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Food-grade fungal pellets as edible scaffolds for bovine stem cell expansion

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ABSTRACT

The scalability of cultivated meat production depends on cost-effective, edible scaffolds that support attachment, proliferation and differentiation of adherent cells whilst meeting food safety and sensory requirements. However, most existing microcarriers are synthetic or of animal-origin, limiting their compatibility with food applications, increasing downstream processing costs, and raising ethical and environmental concerns associated with animal use. For the first time, we present mycelia-based microcarriers derived from food-grade *Penicillium* strains used in cheese production, as scaffolds for cultivated meat. Eight strains, including novel variants developed through non-GMO techniques (sexual breeding and ultraviolet mutagenesis), were screened for cytotoxicity using bovine adipose-derived stem cells. Out of these, four strains (*P. camemberti* Myc1; *P. roqueforti* Myc2, Myc3 and Myc4) were selected for further evaluation based on non-cytotoxic behaviour, ease of handling and pellet size comparable to commercial microcarriers. Morphological characterisation revealed that these strains form highly porous, fibrous pellets with estimated specific surface areas of approximately 4400–5100 cm^2/g, providing a favourable architecture for cell growth. All four microcarriers supported strong initial cell attachment, meeting or exceeding industry benchmarks for mesenchymal stem cells in both serum-containing and animal-free media. Growth kinetics diverged between strains, with Myc3 and Myc4 displaying the highest growth rates ($\geq$2-fold increase; $\mu \approx 0.015\ h^{-1}$), and doubling times of 47–48 h. These findings highlight the great promise of fungal pellets for the development of edible scaffolds for cultivated meat production, helping to address a central bottleneck in bringing affordable, high-quality protein to consumers.

1. Introduction

The world population is expected to reach approximately 9.8 billion by 2054, driving a continued rise in meat demand and, in turn, the need for sustained growth in global meat production (Becker & Fanzo, 2023; UN, 2024). Between 1961 and 2023, the meat output quadrupled, from around 90 million to over 360 million tonnes, and currently is not showing any signs of slowing down (Ritchie et al., 2023). Conventional animal agriculture already accounts for 80% of agricultural land use and contributes between 12 and 19.6% of global greenhouse gas emissions (FAO, 2023; Our World in Data, 2024; Pachauri & Meyer, 2014; Xu et al., 2021). Animal agriculture also consumes around 4387 km^3 of freshwater each year to produce the feed, forage and grazed biomass required by livestock, which is equivalent to 41% of all water used for growing agricultural biomass for human-related purposes, such as food, fibres and biofuels (Heinke et al., 2020). Considering the environmental resource demands of conventional livestock systems, the scale of the projected global appetite for meat raises important questions about the long-term sustainability of current production models. Consequently, alternative proteins, including cultivated animal, plant, fungal and microbial, have gained attention due to their potential to meet this protein demand with lower environmental impact, as well as with improved food safety and reduced ethical concerns (Jafarzadeh et al., 2024; Reynolds et al., 2023). Cultivated meat, produced *via* the *in vitro* expansion and differentiation of animal cells, is a particularly compelling alternative with the potential to decouple meat production from live animal farming (Chapman & Bamezai, 2025).

To achieve commercial viability of cultivated meat, large-scale culture of adherent cells is necessary (Bodiou et al., 2025). It is estimated that 1 ton of cultivated meat requires approximately 10^{13} cells, yet a 0.5

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g tissue biopsy from an animal yields only around 10,000 cells (Post et al., 2020). Roughly 30–40 doublings in a tightly optimised seed train, requiring sufficient surface area to support cell growth, are necessary to achieve those cell numbers. Traditional two-dimensional planar culture systems suffer from limited surface-to-volume ratios and are inherently unscalable. In contrast, microcarriers (MCs), suspended scaffolds that provide a surface for cell attachment when used in conjunction with stirred-tank bioreactors (STBs), offer a high surface-area-to-volume ratio and the ability to further increase cell production through bead-to-bead transfer upon the addition of fresh MCs (Bodiou et al., 2025; Hanga et al., 2020; Hanga et al., 2021; Moutsatsou et al., 2023; Rafiq et al., 2018). Although some progress has been made with aggregate-based systems, these are hindered by challenges in controlling aggregate size and ensuring adequate oxygen and nutrient transfer to the core of the aggregates (Bellani et al., 2020; Malm et al., 2020; Valdoz et al., 2021).

Despite their potential to enable commercial scalability, most existing MCs rely on synthetic or animal-derived materials, such as polystyrene or collagen-coated dextran, respectively (Deshayes et al., 2011; Hillegas et al., 2001). This presents a second set of challenges specific to cultivated meat: material compatibility with food systems. Although biodegradable alternatives have been developed, synthetic and/or biodegradable MCs are typically designed for biomedical use and not intended for consumption (Bomkamp et al., 2022; Handral et al., 2023). They often require removal from the final product, necessitating costly downstream processing and increasing the risk of product loss (Allan et al., 2019). Moreover, their inclusion in products intended for consumption raises regulatory challenges, as even trace amounts of non-edible material may compromise product safety and consumer acceptability, particularly under food legislation that demands full transparency of all ingredients (FSA, 2025; Moslemy et al., 2023; Siegrist et al., 2018).

To address these constraints, there is growing interest in edible, food-safe MCs that remain in the final product, potentially contributing to its nutritional and sensory qualities (Moslemy et al., 2023). However, the field remains nascent; only a few edible MCs have been reported in the literature, with examples including eggshell membrane, gelatin microparticles and decellularised mushroom blends, alongside a limited number of commercially available options (Andreassen et al., 2022; Gelatex, 2026; Norris et al., 2022; Smart MCs, 2026; Yang et al., 2025).

Filamentous fungi, a diverse group of fungi characterised by their thread-like hyphae that form complex networks known as mycelia, have long been used in food applications. For example, *Fusarium venenatum*, the fungus behind Quorn™, has been commercially utilised for decades to create alternative protein products (Wiebe, 2002). More recently, filamentous fungi have been explored as potential edible MCs for cultivated meat applications (Ogawa et al., 2022; Ogawa et al., 2024). In submerged culture, many food-grade strains naturally form spherical pellets and exhibit high specific surface area due to their fibrous hyphal surfaces that promote nutrient and oxygen transfer (Ogawa et al., 2024; Pirt, 1966). Moreover, fungal fermentation is low-cost, scalable and tunable; the three-day process can be adjusted to control the mycelial pellet and fibre size (Berger et al., 2022; Maseko et al., 2025).

Ogawa et al. (2024) presented the first proof-of-concept demonstrating that inactivated mycelial pellets derived from various fungal strains, including both food-grade and non-food-grade species of *Aspergillus* and *Rhizopus*, as well as *Penicillium chrysogenum* (a non-food-grade strain), were capable of supporting the growth of C2C12 mouse myoblasts and bovine satellite cells (bSCs). However, their study focused exclusively on myogenic cell types and relied on foetal bovine serum (FBS) and horse serum, which are not compatible with commercial food production.

The present study probes the suitability of *Penicillium roqueforti* and *Penicillium camemberti* strains as effective mycelia-based microcarriers (MMCs) for cultivated meat production. In particular, we focus on novel strains developed using non-genetically modified (non-GMO) techniques: sexual breeding and ultraviolet (UV) mutagenesis (Böhm et al., 2013; Myconeos, 2022; Ropars et al., 2012). These filamentous fungi are already recognised as safe for human consumption (Coton et al., 2020; Dumas et al., 2020; Ropars et al., 2020) and are widely used in the production of blue and white soft-ripened cheeses. Their established role in the food industry, alongside their filamentous morphology and ability to form pellets in submerged culture, makes them promising candidates for edible fungal-derived microcarriers. Accordingly, this study aims to evaluate the performance of *P. roqueforti* and *P. camemberti* strains as edible MMCs, and to explore how non-GMO methods can provide a practical, regulatory-aligned route for tailoring fungal scaffolds to support cell expansion and improve end-product characteristics.

Eight strains were initially provided by Myconeos Ltd. (UK), including several newly bred strains, created to present new enzymatic activities and traits such as texture, flavour and colour, which may offer additional value when incorporated into cultivated meat products. The strains were first screened for cytotoxic effects, pellet size comparable to commercial MCs and ease of handling. The best four candidates were taken forward for further morphological characterisation and cell attachment and proliferation experiments in static cultures using bovine adipose-derived stem cells (bASCs), a multipotent cell type with the ability to differentiate into fat tissues. In addition to serum-containing media, and to increase industrial relevance, cell growth was also tested in media supplemented with Proliferum® B PB01 (ProB; Multus Biotechnology, UK), an animal component-free (ACF) growth supplement specifically formulated for bovine cell culture.

