

ORIGINAL ARTICLE

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Study on the feasibility of using livestock blood as a fetal bovine serum substitute for cultured meat

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Abstract

Fetal bovine serum (FBS) accounts for the largest portion of the cost of cultured meat production or cell culture experiments and is highly controversial in terms of animal welfare because it is taken from the fetus of a pregnant cow during slaughtering. Nevertheless, FBS is the most important supplement in the cell culture manufacturing process. This study aimed to develop an FBS substitute from slaughterhouse waste blood to reduce the cost of FBS in cultured meat production through various experiments. Our study successfully demonstrated that adult livestock blood obtained from slaughterhouses can effectively replace FBS. Our substitute, when cultured with bovine myosatellite cells, demonstrated cell growth that was either equivalent to or superior to that of commercial FBS. In the process of muscle generation through differentiation, the substitutes from bovine and chicken formed 70%–75% more bovine muscle compared to the control group using FBS. Furthermore, using the FBS substitute can reduce cell culture costs by approximately 61% compared to using commercial FBS. Therefore, the groundbreaking FBS substitute will not only contribute to the development of technology to mass-produce cultured meat using livestock byproducts but will also lower the production cost of media for experimental cell culture or vaccine production.

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KEYWORDS

animal slaughterhouse blood, fetal bovine serum substitute

1 | INTRODUCTION

Cultured meat, which refers to edible meat obtained through cell proliferation without going through the process of raising livestock, has emerged as an alternative technology to solve the concerns and perceptions about animal welfare and the environmental impacts of the livestock industry (Lee et al., 2021; Nielsen & Hawkes, 2019). Many research institutions and start-ups around the world are competitively conducting research on the production of cultured meat, but many problems need to be solved to achieve the industrialization of cultured meat. The main challenges are affirming the safety of cultured meat for human consumption and lowering production costs. O'Neill et al. (2021) suggested that there are technical obstacles still hindering the large-scale commercialization of cultured meat, many of which are related to the cell culture media.

In this regard, fetal bovine serum (FBS) accounts for the largest portion of the cost of producing cultured meat. FBS is the most common media supplement used for *in vitro* culturing of animal and human tissues and organs because it has essential components required for cell attachment, proliferation, and maintenance, such as serum albumin, fetuin, hormones, vitamins, trace elements, and growth factors (Chelladurai et al., 2021). As its name suggests, FBS is the serum fraction of blood taken from normal bovine fetuses harvested from healthy pregnant cows at slaughter (O'Neill et al., 2021). Therefore, the use of FBS is still controversial in terms of animal welfare because serum collection causes unnecessary pain in the fetus (van der Valk et al., 2004), and commercial FBS is expensive. Many researchers are attempting to replace FBS with other materials or develop serum-free media, and technology development that completely replaces FBS or acquires FBS-like materials at low cost stands as an important challenge for the industrialization of cultured meat.

Meanwhile, our research group has been conducting research to acquire and synthesize physiological active substances from livestock slaughter byproducts (Kim et al., 2023; Lee et al., 2019, 2020, 2022; Yoon et al., 2018). Between around 52% and 80% of the live weight of lambs, cattle, chickens, and pigs is discarded as byproducts, which include skin and fat, bone, blood and internal organs, and abdominal and intestinal contents (Limeneh et al., 2022). The majority of these byproducts are disposed of as waste, with severe environmental consequences on local and global scales. Additionally, the additional costs incurred to safely dispose of these byproducts can increase the

price of meat and deteriorate the livestock-rearing environment. Therefore, research has continued to utilize these slaughter byproducts and turn them into resources such as fertilizer, feed, and energy raw materials (Etemadzadeh & Emtiazi, 2021; Lee et al., 2020; Limeneh et al., 2022). Maximizing the utilization of food livestock byproducts as high-added-value products would contribute to attaining sustainability in this sector, which currently faces severe waste issues.

