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Cultured chicken meat developed by structuring cellular spheroids on an edible bacterial nanocellulose bioscaffold

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Cultured meat is considered a sustainable protein alternative. To produce chicken meat in a controlled environment, bacterial nanocellulose (BNC) was used to provide structure similar to traditional chicken meat. BNC was synthesized as a hydrogel from *Novacetimonas hansenii* in vitro culture. Chicken mesenchymal stem cells (MSCs) from pathogen-free eggs were cultivated in both 2D (monolayers) and 3D (spheroids) forms and introduced into BNC. Results showed that the shape, viability, and stemness of 3D spheroids and 2D monolayers were maintained. Notably, 3D platforms better replicate natural cellular environments, enhancing differentiation potential. Differentiated spheroids and monolayers were successfully cultured on both modified and unmodified BNC hydrogels, with tissue-like organization observed mainly in modified BNC. The cultured chicken prototype using nanocellulose proved promising for developing cultured meat products with co-cultivation of 2D and 3D cells. Visual analyses revealed significant similarities between cultured and farmed chicken.

The food industry has presented a wide range of innovations, changes, and possibilities for in vitro cultivating meat settings due to a proportionally expanding global population and an increase in food requirements[1]. Along with demographic and socio-economic imbalances in developing countries, besides increasing longevity in most countries, the Food and Agriculture Organization of the United Nations (FAO) has highlighted the need to boost food production by 70% to meet the demands of an estimated 10 billion people by 2050[2]. Consequently, it is worth exploring alternative protein sources alongside the development of innovative food products.

Cultured meat (CM) is often regarded as a highly promising substitute for conventional meat, as it offers the potential to replicate the structural characteristics of flesh[3]. However, several obstacles must be overcome for CM to reach its full potential, including the development of industrial-scale technological strategies for cell expansion, edible support biomaterials, and cell-to-meat processing[4]. For cell expansion, the establishment of efficient expansion methodologies is a key factor to proliferate cells into biomass. In this context, using cells in spheroid form presents a promising option.

Animal-derived cell lines are primary components for animal protein-based products commonly referred to as CM. Stem cells have great potential for CM. The maintenance of cell stemness is facilitated by the in vivo milieu, which offers growth stimuli along with interactions between cell–cell or cell-extracellular matrix (ECM)[5]. Hence, it is imperative to create cell culture techniques that can effectively enhance both the quantity and quality of stem cell proliferation by mimicking the in vivo environment[6,7]. Maintaining the differentiation potential and stemness of stem cells is more difficult in two-dimensional (2D) cultures than in three-dimensional cell culture environments[7]. Three-dimensional systems can replicate conditions that resemble an in vivo microenvironment, hence facilitating the formation of cell-cell and cell-ECM interaction networks[8]. Therefore, spheroid (3D) stem cell cultures secrete greater quantities of cytokines and chemokines that influence cell viability, proliferation, and migration, as well as angiogenesis, compared to stem cell monolayer cultures[9]. Additionally, cell growth in spheroids enhances the expression of transcription factors that indicate stemness, including Oct-4 and Nanog[10,11].

The cells utilized for CM generation are anchorage-dependent, requiring a substrate for adhesion and proliferation[12]. To support meat production, biomaterials intended for scaffolds must meet food safety standards and provide optimal topography and surface chemistry conducive to the growth and development of the specific cell type being targeted.

Edible ingredients for developing and analyzing bioscaffolds in CM production were previously documented. When selecting a biomaterial, it is crucial to consider its properties and intended use[13]. Bioscaffolds are non-toxic to cells and promote growth and differentiation of target cells in three

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dimensions[14]. Additionally, bioscaffolds can be consumable and offer appealing organoleptic properties, particularly texture.

Considering all the aforementioned attributes and contemplating a cost-effective raw material with expansion potential, bacterial nanocellulose (BNC) was selected. The utilization of BNC in food is not new[15,16] and its interaction with muscle cells was reported more than 15 years ago[17], although recently it gained notoriety for CM[18,19]. The BNC can be synthesized in a variety of forms, including hydrogels, crystals, fibers, pellets[20] and even more complex forms[21]. The diverse range of shapes exhibited by the material can give rise to numerous applications, such as the expansion of bioreactor systems and the prospective fabrication of textured meat pieces. In addition, BNC is a material generally recognized as safe by the U.S. Food and Drug Administration (FDA)[20,22] and it has been applied in biomedical approaches and as a scaffold for tissue engineering and tissue regeneration[23,24].

The current study presents a novel methodology for advancing CM products. This strategy involves the utilization of edible microtissues formed from cellular spheroids combined with BNC. We compared the adipogenic and myogenic differentiation of mesenchymal stem cells (MSCs) cultured in both monolayer (2D) and spheroid (3D) formats, subsequently integrating these cells into a BNC scaffold using two complementary processing methods. In this study, we refer to the laminar forms as *hydrogels* and the three-dimensional macrostructured forms as *scaffolds*, to clearly distinguish between the two. To our knowledge, this is the first study to combine 2D/3D co-cultures with in situ modified BNC scaffolds for the development of engineered meat-like tissues, representing an innovative approach in the field of CM.

Results

Isolation and characterization of chicken MSCs

MSCs were isolated from 9-day' chicken embryos based on their morphology and adherence to a plastic surface[25]. Under DMEM culture, chicken MSCs exhibited a fibroblast-like appearance, similar to that observed in the RPMI medium (Fig. 1B–IV). Additionally, cellular aggregates compatible with CFU were visualized after 7 days in RPMI. Phenotypic characterization confirmed their MSCs properties based on their differentiation potential (Fig. 1A).

Stem cells expressed all tested genes, reflecting their pluripotency. The cOCT4 gene was the most represented, followed by the cSALL4, cNANOG, cLIN28A, cSOX3, cCLDN3, and cKIT genes (Fig. 1E).

Spheroids formation and viability

After 24 h of spheroid formation, they were spherical-shaped and ranged from 200 to 300 µm in diameter, depending on cell number. Spheroid formation was deemed ineffective if no spheroids exceeded a diameter of 100 µm. Since the spheroids were larger than 600 µm, the conditions were deemed unsuitable for producing spheroids of appropriate size for meat cultivation. The area of spheroids prepared from 40,000 cells was 0.306 ± 0.022 mm^2.

Under RPMI media (control), spheroids began to develop regular edges by day 7 (Fig. 1B–IV). Additionally, a strong shrinkage (Table S2) was exhibited from day 5 with an average value of 34.2% when all time points were considered.

The spheroids' viability was assessed by staining with LIVE/DEAD using calcein acetoxymethyl ester and ethidium homodimer-1. Initially 200 µm in diameter, spheroids grew little until 14 days of differentiation induction. LIVE/DEAD staining (Fig. S1) indicated very few dead cells in spheroids smaller than 300 µm, while spheroids 500 µm or larger had a significant number of dead cells. Notably, strong and complete staining for live dye (green, calcein acetoxymethyl ester) was observed on day 20 after the beginning of spheroid development (18 days after initiation of differentiation), suggesting their viability even over long periods. No necrotic cores were observed in spheroids with 7-AAD staining (Fig. S1). The spheroids exhibited some isolated spots of necrosis or apoptosis in the proliferative zone, and a higher concentration of dead cells in the quiescent zone, which is consistent with the normal biology of the spheroids. In addition, complementary analysis with DAPI staining showed strong fluorescence in spheroids cultured for 7 days on laminar BNC hydrogels (Fig. S2), and no dark regions were observed inside them, which excludes the presence of the necrotic core.