2. Materials and methods

2.1. Materials

Bovine adipose-derived stem cells (bASCs) at passage 1 were purchased from Quest Meat Ltd. (UK). The cells were harvested from the intra-muscular fat of a 2-year old female British Blue cow. Newly bred filamentous fungi strains, some developed through UV mutagenesis, were provided by Myconeos Ltd. (UK). Minimum essential medium eagle-alpha modification (α-MEM; Gibco™), Dulbecco's phosphate-buffered saline (DPBS; Gibco™), trypan blue solution (0.4%; Gibco™), penicillin-streptomycin (10,000 U/mL; Gibco™), L-glutamine (200 mM; Gibco™), TrypLE™ Express (1×, phenol red; Gibco™), PrestoBlue™ cell viability reagent, premium foetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and paraformaldehyde solution (4% in PBS) were purchased from Thermo Fisher Scientific (UK). Recombinant human basic fibroblast growth factor (bFGF) was obtained from PeproTech (UK). Alexa Fluor™ 488 Phalloidin (Invitrogen™) and Hoechst 33342 (20 mM; Invitrogen™), LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen™), ethanol (99.8%) and HPLC-grade methanol were sourced from Fisher Scientific Ltd. (UK). Potato dextrose broth (PDB; Merck, UK) and potato dextrose agar (PDA; Merck, UK), used for fungal culture, were purchased from Scientific Laboratory Supplies (UK). Proliferum® B PB01 (ProB), an animal-free medium, was a gift from Multus Biotechnology (UK). Deionised water was used throughout, unless otherwise specified.

2.2. Production of mycelia-based microcarriers (MMCs)

Fresh potato dextrose agar (PDA) slopes were inoculated with spores from eight *Penicillium roqueforti or Penicillium camemberti* fungal strains (Myc1-Myc8; Table 1) using a cotton swab and grown for 7 days at 25 °C. Spores were then harvested and suspended in DPBS. The suspensions were vortexed for 15 s, diluted, and subsequently quantified using an improved Neubauer counting chamber (BLAUBRAND®, Germany).

Spore seeding densities for Myc2-Myc8 were selected to produce pellets with size distributions visually comparable to those of commercial microcarriers (typically ranging from 100 to 400 μm) whilst still being manageable for early-stage experiments (Rafiq et al., 2016). An

Table 1
Overview of the fungal strains investigated in this study. All strains were obtained from Myconeos Ltd. (UK) and are proprietary to the company; therefore, no literature references are available.

Identifier	Species	Comments
Myc1	*Penicillium camemberti*	Used to make camembert cheese.
Myc2	*Penicillium roqueforti*	A UV mutagenesis strain used to make Roquefort cheese; one of Myconeos's first-generation bred strains, characterised by a dark blue pigmentation, medium proteolytic activity and high lipolytic activity.
Myc3	*Penicillium roqueforti*	A UV-mutagenesis strain used to make Roquefort cheese; derived from one of Myconeos's first-generation bred strains, displaying dark blue pigmentation, low proteolytic activity and medium lipolytic activity.
Myc4	*Penicillium roqueforti*	A UV-mutagenesis strain used to make Roquefort cheese, derived from one of Myconeos's first-generation bred strains, characterised by green spores, medium proteolytic activity and medium lipolytic activity.
Myc5	*Penicillium camemberti*	Used to make camembert cheese.
Myc6	*Penicillium roqueforti*	A first generation bred strain used to make Roquefort cheese; characterised by a dark blue pigmentation, medium-low proteolytic activity and medium lipolytic activity.
Myc7	*Penicillium roqueforti*	A first generation bred strain used to make Roquefort cheese; characterised by a dark blue pigmentation, medium-low proteolytic activity and medium lipolytic activity.
Myc8	*Penicillium roqueforti*	A UV-mutagenesis strain used to make Roquefort cheese; derived from one of Myconeos's first-generation bred strains, producing white spores with medium proteolytic and medium lipolytic activity.

additional strain (Myc1), which produced larger pellets under the same fermentation conditions, was also selected to assess the potential handling and performance benefits of an increased pellet size. Spore suspensions were diluted to a final concentration of 2×10^6 spores/mL. Liquid cultures were inoculated using these suspensions in 50 mL of PDB in 250 mL conical flasks and incubated in a SciQuip Benchtop Incubator Shaker (SciQuip, UK) at 25 °C with shaking at 150 rpm for 72 h. The resulting fungal pellets were subsequently autoclaved at 121 °C for 20 min before washing and then stored in sterile DPBS at 4 °C for a maximum of 1 month. To check for inactivation, 10 randomly chosen pellets were assessed for growth by incubating them on PDA agar plates in a MIR-154-PE Cooled Incubator (PHC Europe B.V., UK) for 7 days at 25 °C.

2.3. *Physical characterisation of MMCs*

To calculate the average pellet size, a known quantity of MMCs was suspended in water within petri dishes placed alongside a ruler for scale. Images were taken from a consistent height using a tripod-mounted camera to maintain uniformity, and the diameters of at least 400 individual pellets were measured using ImageJ software (National Institutes of Health, USA). The wet mass of the MMCs was determined by straining the pellets through a fine mesh sieve (Amazon, UK), blotting them on filter paper, weighing them on pre-weighed petri dishes, flash-freezing them at −80 °C overnight and then freeze-drying them in a Lablyo Mini Freeze Dryer (Lablyo, UK) for 4 h at −50 °C under vacuum conditions maintained between 0 and 1 mbar. Dry weights were recorded post-lyophilisation to calculate the wet-to-dry conversion factors for each strain.

Surface area (A) was calculated by treating each pellet as a perfect sphere with a diameter equal to the average measured pellet diameter (d), using **Eq.1**:

$$A = \pi d^2 \tag{1}$$

The surface morphology of the freeze-dried mycelial pellets was examined using a Thermo Scientific Phenom Pro Desktop Scanning Electron Microscope (SEM) (Thermo Scientific, USA). Samples were imaged in high vacuum mode without the application of any conductive coating. Images were obtained at 5–15 kV using a back-scattered electron detector (BSD) and secondary electron detector (SED). The pellets were mounted on aluminium SEM stubs using double-sided carbon adhesive tape. ImageJ software (National Institutes of Health, USA) was employed to measure the fibre diameters, with an average calculated from 100 measurements ($n = 100$).

2.4. *Biological characterisation of MMCs*

2.4.1. *Cell culture*

The bASCs were cryopreserved in freezing medium comprising 10% *v*/v DMSO in FBS under liquid nitrogen and thawed in a bead bath. The cells were subsequently cultured in complete growth medium comprising α-MEM supplemented with 10% (*v*/v) FBS, 1% (v/v) penicillin-streptomycin, 1 ng/mL basic fibroblast growth factor (bFGF) and 2 mM L-glutamine (Hanga et al., 2020). Cultures were maintained at 37 °C with 5% CO₂. Cells between passages 4 and 5 were employed in all experiments. A full medium exchange was performed 24 h after the initial seeding into tissue culture flasks and subsequently every 2 days until cells reached 80–90% confluency (typically around Day 5). Confluency was estimated using an EVOS™ M5000 fluorescent microscope (Thermo Fisher Scientific, UK). Cells were passaged using TrypLE™ Express Enzyme (1×, phenol red) and incubated for 8 min at 37 °C with 5% CO₂, until complete detachment was observed. The enzymatic reaction was neutralised by the addition of growth medium and cells were centrifuged at 200 *g* for 6 min using an Eppendorf 5702 centrifuge (Sigma-Aldrich, UK). The supernatant was carefully discarded and the resulting cell pellet was resuspended in a known volume of culture medium. Cell viability and density were assessed using a 1:1 dilution of the cell suspension with 0.4% (v/v) trypan blue and counted using a haemocytometer (Brightline, Hausser Scientific, USA).

2.4.2. *Cytotoxicity assay*

To assess potential cytotoxic effects, bASCs were seeded into standard 6-well tissue culture-treated plates (Corning™ Costar™) at a density of 5000 cells per cm^2. Once the cells had formed a monolayer, autoclaved mycelial pellets (Myc1-Myc8) were weighed to 1 g per well, placed into Falcon® cell strainer inserts (70 μm pore size; Fisher Scientific, UK) and lowered into the wells. This setup physically separated the scaffolds from the cells to facilitate post-experiment visualisation, whilst allowing hyphal fragments or any residual components released from the inactivated mycelia to diffuse into the culture medium. Cells were incubated with the fungal samples for 48 h at 37 °C with 5% CO₂.

Due to the significant amount of visible fungal debris released into the medium during incubation, each well was washed three times with DPBS prior to imaging. This step helped reduce background interference and allowed for a clearer observation of cell morphology and confluence. Following washing, wells were imaged using an EVOS™ M5000 microscope (Thermo Fisher Scientific, UK). Cytotoxicity was evaluated by comparing test conditions to both positive and negative controls. The negative control included bASCs cultured without any mycelial material, whilst the positive control included wells containing cells and 70% (v/v) ethanol. Strains were classified as non-toxic if bASCs exhibited high densities, elongated morphologies with visible cell-cell interactions and clear signs of substrate attachment and spreading, comparable to the negative control. Strains were considered potentially cytotoxic if cells remained rounded, sparse or failed to adhere.

