


ARTICLE

Scale-up of an intensified bioprocess for the expansion of bovine adipose-derived stem cells (bASCs) in stirred tank bioreactors

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Abstract

Cultivated meat is an emerging field, aiming to establish the production of animal tissue for human consumption in an *in vitro* environment, eliminating the need to raise and slaughter animals for their meat. To realise this, the expansion of primary cells in a bioreactor is needed to achieve the high cell numbers required. The aim of this study was to develop a scalable, microcarrier based, intensified bioprocess for the expansion of bovine adipose-derived stem cells as precursors of fat and muscle tissue. The intensified bioprocess development was carried out initially in spinner flasks of different sizes and then translated to fully controlled litre scale benchtop bioreactors. Bioprocess intensification was achieved by utilising the previously demonstrated bead-to-bead transfer phenomenon and through the combined addition of microcarrier and medium to double the existing surface area and working volume in the bioreactor. Choosing the optimal time point for the additions was critical in enhancing the cell expansion. A significant fold increase of 114.19 ± 1.07 was obtained at the litre scale in the intensified bioprocess compared to the baseline ($**p < .005$). The quality of the cells was evaluated pre- and post-expansion and the cells were found to maintain their phenotype and differentiation capacity.

KEYWORDS

bioprocessing, bioreactors, cultivated beef, microcarriers, scale-up

1 | INTRODUCTION

Over the past couple of years, the concept of cultivated meat has received increased attention due to its undeniable potential as a sustainable food source, as well as its ability to address many of the existing challenges and detrimental effects of livestock meat. Cultivated meat,

also referred to as cultured meat or clean meat is a new food technology that will positively impact animal welfare and the environment, while offering a potentially healthier and safer option for consumers (Mouat & Prince, 2018; Stephens et al., 2018; Tuomisto & de Mattos, 2011).

The first step in the production of any cultivated meat product is the expansion phase of the primary cells used as a cell source for

[†]In memoriam of Prof Christopher J. Hewitt who sadly passed away on 25th of July 2019.

production of fat and muscle. The sourced cells are expanded in high numbers and then differentiated into the respective tissues. It has been estimated that 10^{11} cells are needed to make 1 kg of meat, including all cell types involved (Post et al., 2020). Realistically, such cell numbers are only attainable in bioreactor cultures.

To date, a significant number of published studies have demonstrated the ability to expand human mesenchymal stem cells (hMSCs) at different scales (deSoure et al., 2016; Hanga, Nienow, et al., 2020; Hewitt et al., 2011; Lawson et al., 2017; Rafiq et al., 2013, 2017, 2018). More recently, bovine adipose-derived stem cells (bASCs) as precursors for fat and muscle tissue were also expanded successfully in spinner flasks (Hanga, Ali, et al., 2020). However, that study was carried out in a semi-controlled environment as spinner flasks rely on the temperature and gas control within an incubator. The next step for scalable production is translation to fully controlled bioreactors. This is, to our knowledge, the first report of litre scale expansion of bASCs for cultivated meat production.

Microcarrier culture in bioreactors has a significant advantage over monolayer culture due to their high surface-area-to-volume ratio. In addition, this surface area can be expanded in bioprocesses through the provision of additional microcarriers, thus maximising cell expansion, while maintaining cell growth in the exponential phase for longer. This approach has been reported previously for expansion of human MSCs on microcarriers (Hervy et al., 2014; Rafiq et al., 2018), as well as for the expansion of skeletal muscle satellite cells (Bodiou et al., 2020; Verbruggen et al., 2018). It is believed that a key mechanism for the success of this approach is the phenomenon known as a bead-to-bead transfer. This refers to the migration of adherent cells from one microcarrier to another in suspension culture (deSoure et al., 2016; Rafiq et al., 2018; Takahashi et al., 2017). Bead-to-bead transfer and surface area addition are approaches of particular interest from a manufacturing perspective as they facilitate process intensification, thereby increasing cell yield and reducing process time.

In this study, we investigated the surface area addition approach for process intensification, while translating the bioprocess for expansion of bASCs from spinner flasks to litre scale bench-top bioreactors, thus benefiting from full environmental monitoring and control. The continuous monitoring of process parameters possible in such bioreactors and the offline measurements of important representative metabolites (Moutsatsou et al., 2019) and cell counts performed throughout the culture enabled a better understanding and faster optimisation of the expansion bioprocess of these cells. Such work has not been reported before for bASCs culture and represents a major step forward for this novel use of stem cell culture.

2 | MATERIALS AND METHODS

2.1 | Planar culture of bASC

bASCs were purchased from Cellider Biotech (Spain) and they were cultured as previously reported (Hanga, Ali, et al., 2020) in a growth medium comprising α -Modified Eagle Medium (BE12-169F; 1 g/L glucose; Lonza) supplemented with 10% (v/v) foetal bovine serum (FBS; F7524; Sigma-Aldrich), 2 mM ultra-glutamine (BE17-605E/U1; Lonza) and 1 ng/ml bFGF (100-18B; Peprotech). bASCs between passages 3 and 5 were used for all experiments.

2.2 | Microcarrier culture in spinner flasks

Two different-sized spinner flasks with the same type of impeller were used in this study: 100 ml capacity (Bellco) (Figure 1a) and 500 ml capacity (Wheaton Celstir) (Figure 1b). Before use, all spinner flasks were coated with Sigmacote (Sigma-Aldrich) to prevent cell attachment to the glass (Nienow, Coopman, et al., 2016). SoloHill Plastic microcarriers (Pall) were chosen for this study based on their excellent performance, animal-free characteristics, and commercial availability (Hanga, Ali, et al., 2020; Rafiq et al., 2016). The

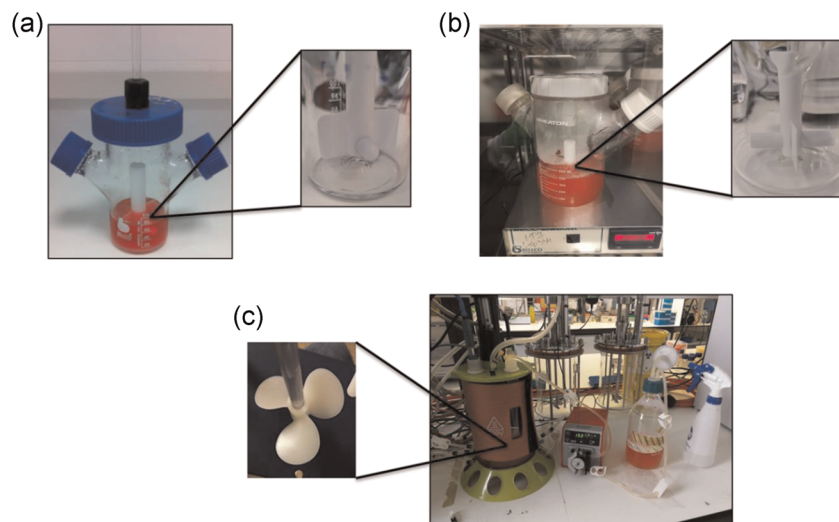


FIGURE 1 Photographs of bioreactors used and their impellers. (a) Bellco spinner flask with a maximum working volume of 100 ml. (b) Wheaton spinner flask with a maximum working volume of 500 ml. Both Bellco and Wheaton spinner flasks are equipped with paddle blade impellers. (c) Litre scale bioreactor assembly comprising Mobius 3 L vessel, peristaltic pump and addition bottle. The Mobius 3 L vessel (with a maximum working volume of 3 L) is equipped with a marine impeller. None of the vessels were equipped with baffles [Color figure can be viewed at wileyonlinelibrary.com]