Bacterial sequencing

A total of 10.6 million reads were sequenced on a NextSeq Illumina platform. After quality control, 7.3 million reads were used to assemble the genome, comprising 61 contigs (3,687,353 bp), with the largest contig length of 428,493 bp and the smallest length of 544 bp. The average of coverage depth was 420x, the N50 and N90 values were 130,303 and 37,095 bp, respectively, and the GC content was 59.5% (Supplementary Data).

The genome annotation resulted in a total of 3323 predicted genes, 3265 coding sequences (CDSs), 47 transfer RNAs (tRNAs), and 4 non-coding RNAs (ncRNAs). Detailed results regarding genome assembly, contig sequences, gene prediction, and functional annotation are available in the NCBI database, BioProject PRJNA1043133.

Comparative genomic analysis using JSpeciesWS identified the closest relationship between our strain and *Novacetimonas hansenii* ATCC 23769, with an average nucleotide identity (ANI) of 99.93% based on BLAST + (ANIb).

Scaffold production and characteristics

We attempted to replicate previous reports on BNC macrostructures, typically formed after 14 days[24]. However, our results showed shape variability within the same flask (Fig. S3), highlighting the need for a more consistent production method. Given the sensitivity of *N. hansenii* BRMSA 3437 to hydrodynamic parameters, we conducted a Design of Experiments (DOE) to optimize conditions for reproducible macrostructures (data not shown). The improved protocol produced visually homogeneous scaffolds 50% faster, with purification starting after 7 days of agitation. The resulting structures, approximately 7.3 × 4.8 ×1.1 cm (L x W x H) (Fig. 2), resembled chicken breast filets and were unaffected by in situ modification, displaying a denser inner region and a translucent outer area, similar to a capsule (Fig. 2 – A, B, D, E).

Laminar BNC hydrogels

To evaluate the interaction between differentiated monolayers and spheroids with our scaffolds, we prepared smaller, circular, laminar BNC hydrogels synthesized by bacterial static culture for 7 days. These hydrogels exhibited no differences in production time or visual characteristics related to sodium alginate presence (data not shown), consistent with observations in larger scaffolds.

Adipocytes differentiation assays for MSC characterization

According to the International Society of Cellular Therapy (ISCT), one criterion for defining MSCs is their ability to differentiate into adipocytes, chondrocytes, and osteocytes under standard in vitro conditions[25]. To fulfill this criterion, chicken MSC spheroids were stabilized in differentiation media for three days before evaluating their size and morphology. These spheroids were compared to monolayer cell culture under identical conditions to assess their efficiency in trilineage differentiation.

Adipocytes. ORO lipid staining revealed that chicken MSC spheroids differentiated into adipocytes more effectively than monolayer cultures, showing positive results at all time points. Spheroids exhibited less shrinkage compared to cultures in basal media (Table S2), with a 55.8% reduction from days 3 to 5 and 23.5% after 18 days. In contrast, monolayer cultures showed only slight positivity by day 14, with pronounced staining by day 18, indicating a lack of mature adipocyte characteristics.

Osteocytes. Alizarin Red S staining identified calcium deposits in MSCs differentiated into osteocytes. Spheroids displayed staining by day 12, whereas monolayers were positive only by day 18. Spheroid shrinkage

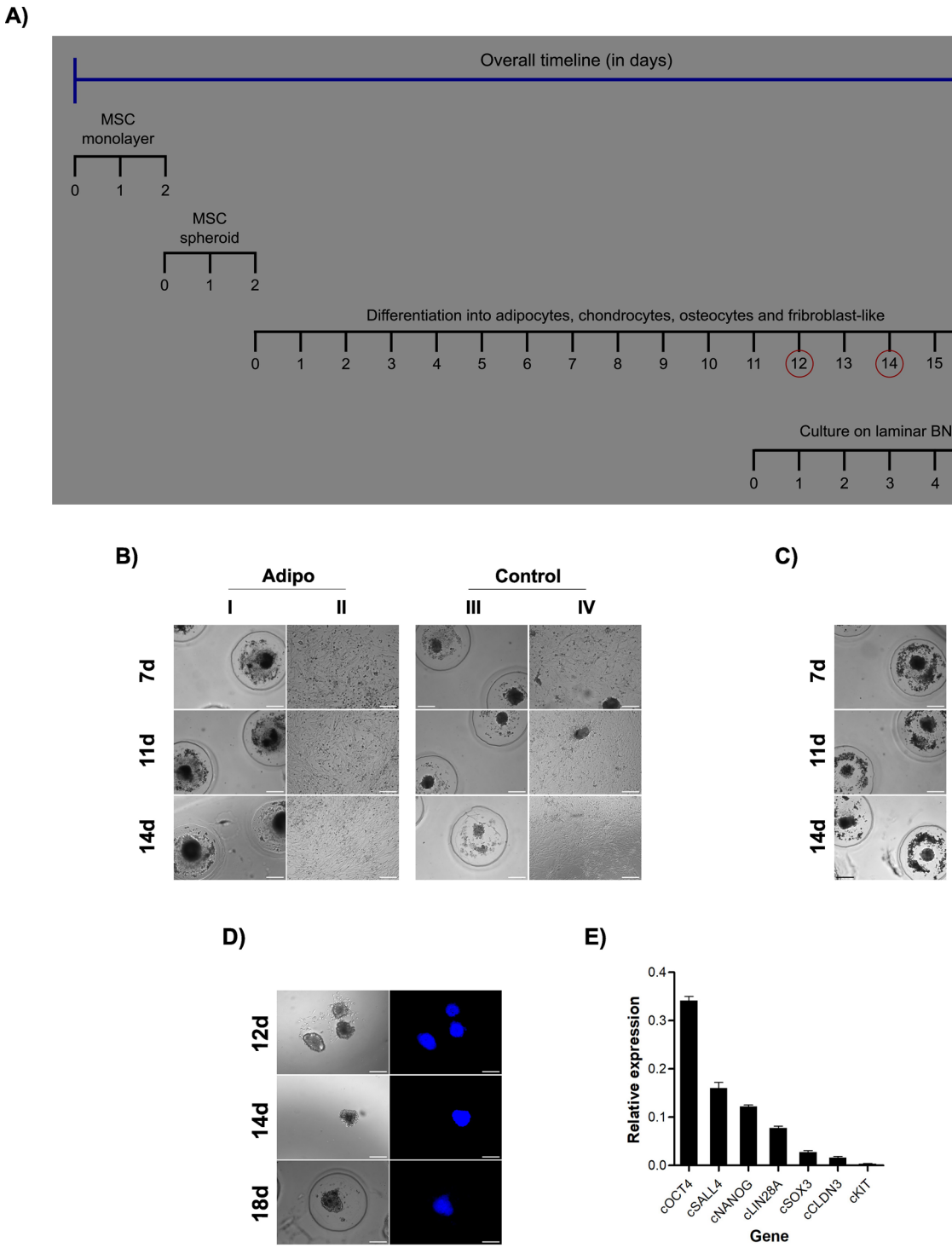

Fig. 1 | Chicken MSCs characterization during the overall timeline. **A** Experiments with chicken mesenchymal stem cells (MSCs) were performed in 22 days and divided into four steps. On the 11th day of the differentiation process, part of the spheroids and monolayer cells were placed onto laminar bacterial nanocellulose (BNC) hydrogels and the remaining ones stayed at their original spot. Red circles represent staining time points. **B** Primary adult monolayer cells (II, and IV) and spheroids (I, and III) during the differentiation process into adipocytes (Adipo). Later time point (day 14) was used for comparison. **C** Spheroids submitted to fibroblasts' differentiation medium. Later time point (day 14) was used for comparison. **D** Nuclear staining of fibroblasts by DAPI (4′,6-diamidino-2-phenylindole), images were acquired using fluorescence microscopy to assess nuclear morphology and exclude the presence of necrotic cores. **E** Expression of important stem genes relative to GAPDH. Scale bar: 200 µm.

was gradual, with a 30% reduction by days 5 and 7, doubling in the following time points. By day 17, spheroids increased in size by 15% compared to controls and appeared darker under bright-field microscopy (data not shown), with nodule-like structures, indicating calcium deposition. These tightly packed arrangements were also seen in monolayer culture (data not shown).