2.4.3. MMC preparation prior to cell seeding

Although live mycelial pellets may hold promise for future co-culture applications, this study focused on evaluating their performance as inactivated scaffolds. Therefore, all of the mycelia-based microcarriers (Myc1-Myc4) were autoclaved prior to cell seeding. This step ensures biosafety and regulatory compliance, whilst also halting further fungal growth and ensuring consistency across experiments. Prior to cell seeding, autoclaved MMCs were washed with sterile DPBS followed by complete growth media, then conditioned by incubation in ultra-low attachment 24-well plates (PrimeSurface® 3D culture; PHC Europe B. V., UK) submerged in growth media at 37 °C and 5% CO_2 for 2 h. To enable reproducibility and meaningful comparison between strains, pellet quantities were standardised by wet mass, with 0.045 g per well empirically selected as it provided adequate base coverage without overcrowding.

2.4.4. Cell proliferation assay

Cell proliferation on the MMCs (Myc1-Myc4) was quantified using PrestoBlue®, a widely used, resazurin-based metabolic assay, which allows for non-destructive, real-time measurements of cell viability and growth. Cells were seeded onto the MMCs at 8.1×10^4 cells/mL, equivalent to 1.8×10^6 cells per gram wet weight of MMCs. Proliferation was assessed over a five-day period. At each timepoint, 10% (*v*/v) PrestoBlue® was added to each well and fluorescence was measured at an excitation of 544 nm and emission of 590 nm using a FLUOstar Omega plate reader (BMG LABTECH, Germany). Following each assay, the residual PrestoBlue solution was aspirated from each well and the scaffolds were gently rinsed twice with DPBS and once with fresh complete medium. Fresh growth medium was then added before returning the plates to the incubator. During the second biological repeat, a parallel comparison was performed to evaluate the performance of ProB animal-free supplement. Each of the four MMC strains (Myc1-Myc4) was cultured under two media conditions (α-MEM supplemented with 10% FBS (control) and α-MEM supplemented with 10% ProB) using the same seeding density and tested in triplicates ($n = 3$).

Key cell growth kinetics were calculated to evaluate MMC performance, based on quantified cell concentrations obtained from PrestoBlue fluorescence readings.

1. Cell attachment efficiency (η_a)

Cell attachment efficiency (**Eq.2**) was calculated by dividing the cell concentration measured at Day 1 (C_{x1}) by the initial seeding density (C_{x0}), providing a measure of the proportion of cells successfully adhering to the MMCs within the first 24 h.

$$\eta_{a=}\frac{C_{x1}}{C_{x0}} \tag{2}$$

2. Fold increase (F_i)

The fold increase (**Eq.3**) was determined by dividing the final cell concentration at Day 5 (C_{x5}) by the cell concentration at Day 1 (C_{x1}), representing the overall expansion of the cell population.

$$F_i = \frac{C_{x5}}{C_{x1}} \tag{3}$$

3. Specific growth rate (μ)

The specific growth rate (h^{-1}) quantifies the exponential growth of the cell population and was calculated using **Eq.4**:

$$\mu = \frac{\ln(C_{x5}) - \ln(C_{x1})}{t}, \tag{4}$$

where C_{x1} and C_{x5} are the cell concentrations at Days 1 and 5, respectively, and t is the elapsed time in hours.

4. Population doubling time (D_t)

The population doubling time was calculated to determine how long it takes for the cell population to double in number, using **Eq.5:**

$$D_t = \frac{\ln(2)}{\mu} \tag{5}$$

2.4.5. Fluorescent imaging

Cell attachment, morphology and distribution across the MMC surfaces was visualised using fluorescent imaging. For the initial two biological repeats assessing bASC proliferation across the four fungal strains, cells were fixed using ice-cold methanol (−20 °C). At the end of the culture period, the media was aspirated, and the samples were gently washed with DPBS. Ice-cold methanol was then added directly to each well and incubated for 10 min at −20 °C. Samples were subsequently washed three times with DPBS and stained with Hoechst 33342 for 7–10 min at room temperature, protected from light. After staining, wells were washed three times with DPBS and imaged immediately. Samples were imaged at 10× magnification in the DAPI and TRANS channels of a fluorescent microscope (EVOS M5000, Thermo Fisher Scientific, UK). Cells fixed and permeabilised served as negative controls. Images were merged and processed using ImageJ software (National Institutes of Health, USA).

For the experiment comparing FBS and ProB, cells were fixed using 4% PFA. Culture media was aspirated and 4% PFA solution was added to each well and incubated for 15 min at room temperature. Samples were then washed three times with DPBS and permeabilised using 0.1% Triton X-100 in DPBS, incubating for 15 min at room temperature, followed by three DPBS washes. Dual staining was performed using Hoechst 33342 for nuclei (1:2000 dilution) and Alexa Fluor™ 488-conjugated phalloidin for F-actin. Samples were incubated with phalloidin for 45 min at room temperature in the dark, washed three times with DPBS and imaged immediately. Samples were imaged at 10× magnification in the DAPI, GFP and TRANS channels of a fluorescent microscope (EVOS M5000, Thermo Fisher Scientific, UK). Cells fixed and permeabilised served as negative controls. Images were merged and processed using ImageJ software (National Institutes of Health, USA).

2.5. Statistical analysis

All primary cells used in this study were derived from the same donor animal. Experimental assays were performed with three replicates and repeated twice to generate two biological replicates. Statistical analysis was conducted using JMP® Student Edition (JMP Statistical Discovery LLC, USA). Results are expressed as the sample mean, calculated as the sum of all data points divided by the total number of data points, and the standard error of the mean (SEM), defined as the standard deviation (SD) divided by the square root of the sample size. Differences between groups were assessed using one-way analysis of variance (ANOVA), with a p-value of <0.05 considered statistically significant. Where significant differences were found, Tukey's honestly significant difference (HSD) test was applied to identify pairwise comparisons. For boxplot visualisations, JMP defines outliers according to Tukey's method: data points lying more than 1.5× the interquartile range beyond the upper or lower quartile are displayed as outliers.

3. Results and discussion

This study explored the potential of food-grade mycelial pellets as scalable, edible microcarriers for the attachment and proliferation of bASCs. Following an initial screening of eight fungal strains, four suitable candidates were identified for further development, based on cytotoxicity data and ease of handling. The selected MMCs were further characterised for size and morphology and then used to culture bASCs under static conditions in serum-supplemented media. The microcarrier ability to support bASCs attachment and proliferation was also tested in animal component-free (ACF) media, to assess the industrial relevance of these MMCs under xeno-free conditions.

3.1. Cytotoxicity screening of fungal strains

Cytotoxicity screening was conducted on eight food-grade filamentous fungal strains to evaluate their suitability as MMCs. As noted by Antinori et al. (2021), autoclaved fungi may release residual metabolites or surface compounds into the culture environment. Therefore, testing for potential toxic effects is a logical first step in the initial screening of the strains, since it can help predict future issues with cell performance, as well as ensure the safety and acceptability of these materials as part of a cultivated meat product.

Strains were classified as non-cytotoxic if, after 48 h of co-incubation, they supported healthy bASC morphology, characterised by elongated, spindle-like shapes with evidence of cell-cell interaction, comparable to the negative control (bASCs cultured without mycelial pellets; **Fig. S1**), with ≥70% viability as defined by ISO 10993-5. All of the strains tested (Figs. 1 and 2) demonstrated non-cytotoxicity, and, in some cases, despite thorough DPBS washes, hyphal fragments appeared to remain attached to the cells (**Fig. S2**), indicating the presence of cell-surface interactions. Strains Myc5, Myc6, Myc7 and Myc8 (Fig. 2) were not carried forward for further experiments. The size distribution of Myc5 was very wide, whereas Myc8 was in the form of big clumps. For Myc6 and Myc7, there were no available fresh slopes from Myconeos Ltd. at the time of the experiments and therefore it was not possible to culture additional pellets for subsequent studies. Strains Myc1-Myc4 (Fig. 1) were therefore carried forward for additional evaluation, based on their non-cytotoxic behaviour, ease of handling and average pellet size comparable to commercial microcarriers (achieved through optimised fermentation conditions). These properties are highly desirable for future scalability of production and regulatory approval.

Safety is an important factor when evaluating fungal-derived materials intended for use in cultivated meat production. Unlike conventional biomedical MCs, MMCs are expected to remain within the final food product; therefore, regulatory compliance must be considered from an early stage of material selection. Importantly, the use of strains already established within the food industry represents a key advantage, as prior use provides a degree of safety precedent and may reduce regulatory uncertainty compared with entirely novel biomaterials. In addition, the autoclaving step used prior to cell culture renders the mycelial pellets biologically inactive, reducing risks associated with viable fungal material, including further growth or spore production. The absence of observable cytotoxic effects following co-culture suggests that no acutely harmful effects were evident under the conditions tested, supporting the suitability of these strains for further development. Whilst regulatory frameworks for cultivated meat emphasise the overall safety and traceability of ingredients (FSA, 2025), cytotoxicity screening represents an early and relevant step within a broader safety-by-design approach.