microcarriers were weighed to achieve the surface area needed and then suspended in D-PBS without calcium and magnesium (BE17-516F; Lonza) for autoclaving. The surface area concentration used was 5 cm²/ml of medium. bASCs were seeded at the optimal seeding density of 1500 cells/cm² established in our previous study (Hanga, Ali, et al., 2020). The feeding regime previously established to be optimal at 80% medium exchange was carried out on Day 3 and then every other day until the end of culture (Hanga, Ali, et al., 2020). Briefly, the agitation was stopped to allow microcarriers to settle (~5 min), then the volume was removed and replaced with a fresh medium. The spinner flasks were then transferred back to the incubator and the agitation was restarted. All spinner flask cultures were agitated at the minimum speed required to just suspend the microcarriers (N_{JS}). N_{JS} is usually assessed visually as the speed at which no particles remain static on the base of the vessel at any point for more than 1 to 2 s (Zwietering, 1958). At this speed, all cells attached to microcarriers are able to access nutrients from the medium and pass metabolites into it. Higher speeds do not enhance those processes, but greatly increase the potential for damage to cells from microcarrier-microcarrier or microcarrier-impeller impacts and enhanced turbulent stresses (Nienow, Rafiq, et al., 2016). The first time the use of N_{JS} was proposed as a preferred operating criterion for the culture of MSC was by Hewitt et al. (2011) for spinner flasks and it was later shown to be appropriate for a range of bioreactors by Nienow, Coopman, et al. (2016). For both types of spinner flasks used in this study, this speed was visually assessed to be 30 rpm, which was kept constant throughout the culture. The spinner cultures were carried out for 11 days when the full harvest was done by using a previously published protocol (Nienow, Hewitt, et al., 2016). Three different combinations of parameters were investigated for the ability to intensify the bioprocess and these are shown in Table 1. Briefly, the "Baseline" experiments were performed in the Bellco spinner flasks at the constant volume of 100 ml and constant surface area of 500 cm². The "microcarriers (MC) only addition" experiments were also carried out in the Bellco spinner flasks at the constant volume of 100 ml, but with the doubling of surface area from 500 to 1000 cm². The "microcarriers (MC) + volume addition" experiments were carried out in the Wheaton spinner flasks which have a maximum capacity of 500 ml. The geometry of the Wheaton spinner flask doesn't allow operation at 100 ml due to the positioning of the impeller. As a result, they were operated at the starting volume of 200 ml with the surface area adjusted to 1000 cm² up to Day 5 to maintain the same microcarrier concentration, after which both the microcarrier surface area and the volume were doubled to a surface area of 2000 cm² in a volume of 400 ml.

2.3 | Microcarrier culture in bioreactors

A single-use, disposable stirred tank bioreactor vessel (Mobius Cell Ready 3L, Millipore) of a 3 L nominal size was used for all experiments. The bioreactor controller (EZ-Control), probes (temperature,

TABLE 1 Varying process conditions used in the experimental runs and outputs

Experimental run	Bioreactor vessel	Initial cell seeding density (cells/cm ²)	Volume at the start (ml)	Volume at the end (ml)	Surface area at the start (cm ²)	Surface area at the end (cm ²)	MC addition	MC addition time point (day)	Surface area at the end (cm ²)	Final cell concentration (cells/ml)	Final cell density (cells/cm ²)	Total volume of medium used (ml)	Volume of medium used per 1×10 ⁶ cells (ml)
Baseline	Bellco	1500	100	100	500	500	No	N/A	500	2.78 × 10 ⁵	55,600	420	15.1
MC only addition (D5)	Bellco	1500	100	100	500	500	Yes	5	1000	3.23 × 10 ⁵	32,350	420	13
MC only addition (D7)	Bellco	1500	100	100	500	500	Yes	7	1000	2.31 × 10 ⁵	23,090	420	18.2
MC + volume addition	Wheaton	1500	200	400	1000	1000	Yes	5	2000	4.37 × 10 ⁵	87,490	840	4.8
Baseline	Mobius	1500	1000	1000	5000	5000	No	N/A	5000	3.02 × 10 ⁵	60472	3000	9.9
MC + volume addition	Mobius	1500	1000	2000	5000	5000	Yes	5	10,000	5.053 × 10 ⁵	101,063	5000	4.9

Abbreviation: MC, microcarriers.

pH, DO), and other relevant accessories (heating mantle, motor adapter) were purchased from Applikon. A 2-point calibration was performed on the pH probe which was then sterilised by autoclaving. The DO probe was first autoclaved and then calibrated post-assembly in the bioreactor using saturation with air supplemented with 5% CO₂ as 100% DO. Aeration was achieved through the headspace using air supplemented with CO₂ which has been previously established to be sufficient for the oxygen demand of human MSCs up to the 5 L scale (Nienow, Rafiq, et al., 2016; Rafiq et al., 2013).

SoloHill Plastic microcarriers (Pall) were also used for the bioreactor cultures and were weighed to provide a surface area of 5000 cm²/L. The microcarriers were sterilised by autoclaving before their addition to the bioreactor vessel. Cells were inoculated on the microcarriers at the established optimal seeding density of 1500 cells/cm² (Hanga, Ali, et al., 2020). The “baseline” bioreactor runs were initially operated at a working volume of 1 L containing a surface area of 5000 cm² throughout the culture. The “MC + volume addition” runs started with 5000 cm² in 1 L up to Day 5 after which both surface area and volume were doubled to achieve 10,000 cm² in 2 L (Table 1). For all bioreactor experiments, the agitation speed was kept constant throughout culture at the N_{js} , which for this vessel-impeller combination was assessed by visual observation and it was found to be 60 rpm. The bioreactor cultures were also maintained for 11 days when the harvest was performed. Medium exchanges in the bioreactor were carried out on the bench using the peristaltic pump (Watson-Marlow 120S), pre-sterilised glass bottles, and a sterile welder (Terumo TSCD-II) for establishing sterile connections (Figure 1c). The medium exchange % was limited to 50% when the bioreactor was operated at the 1 L volume because of the positioning of the sampling port on the Mobius vessel, which only allows the removal of a maximum volume of 500 ml. For the “MC + volume addition,” the 50% medium exchange was maintained for consistency. The first medium exchange was performed at Day 3, then every other day until the end of the culture. Briefly, the agitation was stopped to allow microcarriers to settle (~5 min), then the volume was removed and replaced with fresh medium. The agitation was then restarted. During all processing steps, aeration and temperature were controlled at all times.

