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ARTICLE

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Bioprocess development for scalable production of cultivated meat

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Abstract

Traditional farm-based products based on livestock are one of the main contributors to greenhouse gas emissions. Cultivated meat is an alternative that mimics animal meat, being produced in a bioreactor under controlled conditions rather than through the slaughtering of animals. The first step in the production of cultivated meat is the generation of sufficient reserves of starting cells. In this study, bovine adipose-derived stem cells (bASCs) were used as starting cells due to their ability to differentiate towards both fat and muscle, two cell types found in meat. A bioprocess for the expansion of these cells on microcarriers in spinner flasks was developed. Different cell seeding densities (1,500, 3,000, and 6,000 cells/cm^2) and feeding strategies (80%, 65%, 50%, and combined 80%/50% medium exchanges) were investigated. Cell characterization was assessed pre- and postbioprocessing to ensure that bioprocessing did not negatively affect bASC quality. The best growth was obtained with the lowest cell seeding density (1,500 cells/cm^2) with an 80% medium exchange performed ($p < .0001$) which yielded a 28-fold expansion. The ability to differentiate towards adipogenic, osteogenic, and chondrogenic lineages was retained postbioprocessing and no significant difference ($p > .5$) was found in clonogenicity pre- or postbioprocessing in any of the feeding regimes tested.

KEYWORDS

bioprocessing, bovine adipose-derived stem cells, cultivated meat, microcarriers, spinner flasks

1 | INTRODUCTION

There is an increased need for sustainable protein-rich food sources to support the rapidly growing population (Arshad et al., 2017). There is also a direct correlation between increasing per capita income and meat consumption (Gerbens-Leenes, Nonhebel, & Krol, 2010; Stephens et al., 2018) so that developing countries are expected to significantly impact the global demand for meat products. The food and agriculture organization (FAO) of the United Nations report from 2016 predicts an increase of 48 Mt, for meat demand by 2025 with 73% of this increase coming from developing countries such as Brazil and China (OECD-FAO Agricultural Outlook, 2016). With animal agriculture currently occupying 70% of arable land, generating 14.5% of anthropogenic greenhouse emissions (Grossi, Goglio, Vitali, & Williams, 2019) and consuming 27% of freshwater resources just for livestock feed production (Gerbens-Leenes, Mekonnen, & Hoekstra, 2013), it becomes evident that conventional

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animal agriculture and meat production methods cannot sustain such growth in meat demand. Alternative food technologies such as cultivated meat might provide a solution to this growing problem (Fan, Almanza, Mattila, Ge, & Her, 2019; Salonen & Helne, 2012; Stephens et al., 2018), as initial projections show that it will require 45% less energy, 99% less land and will emit 78–96% less greenhouse gas emissions (Stephens et al., 2018; Tuomisto & de Mattos, 2011). Other life cycle analyses of cultured meat production showed that the environmental impact is highly dependent on the type of meat produced (Mattick, Landis, Allenby, & Genovese, 2015), as well as the method of energy production employed in the manufacturing (Smetana, Mathys, Knoch, & Heinz, 2015). As the production of cultivated meat will most likely require the use of bioreactors to achieve the necessary scale, the energy required for the operation of bioreactors could have a significant environmental impact. However, this problem could be minimized in the future if the decarbonization of energy production can be achieved (Bodiou, Moutsatsou, & Post, 2020). Cultivated meat can undoubtedly have a positive impact on animal welfare and can also offer a potentially healthier and safer option for consumers as the production process can be closely controlled and possibly tuned to produce meat that is free from antibiotics, free from zoonotic bacteria and viruses, and with a specific desired nutritional profile (e.g., enriched in omega fatty acids and reduced cholesterol).

In 2013, the worlds' first cultivated meat burger was produced by Prof Mark Post as a proof of concept, however with a cost of approximately £250,000 (Mouat & Prince, 2018). In the past couple of years, the interest in cultivated meat and its potential benefits has increased significantly. Now there is a race to bring commercially viable cultivated meat products to the market, but to achieve this, research to increase product output, that is, yield and to decrease cost must be conducted. The underpinning biological knowledge needed to produce a cultivated meat product is somewhat understood. The first step in cultivated meat production is the choice of the starting cell source. Some research groups in the field of cultivated beef use bovine satellite cells which are dedicated muscle progenitors (Kadim, Mahgoub, Baqir, Fave, & Purchas, 2015; Verbruggen, Luining, van Essen, & Post, 2017). However, these cells can only differentiate towards muscle. Meat is complex and comprises several types of tissues (e.g., muscle, fat, and connective tissue; Listrat et al., 2016), thus using satellite cells as starting material is not sufficient as it requires the addition of other cell types (e.g., fat) from other sources. Mesenchymal stem cells (MSCs), on the other hand, have the ability to differentiate towards both adipogenic and myogenic lineages (Bosnakovski et al., 2005; Okamura et al., 2018), thus being able to produce both cell types of interest (e.g., muscle and fat) for production of cultivated meat. In addition, MSCs are easy to isolate from a variety of tissues, such as bone marrow, adipose, umbilical cord, placenta, and fetal fluids (Hill, Bressan, Murphy, & Gracia, 2019). In this study, bovine adipose-derived stem cells (bASCs) were chosen as a starting cell source.

The first step in the production of cultivated meat is the expansion phase, that is, the production of quality starting cells. It has been estimated that the number of cells required for producing 1 kg of protein from muscle cells will be of the order of 2.9×10^{11} (Allan, De Bank, & Ellis, 2019) to 8×10^{12} cells (Stephens et al., 2018). Such cell numbers are in any realistic sense unattainable from monolayer culture, but microcarriers used in conjunction with stirred bioreactors are a much more feasible option. For example, one 5-L stirred bioreactor with a 5,000 cm^2 surface area provided by microcarriers per liter was able to produce as many human mesenchymal stem cells (hMSCs) as 65 T-flasks at the confluence (Rafiq, Brosnan, Coopman, Nienow, & Hewitt, 2013). To date, there are many other published studies focused on the use of stirred bioreactors for the expansion of hMSCs at different scales (Heathman et al., 2015; Hewitt et al., 2011; Lawson et al., 2017; Rafiq et al., 2013, 2017, 2018; de Soure, Fernandes Platzgummer, da Silva, & Cabral, 2016). However, there is no available literature on the development of bioprocesses in stirred bioreactors for the expansion of bovine analogs.