3.2. Characterisation of fungal pellets

The morphology and surface topography of microcarriers can have a major influence on key aspects of cell behaviour, including attachment, migration, proliferation and differentiation, as well as on oxygen and nutrient transfer, and mixing performance inside the stirred tank bioreactor environment. In the case of MMCs, pellet morphology is influenced by multiple factors, including fungal species and fermentation parameters such as inoculum concentration and agitation rate (Cairns et al., 2022; Veiter et al., 2018). As can be seen from Fig. 3, there is considerable variability between the four selected MMCs, with Myc1 having the largest average pellet diameter (1230 μm ± 412 μm), whilst Myc3 formed the smallest pellets (583 μm ± 163 μm). Since each fungal strain was cultured under standardised fermentation conditions, differences in species identity likely contributed to the observed variation in pellet size and structure.

Furthermore, Myc1 (Fig. 3A) exhibited the widest distribution of pellet diameters amongst the strains tested, with sizes ranging from 460 μm to 3270 μm, and an interquartile range (IQR) of 610 μm (Q1 = 900 μm, Q3 = 1510 μm), indicating substantial variability. In contrast, Myc3 (Fig. 3C) showed the narrowest size distribution, with diameters between 390 μm and 1560 μm and an IQR of just 170 μm (Q1 = 480 μm, Q3 = 650 μm). This narrow spread, with only 14 outliers above 905 μm (3.45% of the dataset), also suggested low batch-to-batch variability, and therefore reproducibility of manufacture which is highly desirable. For Myc2 (Fig. 3B) the high number of smaller pellets, resulted in a

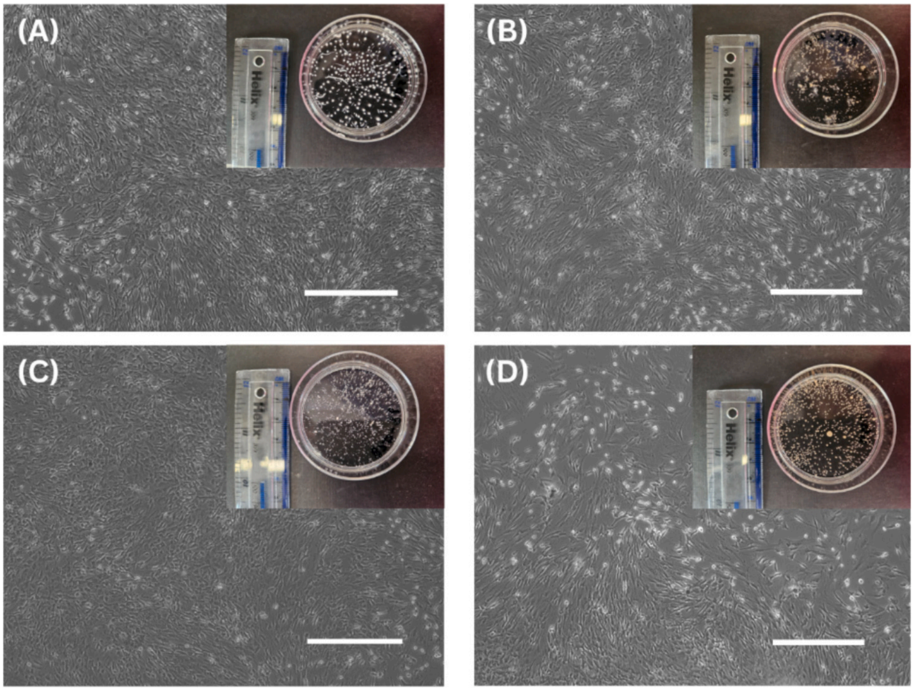

Fig. 1. Microscopy images of bASCs following 48 h of contact with MMCs, and photographs of the mycelial pellets (inserts): (A) Myc1; (B) Myc2; (C) Myc3; (D) Myc4. Scale bars: 700 μm.

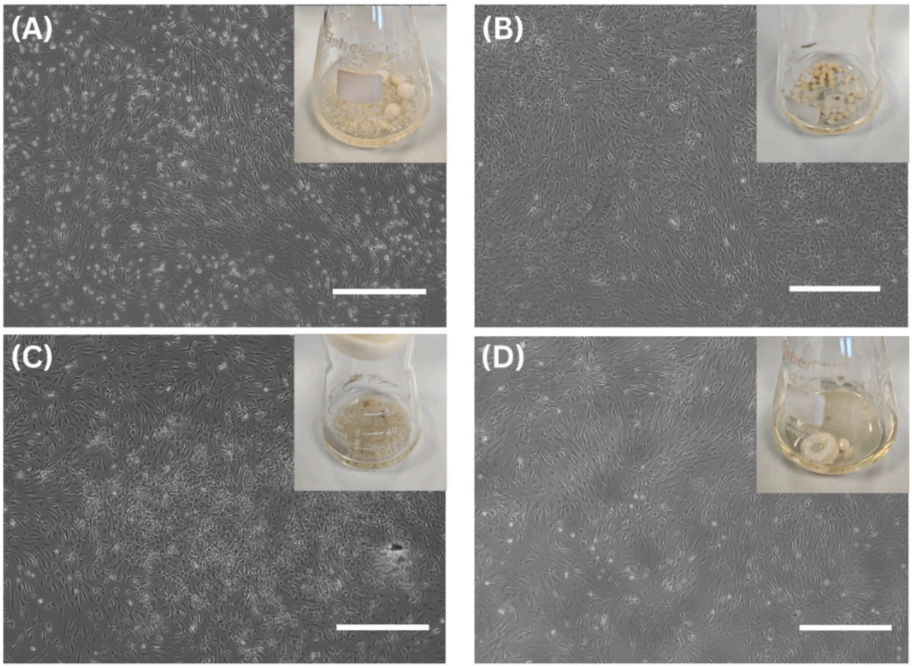

Fig. 2. Microscopy images of bASCs following 48 h of contact with MMCs, and photographs of the mycelial pellets (inserts): (A) Myc5; (B) Myc6; (C) Myc7; (D) Myc8. Scale bars: 700 μm.

right-skewed distribution, with a high concentration of smaller pellets (Q1 = 500 μm, Q3 = 790 μm) but a broader tail extending up to 2310 μm. Myc4 (Fig. 3D) presented a moderately symmetrical distribution, with most pellet diameters falling between 712 and 900 μm, indicating more consistent sizing but with some notable outliers. Some degree of heterogeneity can be beneficial, particularly in dynamic suspension systems, where variation in pellet size may enhance flow dynamics (Markvicheva & Grandfils, 2004). However, excessive variation risks inconsistent cell seeding and uneven nutrient diffusion (Veiter et al., 2018). From a bioprocessing perspective, the more uniform size distributions observed in Myc3 and Myc4 could be advantageous, as they offer greater standardisation and may reduce batch-to-batch variability.

To quantify the specific surface area of the MMCs, pellet diameter measurements were used to estimate surface area under the assumption of smooth spherical geometry, an approach similarly adopted by Ogawa et al. (2024). However, this assumption likely underestimates the actual surface area of the MMCs, due to the fibrous and irregular outer architecture of the pellets, which cannot be easily captured by standard surface area calculations. As such, whilst this method allowed for comparisons between the strains tested, it remains a limitation when benchmarking against commercial MCs. The values were subsequently normalised using the average dry mass per pellet, yielding the following estimated specific surface areas (total surface area per gram of dry material): 4533 cm^2/g (Myc1); 5098 cm^2/g (Myc2); 4463 cm^2/g (Myc3); 4389 cm^2/g (Myc4). For comparison, Cytodex 3, a commercially available MC commonly used to support the growth of MSCs, offers a surface area of 2700 cm^2/g (Cytiva, 2026; Rafiq et al., 2016). This enhanced surface area per unit mass is highly desirable when scaling up microcarrier-based systems because of its influence on cell attachment and subsequent yields (Van Beylen et al., 2021).

SEM was employed to further characterise the topography of the MMCs (Echeverria Molina et al., 2021; Xiao et al., 2023) and the images are presented in Fig. 4. The fungal pellets are highly porous, fibrous spheres composed of hyphae with diverse branching patterns, giving the MMCs a distinctive 'hairy' appearance. These fine structures significantly increase the functional surface area available for cell attachment. The hyphae fibres seem well-preserved following autoclaving, indicating some level of structural integrity and suitability for use as edible MCs. The irregular fibrous morphology of MMCs may also provide a more favourable topography compared to the high curvature of conventional MCs, which is known to negatively influence cell attachment and growth (Lee & Yang, 2017; Werner et al., 2017).