2.4 | Cell harvest

Cell harvesting from microcarriers in spinner flasks was carried out using the protocol previously developed by Nienow, Hewitt, et al. (2016). For cell harvest from microcarriers in the bioreactor, the same protocol was adapted to the litre scale with the difference that all steps were carried out directly in the bioreactor on the bench using the peristaltic pump and sterile welder rather than transferring to the biological safety cabinet. Briefly, the agitation was stopped and the microcarriers were allowed to settle for 5 min before removing 50% of the spent medium and replacing it with DPBS for washing under agitation at the

established N_{js} . These steps were repeated three times, followed by removal of 50% of the volume and replacing with 0.25% trypsin-EDTA (25200072; Gibco, Thermo Fisher Scientific). The incubation with the enzyme was carried out for up to 20 min at 37°C, while stirring at 150 rpm. For “Baseline” and “MC addition only” conditions, the exposure time to the proteolytic enzyme was 15 min, while for the “MC + volume addition” conditions where large cell densities were obtained, 15 min was not deemed satisfactory and thus, it was extended to 20 min. A sample was then taken for microscopic evaluation of complete cell dissociation from microcarriers. Once this was confirmed, the enzyme was then inactivated with growth medium to double the volume. For spinner flasks, the total volume of 100 ml was then taken post-dissociation, while for the bioreactor culture, only a sample of 200 ml was taken post-dissociation for the filtration step to remove the microcarriers. This was achieved using Steriflip filtering devices (Millipore; 90 µm pores). The cell suspension was then centrifuged at 250g for 5 min to obtain a pellet.

2.5 | Process analytics

wSamples were taken at the same time points when feedings were performed and were used for cell imaging, cell counting and glucose, and lactate measurements before and after medium exchanges. Live/dead staining kit (L3224; Thermo Fisher Scientific) comprising calcein-AM for live cells and ethidium homodimer for dead cells was used following the manufacturer's instructions to assess cell viability on microcarriers using a fluorescent microscope (Evos FL, Thermo Fisher Scientific). Spent medium samples were collected before and after medium exchanges and were analysed for glucose and lactate concentrations on the AccuTrend Plus meter (Roche). Fresh growth medium was used as the baseline control. Cell counts were performed directly onto microcarriers using the reagent A100 and reagent B protocol on the Nucleocounter NC-3000 (Chemometec). Briefly, the cell-microcarrier suspension was diluted to a 1:3 ratio with reagent A100 (lysing agent) and reagent B (stabilising agent). The resulting suspension was then loaded onto a Via-1 Nucleocassette pre-loaded with acridine orange and 4',6-diamidino-2-phenylindole (DAPI).

Based on cell counts, the following parameters were calculated: μ (specific growth rate, h⁻¹) $\left[\mu = \frac{\ln(Cx(t)/Cx(0))}{\Delta t} \right]$, t_d (doubling time, h) $\left[td = \frac{\ln 2}{\mu} \right]$ and FI (fold increase) $\left[FI = \frac{Cx(t)}{Cx(0)} \right]$. $Cx(t)$, $Cx(0)$ are cell numbers at the end and the start of the culture.

2.6 | Cell product characterisation

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Cells from both spinner flasks and bioreactor cultures were harvested at the end of culture as described in Section 2.4 and used for characterisation.

2.6.1 | Tri-lineage differentiation potential

The StemPro differentiation kits (Thermo Fisher Scientific) were used to drive differentiation of bASCs to adipogenic (A1007001), osteogenic (A1007201), and chondrogenic (A1007101) lineages. bASCs were seeded at 5,000 cells/cm² for osteogenic and 10,000 cells/cm² for adipogenic differentiation. For chondrogenic testing, the micromass method was used (Hanga et al., 2017; Hanga, Ali, et al., 2020; Rafiq et al., 2017). Briefly, 5 µl droplets of a highly concentrated bASCs suspension (1×10⁷ cells/ml) were seeded in an empty well plate (Corning) and incubated for 1–2 h. The chondrogenic differentiation medium was then carefully added dropwise. All cultures were kept in their respective media (StemPro, Thermo Fisher Scientific) for 21 days with a medium change every 3–4 days. The cells were then fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature and stained with their corresponding stains. Oil Red O was used for confirming adipogenic differentiation. Von Kossa stain was used to assess osteogenic differentiation, while the chondrogenic differentiation was assessed using Alcian blue.

2.6.2 | Cell surface marker expression

The expression of two cell surface markers with bovine reactivity (CD73—ab231643, CD90—ab212885; Abcam) was assessed using immunocytochemistry staining. All stains were used at the manufacturer's recommended dilutions. Briefly, the cells were fixed as above and then permeabilised for 5 min using Perm Wash (421002; Biolegend). Two washes with cell staining buffer (420201; Biolegend) were performed, followed by an incubation step with normal serum block (927502; Biolegend) for 45 min in the dark. The primary antibody was then added at the recommended dilution and incubated overnight at 4°C. The secondary antibody containing the fluorophore was then incubated with the pre-stained cells for 2 h at room temperature in the dark. DAPI was used for nuclei staining at the 300 nM working concentration. Phalloidin (94072; Sigma-Aldrich) was used to stain the cytoskeleton. Stained cells were then imaged on a fluorescence microscope (Evos FL; Thermo Fisher Scientific).

2.7 | Statistical analysis

All spinner flask experiments were performed in four biological repeats, while bioreactor experiments in triplicates. Cell counts were obtained from two independent samples from each repeat. Data were expressed as mean ± SD. Statistical analysis was carried out using Graph Pad Prism 9. For comparison of multiple data sets, significance was determined by one-way analysis of variance. Statistical significance was considered when $p < .05$.

3 | RESULTS AND DISCUSSION

The aim of this study was dual: (1) to investigate the potential for process intensification through surface area addition by utilising the bead-to-bead transfer phenomenon and (2) to translate the improved bioprocess to litre scale in controlled bioreactors. To the best of our knowledge, no such study has been done using bovine stem cells. The assumption was that the bead-to-bead transfer phenomenon would be applicable to the bovine-derived MSCs and would lead to higher cell yields.

3.1 | Bioprocess intensification for bASCs expansion in spinner flasks

The first objective was to investigate if, through fresh microcarrier addition and utilising the bead-to-bead transfer phenomenon, the freshly added microcarriers would be populated with attached cells, thereby maintaining cell growth and enhancing total cell numbers over the same culture period. Enhanced total cell numbers in the same timeline is an indication of process intensification, which is highly advantageous leading to an increased cell number while reducing process time. Bead-to-bead cell transfer is a phenomenon that can be influenced by multiple factors, including medium composition, attachment substrate (i.e., type of microcarrier), feeding regime, time point for fresh microcarrier addition, agitation intensity and possibly other parameters (deSoure et al., 2016; Ferrari et al., 2012; Hervy et al., 2014; Rafiq et al., 2018; Takahashi et al., 2017). Many of these parameters were kept constant between the baseline and the microcarrier addition bioprocesses reported in this study.