Before moving to the liter scale bioreactors, due to cost considerations, process development for MSCs is typically carried out in spinner flasks which are essentially small stirred tank bioreactor vessels operated at the 100–250 ml scale (Bardy et al., 2013; Goh et al., 2013; Hewitt et al., 2011; Schirmaier et al., 2014). Spinner flasks are easy to use, provide a dynamic environment, and are relatively easily translatable to liter scale-stirred tank bioreactors (Rafiq et al., 2013). This study focuses on the development of a scalable bioprocess in spinner flasks for the expansion of bASC as the first step in cultivated beef production. The objectives of this study were: (a) to establish bASCs cultures examining growth and cell product quality attributes; (b) to develop a scalable bioprocess in spinner flasks for bASCs expansion, and (c) to investigate if cell quality is retained postbioprocessing.

2 | MATERIALS AND METHODS

2.1 | Planar culture of bASC

bASCs were purchased from Cellider Biotech (Spain) and stored in liquid nitrogen. Upon thawing, the cells were cultured in T-flasks using a growth medium comprising of α-modified Eagle's medium (1 g/L glucose; Lonza, UK) supplemented with 10% (vol/vol) fetal bovine serum (Sigma-Aldrich, UK), 1 ng/ml bFGF (PeproTech, UK) and 2 mM UltraGlutamine (Lonza). bASCs between passage numbers 2 and 10 were used for the planar study, while for the microcarrier cultures, only cells between passage numbers 2 and 5 were used. The cells were stored in a humidified incubator, at 37°C and 5% CO_2. A complete medium exchange was performed after 72 hr in culture and cells were passaged at Day 5 when ~80% confluency was achieved. Cell passage was performed with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco; Thermo Fisher Scientific, UK) for 8 min at 37°C until complete cell detachment. The enzyme was then inactivated by the addition of growth medium and the cell suspension was centrifuged at 250 *g* for 5 min.

2.2 | Microcarrier culture in spinner flasks

The inside of the spinner flasks (100 ml working volume with a magnetic rod impeller equipped with a paddle [Figure 3a]; Belco)

were first coated with Sigmacote (Sigma-Aldrich) to prevent cells attaching to their inner surface. Plastic microcarriers (Pall, UK) were weighed to achieve a surface area of 5 cm^2/ml in the spinner, which were then sterilized by autoclaving. bASCs between passage numbers 2 and 5 were seeded at different seeding densities of 1,500, 3,000, and 6,000 cells/cm^2 of microcarrier surface. Upon seeding, the spinner flasks were stored in a humidified incubator at 37°C and 5% CO_2. Different feeding regimes ranging from 50% to 80% medium exchanges were investigated with the first carried out at Day 3 and then every other day. Samples were taken regularly during culture to enable cell imaging, cell counts, and analysis for glucose and lactate. The spinner flask cultures were agitated at the minimum speed required for microcarrier suspension, N_{js} (30 rpm) for the first 3 days (Hewitt et al., 2011; Nienow, Coopman, Heathman, Rafiq, & Hewitt, 2016), followed by an incremental increase of 10 rpm every 2 days thereafter to ensure suspension was maintained and to control aggregation of microcarriers, as cells on them were growing more confluent. The cultures were kept for up to 9 days when the full harvest was performed by using a previously described protocol (Nienow, Hewitt et al., 2016). Briefly, two washes with Dulbecco's phosphate-buffered saline were performed while stirring, followed by incubation with 0.25% trypsin-EDTA at 37°C while stirring at 150 rpm for 10 min. During the last 10 s of incubation, the agitation was increased to 250 rpm. The aim of this approach was to use the high-specific energy dissipation rate to enhance the detachment of the cells from the microcarriers. The advantage of this period of high agitation intensity is that essentially 100% of the cells are detached from the microcarriers and the time during which potential damage to the cells could occur from the detachment enzyme is greatly reduced. In addition, as soon as the cells are detached, they are smaller than the microscale of turbulence and the cells do not get damaged by fluid dynamic stresses (Nienow, Coopman et al., 2016, Nienow, Hewitt et al., 2016). Once the cells were completely detached from the microcarriers, the cell-microcarrier suspension was passed through an 80-μm filter (Steriflip), which removed the microcarriers and the cell suspension was then centrifuged at 250 *g* for 5 min.

2.3 | Cell characterization

2.3.1 | Trilineage differentiation potential

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Stem cells were differentiated towards adipogenic, osteogenic, and chondrogenic lineages using StemPro Differentiation Kits (Thermo Fisher Scientific). A 12-well plate was used for all experiments. bASCs were seeded at 5,000 cells/cm^2 for osteogenic differentiation and 10,000 cells/cm^2 for adipogenic differentiation. For chondrogenic differentiation, the macromass method was used (Heathman et al., 2015). Briefly, 5 μl droplets of a highly concentrated bASCs suspension (1×10^7 cells/ml) were seeded in an empty well plate and allowed to attach for 1–2 hr at 37°C in a CO_2 incubator. Upon cell attachment, the chondrogenic differentiation medium (Stempro) was added. For all differentiation cultures, the cells were grown in their respective media for 21 days with a medium change performed every 3–4 days. At the end of 21 days, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Adipogenic differentiation was assessed by using LipidTox green (Thermo Fisher Scientific) to stain lipid vesicles in adipogenic differentiated cells. The stain was used according to the manufacturer's instructions. Osteogenic differentiation potential was assessed using von Kossa stain as previously described (Hanga et al., 2017). Silver nitrate was used to stain bone mineralization, while also assessing alkaline phosphatase expression. Chondrogenic differentiation was assessed by Alcian blue staining (Hanga et al., 2017).

2.3.2 | Clonogenicity

Three hundred cells were seeded into a T-25 flask in growth medium and placed into an incubator set to 37°C and 5% CO_2. The medium was changed every 3–4 days and the flasks were kept for up to 14 days. The medium was then removed and cells were fixed and stained with 1% crystal violet (Sigma-Aldrich) for 30 min at room temperature. The stained colonies were then manually counted.