Through visual observation, all strains demonstrated rapid sedimentation rates, which is advantageous for efficient solid-liquid separation processes in bioreactor applications (Hewitt et al., 2011). It was also noted that occasionally, following autoclaving, there was some pellet fragmentation, which led to the presence of wispy debris and some irregular MMC shapes, that persisted throughout the culture period despite repeated washing steps. In filamentous fungal systems, pellet morphology and density are known to influence resistance to shear and collision-induced breakage, and fragmentation often occurs when mechanically stressed hyphae have first undergone structural or physiological weakening (Papagianni, 2004). In the present study, thermal sterilisation may have contributed to this effect by reducing inter-hyphal cohesion, thereby increasing the likelihood of peripheral hyphal detachment during subsequent handling. In some cases, pellets were sterilised more than once to enable storage and repeated downstream handling, which may have further altered structural integrity; although this effect was not quantified here, it highlights a potential process variable that should be minimised or carefully controlled in future studies.

From a bioprocessing perspective, excessive fragmentation may reduce reproducibility and complicate downstream processing. This is particularly relevant for future translation to stirred bioreactor systems, where hydrodynamic shear and particle-particle collisions are expected to impose additional mechanical stresses on the MMC structure. However, limited hyphal shedding may not necessarily be detrimental. Fragmentation may also increase the total available surface area within the culture, potentially creating additional sites for cell attachment and growth during the bioprocess. In MC-based suspension cultures, bead-to-bead transfer (the migration of adherent cells between carriers) is considered an important mechanism for process intensification, enabling increased cell yield and reduced process time through efficient surface area utilisation (deSoure et al., 2016; Hanga et al., 2021; Rafiq et al., 2018; Takahashi et al., 2017). It is therefore plausible that detached hyphal fragments could act as transient bridging structures,

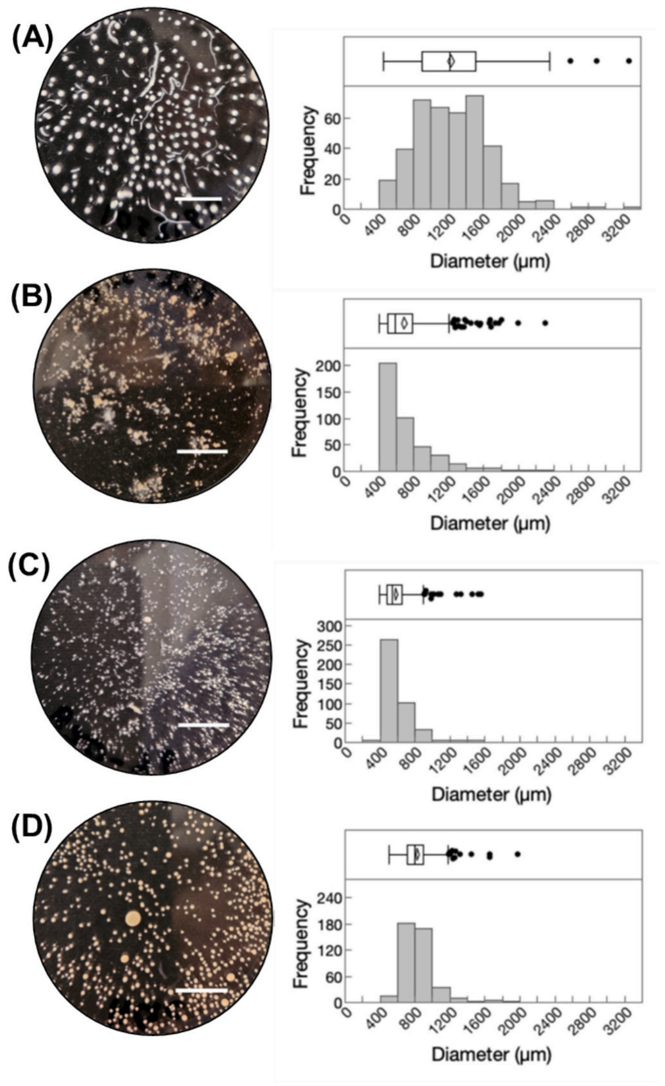

Fig. 3. Light microscopy images (left-hand side) and accompanying histograms and box plots (right-hand side) displaying size distribution of mycelial pellets for (A) Myc1, (B) Myc2, (C) Myc3 and (D) Myc4. Mean pellet diameters were 1230 ± 412 μm, 693 ± 285 μm, 583 ± 163 μm and 827 ± 167 μm respectively (n = 400). In the boxplots, the central line indicates the median, the box represents the interquartile range, whiskers show the data range excluding outliers, the diamond denotes the sample mean and the outliers are displayed as black dots. Scale bars: 1 cm.

potentially facilitating inter-pellet cell transfer under stirred conditions. Nevertheless, achieving an appropriate balance between structural robustness and controlled fragmentation will be important for future optimisation and scale-up under hydrodynamic shear. Cheng et al. (2023) suggested that modifying the media composition during liquid fermentation can enhance structural uniformity, should mycelia breakage become an issue. They demonstrated that altering the composition of the media influenced pellet rigidity and helped minimise fragmentation following autoclaving. Although this is beyond the scope of the present study, assessing mycelial microcarrier rigidity under dynamic conditions will form part of our future work on scale-up to spinner flasks.

The size distributions of the hyphal diameters for the four selected MMCs are displayed in Fig. 5. Myc1 exhibited the narrowest distribution, with a mean fibre diameter of 1.84 ± 0.46 μm and only two outliers above the upper whisker, suggesting a relatively homogeneous fibrous structure. In contrast, Myc4 had the thickest fibres on average (2.78 ± 0.67 μm) and demonstrated a more symmetrical, bell-shaped distribution. Myc2 and Myc3 displayed intermediate values, with slightly broader distributions and a small number of high-end outliers.

Across all strains, hyphae appeared randomly orientated in the SEM images (Fig. 4), contributing to a highly porous, mesh-like structure. These differences in fibre thickness and arrangement may influence the compactness and porosity of the pellets (Papagianni, 2004; Pirt, 1966), which subsequently govern key bioprocess parameters such as nutrient and oxygen diffusion, waste removal and overall mass transfer efficiency within the scaffold structure (Dehghani & Annabi, 2011). From a mechanical perspective, more compact architectures may offer improved structural stability but reduced accessible surface area, whereas more porous networks may enhance cell accessibility at the expense of robustness under agitation. Fibre thickness may also influence cell-material interactions at the microscale, as thinner fibres generate higher local curvature and a more complex topography, whilst thicker fibres may provide flatter attachment regions that support greater cell spreading (Lee & Yang, 2017). Since surface topography and mechanical cues are known to modulate cell adhesion and cytoskeletal organisation (Xiao et al., 2023), variation in hyphal architecture may partially explain the strain-dependent variation in bASCs performance observed in subsequent culture assays.

3.3. *Cell culture performance on mycelia microcarriers in serum-containing media*

Whilst factors such as pellet size, fibre thickness and surface topography are known to influence mass transfer and cell behaviour, these features must ultimately translate into significant cell growth to be industrially relevant. Therefore, we next evaluated how these physical differences impacted biological performance, focusing on the following key metrics: cell attachment efficiency, fold increase in metabolically active cells, specific growth rate and doubling time. Given the novelty of MMCs and their structural divergence from conventional microcarriers, these experiments were designed to benchmark performance across strains rather than against commercial standards. Nevertheless, the growth profiles observed across all four strains suggest that MMCs may offer performance comparable to established systems.

All four MMCs demonstrated strong initial cell attachment on Day 1 (Fig. 6), with efficiencies that matched or exceeded commonly reported industry benchmarks for MSCs (typically 70–90%; Chen et al., 2011; Chui et al., 2018; Tsai et al., 2020). This suggests that, despite their lack of surface functionalisation, the MMCs offer a compatible surface for bASCs adhesion. Myc2 exhibited the highest attachment efficiency of 0.96, followed by Myc3 (0.91), whilst Myc1 demonstrated the lowest (0.72). The high initial attachment observed for Myc2 and Myc3 may be attributed to differences in cell-surface interaction sites, such as the abundance or accessibility of adhesion-promoting motifs like arginine-glycine-aspartic acid (RGD), which are widely found in the Fungi kingdom (Teo et al., 2023) and facilitate integrin-mediated binding (Bodiou et al., 2020; Derakhti et al., 2019; Ruoslahti & Pierschbacher, 1986). Such differences could arise from strain-specific variations in surface protein expression or hyphal structure. Myc3 and Myc4 are both derived from the same parent strain yet displayed distinct attachment efficiencies, suggesting that even closely related strains can exhibit markedly different cell-interactive properties. By contrast, Myc1 (a different species) may present fewer accessible binding motifs or a less favourable surface chemistry for rapid adhesion.