Although cattles, pig, and chicken slaughter byproducts, including blood, are mostly being discarded, blood contains a variety of bioactive substances, such as hormones, growth factors, protein, amino acids, glutamine, vitamins, or iron. Although research is underway to replace FBS, its use is still significantly effective in cell culture (Kolkmann et al., 2020; Skrivergaard et al., 2023). In addition, even in cases where FBS is excluded, not only albumin, growth factors, and fetuin are added to prepare serum-free media, but also matrigel, collagen, gelatin, and laminin, coating agents used on culture dishes or scaffolds for cell attachment, also originate from animals (Kolkmann et al., 2020; Liu et al., 2024; Skrivergaard et al., 2023; Wu et al., 2020). From this perspective, the efficient use of byproducts generated during meat production will not only reduce waste disposal costs and environmental pollution while improving the added value of byproducts but also help improve animal welfare by resolving the controversy over fetal blood collection. In addition, the development of FBS substitutes is expected to be used not only for mass production of cultured meat but also for various types of cell line cultivation or vaccine production, which will lead to a reduction in production costs. Therefore, the purpose of this study is to develop an FBS substitute using slaughterhouse waste blood, which will reduce the reliance on bovine fetuses and the cost of cultured meat production.

2 | MATERIALS AND METHODS

2.1 | Manufacturing of FBS substitute

2.1.1 | Acquisition of livestock slaughterhouse blood

Blood from bovine, porcine, and chicken sources was obtained from Chung-Ang University's Farm and Woo Gyeong Livestock for the development of FBS substitute. Bovine blood was collected from male (38 months) and female (54 months) Korean cattle (one head for each sex).

Porcine blood was collected from slaughtered sows (LYD crossbreed, three female heads) aged 180 days. Chicken blood was collected from slaughtered broilers (ROSS 308 breed, 40 female heads) aged 40–43 days.

2.1.2 | Preparation of FBS and FBS substitute

Commercial FBS (35-015-CV) was purchased from Corning. The purchased FBS was inactivated in a water bath at 55°C for 30 min (based on 500 mL), subdivided, and stored frozen. The process of manufacturing FBS substitutes using slaughterhouse waste blood from bovine, porcine, and chicken sources is presented in Figure 1. All blood samples were centrifuged (Combi 514R, Hanil Scientific) at 2,691 g for 15–30 min after sufficient coagulation had occurred. The recovered sera were stored frozen. Three pretreatment methods were used to prepare FBS substitutes (originating from bovine, porcine, and chicken serum, respectively). The thawed serum was again subjected to two different centrifugation steps to remove unnecessary impurities. The first product (referred to as Raw) was the as-recovered serum. The second product had undergone sonication treatment (referred to as Sonication). To this end, the thawed sample was repeatedly processed for 5 s on and 5 s off for 5–20 min using an ultrasonicator (KFS-250N, Korprotech), during which time, the sample temperature did not exceed 40°C. The third product (referred to as Inactivation) was obtained by heating the thawed sample at 55–65°C for 15–30 min. The treated samples were filtered sequentially two times through a 0.1–0.22 µm filter (Millipore®) and kept frozen until immediately before use. The FBS substitute used was confirmed to be usable for cell culture using the mycoplasma detection kit (SMD0172, Biomax) and Petrifilm (6406 (Aerobic bacteria), 6414 (*Escherichia coli*), 6424 (*Staphylococcus aureus*), 3 M).

2.2 | Production of cultured meat

2.2.1 | Preparation before cell culture and cell acquisition

The culture medium for culturing myocytes was prepared with Ham's F-10 medium, FBS (both commercial and substitute), and antibiotics (penicillin, streptomycin, and amphotericin-B: P/S/A). The control medium (growth medium) of cell culture was made by adding commercial FBS (20%) and P/S/A (1%; 03-033-1B, Biological Industries) to the basal medium. The cell culture dish was coated before use. The coating solution (0.1% collagen) was sterilized using an autoclave after completely dissolving collagen from calf skin (C9791, Sigma-Aldrich) in water. The subdivided solution was stored frozen and then thawed

before use. Collagenase D (11088858001, Sigma-Aldrich) and Dispase II (4942074800, Roche) solutions were prepared (3.00 and 4.48 U/mL 1× DPBS (Dulbecco's Phosphate Buffered Saline), respectively) and stored frozen. Calcium chloride (CaCl₂) was dissolved (100 mM), filtered through a 0.22 µm syringe filter, and stored refrigerated. The enzyme cocktail solution for isolating cells from the tissue contained collagenase D (1 mL), Dispase II (1 mL), and CaCl₂ (50 µL, 100 mM), which were mixed immediately before use.