2.3.3 | Cell surface marker expression

Immunofluorescence staining was performed to assess the expression of a panel of three cell surface markers identified as indicative of MSCs and these were: CD73 (Abcam), CD90 (Abcam), and CD105 (Thermo Fisher Scientific). Cells were fixed using 4% paraformaldehyde for 20 min at room temperature and then permeabilized using Perm Wash 1X (BioLegend) for 5 min. Cells were then incubated with 10% normal goat serum (Thermo Fisher Scientific) for 45 min in the dark to block any nonspecific binding, followed by incubation overnight at 4°C with the primary antibody at the recommended dilution and then incubation for 2 hr at room temperature with the secondary antibody at the manufacturer's recommended dilution. 4′,6-Diamidino-2-phenylindole (DAPI) was used for staining the nuclei and Phalloidin (Sigma-Aldrich) was used to stain the cytoskeleton. Cells were then visualized and imaged under a fluorescence microscope (EVOS FL; Thermo Fisher Scientific).

2.4 | Analytical techniques

Imaging was performed by either phase-contrast microscopy or fluorescence microscopy. Live/Dead Staining (calcein-AM/ethidium Homodimer) Kit (Thermo Fisher Scientific) was used to assess cell viability on microcarriers following manufacturer's instructions. Cell counts were performed using the NucleoCounter NC-3000 (ChemoMetec). For the spinner flasks cultures, cell counts were performed directly onto microcarriers using the reagent A100 and reagent B protocol. Briefly, the cell-microcarrier suspension was

diluted in a 1:3 ratio with reagent A100 (lysing agent) and then reagent B (stabilizing agent). The resulting suspension was then loaded onto a NucleoCassette that contains acridine orange and DAPI. Spent medium samples were collected before and after medium exchanges and analyzed for glucose and lactate concentrations on a Roche Accutrend Plus meter. Fresh growth medium was used as baseline control. On the basis of cell counts, the following parameters were calculated:

1. Specific growth rate

$$\mu = \frac{\ln\left(Cx\left(t\right)/Cx\left(0\right)\right)}{\Delta t}, \tag{1}$$

2. doubling time

$$t_d = \frac{\ln 2}{\mu}, \tag{2}$$

3. fold increase

$$\mathrm{FI} = \frac{Cx\left(t\right)}{Cx\left(0\right)}, \tag{3}$$

4. cumulative population doublings

$$\mathrm{CPDL} = \left[\frac{1}{\log 2} * \mathrm{LOG}\left(\frac{Cx\left(t\right)}{Cx\left(0\right)}\right)\right], \tag{4}$$

where μ is the specific growth rate (h^{-1}); t_d is the doubling time (hr); FI is the fold increase; CPDL is a cumulative population doubling; $Cx(t)$ and $Cx(0)$ represent cell numbers at the end and the start of the culture; and t represents time in culture (hr).

2.5 | Statistical analysis

All experiments were performed in triplicates with primary cells from the same animal. Cell counts were acquired from two independent samples from each replicate. Data were expressed as mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism 8. For comparison between two datasets, statistical significance was determined by Student's two-tailed t test. For comparison of multiple datasets, significance was calculated by one-way analysis of variance. Significance was determined at $p < .05$.

3 | RESULTS AND DISCUSSION

3.1 | Establishing a baseline for bASCs expansion

The first step was to establish a baseline for the growth and quality of the bASCs. For this, the cells were cultured for 10 consecutive passages. bASCs morphology was monitored throughout the entire duration of the continued culture and it was found to be fibroblast-like and similar to that reported for hMSCs (Heathman et al., 2016; Figure 1a). In comparison to hMSCs, bASCs were found to be smaller with sizes ranging from 13 µm at the earlier passages to 16 µm at the later passages (Figure 1c). An increase in cell size for MSCs is usually associated with senescence and slowdown of growth (Wagner et al., 2008) which was also found applicable to bASCs. Passage 2 cells had a doubling time of 42.32 ± 0.83 hr at a cumulative population doubling of 5.99 ± 0.06, while passage 8 cells were found to have a doubling time of 199.55 ± 30.16 hr at a cumulative population doubling of 20.67 ± 0.09. Beyond Passage 8 (at ~20 population doublings), cell growth slowed down (Figure 1b). Knowing this profile is important as it dictates the flexibility in handling bovine cells before senescence is reached and cells become old and unusable. This information also shows what range of cell passages can be used for bioreactor inoculation to avoid reaching senescence. Guidelines for hMSCs characterization were previously published by Dominici et al. (2006); however, no such quality guidelines have been set for their bovine analogs. As a result, the same Dominici guidelines were used here. The quality baseline for bASCs was assessed through differentiation towards the three lineages (adipogenic, osteogenic, chondrogenic), expression of cell surface markers (CD90, CD73, and CD105) and clonogenicity potential. hMSCs have a positive expression of markers such as CD73, CD90, and CD105 and lack expression of markers such as CD34, CD45, HLA-DR, CD14, CD11b, and CD19. Previous studies have shown that bovine MSCs have a positive expression of some of the same markers as their human analogs (e.g., CD73, CD90, and CD105), but they also express additional markers, such as CD29, CD166, and CD44 and do not express CD45 or CD34 (Gao et al., 2014; Hill et al., 2019). Here, the expression of CD73, CD90, and CD105 (Figure 1d–f) was assessed and was found to be positive.

Differentiation to osteogenic lineage was deemed successful as bone mineralization was visibly stained in black, while alkaline phosphatase expression which is specific to osteocytes (Van der Plas et al., 1994) was evident through the red staining (Figure 2b). Similarly, chondrogenic differentiation was also deemed successful as macromasses were formed and stained in blue (Figure 2c) indicating the strong formation of GAGs representative to cartilage (Kuiper & Sharma, 2015). However, adipogenic differentiation was limited as indicated by the limited green fluorescence shown in Figure 2a indicating lipid vesicles typically present in adipocytes. The adipogenic differentiation efficiency (calculated as the percentage of cells presenting lipid droplets per total number of cells) was found to be 12.1 ± 2.8% (n = 1,982 cells analyzed). This limited differentiation efficiency could be a direct result of the formulation of differentiation medium used here which was the commercially available Stempro (Thermo Fisher Scientific). As stem cells have an increased potential to cure diseases, their applicability is highly popular in human cell therapies (Watt & Driskell, 2010). As a result, there is a huge market for reagents specifically formulated for human cells. Cultivated meat, on the other hand, is a