Since initial attachment directly impacts the number of cells available for subsequent proliferation, strong early adhesion is generally considered advantageous for expansion (Bock et al., 2009). However, in our study herein, high initial attachment did not always translate into sustained growth. Despite Myc2 exhibiting the highest Day 1 attachment, it supported comparatively limited proliferation over the following days (**Fig. 7**). This suggests that whilst initial cell attachment to surfaces is important, it does not solely determine the ability of an

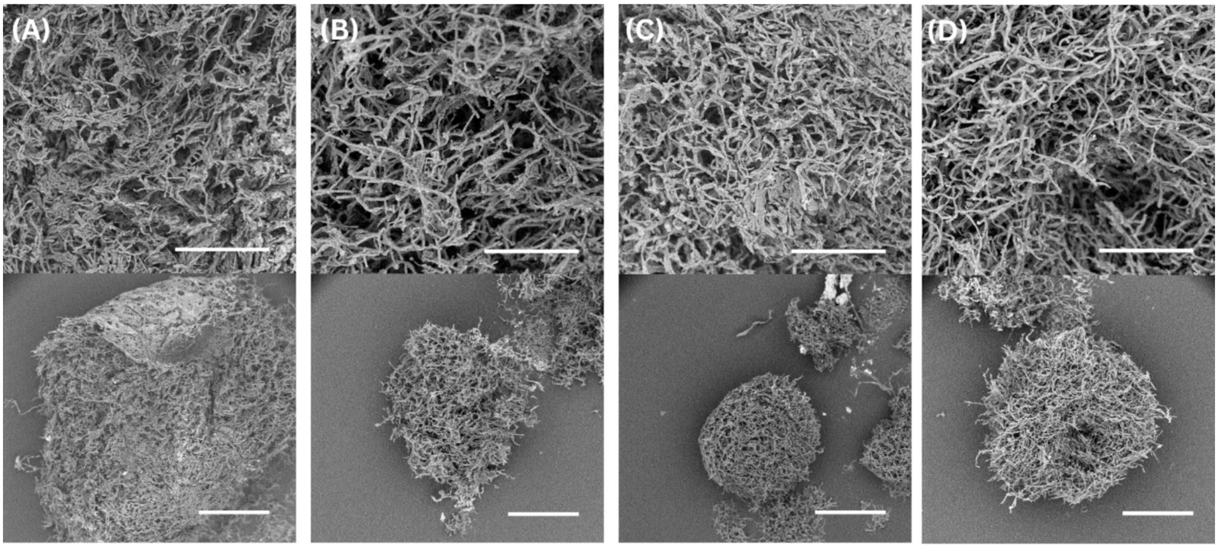

Fig. 4. SEM images of hyphal fibres (top) and whole mycelial pellets (bottom): (A) Myc1; (B) Myc2; (C) Myc3; (D) Myc4. Scale bars: 100 μm (top), 300 μm (bottom).

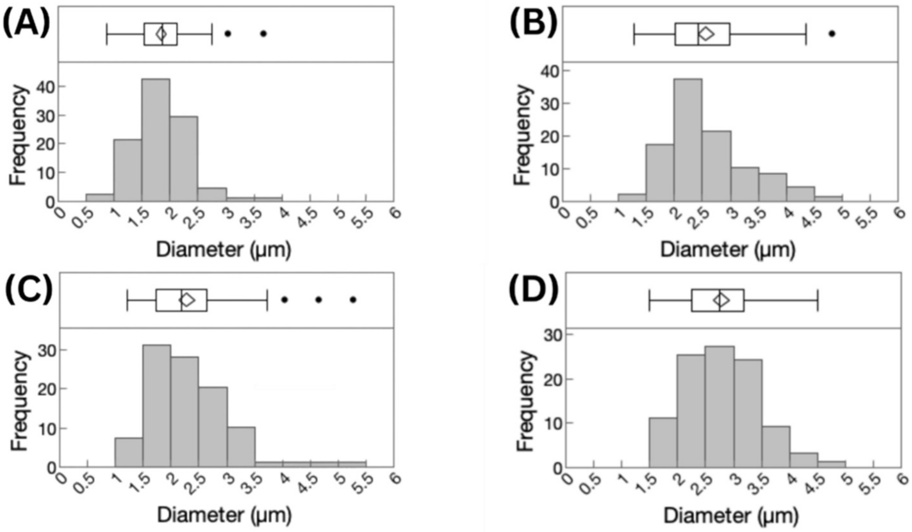

Fig. 5. Histograms and boxplots displaying size distribution of fibres for (A) Myc1, (B) Myc2, (C) Myc3 and (D) Myc4. Mean fibre diameters were 1.8 ± 0.5 μm, 2.6 ± 0.7 μm, 2.3 ± 0.7 μm and 2.8 ± 0.7 μm respectively (n = 100). In the boxplots, the central line indicates the median, the box represents the interquartile range, whiskers show the data range excluding outliers, the diamond denotes the sample mean and the outliers are displayed as black dots.

MMC to support expansion. Other factors, such as biophysical and biomechanical cues, likely play a prominent role in guiding sustained growth on MMCs (Naqvi & McNamara, 2020). For example, Myc2 exhibited a comparatively larger estimated surface area and relatively thick hyphal fibres, which may initially promote high attachment by providing abundant accessible surface. However, they may also result in excessive spatial dispersion of cells across the carrier surface. Increased cell spacing can limit cell-cell contact and reduce the local accumulation of paracrine signalling factors, both of which are important for sustaining MSC proliferation (Boland et al., 2013; McMurtrey, 2017). In addition, thicker fibres and a highly porous structure may alter local cell spreading and cytoskeletal tension, potentially negatively influencing downstream proliferation responses (Engler et al., 2006).

All strains showed a significant increase in metabolically active cells from Day 1 to Day 3 (**$p < 0.01$ or ***$p < 0.001$), followed by a plateau between Day 3 and Day 5 (Fig. 7). ANOVA and Tukey's HSD tests confirmed that this trend was statistically consistent across all selected strains. The proliferation plateaus observed across all four selected strains may indicate limitations in expansion capacity under the given experimental conditions or alternatively, reflect the onset of confluency, where available surface area becomes saturated with adherent cells. Seeding density calculations revealed that all MMCs were seeded at very low densities (~350–410 cells/cm^2), which are considerably less than the 3000–5000 cells/cm^2 typically used with commercial MCs such as Cytodex 3, and future studies will explore whether optimising the seeding density could enhance proliferation. It is also plausible that the large surface area of the MMCs, as previously discussed, caused excessive dispersion of cells, limiting cell-cell contact and reducing proliferation cues such as paracrine factor accumulation (Boland et al., 2013). Inadequate intercellular communication is a known constraint for mesenchymal stem/stromal cells, which rely on direct contact and local biochemical gradients to sustain proliferation (McMurtrey, 2017).

Growth kinetics analysis further highlighted the strong performance of Myc3 and Myc4 compared to the other two selected MMCs. These strains exhibited the highest fold increases (≥2.0) and specific growth rates (~0.015 h^{-1}), with the shortest doubling times (~47–48 h)

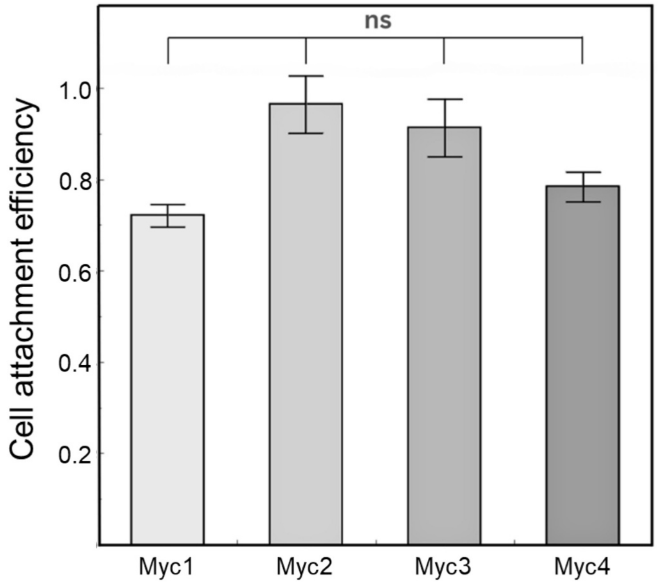

Fig. 6. Cell attachment efficiency for Myc1-Myc4 measured on Day 1 from the starting cell seeding density (1.8 × 10^6 cells/g); ns indicates no significant differences. Data displayed as the sample mean ±SEM (n = 6).

(Fig. 8). In contrast, Myc1 and Myc2 supported slower cell growth, despite high early attachment in the case of Myc2. Myc3 and Myc4 doubling times were comparable to those reported in previous bASCs expansion studies using commercial MCs (*e.g.* 42.3 ± 0.8 h, Hanga et al., 2020; 40–60 h, Dagès et al., 2025). Although a ~ 2-fold increase over five days is modest compared to 5-10× expansion commonly achieved using optimised commercial systems, it can be attributed to the 10-fold lower initial cell seeding densities employed in these studies.