2.2.2 | Acquisition of bovine muscle cells (bovine myosatellite cells)

Bovine muscle for cell isolation was from a 34-month castrated Korean cattle beef (Hanwoo) and provided by Chungbuk National University. All procedures involving the cattle were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (approval number 202,301,020,027). All bovine cell acquisition procedures were conducted in compliance with livestock slaughter regulations. The cell acquisition process was modified by referring to our previous papers (Lee et al., 2021; Yun et al., 2023). After washing with 70% ethanol to prevent further contamination, the tissue proceeded to the next step. Tissues were minced using dissecting scissors and a scalpel while immersed in cold 1× DPBS containing P/S/A (1%). In this process, connective tissue, blood, and fat were removed, leaving only muscle tissue. The minced tissue was reacted in a 37°C water bath by adding the prepared enzyme solution (Section 2.2.1) to the tissue (1:1.5 w/v) just before use. The enzyme reaction was inverted once every 10 min during incubation (30–90 min). To muscle tissue with sufficient decomposition, 1× DPBS was added more than four times or more the total volume to stop the enzymatic reaction, and then the mixture was shaken well to dilute the agglomerated tissue. Undecomposed tissue was sequentially filtered using a cell strainer (100–30 µm). After centrifuging the filtered cell solution, the pellet was dissolved in basal media. The cell solution was placed in an uncoated dish and left in a 37°C/5% CO₂ incubator for 30–60 min. Only the supernatant of the cell culture solution was collected in a dish. The supernatant was again dissolved in basal media containing inactivated-FBS (20%) and P/S/A (1%) and incubated in a coated dish (Primary cells). The medium was replaced on the first day of culture, and the primary cells were collected once they had proliferated sufficiently. The harvested cells were treated with antibodies CD29 (APC anti-human CD29 Antibody, 303008, BioLegend) and CD56 (CD56 PE-CyTM7, 335826, BD Biosciences) to label muscle satellite cells and were then sorted using Fluorescence Activated Cell Sorting (FACS). In subsequent experiments, only cells positive for CD29 + CD56 were proliferated and used (data not shown).