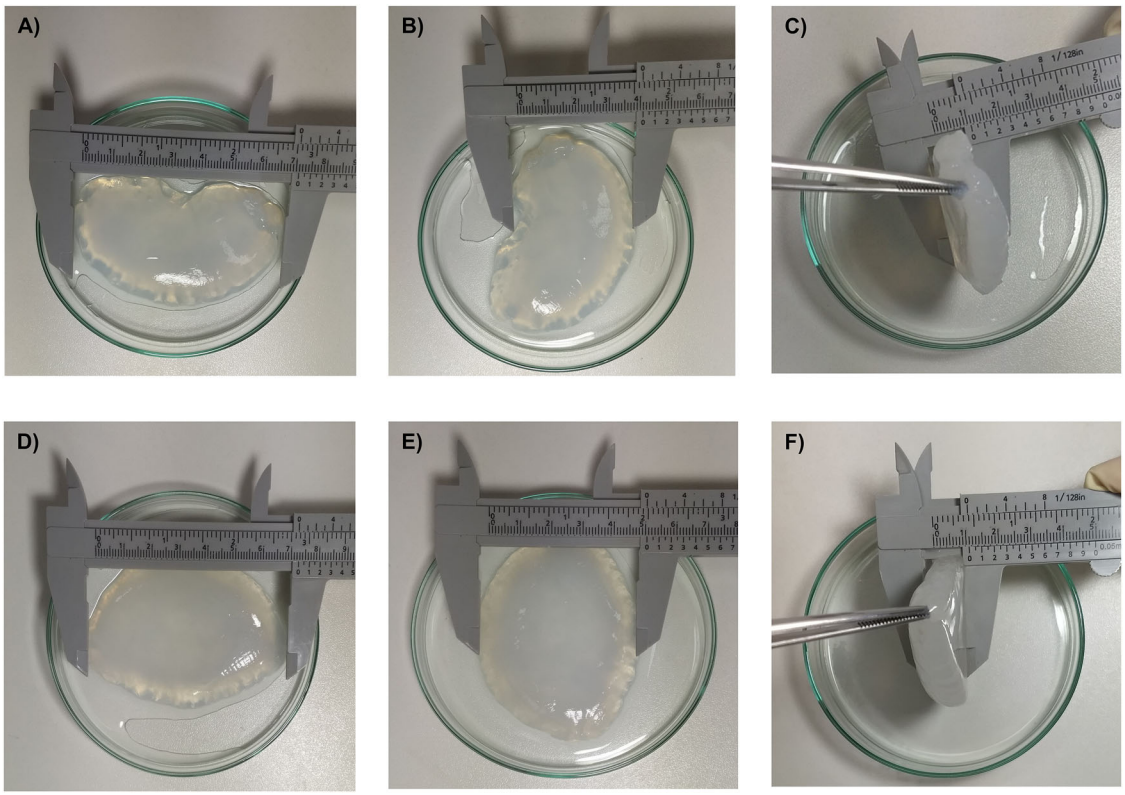

Fig. 2 | Cellulose macrostructures (scaffolds) used to prepare cultured chicken breast fillets. Scaffolds synthesized by *N. hansenii* BRMSA 3437 after optimal parameters. **A–C** Control scaffolds (no modification in situ). **D–F** Scaffolds modified in situ (sodium alginate).

Chondrocytes. Spheroids induced to differentiate into chondrocytes became darker under bright-field microscopy (data not shown) and increased in area at later time points (days 17 and 18, data not shown). Unlike adipocytes and osteocyte spheroids, chondrocyte differentiation was not significantly enhanced in three-dimensional culture compared to 2D culture. Alcian blue staining did not show any difference in the amount of proteoglycan that was deposited between spheroids and monolayers. The results were positive for both cultures at all time points, which means that early proteoglycan deposition happened in both systems.

Fibroblast-like differentiation assays

A regular edge was visible after 7 days in culture (Fig. 1C). However, similar to spheroids cultured in RPMI, strong disaggregation occurred, with shrinkage values varying from 59.9% on day 5 to 80.3% on day 18 (Table S2). All tested spheroids were successfully dyed by DAPI and no necrotic areas were seen (Fig. 1D).

Spheroids cultured on laminar BNC hydrogels

To evaluate our model performance, we cultured a combination of differentiated-induced MSC spheroids and monolayer cells on BNC hydrogels and compared them with cultures consisting of spheroids-only and monolayers-only (Table S1), followed by cultures on the scaffold. We also assessed the effects of modified versus unmodified BNC.

Adipocyte spheroids derived from MSC successfully adhered to in situ modified laminar BNC hydrogels, being able to migrate as early as one day after seeding. The spheroid-detached cells maintained a circular morphology throughout the entire analysis period. On the other hand, it was not possible to observe intact spheroids on unmodified laminar BNC hydrogels and only spheroid-detached cells were seen. These differences are related to the technical limitations instead of the hydrogel in situ modifications since the spheroids were collected individually, a time-consuming approach that impacts their number and integrity. Monolayer cells also displayed a round shape morphology for both laminar hydrogels, which is in accordance with adipocytes' morphology and indicates a good environment for their growth[26].

Intact chondrocyte spheroids attached to modified laminar BNC hydrogels successfully, and migrating cells were observed on day two after seeding. Non-modified hydrogels, however, showed only detached chondrocyte spheroids. Regardless of BNC modifications, monolayer chondrocytes and spheroid-detached cells exhibited spherical morphology and agglomerated.

In spite of osteocyte spheroids on BNC, we observed tightly packed structures in both laminar BNC hydrogels, similar to those already observed in non-hydrogel cultures (data not shown). This observation is also true for monolayer osteocytes. Finally, fibroblast-like spheroids adhered and grew on BNC as well as other cell type,s but their distribution in the laminar hydrogels was not uniform. These results demonstrate that MSCs retained their multipotent potential and were successfully induced to differentiate after being seeded onto the BNC hydrogels.

No significant differences were observed between modified and unmodified laminar hydrogels in either the spheroids-only (Fig. 3) or monolayers-only groups (Fig. 3). However, tissue-like structures (Fig. 3 spheroid's pool + monolayers' pool) were observed exclusively in cultures combining spheroids and monolayers, where cells began migrating from spheroids on day two and formed tissue-like structures by day six (Fig. S4), and remained viable for the entire time of analysis, a phenomenon absent in the spheroids-only group. This demonstrates that only the co-culture of spheroids and monolayers promoted cell migration and the formation of organized tissue-like structures, outcomes that were not achieved in single cultures.