Fluorescence microscopy revealed the presence of small cellular aggregates around the surface of the MMCs, particularly for Myc1, none exceeding 100 μm in diameter (Fig. 9). This suggested that the fibrous architecture of the MMCs may support the formation of controlled microtissues without the diffusion limitations associated with larger spheroids, potentially by physically trapping cells between fibres or near the pellet surface, an observation consistent with those reported by Ogawa et al. (2024). These small aggregates are still capable of supporting cell proliferation and differentiation (McMurtrey, 2016; Norris et al., 2022), and their formation around the MMCs could therefore offer a valuable strategy for controlled cell expansion in a bioreactor environment.

3.4. *Cell culture performance on mycelia-based microcarriers (MMCs) in animal component-free media*

To demonstrate industrial relevance, bASCs were also cultured on Myc1-Myc4 using ProB-supplemented media as an animal-free alternative. Cell proliferation was assessed over five days using PrestoBlue® metabolic activity assay. Across all four selected MMC types, ProB consistently supported significantly higher metabolic activity and greater fold increase than FBS-containing medium (Fig. 10). On Day 1, the number of metabolically active cells was already higher under ProB conditions for all strains, suggesting more efficient early attachment. This trend persisted throughout the culture period, with ProB outperforming FBS by Day 5 on every MMC type. Myc2 exhibited the most pronounced difference between the two growth conditions, with a 2.2-fold increase in ProB compared to 1.7-fold in FBS.

The superior performance observed with ProB media may arise from differences in how animal component-free formulations interact with the fungal surface compared to serum-containing conditions. Cell attachment to biomaterials is influenced by adsorbed proteins, and defined media formulations can produce more consistent and favourable conditioning layers compared with the highly variable protein composition of FBS (Wilson et al., 2005). It is therefore plausible that components within ProB adsorb onto the fibrous MMC surface, thereby enhancing the presentation of adhesion cues and supporting more efficient early attachment. According to Multus Biotechnology's specification sheet, ProB contains a proprietary combination of components tailored to support bovine cell growth without animal-derived ingredients, although the precise formulation is undisclosed. Nevertheless,

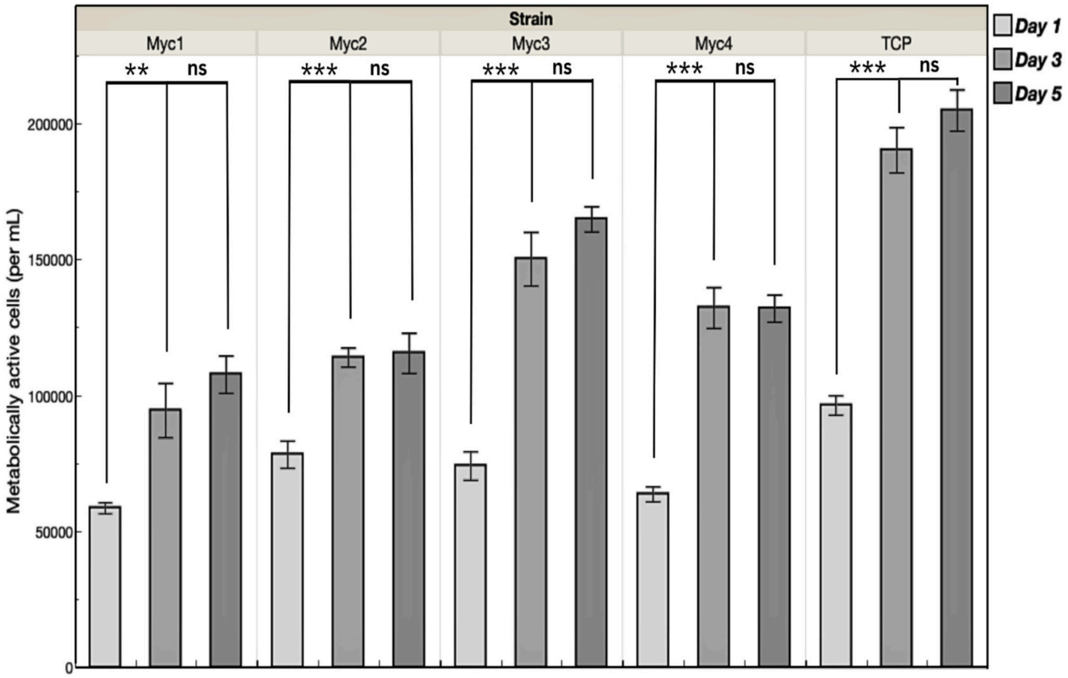

Fig. 7. bASC proliferation on different strains over 5-days, grown in media supplemented with FBS;
** indicates significant differences at $p < 0.01$, *** at $p < 0.001$ and ns indicates no significant differences. Data displayed as the sample mean ± SEM (n = 6).

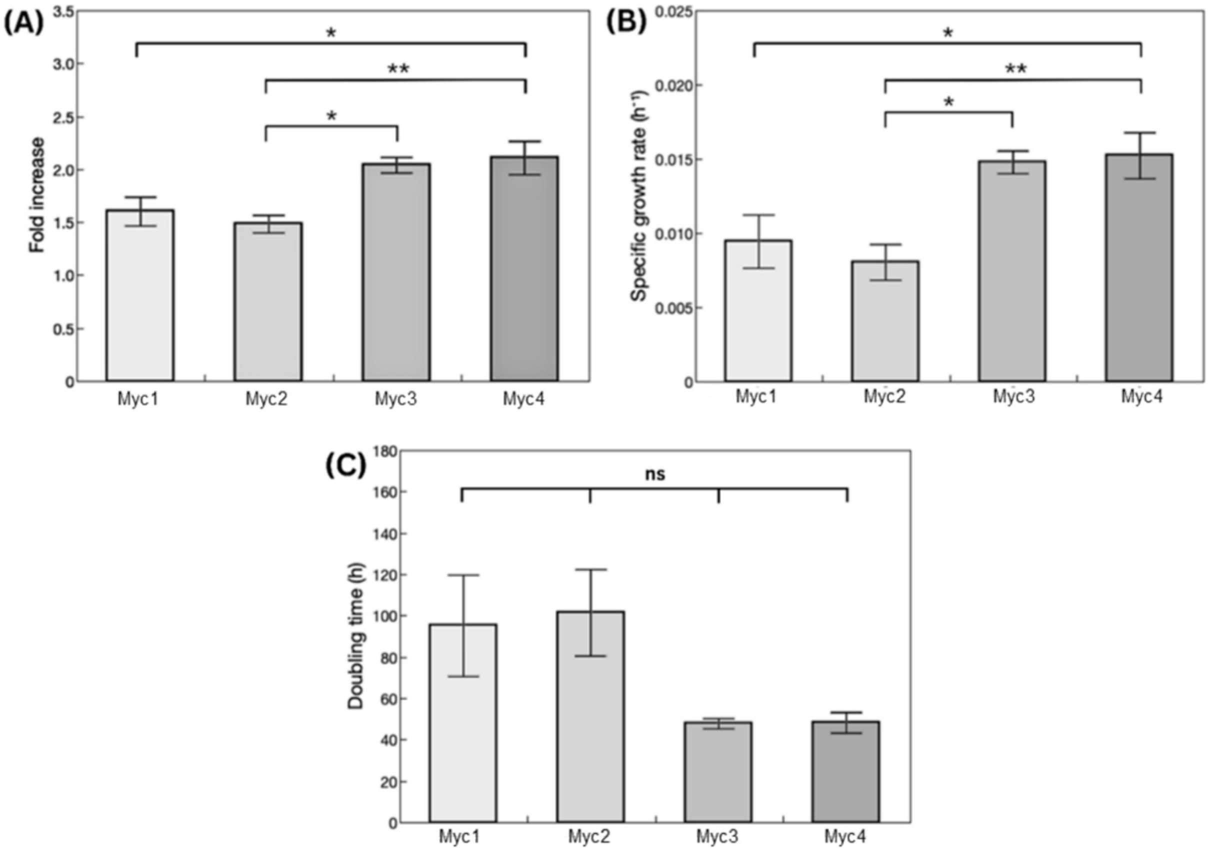

Fig. 8. Cell growth kinetics for bASCs grown on fungal strains Myc1-Myc4 using media supplemented with FBS: (A) Fold increase of cell concentration from the starting cell density; (B) bASC specific growth rate measured from Day 1 to Day 3; (C) bASC doubling rate measured from Day 1 to Day 3; * indicates significant differences at $p < 0.05$, ** at $p < 0.01$ and ns indicates no significant differences. Data displayed as the sample mean ± SEM ($n = 6$).

