). Energy metabolism and NAD-NADH redox state in brain slices in response to glutamate exposure and ischemia. *Metabolic Brain Disease* 14, 33-43.

Ladak, A. and Anthis, J. R. (2021). Animals, Food, and Technology (AFT) Survey: 2020 Update.

Maalouf, M., Sullivan, P. G., Davis, L., Kim, D. Y. and Rho, J. M. (2007). Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. *Neuroscience* 145, 256–264.

Mcfarland, D. C., Ane, J., Pesall, E., Norberg, J. M. and De, M. A. D. (1991). Proliferation of the turkey myogenic satellite cell in a serum-free medium. *Comp. Biochem. Physiol.* 99A, 163–167.

Murakami, H. and Masui, H. (1980). Hormonal control of human colon carcinoma cell growth in serum-free medium. *Proc. Natl. Acad. Sci. USA* 77, 3464–3468.

Ng, J. Y., Chua, M. L., Zhang, C., Hong, S., Kumar, Y., Gokhale, R. and Ee, P. L. R. (2020). Chlorella vulgaris Extract as a Serum Replacement That Enhances Mammalian Cell Growth and Protein Expression. *Frontiers in Bioengineering and Biotechnology* 8, 564667.

Okamoto, Y., Haraguchi, Y., Sawamura, N., Asahi, T. and Shimizu, T. (2020). Mammalian cell cultivation using nutrients extracted from microalgae. *Biotechnology Progress* 36, e2941.

461 Penttinen, K. and Saxén, E. (1959). Growth-Controlling Action of Human Serum in Cell Culture.
462 *Nature* 184, 1570–1571.

463 Reiman, R. E., Huvos, A. G., Benua, R. S., Rosen, G., Gelbard, A. S. and Laughlin, J. S. (1981).
464 Quotient Imaging with N-13 L-Glutamate in Osteogenic Sarcoma: Correlation with Tumor
465 Viability. *Cancer* 48, 1976–1981.

466 Schlee, M. and Hartmann, G. (2016). Discriminating self from non-self in nucleic acid sensing.
467 *Nature Reviews Immunology* 16, 566–580.

468 Setlow, R. and Boyce, R. (1960). The ultraviolet light inactivation of phiX174 bacteriophage at
469 different wave lengths and pH's. *Biophysical Journal* 1, 29–41.

470 Specht, L. (2020). An analysis of culture medium costs and production volumes for cultivated
471 meat. The Good Food Institute.

472 Takano, T., Lin, J. H. C., Arcuinoo, G., Gao, Q., Yang, J. and Nedergaard, M. (2001). Glutamate
473 release promotes growth of malignant gliomas. *Nature Medicine* 7, 1010–1015.

474 Talbot, N. C., Powell, A. M. and Caperna, T. J. (2004). Comparison of Colony-Formation
475 Efficiency of Bovine Fetal Fibroblast Cell Lines Cultured with Low Oxygen, Hydrocortisone,
476 L-Carnosine, bFGF, or Different Levels of FBS. *Cloning and Stem Cells* 6, 37–47.

477 Talley, D. J., White, L. H. and Schmidt, R. R. (1972). Evidence for NADH-and NADPH-specific
478 isozymes of glutamate dehydrogenase and the continuous inducibility of the NADPH-
479 specific isozyme throughout the cell cycle of the Eucaryote *Chlorella**. *Journal of Biological*
480 *Chemistry* 247, 7927–7935.

481 Tatsumi, C., Yorifuji, S., Takahashi, M. and Tarui, S. (1989). Decreased viability of skin fibroblasts
482 from patients with glutamate dehydrogenase deficiency. *Neurology* 39, 451-452.

483 Tran, D. H., Kesavan, R., Rion, H., Soflaee, M. H., Solmonson, A., Bezwada, D., Vu, H. S., Cai, F.,
 484 Phillips, J. A., DeBerardinis, R. J., et al. (2021). Mitochondrial NADP⁺ is essential for proline
 485 biosynthesis during cell growth. *Nature Metabolism* 3, 571–585.

486 Tuomisto, H. L. and Teixeira De Mattos, M. J. (2011). Environmental impacts of cultured meat
 487 production. *Environmental Science and Technology* 45, 6117–6123.

488 Yamaguchi, I., Yoshimura, S. H. and Katoh, H. (2020). High cell density increases glioblastoma
 489 cell viability under glucose deprivation via degradation of the cystine/glutamate
 490 transporter xCT (SLC7A11). *Journal of Biological Chemistry* 295, 6936–6945.