Both monolayer cells and MSC spheroids adhered effectively to the modified BNC scaffold in situ (Fig. 3). The treated BNC scaffold showed a higher cell proliferation rate compared to the untreated ones, as evidenced

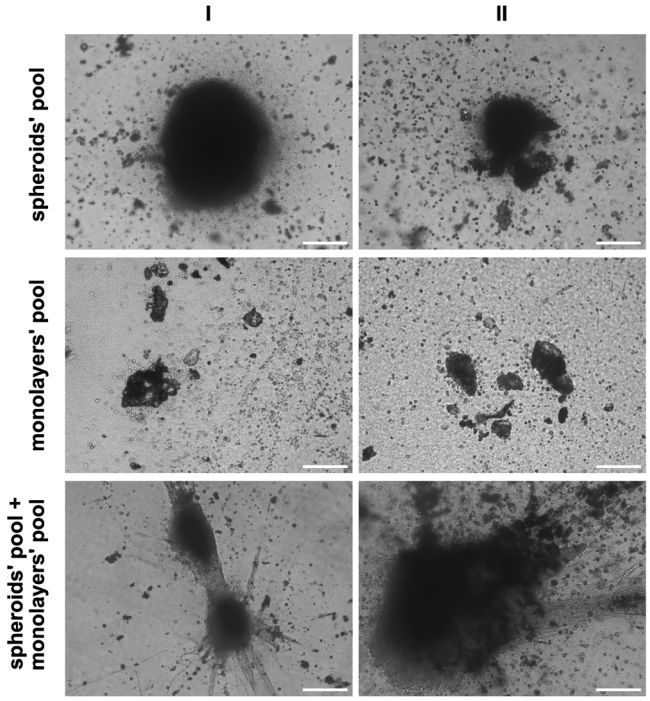

Fig. 3 | Culture Culture of differentiated cells (spheroids and monolayers) on bacterial nanocellulose (BNC) laminar hydrogels and scaffolds. Representative images of differentiated cells seeded as monolayers and spheroids on BNC hydrogels, either in situ modified with sodium alginate **(I)** and unmodified **(II)**, after 6 days of culture. Cultures include adipocytes, chondrocytes, and osteocytes derived from mesenchymal stem cells (MSCs). Scale bar: 200 µm.

by the increased density of monolayer cells, as well as a greater amount of cells migrating from spheroids in the treated hydrogels. The differentiated cells and their respective spheroids showed a pattern similar to the cells and spheroids of MSCs.

After confirming that MSCs retained their multipotency, with preserved differentiation potential toward adipogenic, chondrogenic, and osteogenic lineages, target cell types relevant for CM production, such as myoblasts and adipocytes, were evaluated. Given the more favorable results obtained with MSCs on treated BNC, subsequent tests with myoblasts and adipocytes were conducted using the treated BNC hydrogels and scaffolds (Fig. 4). Myoblast spheroids adhered successfully to in situ modified laminar BNC hydrogels and scaffolds, exhibited early migratory behavior, with cell dispersion observed as early as 24 hours post-seeding. The myoblasts adhered, proliferated, and maintained their differentiation capacity; after seven days in culture, immunostaining revealed positive expression of MyF5 and MyHC (Fig. 5A), with more intense staining observed in spheroid-derived cells compared to monolayer cultures seeded on the BNC scaffold.

LipiTox™-labeled adipocyte spheroids exhibited strong cytoplasmic fluorescence, confirming the presence of intracellular lipid droplets characteristic of adipogenic differentiation (Fig. 5B). The lipophilic dye selectively stained neutral lipids within the spheroids, allowing clear visualization of lipid accumulation in three-dimensional structures, such as BNC. The staining revealed that adipocytes adhered, proliferated, differentiated, and accumulated lipids. Adipogenic cells were well organized and densely packed, with abundant lipid storage, corroborating the functional maturation of adipocytes within the BNC spheroids.

Cooking experiments, physico-chemical and microbiological assessments

Drip tests ($n = 12$) evidenced that the structure begins losing water immediately after removal from the environment (water content: 97.86%), necessitating reinforcement for cooking tests (Fig. 6A). After cooking in the air fryer, sample retained an appearance similar to that of a chicken filet (Fig. 6B). The average scaffold mass ($n = 6$) was 18.99 g when thawed, and the BNC structure lost 57% of its mass after 35 min. Cells were manually injected into the BNC using a syringe at a density of 1×10^6 cells/mL for adipocytes, chondrocytes, osteocytes, and fibroblasts. However, the resulting cell quantity was below the detection threshold of the protein quantification method used.

Samples of our farmed chicken were tested and analyzed by PCR, in an accredited laboratory of the Ministry of Agriculture and Livestock of Brazil. The analysis showed no detectable presence of *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Staphylococcus aureus*, Influenza Virus Type A, Newcastle Disease Virus-1, and *Salmonella* in the samples.

Discussion

In vitro meat production utilizes stem cell technology and tissue engineering to efficiently generate CM by differentiating stem cells into muscle cells and adipocytes. These cells are grown on scaffolds, which are essential for developing the final CM product. Research is advancing steadily, aiming to replicate the characteristics of traditional meat, with a focus on creating biodegradable, cost-effective edible scaffolds.

The scaffold plays an important role in ensuring the efficient transport of oxygen, nutrients, and waste products to cells[18,21,27]. Furthermore, a scaffold is a substantial component of the ultimate product and contributes to the distribution and geometry of the cells. Plant-derived proteins, as well as mammalian biopolymers like gelatin, collagen, hyaluronic acid, and alginate, have been proposed as potential supporting materials for CM production. Acknowledging the significance of edible scaffolds in CM production, this study investigates the formulation and fabrication of an edible nanocellulose scaffold. Cellulose is "generally recognized as safe" (GRAS) by the FDA, and despite the absence of nutritional value, it is suitable as a dietary fiber[28] whose use as a scaffold in the CM field was recently discussed[19].

Additionally, it is crucial to investigate the biological interactions between cells and the scaffold structure to promote cell adhesion, proliferation, and activation[29]. To achieve this, the scaffold's manufacturing material must meet requirements such as intrinsic biocompatibility and adequate chemistry to induce molecular biorecognition of cells. In the current study, we selected BNC as the material and analyzed cellular behavior on this substrate. This nanocellulose is naturally secreted by *Novacetimonas* genus bacteria, which features a macrostructure that can be modified according to culture parameters. BNC was already used for cultures of monolayer adipocytes[26], chondrocytes[30], and osteocytes[31], among other cell types[21]; however, research on spheroid cultures in BNC remains scarce.

Multipotential stromal cells, or MSCs, were initially obtained as collections of fibroblast-like cells that stuck to plastic surfaces. These cells have the ability to differentiate into multiple cell types both in vitro and in vivo[32]. This study proposes the use of MSCs as a cell source for CM production. The approach involves large-scale expansion of MSCs, followed by direct differentiation into desired cell types. This differentiation process can be induced using cells cultured either as monolayers or in spheroid formations, providing flexibility and scalability in production.