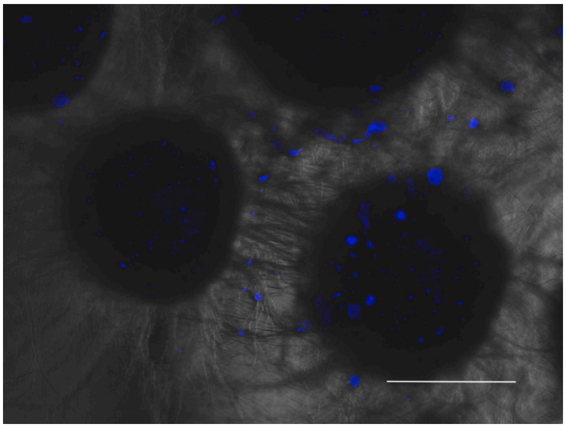

Fig. 9. Fluorescent micrograph of bASCs cultured on Myc1 pellets, stained with Hoechst (blue) to visualise cell nuclei. Nuclear aggregates are visible at the fibrous tips of the MMCs. Scale bar: 700 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

its effectiveness on fungal-derived MMCs aligns with industry goals to move towards animal component-free scalable production. ProB is also more economical than FBS, augmenting its appeal for cultivated meat applications.

Cell viability was further assessed using fluorescent microscopy. Cells exhibited healthy morphology, particularly when supplemented with ProB, appearing to grow along the surface of the fibres and forming intercellular connections (Fig. 11). The complex three-dimensional architecture of the MMCs, attributable to the abundance of fibrous projections and curved pellet geometry, introduced multiple focal planes, making it challenging to capture cells growing beyond the outer hyphal tips. In addition to supporting bASCs attachment and proliferation, the healthy morphology and intercellular interactions demonstrate that MMCs maintain high cell viability, especially when used with ACF media. These results directly address the primary aim of this study: to examine the potential of food-safe fungal materials as MMCs for bovine stem cell expansion.

4. Conclusions

This work provides the first evaluation of edible MCs derived from *Penicillium roqueforti* and *Penicillium camemberti* species for the expansion of bASCs, marking a novel contribution to the development of food-safe, animal-component-free bioprocesses for cultivated meat. Four MMC strains (Myc1-Myc4) were selected from an initial panel of eight, based on their lack of cytotoxicity, their consistent growth into relatively uniform pellets under the same culture conditions and their ease of handling. These edible scaffolds exhibited porous, fibrous morphology and strain-dependent variability in pellet and fibre size. When cultured in static conditions, proliferation assays revealed that Myc3 and Myc4 outperformed the other strains, achieving ~2-fold increases in metabolic activity and doubling times of ~47–48 h under non-optimised conditions and without the need to functionalise surfaces. These values are comparable to those reported for commercial MCs, supporting the viability of MMCs for cultivated meat applications. The performance of all MMCs was significantly improved when cultured in ProB, an ACF medium formulated specifically for bovine cell culture, demonstrating their industrial relevance.

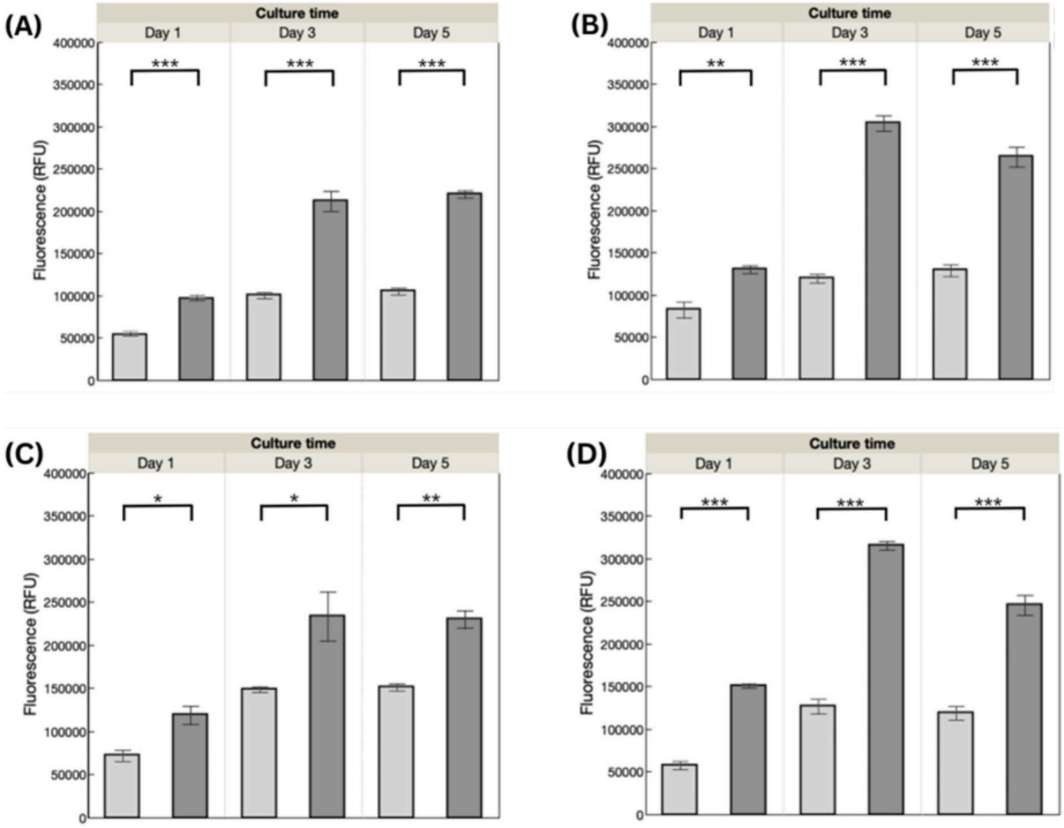

Fig. 10. bASC proliferation on different strains over 5-days, grown in media supplemented with FBS (light grey bars) or ProB (dark grey bars): (A) Myc1; (B) Myc2; (C) Myc3; (D) Myc4. Data displayed as the sample mean ± SEM (n = 3).

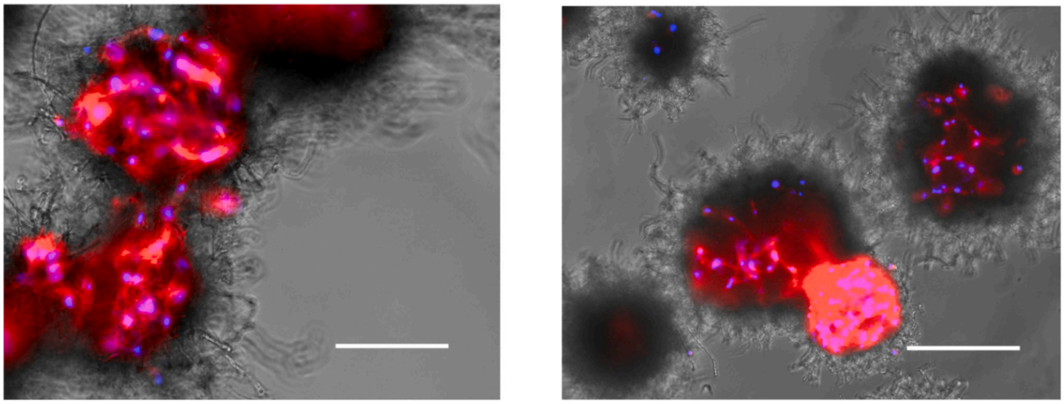

Fig. 11. Fluorescent micrographs of bASCs attached to the fibrous surfaces of autoclaved Myc3 pellets after being cultured in ProB-supplemented media, stained with Hoechst (blue; nuclei) and phalloidin (red; F-actin cytoskeleton). Scale bar: 250 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Myc3 and Myc4, both newly bred strains, demonstrate how non-genetically modified sexual breeding techniques can be used to refine MMC design. These methods allow for the selection of strains with specific traits, such as improved cell attachment or favourable nutritional and sensory characteristics, whilst remaining compatible with regulatory and consumer expectations regarding genetic modification. This approach provides a practical route to tailoring fungal strains for particular cell types or improving expansion efficiency.

Ongoing and future investigations will focus on enhancing MMC performance through optimised seeding densities and adaptation to stirred-tank bioreactor environments. Given the fibrous and highly porous architecture of the MMCs, further characterisation of internal cell distribution will be pursued using cryoembedding and microtome sectioning, coupled with high-resolution imaging techniques such as atomic force microscopy. These methods will enable visualisation of cell growth beyond surface-level attachment and provide deeper insights into cell-matrix interactions, as well as the stiffness of individual hyphal fibres. Collectively, this future work has the potential to translate MMCs into a viable industrial solution for large-scale, food-grade cell culture.

CRediT authorship contribution statement

Alice Millbank: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amélie Savers:** Writing – review & editing, Resources, Methodology. **Paul D. Topham:** Writing – review & editing, Supervision. **Mariana Petronela Hanga:** Writing – review & editing, Supervision. **Jean-Baptiste R.G. Souppez:** Writing – review & editing, Supervision. **Eirini Theodosiou:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: (Alice Millbank reports equipment, drugs, or supplies was provided by Myconeos Ltd. Alice Millbank reports equipment, drugs, or supplies was provided by Multus Biotechnology. Amelie Savers reports financial support was provided by Myconeos Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2026.104518.

Data availability

Data will be made available on request.

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