491

492

Figure Legends

Figure 1. Comparative characterization of *Chlorella* extracts versus FBS by LC-MS

(a) Perspective 3D surface plots are shown comparing UV chromatograms of FBS, CVE and CGF samples (wavelengths 200-400 nm); each plot shows the mean normalized absorbance spectra of three replicate injections of the same sample type. Arrows indicate large, broad peaks around 260 nm present in CVE and CGF samples. (b) Normalized total ion chromatograms (TICs) are shown as the sum of signals of all m/z values from 400-1200 for FBS, CVE and CGF samples; representative data shown are averaged spectra from three injections of the same sample type. (c) Scatter plot shows results of principal component analysis (PCA) on panel b MS TIC data; sample types are indicated by different colors with PC2 (circles) and PC4 (diamonds) values plotted on the same y-axis scale. (d) Perspective 3D surface plots show normalized mass spectra of FBS, CVE and CGF samples; data shown are averages of three replicate injections. Dashed box indicates region of interest where signal location/intensity appears similar across all three sample types (i.e., retention times from 15-21 minutes and m/z values from 800-1200). (e) Aligned mass spectra show the sum of all signals for retention times from 15-21 minutes at each whole integer m/z value from 800-1200; data are shown as mean normalized spectra of three replicate injections of the same sample. Red vertical lines indicate signals of relatively high abundance shared across all three sample types; table lists potential parent species for corresponding signals based on top candidates identified by database (MassBank.eu) search.

Figure 2. *Chlorella* extracts alone slightly improve cell viability in the absence of FBS.

(a) Bar plots show relative viability of HEK-293 cells cultured with a range of concentrations of CVE or CGF at day 1, 3 and 7, compared to 10% FBS and no serum controls; panel (b) shows that of HEK-293 (blue) and EBTr (yellow) cells cultured with 10-fold higher CVE and CGF at day 3. Statistical significance was determined by *t*-test with Welch's correction in which experimental groups were compared to respective no serum control groups; significance levels are indicated as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant, n=6.

Figure 3. Nuclease-treated *C. vulgaris* extract and its insoluble fraction decrease cell viability.

Bar plots shows relative viability of EBTr (a) and HEK-293 (b) cells cultured with different fractions of 10 g/L CVE relative to control cells cultured in 10% FBS. Statistical significance was calculated by *t*-tests with Welch's correction in which experimental CVE fractionation groups were compared with versus without nuclease treatment; significance levels are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=not significant, n=6.

Figure 4. Select growth factor and insulin supplemented *C. vulgaris* extract improves cell viability in the absence of FBS.

(a) Box plots show relative viability of EBTr (yellow) and HEK-293 (blue) cells cultured in serum-free media, supplemented with 10 or 100 ng/mL fibroblast growth factor 2 (FGF) or 1 or 10 ng/mL transforming growth factor beta1 (TGF), relative to controls cultured in 10% FBS. Statistical comparisons were between experimental groups and respective no serum control groups. Panel **(b)** shows relative viability of EBTr and HEK-293 cells cultured in serum-free media, supplemented with growth factors (GFs) or 3 μ M bovine insulin or 0.2 mM L-ascorbate (LA) or 80 nM selenium dioxide (Se) alone, or combinations thereof, relative to controls cultured in 10% FBS. Growth factors were used at 30 ng/mL (FGF) and 10 ng/mL (TGF). Statistical comparisons were between experimental groups and respective no serum control groups. Panel **(c)** shows relative viability of EBTr cells cultured in serum-free media, supplemented with 10 g/L CVE or 10% CGF alone, or GFs and insulin with and without LA and Se, or combinations thereof, compared to controls cultured in 10% FBS. Concentrations of GFs, insulin, LA and Se were the same as that for panel b. Statistical comparisons were between groups treated with CVE alone and CVE plus other supplements. Statistical significance was determined by *t*-tests with Welch's correction; significance levels are indicated as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant, n=6.

Figure 5. Growth factor and insulin supplemented *C. vulgaris* extract stimulates cell growth in the absence of FBS.

Line plots show live (a) and percent dead/non-intact (b) EBTr cells after 0, 2, 4 and 8 days in culture. Different media conditions are indicated by different colors; all counts were performed in duplicate. (c) Brightfield images show representative EBTr cell density and morphology for each condition and time point; white scale bar indicates length of ~100 μ m. CVE was used at 10 g/L and CGF at 10% by volume. Supplements include 10 ng/mL TGF, 30 ng/mL FGF, 3 μ M bovine insulin, 0.2 mM L-ascorbate, and 80 nM selenium.

Figure 2

a **b**

Figure 3

a

EBTr

b

HEK-293

Figure 4

a **b** **c**

a **b** **c**