Obtaining and expanding MSCs in vitro is a reasonably simple process[33]. Traditionally, the conventional method for expanding cells in vitro for CM production has been through 2D adherent culture conditions. Conversely, 3D cultures, such as spheroids, have been applied in tissue bioengineering, as they better maintain cellular characteristics[34], recreate the tissue microenvironment in vitro[35–37] and are seen as more physiologically relevant. Both forms of cell cultivation, 2D and 3D, should be considered for MSC cell production. Our results demonstrated that the shape, viability, and stemness of the spheroids and monolayer were maintained throughout the experimental period. Long-term 2D culture of stem cells has been shown to cause some changes, such as cellular senescence, immunogenicity, and a loss of stemness and paracrine activity[38–40]. However, the major advantage is that

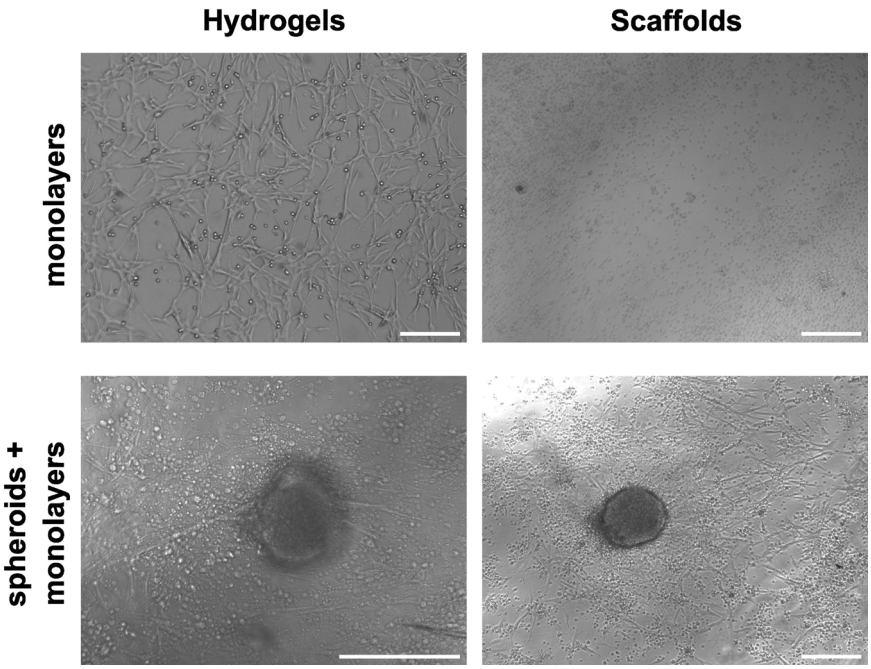

Fig. 4 | Culture of myoblast cells (spheroids and monolayers) on bacterial nanocellulose (BNC) hydrogels and scaffolds. Representative images of chicken myoblast cells seeded as monolayers and spheroids and cultured on BNC hydrogels modified with sodium alginate. The modified scaffolds exhibited higher cell density and enhanced migration from spheroids. The combination of 2D/3D co-culture on in situ modified BNC scaffolds proved more effective than conventional 2D culture. Scale bar: 200 µm.

the 3D platforms replicate the natural cellular environments found in living organisms[40].

Interestingly, our findings showed that the ability to differentiate into adipogenic lineages, as well as the potential of stem cells, were improved in a spheroid culture setting, which supports previous findings on tissue engineering[41]. During the three cellular differentiations, our study revealed that MSCs cultured in spheroids showed enhanced vitality and a stronger capacity to differentiate into adipocytes compared to MSCs cultured in conventional 2D. A similar pattern was observed for myogenic differentiation, with spheroid-derived cells showing more robust myoblast/myotube marker expression than monolayer cultures on BNC scaffolds. The reason for this is that MSCs cultivated in spheroids are spherical internally and elongated externally, resulting in an overall decrease in cytoskeletal and ECM molecules. Our findings indicate that 3D culture significantly enhances the yield of differentiated MSCs compared to traditional methods.

The increased rate of cellular differentiation observed in 3D spheroid cultivation may have also been influenced by enhanced secretome stimulation. One of the methods to stimulate the secretome in stem cells is the 3D culture system[42]. The stem cell secretome refers to extracellular biomolecules, which encompass a diverse array of proteins, soluble factors (transcription factors, growth factors, angiogenic molecules, lipid mediators, and hormones), cytokines, miRNA, and extracellular vesicles (including exosomes and microvesicles)[43]. Therefore, the coculture method and promotion of MSCs transdifferentiation have potential applications in various fields due to the enhanced functionality of the produced spheroids.

The size of MSCs in the spheroids is significantly smaller than that of cells in the 2D monolayer, resulting in a 75% decrease in the volume of each cell[44]. Cellular morphology is a crucial feature used to determine the cellular phenotypes and fate of MSCs[45]. MSCs that are small and rounded tend to differentiate into adipogenic cells, while MSCs that are large and extended tend to differentiate into osteogenic cells in both 2D and 3D culture methods[46].

Acquiring a substantial quantity of cells for CM production in biomaterials is extremely challenging due to the limited ability of stem cells to multiply in a controlled environment. Our study used the cellular spheroid approach in an additional static culture of cellularized microtissues to induce ECM aggregation and production. Additional cellular analyses in 2D monolayers and spheroids (3D) were performed to test the transdifferentiation potential of MSCs in 2D and 3D cultures. Differentiated spheroids and monolayers were successfully cultured onto in situ modified and unmodified BNC laminar hydrogels. Chondrocyte spheroids were seen at different planes of the hydrogel, suggesting their migration through the pores, as already verified by Svensson et al.[30]. However, this hypothesis needs to be validated, once BNC has a typically sub-micron pore size, which usually prevents cell migration through BNC pore[30].

Despite differentiated cells growing in both modified and unmodified BNC, tissue-like organization with cell alignment was only observed when both differentiated spheroids and monolayers were cultured together. Our findings show that tissue-like organization occurred more rapidly and was more prominent in modified BNC, suggesting that the modifications to the BNC affect the cells differentially. Additionally, adipocyte and myoblast/myotube spheroids and monolayers cultured in a BNC scaffold (i.e., non-laminar BNC) exhibited the same pattern. Thus, combining 2D and 3D cells with scaffold modification may provide a promising approach for achieving the cell alignment required for muscle fiber formation. This finding underscores the synergistic effect of co-culture, as neither 2D nor 3D cultures alone achieved comparable organization. By integrating both systems, cellular alignment and migration were accelerated, directly supporting the structural requirements of muscle fiber formation. These advantages suggest that co-culture strategies may be essential for developing more realistic and functional CM products.

In this study, we demonstrated the potential of combining 2D and 3D cultures of MSCs, adipocytes, and myoblasts/myotubes on modified BNC scaffolds for applications in CM production. Visual analyses revealed striking similarities between the engineered constructs and conventional chicken tissue, supporting the relevance of this approach. Our findings indicate that the use of modified BNC supports cell adhesion, proliferation, and differentiation in both monolayer and spheroid formats, with 3D cultures showing enhanced cellular behavior. Notably, this is the first study to successfully combine 2D/3D co-culture with in situ modified BNC scaffolds for the development of structured CM. Moreover, we observed more efficient cellular differentiation in spheroid models compared to monolayer cultures, reinforcing the advantages of 3D systems in mimicking native tissue environments. Future research should focus on incorporating these differentiated cell types into dynamic culture systems to better assess the scalability and functional outcomes of spheroid- versus monolayer-based strategies. Collectively,