The “baseline” bioprocess for the bASC expansion on microcarriers in the Bellco spinner flasks was developed in our previously published work (Hanga, Ali, et al., 2020) and it was used here as a starting point for the intensification of the expansion bioprocess by keeping cell seeding density, initial surface area, and the feeding regime the same. The first critical parameter for process intensification is the selection of the optimal time point for microcarrier addition. In this study, two time points were investigated: Days 5 and 7. The better microcarrier addition time point was selected based on the observed development of aggregation throughout the culture and its effect on final cell number. At Day 7, cell-microcarrier aggregates of three or more microcarriers were observed in all experiments, while at the earlier time point (Day 5), zero or minimal aggregation was observed in the samples (Figure 2a).

In the “MC only addition” runs, different levels of cell growth were found when the microcarriers were added at either Day 5 or 7. At Day 7, a fold increase of 30.78 ± 4.60 was obtained which was lower than the “baseline” bioprocess that yielded a fold of 37.10 ± 6.73 . On the other hand, when microcarriers were added at the earlier time point in culture (Day 5), it resulted in a slightly higher fold increase of 43.13 ± 9.17 (Figure 3a). It was postulated that when

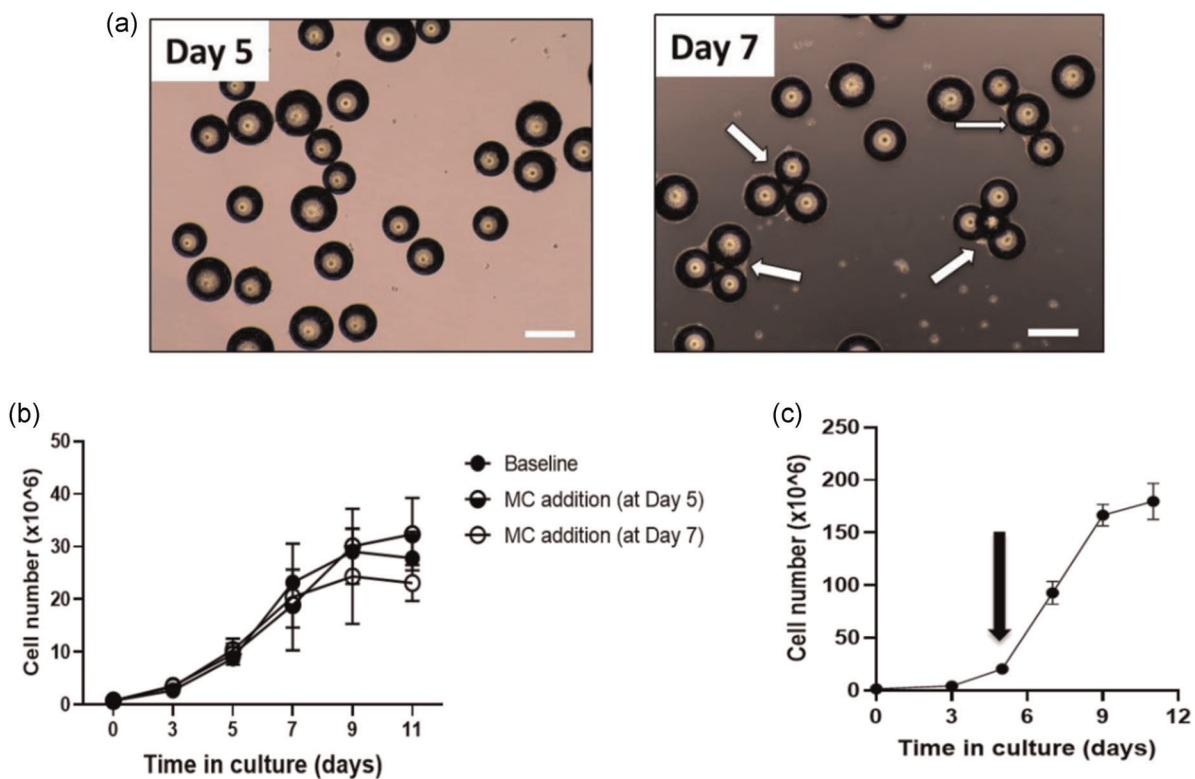


FIGURE 2 Expansion of bASCs in spinner flasks for up to 11 days. (a) Representative phase contrast images taken before microcarrier addition at Days 5 and 7 in culture. White arrows point to cell-microcarrier aggregates. Scale bar = 200 μm . (b) Cell number over time in a baseline bioprocess in the Bellco spinner flasks compared to microcarrier addition at different time points in a constant volume. (c) Cell number over time in the Wheaton spinner flask in which surface area and volume were doubled. The arrow indicates the time point (Day 5) for the addition. Data expressed as mean \pm SD, $n = 4$ [Color figure can be viewed at wileyonlinelibrary.com]

the addition was performed at the later time point, the lower cell growth obtained was linked to the existence of the cell-microcarrier aggregates observed then (Figure 2a). Cell aggregation is a known issue in bioreactor cultures as it can cause heterogeneity in the culture environment and depending on the size of aggregates, it can lead to cell exposure to concentration gradients, especially low oxygen (Ferrari et al., 2012; Wu et al., 2014). The existence of aggregation at Day 7 was assumed to lower the efficiency of the bead-to-bead transfer, thus explaining the diminished growth registered. However, although the early addition of extra microcarriers gave a better measurable performance, the differences compared to the “baseline” were not statistically significant (ns ; $p > .05$). In these runs, there is a possibility that, as known in early work on hMSCs (Hewitt et al., 2011), above a certain concentration of microcarriers per ml of medium, the culture performance deteriorates. More recent work has emphasised quantitatively how the microcarrier collisions with rotating impellers and particularly between themselves increases with concentration (Nienow, Coopman, et al., 2016). As the cells are situated on the surface of the microcarriers, these collisions can cause damage and a reduction in culture performance and thus possibly resulting in cell death. This mechanism might also explain why the difference in cell growth between the “baseline” and the two different addition time points, were not statistically significant.

Another possible cause of this finding could be that nutrients provided in the constant volume were either depleted faster or were not sufficient for the amount of cells now proliferating on the doubled surface area. Alternatively, metabolite production (e.g., lactate) could also have an inhibitory effect on cell growth (Qie et al., 2012; Schop et al., 2009). However, neither of these latter reasons are relevant here as glucose depletion or lactate accumulation didn't occur in either the “baseline” or the “MC only addition” runs (Figure 3d). Other nutrients such as glutamine or metabolites such as ammonia could have been depleted or respectively produced in high concentrations, thus influencing cell growth negatively (Schop et al., 2009). However, these were not measured here.

The next step was to combine microcarrier addition at the determined better time point of Day 5 with volume doubling at the same time (“MC + volume addition”). This new approach yielded approximately 1.8×10^8 cells at Day 11 (Figure 2c) equivalent of a fold increase of 116.65 ± 9.73 . This increase was 3.14 times higher than the “baseline” yield and 2.7 times higher than the “MC only addition” yield ($****p < .0001$) (Figure 3a). The “MC + volume addition” resulted in a significantly higher specific growth rate of $0.0180 \pm 0.00 \text{ h}^{-1}$ ($****p < .0001$) (Figure 3b) and a significantly lower doubling time of $38.48 \pm 0.65 \text{ h}$ ($****p < .0001$) (Figure 3c) compared to the other conditions tested.