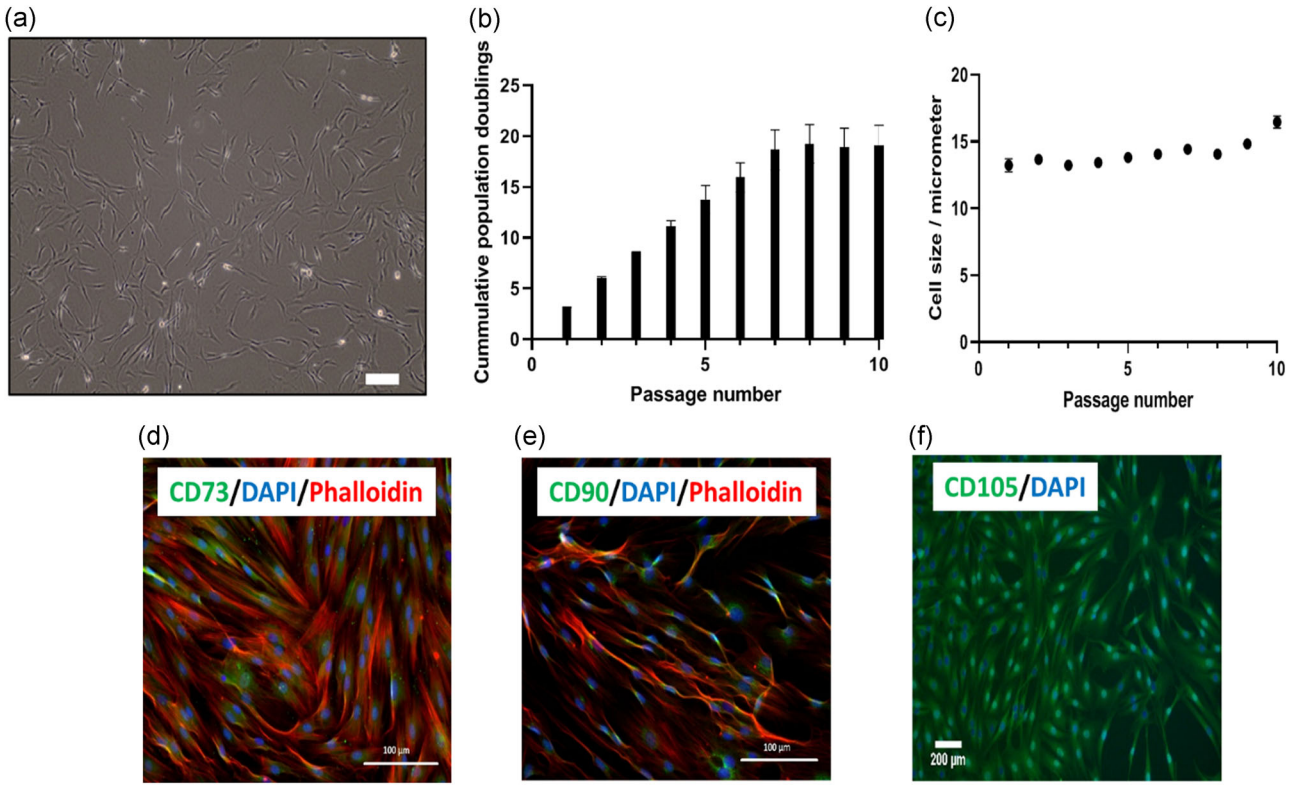

FIGURE 1 (a) Bovine adipose-derived mesenchymal stem cell morphology. The image was taken at Day 2 in culture. Scale bar = 200 µm. (b) Cumulative population doublings over 10 consecutive passages. (c) Cell size (µm) over 10 consecutive passages. Data expressed as mean ± *SD*, *n* = 4. Immunofluorescence staining showing expression of (d) CD73, (e) CD90, and (f) CD105. (d and e) Scale bar = 100 µm, while for (f) scale bar = 200 µm. DAPI, 4′,6-diamidino-2-phenylindole; *SD*, standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

newly developed concept that utilizes stem cells from other species than humans (e.g., bovine, porcine, ovine, and poultry). Unfortunately, as this is a developing area, the availability of reagents specifically formulated for other species than humans is limited. As the Stempro adipogenic differentiation medium is specifically formulated for human cells, the differences in the metabolism of bovine cells and human cells are not taken into consideration (Dodson et al., 2010), thus explaining the limited differentiation potential of bASCs to adipogenic lineage. Clonogenicity potential was also assessed at an early passage (p2) and late passage (p7; Figure 2d) and it was found to be significantly lower at the later passages (****$p < .0001$).

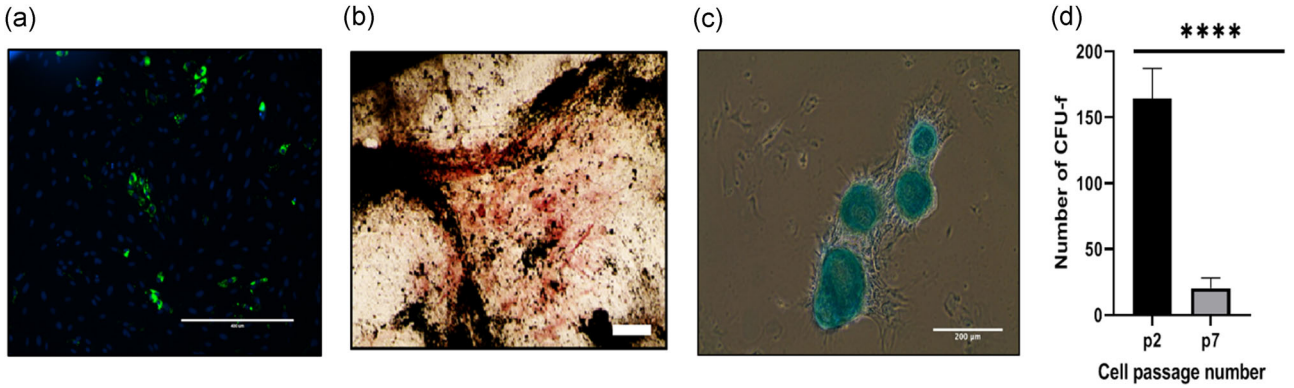

FIGURE 2 Prebioprocessing cell quality assessment. Differentiation staining of bASCs towards (a) adipogenic (LipidTox staining), (b) osteogenic (von Kossa stain), and (c) chondrogenic (Alcian blue) lineages. For adipogenic, scale bar = 400 µm. For osteogenic and chondrogenic, scale bar = 200 µm. (d) Clonogenic potential of bASCs at early (p2) and late (p7) passage numbers. Data expressed as mean ± *SD*, *n* = 5. ****$p < .0001$ (unpaired *t*-tests). bASC, bovine adipose-derived stem cell; CFU, colony-forming unit; *SD*, standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | The initial expansion of bASCs on microcarriers in spinner flasks

Microcarrier cultures were carried out in spinner flasks at the 100 ml scale (Figure 3a). The cells successfully attached to the microcarriers and by Day 5, cell-microcarrier bridges were observed as indicated by white arrows in Figure 3b which is a sign of cell proliferation. Live/dead staining was used here to assess cell viability on the microcarriers, while also visualizing the distribution of cells on their surface. Figure 3c shows increased cell viability on microcarriers at Day 5 with live cells stained in green and dead cells in red.