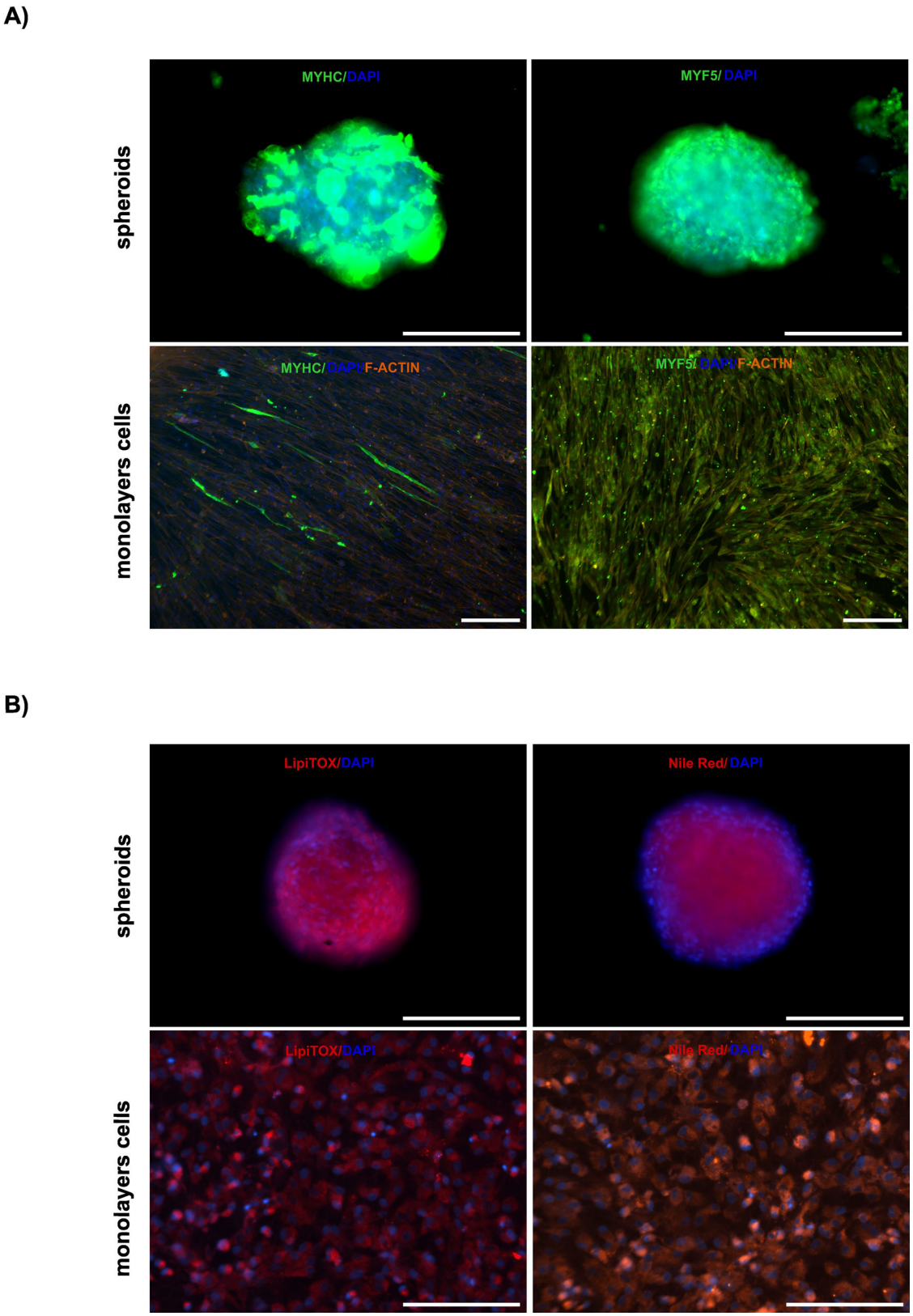

Fig. 5 | Immunofluorescence staining of chicken myogenic and adipogenic cells cultured on bacterial nanocellulose (BNC) hydrogels. A Myoblasts and myotubes derived from mesenchymal stem cell (MSC) spheroids and monolayers were cultured on modified BNC hydrogels and stained for myogenic markers. Myogenic factor 5 (MYF5) and myosin heavy chain (MYHC) were detected using primary antibodies and Alexa Fluor 488-conjugated secondary antibodies (green). F-actin was counterstained with Rhodamine Phalloidin (orange), and nuclei were counterstained with DAPI (blue). **B** Adipocytes derived from MSC spheroids were stained with HCS LipidTOX™ Red Neutral Lipid Stain to detect intracellular lipid droplets (red). Nuclei were counterstained with Hoechst 33342 (blue). Staining confirmed adipogenic differentiation and lipid accumulation within 3D cultures on BNC. Scale bar: 200 µm.

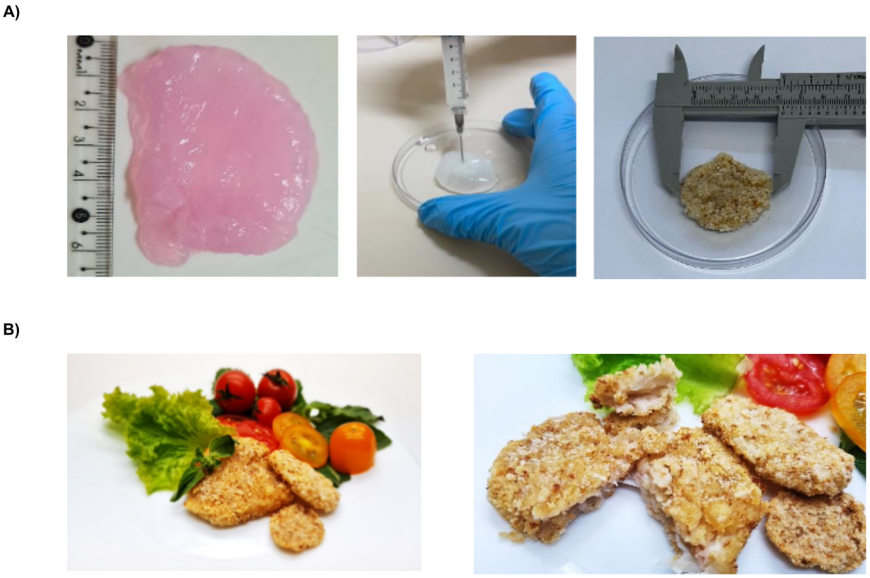

Fig. 6 | Cultivated chicken fillet before and after cooking. A Cultivated chicken fillet (BNC scaffold containing differentiated cells) preparing for cooking. **B** Cultivated chicken fillet after cooking in an air fryer, showing appearance similar to conventional chicken meat. Cooking conditions: 180 °C for 7.5 min, turning halfway through.

our results contribute to the advancement of scaffold design and cellular organization in the emerging field of CM[47].

Methods

Isolation and characterization of MSCs

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Embrapa Suínos e Aves. Embryonated specific-pathogen-free (SPF) chicken eggs were incubated at 38 °C and 50% humidity (Solab, SL-200) for 9 days and selected by ovoscopy (Omnium). The embryonic tissues were collected and dissociated with 0.125% trypsin-0.02% versene (TVP) buffer (in-house). After neutralization with fetal bovine serum (FBS) (Gibco), the cells were collected by centrifugation (Eppendorf, 5810 R). The precipitated cells were resuspended in cell culture medium, which consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) replenished with 10% FBS, 1% penicillin/streptomycin (Life Technologies). Cell suspensions were seeded into 6-well culture plates (Corning) with MSCs as feeding layer[47] at a density of 1×10^4/well, and cultured at 37 °C in 5% CO_2 (Panasonic, MCO-19AICUVH-PA).

For genetic characterization, total RNA was extracted from stem cells using TRIzol (Invitrogen) associated with the RNeasy Mini Kit (Qiagen), following the manufacturer's protocols. DNA digestion was performed on the column with the RNase-Free DNase Set (Qiagen) to eliminate contaminating genomic DNA. RNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) synthesis was conducted with the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen), adhering to the manufacturer's instructions.