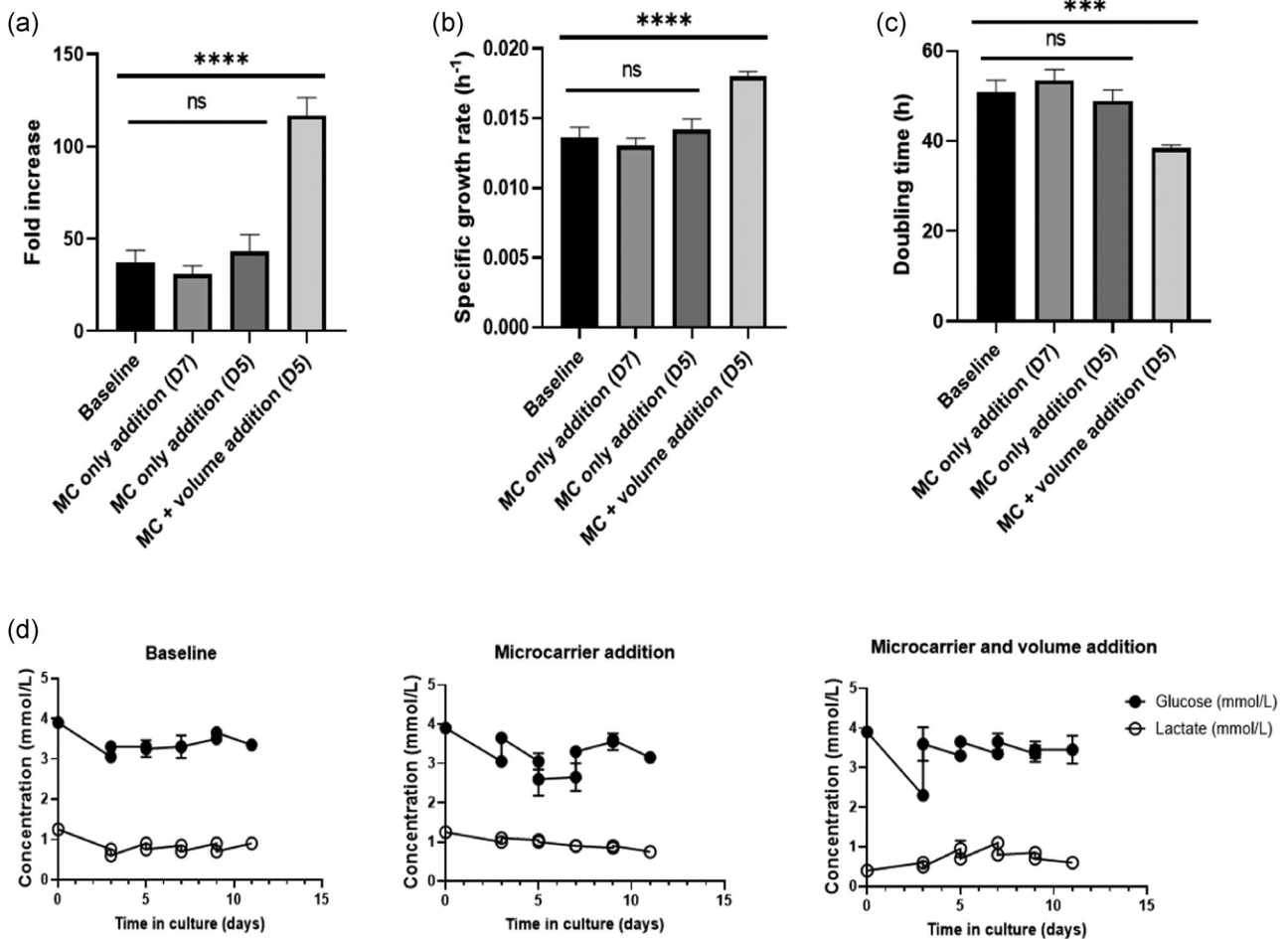
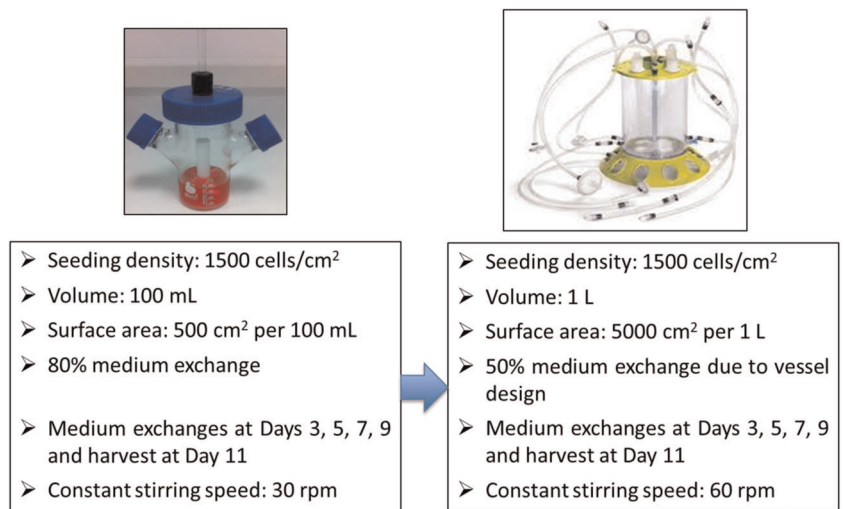


FIGURE 3 Comparison of (a) fold increase, (b) specific growth rate (h^{-1}) and (c) doubling time (h) of the expansion of bASCs in a baseline and microcarrier addition (Bellco spinner flasks) or microcarrier and volume addition (Wheaton spinner flasks) bioprocesses. (d) Glucose and lactate concentrations (mmol/L) over time in the baseline, microcarrier addition at Day 5 and microcarrier and volume addition bioprocesses. Data expressed as mean \pm SD, $n = 4$; ns, not significant; *** $p < .0001$; **** $p < .00001$

FIGURE 4 Bioprocess translation from spinner flasks (100 ml scale) to the litre scale in a fully controlled Mobius bioreactor (1 L scale) [Color figure can be viewed at wileyonlinelibrary.com]



3.2 | Bioprocess translation to litre scale

The “baseline” and “MC + volume addition” bioprocesses developed in spinner flasks were then translated to the litre scale in the single-use, disposable, Mobius bioreactor vessel. The bioprocess parameters translated from spinner flasks to the litre scale bioreactor are shown in Figure 4. The initial cell seeding density, feeding regimes, and concentration of microcarriers per volume of medium (5 cm²/ml) were kept the same. It was not possible to translate the 80% medium exchange from spinner flasks to the Mobius bioreactor when operated at 1L because of the vessel design and positioning of the medium removal port, which creates a dead volume of approximately 500 ml. Nevertheless, earlier work (Nienow, Hewitt, et al., 2016; Rafiq et al., 2013) suggested that similar results could be obtained at 50% medium exchange and with increasing scale, a smaller exchange volume would represent a significant cost saving.