The initial microcarrier expansion was carried out at the same seeding density as in monolayer of 5,000 cells/cm^2 for 5 days. Cell growth on the microcarriers is compared to monolayer and is shown as a fold increase in Figure 4a, specific growth rates in Figure 4b, and doubling times in Figure 4c. No significant difference ($p > .05$) was found between cell growth in monolayer and on microcarriers. Similar fold increase values were obtained after 5 days of culture with 6.05 ± 0.31 in monolayer compared to 6.23 ± 1.69 in microcarrier culture. Similarly, a doubling time of 46.19 ± 1.34 hr was obtained in monolayer compared to 47.12 ± 8.7 hr in microcarrier culture. The similar growth on microcarriers was encouraging as this finding means that the bASCs can be expanded that way with its many advantages. These advantages include a high surface-area-to-volume ratio (provided that the microcarriers are adequately suspended, that is, at not less than N_{JS} [Heathman et al., 2018]) on which the cells can grow. It also facilitates the scalability of the bioprocess in other ways: a highly homogeneous environment leading to minimal gradients and the potential control of pH, temperature, and concentration combined with enhanced oxygen transfer. These advantages minimize culture variability, reduce cell quality variation, and lead to enhanced cost efficiency through optimized feeding strategies thereby maximizing cell growth, while minimizing medium use.

3.3 | Bioprocess enhancement for microcarrier expansion of bASCs in spinner flasks

Bioprocess improvement was initiated with studies of different cell seeding densities, which have previously been found to be important (Hewitt et al., 2011) ranging from 1,500 to 6,000 cells/cm^2. The microcarrier cultures were carried out for up to 9 days. At the highest cell density (6,000 cells/cm^2), the cell number increased exponentially up to Day 5. However, beyond this time point, cell loss was observed (Figure 5a). This loss could be attributed to aggregation observed from Day 5 onwards (Figure 5b) when most of the available surface areas provided by the microcarriers were utilized leading to contact inhibition and arrest of cell growth (Eagle & Levine, 1967). At the intermediary cell seeding density tested (3,000 cells/cm^2), a slight lag phase was observed until 72 hr followed by a constant increase in cell number, while at the lowest seeding density of 1,500 cells/cm^2, no lag phase was observed (Figure 5a). At the highest cell seeding

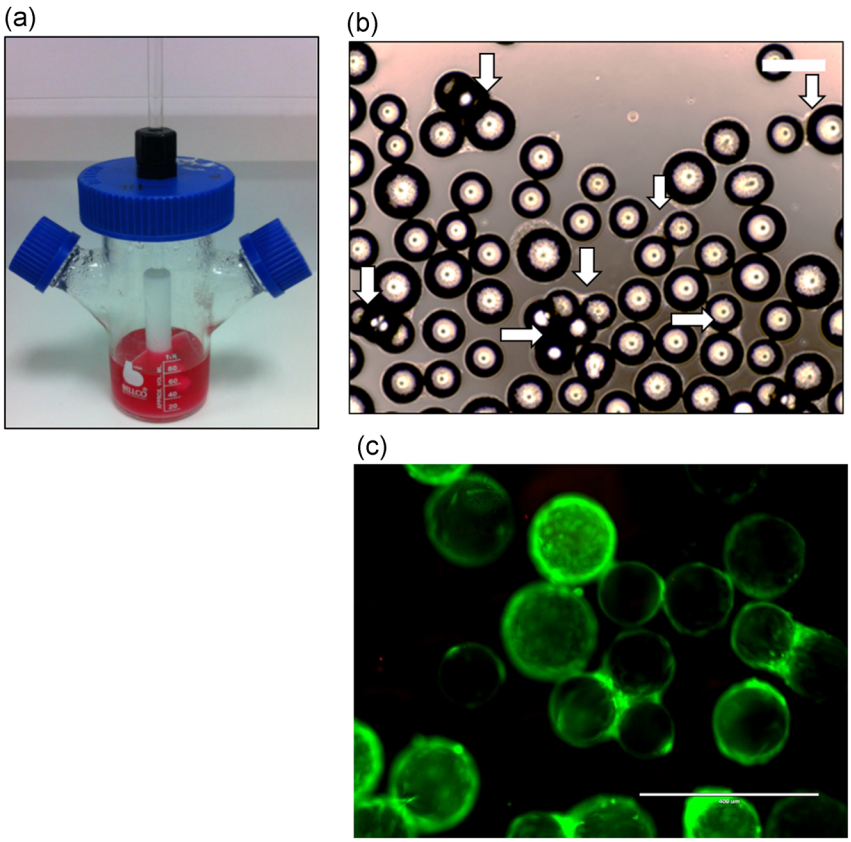

FIGURE 3 (a) Spinner flask. (b) Phase-contrast image taken at Day 5 in culture showing bovine adipose-derived stem cells (bASCs) attached to microcarriers forming cell-microcarrier bridges. White arrows indicate cell bridging. Scale bar = 200 µm. (c) Live (green)/dead (red) staining of bASCs on microcarriers at Day 5 in culture. Scale bar = 400 µm [Color figure can be viewed at wileyonlinelibrary.com]