RT-qPCR reactions were carried out using the QuantiNova SYBR Green PCR kit (Qiagen), with primer concentrations adjusted accordingly. The chicken genes evaluated included cOCT4, cSOX3, cNANOG, cSALL4, cCLDN3, cKIT and cLIN28A using primer pairs described by Giotis et al.[48] and by Han et al.[49]. The reactions were run on an ABI 7500 Real-Time PCR System (Applied Biosystems), with each sample amplified in triplicate using 50 ng of cDNA. Relative gene expression was determined by normalizing to the endogenous gene cGAPDH, using the $2^{-\Delta Ct}$ method.

Cell culture and spheroid formation

The synthesis of MSC spheroids was accomplished via the liquid overlay method[50]. To achieve this, 2% microwave-melted agarose gel (Sigma-Aldrich) was introduced into each well of a 24-well plate (Corning) using micro-molds (MicroTissue® 3D Petri Dish®, 256 positions, Sigma-Aldrich). Following 30 min of gel polymerization at ambient temperature, 1×10^6 MSCs cells (passage 1) in 190 µL were seeded onto the gel. Subsequently, the cells were incubated in a humidified atmosphere supplemented with 5% CO_2 (Panasonic, MCO-19AICUVH-PA). Thus, the implanted cells spontaneously aggregated into three-dimensional spheroids, collected two days later (Fig. 1A). On the other hand, detached cells were introduced into 24-well culture plates (Corning) at a density of 1×10^5 cells for the monolayer culture. Spheroids and monolayer cultures were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) media supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich) until subsequent processing. Additionally, chicken MSCs were cultured at passage 2 at a density of approximately 4.0 × 104 cells/spheroid (1 × 106 cells/micro mold 256 positions) in a supplemented RPMI medium.

Preparation of BNC scaffolds and hydrogels

Novacetimonas hansenii BRMSA 3437 (formerly *Komagataeibacter hansenii*) was activated in static Mannitol media[24] (pH 6.5) at 30 °C (Lab-Line, V.I.P. Imperial) for 3–4 days. After this, a thin cellulose pellicle formed at the air-liquid interface was harvested, sectioned, and transferred to fresh Mannitol media containing 0.125% sodium alginate for in situ modification. The culture was then agitated at 150 rpm for 3 days (Marconi, MA410/CF). Control scaffolds without sodium alginate were prepared to assess the impact of the modification. The resulting BNC macrostructures, resembling small chicken breasts, were collected, washed, and purified using an adapted Recouvreux et al.[24] method. This involved treatment with 0.1 M NaOH (ACS) at 80 °C (Novatecnica) for 30 min, followed by extensive washing until neutral pH was achieved. The purified BNC was autoclaved (Phoenix, AV-75 Plus) and stored at 2 °C–8 °C.

For some analyses, laminar hydrogels were produced by static culture of *N. hansenii* BRMSA 3437 for 7 days at 30 °C (Lab-Line, V.I.P. Imperial). The cellulose was crushed and homogenized, and an inoculum was prepared with 2.5% bacteria in situ-modified Mannitol media, with non-modified media used as control. To prevent drying, 6 mL of inoculated media was added to each well of a 6-well plate (Corning), and the hydrogels were retrieved and purified after incubation.

Characteristics of scaffolds

The reproducibility of the BNC scaffold fabrication process was evaluated using caliper measurements. Briefly, excess water was drained from the scaffold by gently placing it in a sieve for 20-30 seconds. The scaffold was then transferred to a Petri dish and measured in all dimensions (length, width, and height). All measurements were conducted on three samples unless stated otherwise.

Bacteria sequencing

To certify the genetic identification of our strain, the sample DNA was extracted and sequenced. For the sequencing, the DNA was isolated using TRIzol™ reagent (Thermo Fisher Scientific) as indicated by the manufacturer and resuspended in ultra-pure water. Concentration and quality were measured using a BioDrop spectrophotometer (Biochrom) and a Qubit 2.0 fluorometer (Invitrogen). Nucleic acid was further verified by electrophoresis on a 1.5% agarose gel (Invitrogen).

Whole genome sequencing (WGS) of *Novacetimonas hansenii* BRMSA 3437 was performed using an Illumina NextSeq platform with a paired-end library (2×300 bp). Quality control involved adapter trimming and removal of short (<100 bp), low-quality (Qphred <20), and duplicate reads using Fastp (v. 0.23.4)[51]. Sequences were assembled using Spades (v. 3.15.5)[52] and their quality evaluation was performed by Quast (v. 5.3)[53]. Gene prediction and annotation were carried out using the NCBI prokaryotic genome annotation pipeline (PGAP, v. 6.6)[54]. We determined the BRMSA 3437 genome similarity among other species by using the ANI calculated by BLAST + (ANIb) on the JSpecies Web Server[55].

Adipogenic, chondrogenic, and osteogenic assays

To evaluate their multipotent nature, chicken MSCs, both monolayers and spheroids, were differentiated into adipogenic, chondrogenic, and osteogenic cell lines using standard induction media according to the manufacturer's instructions (StemPro Adipogenesis, StemPro Chondrogenesis, StemPro Osteogenesis; Invitrogen). MSCs were plated at a density of 1×10^4 cells/cm^2 in 10 cm^2 plates and cultured for 14 days for adipogenic and chondrogenic differentiation and for 21 days for osteogenic differentiation. Cells were maintained at 37 °C in a humidified 5% CO_2, with media changes every two days. Cells were harvested at designated time points (Fig. 1A) for seeding on laminar BNC hydrogels or for characterization. Differentiation was assessed using Oil-Red-O (Sigma-Aldrich), Alizarin-Red (Sigma-Aldrich), and Alcian Blue (Sigma-Aldrich) staining[56]. Visual differences between differentiated and non-differentiated spheroids, as well as between spheroids and monolayers were observed under light microscopy (EVOS M7000 Imaging System, Thermo Fisher) and analyzed using FIJI (version 2.14.0/1.54 f).

Fibroblast-like assays and cell differentiation

Chicken MSC was isolated from three unincubated fertile eggs, following protocols from our previous study[57]. These cells were used for adipogenic, muscular and fibroblast proliferation and differentiation.

As DMEM supplemented with 10% FBS is the common media used for adherent cells and allows higher proliferation rates of fibroblasts[58] and fibroblast-like cells, part of the prepared spheroids were maintained in this media after initial proliferation at RPMI 1640 medium as indicated above. Similar to other differentiation cultures, cells fed with DMEM were maintained for 18 days, with 30% exchange every 3 days. Additionally, harvesting was performed at designated time points for seeding on laminar BNC hydrogels, or for further characterization (Fig. 1A).

Myoblasts cells were selected from MSC by selective adhesion as previously described by our group[57]. For cell differentiation, when the myoblasts reached 90% confluence, they were cultured in a differentiation medium composed of DMEM high glucose medium (Thermo Scientific) supplemented with 2% FBS (Thermo Scientific) and 1% antibiotic-antimycotic (Thermo Scientific), at 41 °C under 5% CO_2, to induce the formation of myotubes and myofibers. Chicken myoblasts and myotubes were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100, and blocked with 5% goat serum or 3% BSA for 1 h. Cells were incubated overnight at 4 °C with primary antibodies targeting MyHC (MF20, ThermoFisher) and MyF5 (Abcam) followed by Alexa Fluor 488-conjugated secondary antibodies (1:800, ThermoFisher) for 1 h at 37 °C. F-actin was counterstained with Rhodamine Phalloidin (1:500, ThermoFisher), and nuclei with DAPI (ThermoFisher). The stained wells were scanned using an EVOS M7000 fluorescence microscope (light cubes: Cy5 and DAPI, objective: 10× or 20×). Pseudo colors were assigned to the images using the LUT color scheme available in the ImageJ software (Fiji 2.14.0).