bASCs cultured on Plastic microcarriers in the Mobius bioreactor using the “baseline” operating parameters grew successfully, achieving an average yield of 3.02×10^8 cells in 11 days of culture, which was the equivalent of a fold increase of 40.31 ± 3.53 (Figure 5a). Moreover, the cells remained viable on the microcarriers as shown by the live/dead staining (Figure 5b). Even with the increased levels of aggregation which occurred in the last days of culture, cell viability was maintained high with only a small number of dead cells (red) observed (Figure 5b). As anticipated, following the smaller scale studies in the spinner flasks, the “MC + volume addition” bioprocess also yielded a significantly higher number of cells (1.01×10^9 cells) compared to the “baseline” ($****p < .0001$) (Figure 6a). The equivalent fold increase of 134.75 ± 36.94 was 3.34 times higher than the “baseline” bioprocess ($*p < .05$) (Figure 6b). The

specific growth rate (h^{-1}) was calculated as $0.0184 \pm 0.00004 h^{-1}$ in the “MC + volume addition” bioprocess compared to the “baseline” which was only $0.0140 \pm 0.0003 h^{-1}$ ($**p < .005$) (Figure 6c); and the doubling time was significantly lower in the same bioprocess at $37.56 \pm 1.93 h$ compared to $49.55 \pm 1.13 h$ ($**p < .005$) in the “baseline” (Figure 6d).

The metabolic analysis showed many differences between the “baseline” and the “MC + volume addition” bioprocesses, particularly towards the end of the culture (Figure 7). These differences were observed in both glucose consumption and lactate production. In the baseline, glucose depletion was not reached at any time point in culture with a minimum of 1.36 mmol/L reached (Figure 7a1), while lactate concentration reached a maximum of 3.73 mmol/L as an average (Figure 7a2). On the other hand, the “MC + volume addition” bioprocess resulted in almost two times higher lactate concentration at Day 11 with a steep increase from Day 5 onwards despite the doubling of volume (Figure 7b2). The glucose concentration also showed a steep decline from Day 5 onwards, reaching low levels (<1 mmol/L) at the final day of culture (Figure 7b1). From Day 9 onwards, increasing the glucose concentration in the culture medium proved difficult with the 50% exchange and feeding regime at the frequency adopted, which could explain the small drop in total cell numbers at Day 11. As the lactate concentration reached levels of over 7 mmol/L, to avoid inhibitory concentrations, medium refreshment might be needed. The metabolite inhibitory levels are cell line and type dependent (Schop et al., 2009) and to the best of our knowledge, no published study has investigated those levels for bovine ASCs. Overall, the glucose and lactate trends correlated well with the cell growth obtained in both bioprocesses. pH and dissolved oxygen (DO, %) were continuously monitored throughout the bioreactor cultures. In the “baseline” bioprocess, the pH was recorded and maintained within the

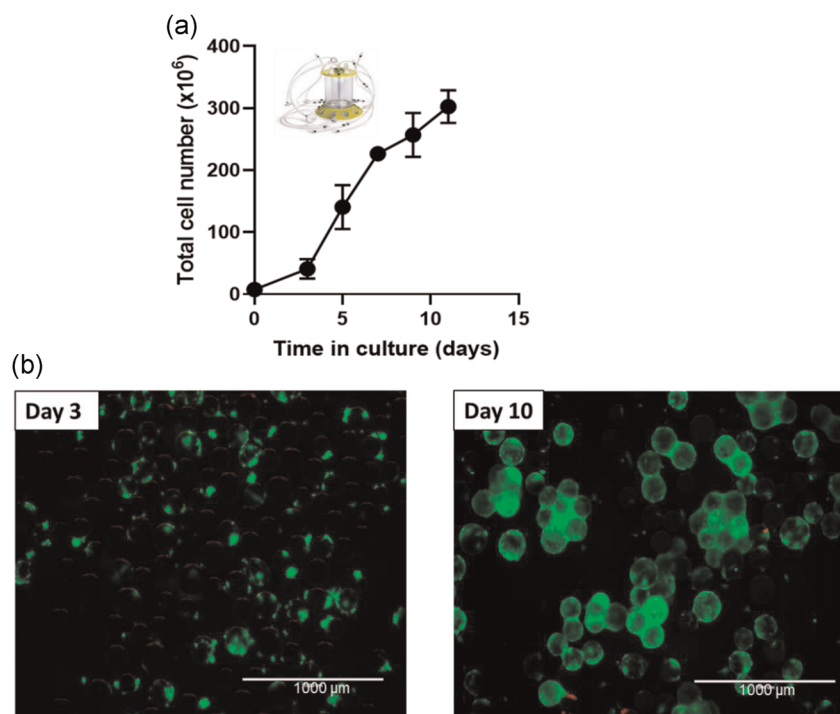


FIGURE 5 bASC expansion overtime on Plastic microcarriers in the fully controlled environment of the Mobius bioreactor vessel in a working volume of 1 L. (a) Total cell number over time. Data expressed as mean \pm SD, $n = 3$. (b) Live/dead staining of samples taken from the Mobius bioreactor at Days 3 and 10 in culture. Live cells appear green, while dead cells appear red. Scale bar = 1000 μ m [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 6 Comparison of bASC expansion over time in the Mobius bioreactor vessel in a baseline versus a microcarrier and volume addition bioprocess at the litre scale. (a) Total cell number over time. (b) Fold increase. (c) Specific growth rate (h^{-1}). (d) Doubling time (h). Data expressed as mean \pm SD, $n = 3$; * $p < .05$; ** $p < .005$