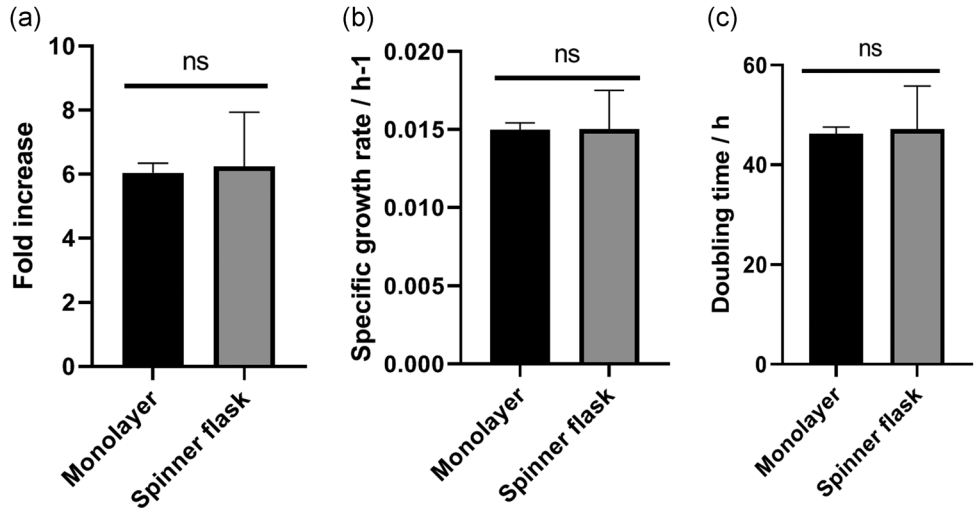

FIGURE 4 Comparison between monolayer and microcarrier culture in spinner flasks of bovine adipose-derived stem cells at Passage 3. (a) Fold increase. (b) Specific growth rate. (c) Doubling time. Data shown as mean ± standard deviation, $n = 3$. *ns*, not significant (unpaired *t* tests)

density (6,000 cells/cm^2), glucose concentration constantly decreased up to Day 5 in line with the increase in cell number, followed by an increase in concentration as the drop in cell number occurred. Related to these changes, the lactate concentration increased for the first 5 days, followed by a decrease thereafter (Figure 5c). At the lowest cell seeding density tested (1,500 cells/cm^2), there was a sudden drop in glucose concentration to 2.47 mmol/L up to Day 3 compared to 3.13 mmol/L obtained at the same time point for 6,000 cells/cm^2, followed by a subtle decrease until the end of the culture. During this time, the lactate concentration for this seeding density increased to 2.57 mmol/L at Day 5 and remained constant thereafter (Figure 5e). These trends (Figure 5c–e) mirrored the cell growth profiles for each cell seeding density tested (Figure 5a). It is also worth noting that in all of the growth conditions tested, inhibitory levels of lactate were not reached, that is, >15 mM/L (Schop et al., 2009), so the slowdown in cell growth observed at the highest

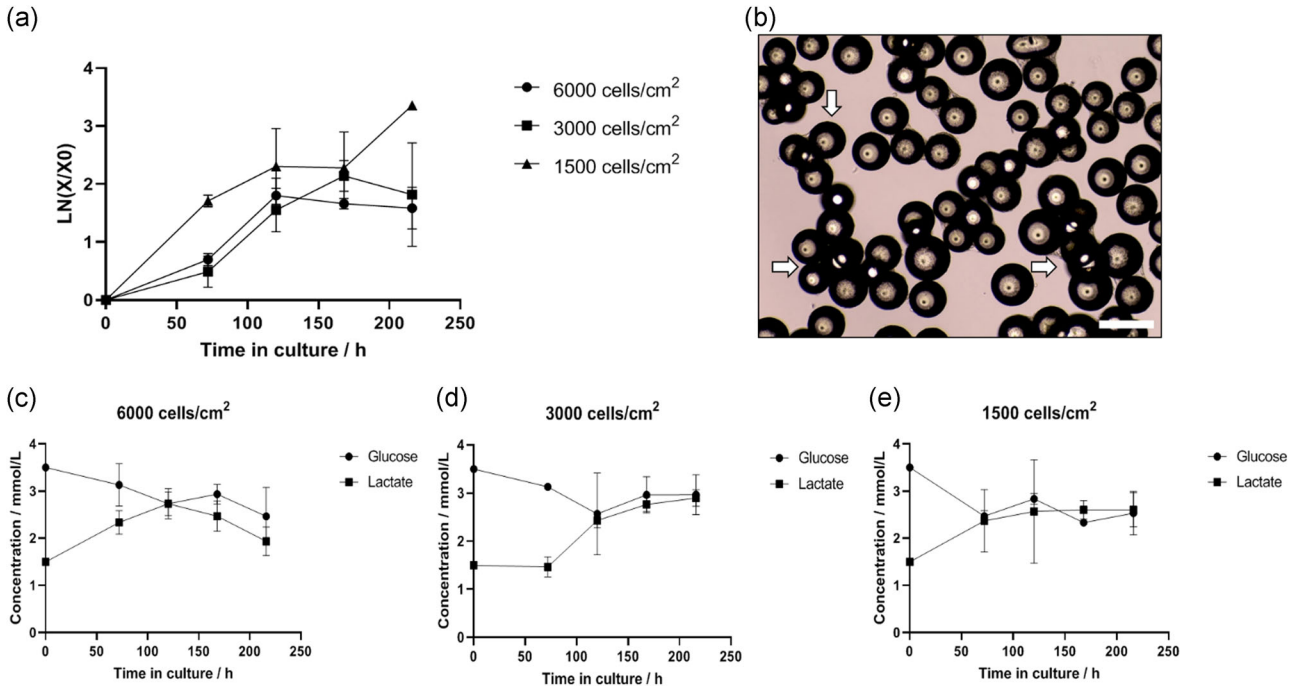

FIGURE 5 Bovine adipose-derived stem cells expansion in spinner flasks at different cell seeding densities. (a) Specific growth at different cell seeding densities over 9 days in culture. (b) Aggregation at Day 5 as indicated by white arrows when using 6,000 cells/cm^2 as a seeding density. Scale bar = 200 µm. Glucose and lactate profiles for (c) 6,000 cells/cm^2, (d) 3,000 cells/cm^2, and (e) 1,500 cells/cm^2. Data shown as mean ± standard deviation, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

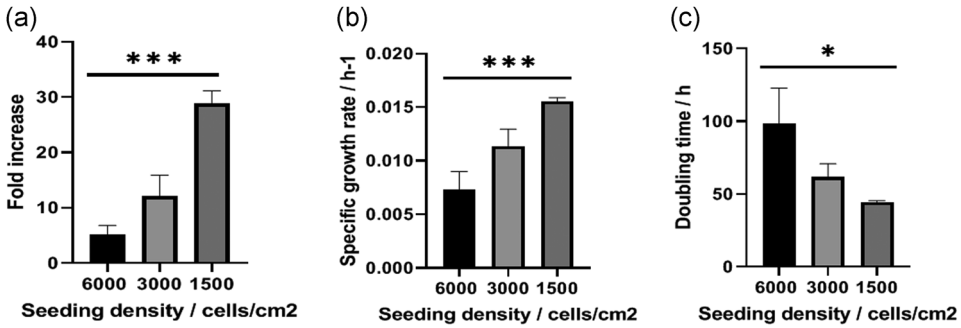

FIGURE 6 Growth kinetics comparison at different cell seeding densities. (a) Fold increase, (b) specific growth rate, and (c) doubling time. Data shown as mean ± standard deviation, $n = 3$. ***$p < .0001$, *$p < .05$ (one-way analysis of variance)

cell seeding density (6,000 cells/cm^2) can only be attributed to confluency being reached on the microcarriers.