The adipogenic cells to be co-plated on BNC scaffolds alongside muscle cells were derived from chicken fibroblasts. These fibroblasts were seeded at a density of 4×10^4 cells/cm^2 under adherent conditions. Transdifferentiation was induced using DMEM supplemented with 10% FBS, 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich), 10 µg/mL insulin (Sigma-Aldrich), and 0.1 µM dexamethasone (Sigma-Aldrich) for 3 days, followed by continued culture with 10 µg/mL insulin alone for an additional 4 days. For adipocytes, differentiated cells were further fixed with 4% paraformaldehyde for 30 min on day 21. Lipid staining was performed using the neutral lipid stain HCS LipidTOX Red (Thermo Scientific), diluted 1:1000 according to the manufacturer's protocol, and Nile Red was prepared by diluting the stock solution (1 mg/mL) to a working concentration of 0.5 µg/mL in DPBS. Cells were counterstained with Hoechst 33342 (Thermo Scientific) at 1 µg/mL in DPBS for 10 min at room temperature. After staining, cells were washed with DPBS and imaged under fluorescence light microscopy (EVOS M7000 Imaging System, Thermo Scientific).

Myoblasts cells cultured into BNC scaffolds

To verify whether BNC scaffolds support MSC and myoblasts growth, the isolated cells were seeded in both modified and unmodified BNC at a concentration of 5×10^5 cells/scaffold/mL of media within cylindrical scaffolds (1.5 cm diameter × 1.0 cm height), which were placed in a plate. Each scaffold was seeded with 40 undifferentiated spheroids by means of a dynamic seeding procedure. After 7 days in DMEM culture, we imaged the structure using an inverted microscope (Carl Zeiss, Axio Vert. A1).

Cell viability of MSCs spheroids

To confirm that our method would allow MSC spheroids to grow for the entire period of differentiation (18 days), we imaged spheroids cultured either in RPMI 1640 or low DMEM media every 2-3 days. Additionally, cells were stained with DAPI 1/2000 for 2 h and 30 min and LIVE/DEAD cell (Thermo Scientific) viability staining was used to assess live and dead cells, according to the product manual. To achieve this, spheroids were rinsed twice with PBS and fixed with 4% (w/v) paraformaldehyde (Synth) for 2 h. Monolayer cultures were used for comparison. Nonetheless, fixing and staining were performed for 30 min, each. All images were taken in an inverted microscope (Carl Zeiss, Axio Vert. A1) and analyses were carried out on ImageJ (version 1.53t).

Morphology of differentiated spheroids

Differentiated spheroids were characterized through morphological analysis by imaging them every 2-3 days through an inverted microscope. These images were compared with spheroids cultured on either RPMI 1640 or low DMEM at the same time points.

Spheroid dimensions were tracked by comparing their area on day 3 with days 5, 7, 11, 14, 17, and 18 following the initiation of differentiation media. The shrinkage percentage was calculated by Eq. (1):

$$\%\, shrinkage = \frac{area\ on\ day3 - area\ on\ dayX}{area\ of\ day3} x100$$

Imaging analyses were performed on ImageJ (version 1.53t).

Adipocytes and myoblasts cells cultures on laminar BNC hydrogels

The ability of adipocyte, chondrocyte, and osteocyte spheroids to adhere and grow on in situ modified laminar BNC hydrogels was tested and compared with non-modified hydrogels (Table S1). Before cell seeding, BNC hydrogels were rinsed with PBS, incubated in RPMI medium at 37 °C and 5% CO_2 for 30 min, and then seeded with spheroids. Adherence between BNC and cells was ensured by incubating the hydrogels without media at 37 °C and 5% CO_2 for 30 min. Following this, 800 µL of RPMI was

gently added, and the cells were incubated overnight. The culture was maintained for 7 days with 30% media replacement every 2 days. Monolayers followed the same protocol. Daily or alternate-day imaging was conducted, and at the end of the period, adipocytes, and muscle cells were stained according to established protocols, while fibroblast-like spheroids were stained with DAPI. RPMI 1640 cultures were used as a control and stained with DAPI for comparison.

Interaction between differentiated spheroids and monolayers cultured on laminar BNC hydrogels

In order to comprehend the interaction between different spheroids and monolayers in laminar BNC hydrogels, we mimicked the cellular components of a chicken breast filet. We prepared pools of adipocyte and myoblast cell monolayers, and adipocyte, myoblast cell and fibroblast spheroids. These pools were seeded individually or together (Table S1) onto the porous surface of laminar BNC hydrogels. Cultures were maintained for 7 days, with 30% media replenished every 2 days. Images were captured daily or every other day. At the end of the period, hydrogels were fixed and stained with DAPI.

Drip loss

Drip loss was realized according to Boccard et al.[59] and adapted by Bridi and Silva[60]. Briefly, samples underwent a cutter until a cylindrical shape was obtained. Then, they were placed in plastic collectors (specific for drip testing) in a specific tray. Samples remained refrigerated at 4 °C for 48 h before being weighed on a semi-analytical scale.

Protein and moisture determination

Total protein was quantified via Dumas method, according to AOAC Official Method 992.15[61]. This technique measures the amount of nitrogen in organic samples. It is a rapid and accurate method based on the combustion of the sample and analysis of the resulting gases. The combustion is performed in an environment with excess oxygen, in which the samples' nitrogen is converted into nitrogen gas, which is analyzed and converted into the amount of nitrogen in the sample. Moisture content at 105 °C was determined by the gravimetric method until constant weight[62].

Cooking in an air fryer

Cooking experiments were conducted after the injection of cells (adipocytes, chondrocytes, and osteocytes) into the BNC scaffolds until complete saturation by using a 5 mL disposable syringe with a needle (0.7 x 25 mm) equipped with a Luer Lock tip and thread attachment. The breaded cultivated chicken was prepared by dissolving 1% of equal quantities of isolated soy protein, coconut milk powder, and thickener (xanthan gum) in distilled water to assist in structuring, which was necessary due to the scaffold's high water content. Subsequently, the scaffolds were coated with breadcrumbs and deep-fried at 180 °C for 7.5 min, rotating the pieces halfway through.

Data availability

The data that support this study are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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Author contributions

K.P.V.O. (ORCID [0000-0003-4144-1163]): conceived, planned, and performed scaffold experiments; planned and performed most cell experiments; prepared samples for NGS and helped in sequencing analysis; drafted the manuscript and designed figures; A.P.B. (ORCID [0000-0002-0873-1639]): drafted the manuscript, cell viability analysis, conceived and designed the experiment; A.L.C.B. (ORCID [0000-0003-0245-122X]): stem cell standardization; F.A.V. (ORCID 0000-0002-5149-0359): performed most cell experiments; V.G. (ORCID [0000-0003-3162-2630]): cooking experiments and physico-chemical analysis; V.L.K. (ORCID [0000-0003-0586-0985]): physico-chemical analysis; V.H. (ORCID [0000-0001-5399-4107]): cell genetic analysis; performed cell isolation, myogenic and adipogenic differentiation assays, and cell viability analysis; A.C.J. (ORCID 0000-0002-4808-080X): physico-chemical analysis; M.E.C. (ORCID 0000-0002-5988-6542): sequencing analysis; K.R.D.S (ORCID 0000-0002-7782-5225):

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Competing interests

The authors declare no competing interests.

Additional information

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