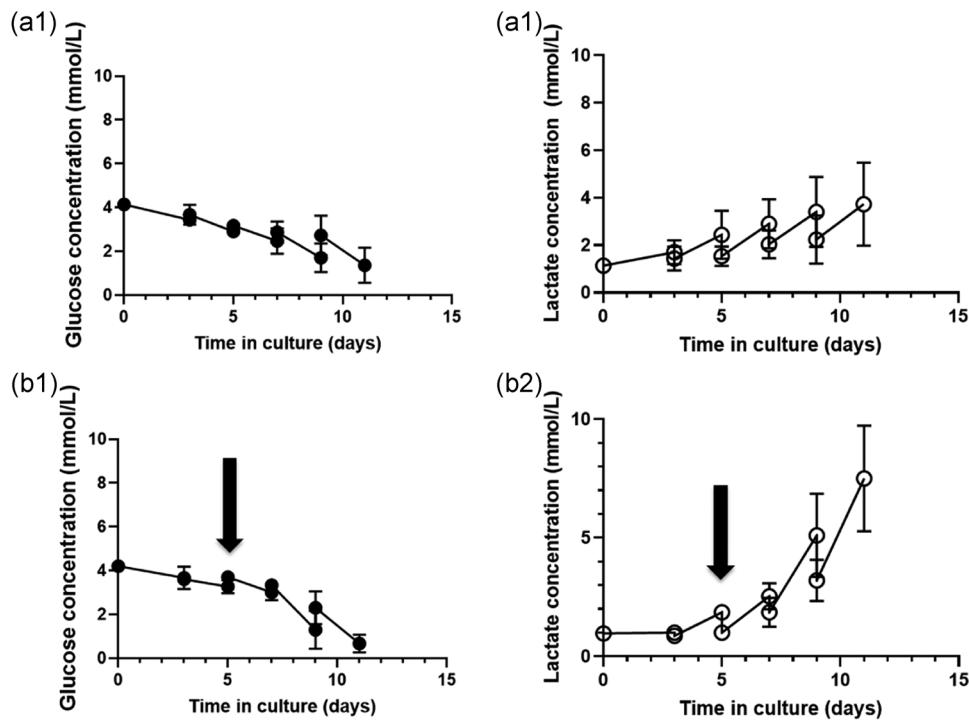
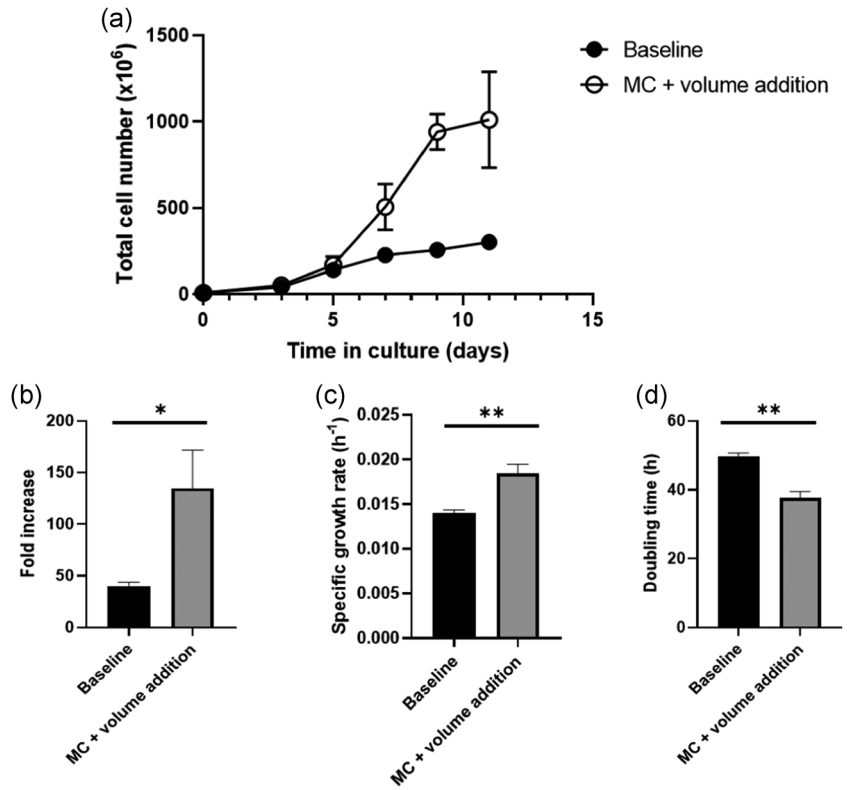


FIGURE 7 Glucose (a1; b1) and lactate concentrations (mmol/L) (a2; b2) measured when bASCs were expanded at the litre scale in the Mobius bioreactor. (a) In the baseline bioprocess. (b) In the microcarrier and volume addition baseline. The arrow shows the time point in culture for microcarrier and volume addition (Day 5). Data expressed as mean \pm SD, $n = 3$

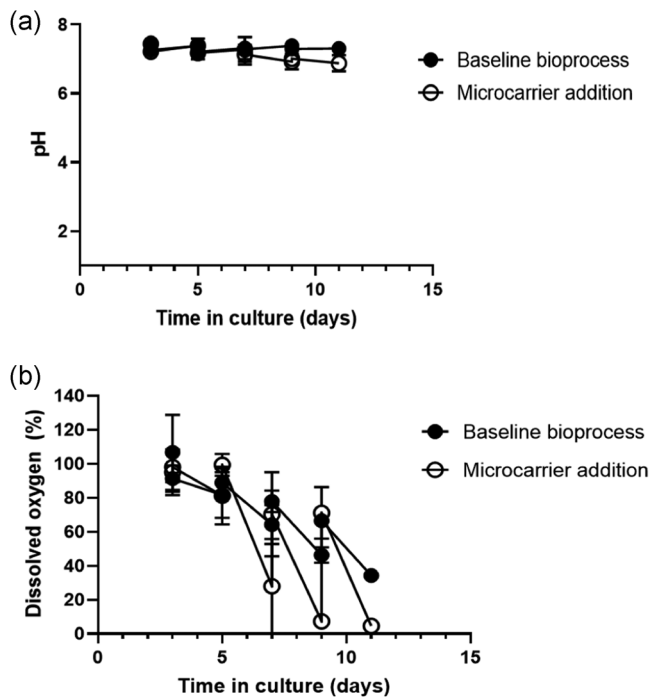


FIGURE 8 Process parameters monitored during the bASCs expansion on microcarriers in the Mobius bioreactors. (a) pH and (b) DO (%). Data expressed as mean \pm SD, $n = 3$

range of 7.3 to 7.4. However, for the “MC + volume addition” bioprocess, the pH range recorded was wider, from 6.88 to 7.3 (Figure 8a) and it was outside the physiological pH range, which also may have impacted the cell number towards the end of the culture. The DO levels showed a steep decrease over time in both the baseline and the intensified bioprocesses. In the “baseline” bioprocess, the lowest DO level was 34.2% (Figure 8b). Such a level would not be expected to cause a problem with the cells; indeed there is some evidence to suggest that even lower values are beneficial (Heathman et al., 2019). However, that study was done using human MSCs and not bovine. The “MC + volume addition” bioprocess reached low levels of DO (4.63%) meaning there was a higher oxygen demand which correlates with higher cell numbers. The “MC + volume addition” conditions in both spinner flasks and the Mobius bioreactor resulted in higher cell concentrations of 4.37×10^5 and 5.05×10^5 cells/ml, respectively, compared to maximum 3×10^5 cells/ml for baseline and “MC addition only” conditions (Table 1, column 10). Moreover, the volumes of media required to produce 1×10^6 cells were the lowest for the “MC + volume addition” conditions with only 4.8 ml needed in spinner flasks and 4.9 ml in the Mobius bioreactor compared to 15.1 ml (spinners) and 9.9 ml (Mobius) for baseline (Table 1, column 13). This suggests that the intensified bioprocesses would be more cost-efficient particularly given that the media is the main contributor to the overall manufacturing cost. Overall, the volume of media required to produce 1×10^6 cells was lower in the bioreactor at the litre