At the end of the culture, there was a consistent trend with the lowest cell seeding density of 1,500 cells/cm^2 generating a significantly higher fold increase of 28.8 ± 2.29 compared to 10.4 ± 3.39 for 3,000 cells/cm^2 and 5.08 ± 1.7 for 6,000 cells/cm^2 (***$p < .0001$; Figure 6a). Similarly, the lowest cell seeding density also generated a significantly higher specific growth rate (***$p < .0001$; Figure 6b) and significantly lower doubling time (*$p < .05$; Figure 6c). A lower starting cell density is beneficial from a bioprocessing and handling point of view, as less two-dimensional processing steps are required to get enough cells to seed a bioreactor culture, smaller cell banks can be used, and more important, more doublings can be achieved in the same bioreactor size, allowing for fewer steps during the seeding train, and, therefore, leading to lower production costs. On the basis of these results, the cell seeding density of 1,500 cells/cm^2 was selected for the next set of experiments investigating the impact of the feeding strategy.

In spinner flask cultures, a 100% medium exchange is not possible if the aspiration of microcarriers is to be avoided leading to a loss of cells. In this study, four different medium exchange strategies were investigated ranging from 50% to 80% exchange throughout culture and a combination of 80% exchange for the first feeding point at Day 3 followed by 50% exchange for the rest of the culture. For all investigated feeding strategies, medium exchanges were performed at Day 3 and every other day thereafter. In addition, the cell seeding density of 1,500 cells/cm^2 was used for all runs. The 80% medium exchange yielded the highest cell number

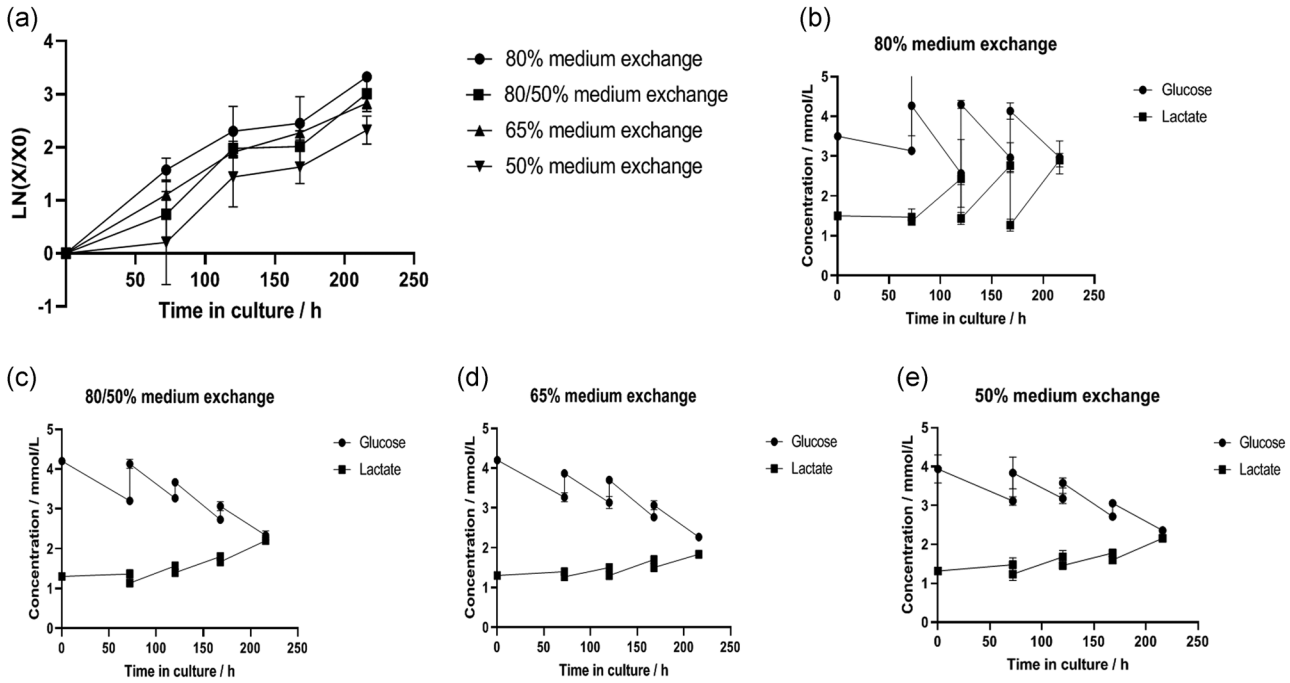

FIGURE 7 (a) The effect of different feeding strategies on cell growth. Glucose and lactate profiles for different feeding strategies: (b) 80% Medium exchange; (c) 80%/50% medium exchange, (d) 65% medium exchange, and (e) 50% medium exchange. Data expressed as mean ± standard deviation, $n = 5$

TABLE 1 Growth kinetics for bASC expansion on microcarriers in spinner flasks when different feeding strategies were employed

Medium exchange/ calculated parameter	80%	80%/50%	65%	50%
Fold increase	28.01 ± 2.59	21.12 ± 5.69	17.02 ± 1.9	10.49 ± 2.95
Specific growth rate (hr^{-1})	0.0154 ± 0.0004	0.0139 ± 0.002	0.0131 ± 0.001	0.0107 ± 0.001
Doubling time (hr)	45 ± 1.28	50.31 ± 6.57	52.95 ± 2.03	65.15 ± 7.15

Note: Data presented as mean ± *SD* ($n = 5$).
Abbreviations: bASC, bovine adipose-derived stem cells; *SD*, standard deviation.

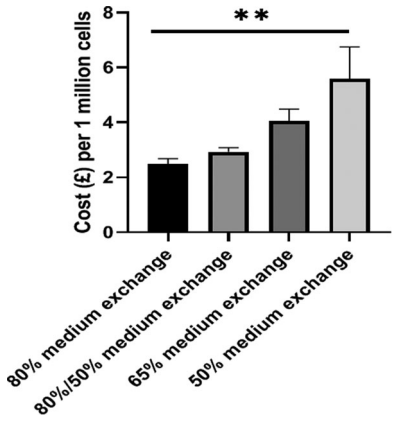

FIGURE 8 Cost (£) per million of cells produced when using different feeding strategies. Data expressed as mean ± standard deviation, $n = 3$. $^{**}p = .0014$ (one way analysis of variance)

throughout culture (Figure 7a) with a fold increase of 28.01 ± 2.59 ($^{****}p < .0001$), while the 50% exchange yielded a fold increase of only 10.49 ± 2.95. A doubling time of 45 ± 1.28 hr was obtained when 80% exchange was performed, while a 50% exchange produced a doubling time of only 65.15 ± 7.15 hr (Table 1). The glucose consumption and lactate production profiles for the different feeding strategies tested are shown in Figure 7b–e. When the 80% exchange was used, there was a continuous drop in glucose concentration and an increase in lactate concentration indicating good cell growth (Figure 7b). On the other hand, when a 50% exchange was used, the increase in lactate concentration was minimal to only 2.16 ± 0.055 mmol/L (Figure 7e) compared to 2.9 ± 0.17 mmol/L for 80% exchange at the end of the culture when the highest cell number was recorded. The glucose and lactate profiles showed a good correlation to the growth kinetics of bASCs at different exchange strategies.

It is well established that culture medium represents a large proportion of the manufacturing cost due to the large volumes needed when scaling up a bioprocess. Several strategies can be applied to reduce this cost and these include altering the medium composition, recycling medium, and some of its components or devising feeding strategies to minimize medium consumption and to maximize cell production (Stephens et al., 2018; Van der Weele & Tramper, 2014). The latter was investigated here. Thus, a cost analysis of consumables and reagents required for carrying out microcarrier culture in spinner flask at the working volume of 100 ml for 9

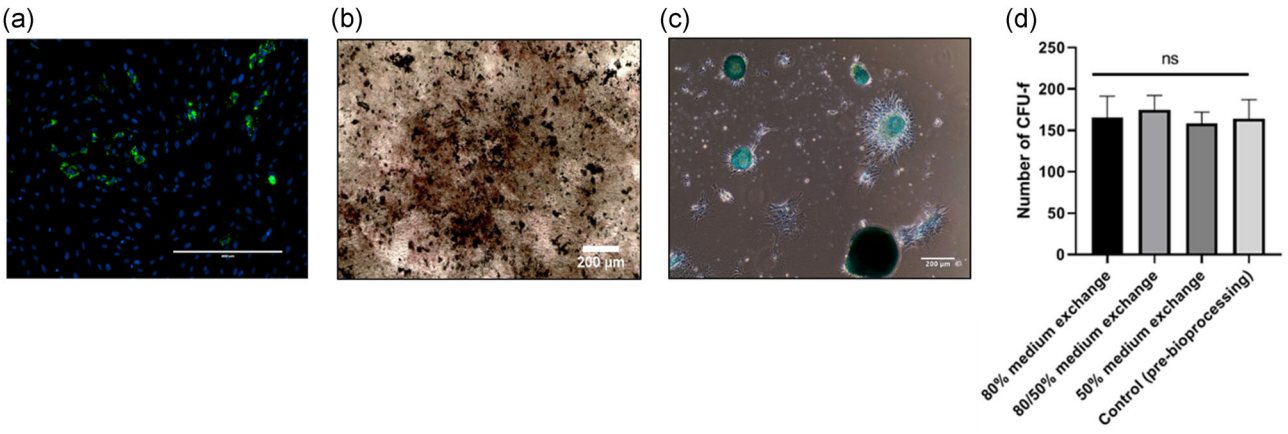

FIGURE 9 Postbioprocessing cell quality assessment. Differentiation staining of bASCs towards (a) adipogenic (LipidTox staining), (b) osteogenic (von Kossa stain), and (c) chondrogenic (Alcian blue) lineages. For adipogenic, scale bar = 400 µm. For osteogenic and chondrogenic, scale bar = 200 µm. (d) Clonogenic potential of bASCs postexpansion when using different feeding strategies. Data expressed as mean ± *SD*, $n = 5$. bASC, bovine adipose-derived stem cell; CFU, colony-forming unit; *ns*, not significant; *SD*, standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

days was carried out. The main variable in this cost analysis was the volume of the medium used as a result of different exchange strategies. The lowest cost found in relation to the cell number obtained was associated with the 80% medium exchange which was significantly lower (**$p < .001$) than the cost obtained for 50% medium exchange, but insignificant compared to the 80%/50% medium exchange where only the first medium exchange was 80% and the rest 50% (Figure 8). This analysis suggests that in the early stages of culture, nutrient availability and lack of metabolites are very important for cell growth.

3.4 | Assessment of postbioprocessing cell quality

It is important to acknowledge that exposure of the cells to the culture environment may have a detrimental effect on cell quality. As such, cell characterization needs to be performed before and after bioprocessing to ensure that none of the steps taken during the bioprocess impacts negatively on cell quality. Postbioprocessing, the bASCs expanded in spinner flasks retained their ability to differentiate towards adipogenic, osteogenic, and chondrogenic lineages (Figure 9a–c). Moreover, there was no significant difference ($p > .5$) in the clonogenicity before and after bioprocessing at any of the feeding regimes tested (Figures 9d).

4 | CONCLUSION

Meat is composed of several different types of cells (fat, muscle, and connective cells). bASCs are a suitable cell source for the production of cultivated meat as they have the ability to differentiate to both adipogenic and myogenic lineages. The first step in the production of cultivated meat is the scalable expansion of starting cells. This study describes the development of a bioprocess in small stirred tank bioreactors (e.g., spinner flasks) for the expansion of bASCs. The best bioprocess developed included using a low cell seeding density of 1,500 cells/cm^2 of surface area, a feeding regime of 80% medium exchange, and an agitation strategy of incremental increase every 2 days. These conditions yielded a 28-fold increase in cell number. The cells retained their cell surface marker expression and their ability to differentiate towards adipogenic, osteogenic, and chondrogenic lineages without any change in their clonogenicity. This bioprocess can serve as a starting point for translating the manufacturing of these bASCs to the liter scale and beyond.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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