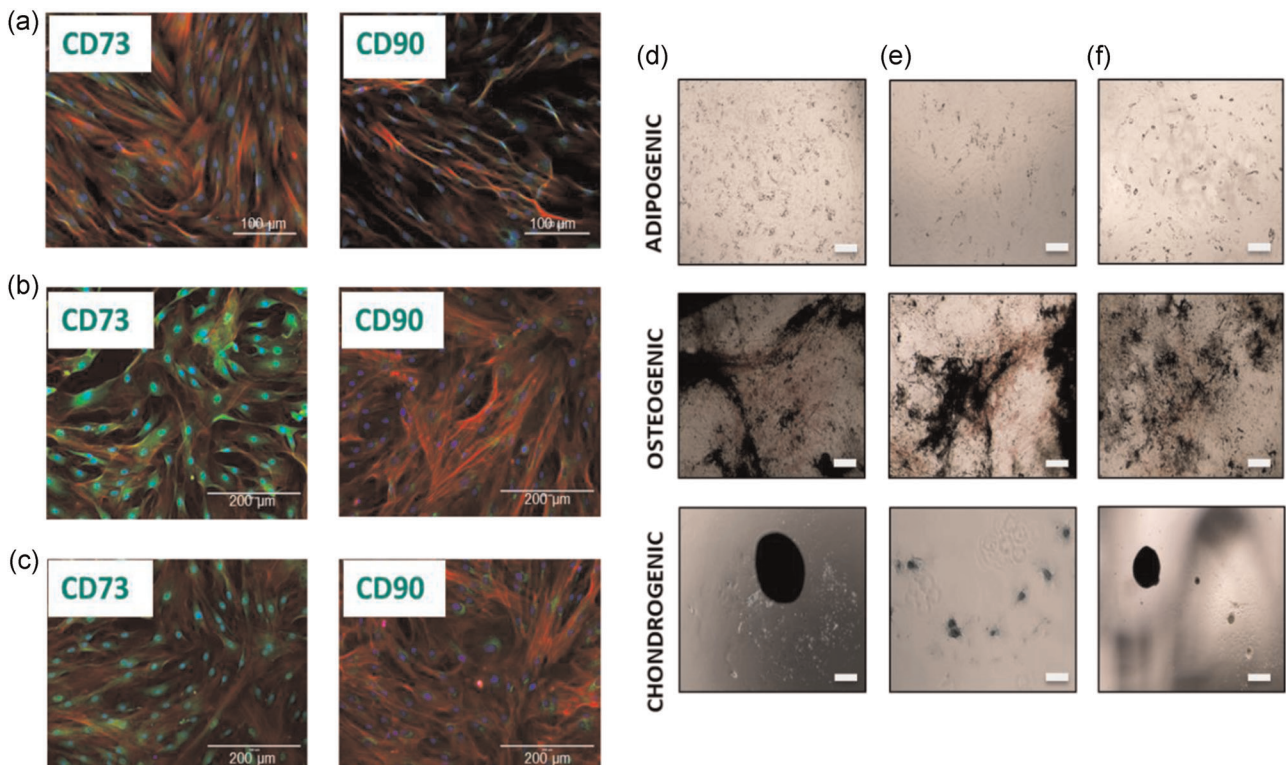


FIGURE 9 Cell quality assessment of bASCs pre- and post-expansion in the Mobius bioreactor. Immunocytochemistry staining for CD73 and CD90 markers expression (green), phalloidin (red) for cytoskeleton, and DAPI (blue) for nuclei for (a) pre-bioprocessing. Scale bar = 100 μm. (b) Post-bioprocessing in the “Baseline” bioprocess. Scale bar = 200 μm. (c) Post-bioprocessing in the “MC + volume” bioprocess. Scale bar = 200 μm. Differentiation towards the three lineages: adipogenic, osteogenic and chondrogenic for (d) pre-bioprocessing; (e) “Baseline” bioprocess and (f) “MC + volume” bioprocess. All scale bars for differentiation are 200 μm [Color figure can be viewed at wileyonlinelibrary.com]

scale compared to the spinner flasks. In the Mobius bioreactor, only a 50% medium exchange was possible due to the vessel configuration which resulted in media saving without compromising the final cell yield. Additionally, the final cell densities (cells/cm²) followed a similar pattern with the highest obtained at the litre scale and in the “MC + volume addition” conditions (Table 1, column 11) suggesting that in the intensified bioprocesses, better utilisation of surface area was achieved.

3.3 | Potential ways to further improve the intensified culture

Several issues were identified in these experiments when scaling up the “MC + volume addition” bioprocess in the Mobius bioreactor even though this process still gave the best performance. From Day 9 onwards, the frequency of feeding with only a 50% medium exchange could not prevent the decrease in glucose concentration to almost depletion. However, this issue could be easily resolved by either increasing the % of medium exchange from 50% to ~75% which with a 2 L operating volume would be possible in the Mobius bioreactor (and probably in other bioreactor designs). Alternatively, supplementation with glucose and/or other depleted nutrients could also be used, with the latter being more cost-effective. The pH was also found to drop below the physiological range in the intensified bioprocess particularly towards the end of the culture. However, this difficulty is again easy to address by controlling the pH through the addition of a base or acid, rather than just relying on the buffer system present in the medium. The very low DO levels reached towards the end of the intensified bioprocess could also be an issue. Aeration in these experiments was done via the headspace with no further supplementation with oxygen. This approach was found sufficient until Day 9 of culture, but not beyond when in the final stages of the culture, oxygen supplementation is necessary. However, it is also possible that the lower DO was one of the reasons for the improvement as reported in the growth of hMSCs and is certainly worthy of further investigation.

3.4 | Cell quality assessment pre- and post-bioprocessing

In the absence of tests proscribed for bovine ASCs and as discussed and justified in our earlier paper, cell quality was assessed using the guidelines proposed by Dominici et al. (2006). Pre- and post-bioprocessing, the bASCs retained their ability to differentiate towards adipogenic, osteogenic, and chondrogenic lineages. Moreover, in all conditions tested, the cells retained their expression of CD90 and CD73 markers (Figure 9).

4 | CONCLUSIONS

The first step in the production of cultivated meat is the expansion of the sourced cells. This study focused on the translation to the litre scale and intensification of a bioprocess for the expansion of bASCs as a source of

fat and muscle. This aim was achieved through a combination of surface area and volume addition during the culture. This approach took advantage of the bead-to-bead transfer phenomenon in which cells already attached to microcarriers have the ability to populate fresh microcarriers when added during culture. However, the time point for the addition is critical as the cells lose this ability when the level of aggregation is too high. Here, we found Day 5 to be the better time point for surface area addition compared to Day 7. Microcarrier addition was much more efficient when combined with volume addition rather than when the volume was kept constant, probably because with the “surface only” addition, the increased concentration of microcarriers per ml resulted in an increased probability of microcarrier collision during agitation with cell damage as a consequence. The “surface + volume addition” approach lead to significantly higher cell numbers in the same timeline in a spinner flask and to an even better performance in the well-instrumented Mobius bioreactor. Overall, intensification using the “surface + volume addition” in the bioreactor lead to the highest number of cells/cm² and the highest cells/ml and reduced the volume of media required for the production of 1×10^6 cells from about 15 ml in the baseline in the spinner flask to just under 5 ml. This bioprocessing approach could be implemented with other adherent cell types and other species for applications not only in cultivated meat production but also cell manufacturing for therapeutics.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Hanga and de la Raga have performed the experimental work and data analysis. Hanga wrote the first draft of the manuscript. All authors (except Hewitt) reviewed this manuscript, contributed, and approved the final manuscript. Hewitt provided guidance and advice on the experimental plan before his passing.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author (Hanga) upon reasonable request.

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