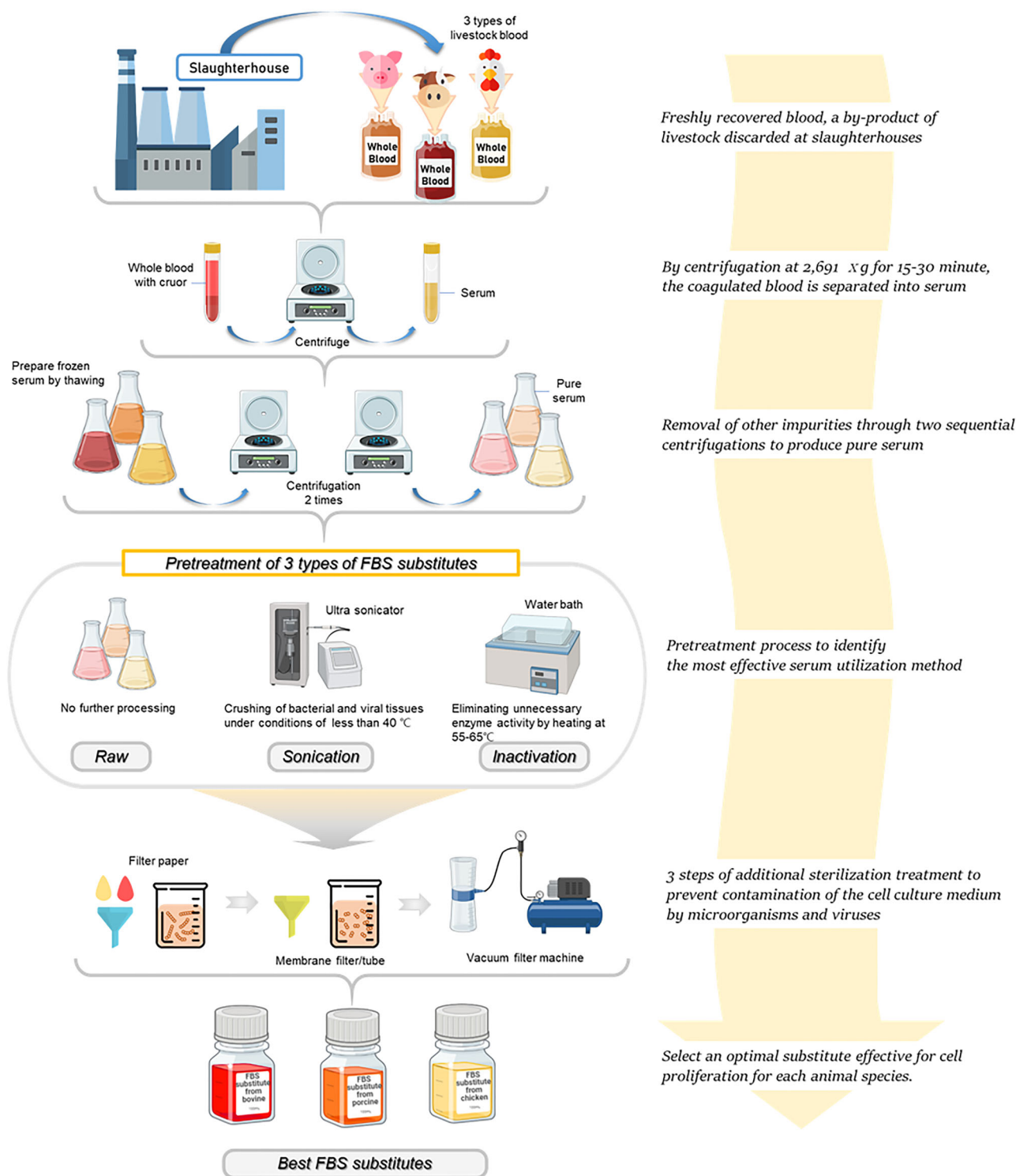


FIGURE 1 Overview of the preparation of fetal bovine serum (FBS) substitutes obtained from livestock waste blood in slaughterhouses.

2.2.3 | Subculture of bovine myosatellite cells

Cells cultured in the coated dish were subcultured when the cells had proliferated sufficiently. During culture, the medium was replaced every 2–4 days. During subculture, the existing medium was removed and washed with

1 $\times$ DPBS. Then, the cells were detached by incubation with trypsin solution (0.25%). Trypsin was subsequently inactivated by adding 1 $\times$ DPBS, and the cells were collected. The cell fluid was centrifuged, and the collected cell pellet was suspended in the cell culture media. The cells were placed in a collagen-coated dish with growth

medium (5–10 mL). All treatment groups, including the control group, were supplemented with cell culture additives CAU- α [®] and CAU- β [®]. CAU- α contains a mixture of lipid-rich bovine serum albumin (BSA) and insulin-transferrin-selenium, whereas CAU- β contains a mixture of three types of growth factors, including basic fibroblast growth factors. This study did not present more detailed manufacturing methods and ratios due to the use of patent-pending products. Cell was incubated in a 37°C/5% CO₂ incubator.

2.2.4 | Inducing differentiation of bovine myosatellite cells to muscle

Differentiation of myosatellite cells can be induced with myogenic medium, and the composition of this medium is changed over two stages. Differentiation induction was started with DMEM (Dulbecco Modified Eagle Medium) containing P/S/A (1%) and horse serum (2%) and then replaced with DMEM containing P/S/A (1%) and horse serum (10%).

2.3 | Analysis of cell viability with FBS substitutes derived from slaughterhouse blood

In an experiment to confirm the threshold, an FBS substitute was used to replace FBS in the cell proliferation medium. Quanti-Max™ WST-8 Cell Viability Assay Kit (QM2500, Biomax) was used. For the analysis, cells (5–10 × 10³) were put in a 96-well plate coated with collagen (0.1%), and the FBS substitute medium prepared for each concentration was added and incubated. FBS substitutes were added from a minimum of 20% (equivalent to commercial FBS) to a maximum of 40%. After 2–3 days, when the cells were sufficiently grown, WST reagent was added. The plate was wrapped in foil and reacted in a 37°C/5% CO₂ incubator. After 3 h, the absorbance was measured at 450 nm using a microplate reader (SpectraMax 190, Molecular Devices).

The thiazolyl blue tetrazolium bromide (MTT) assay was performed to confirm the viability of the produced FBS substitutes. For the analysis, cells (5–10 × 10³) were put in a 96-well plate coated with collagen (0.1%), and the FBS substitute medium prepared for each concentration was added and incubated. After 2–3 days, when the cells were sufficiently grown, the supernatant was removed. MTT (M2128, Sigma-Aldrich) reagent was prepared (5 mg/mL 1× PBS) in the dark, diluted in a ratio of 1:9 with medium, and added. The plate was wrapped in foil and reacted in a 37°C/5% CO₂ incubator. After 4 h, the supernatant was removed, DMSO was added, and the reaction was performed in the dark for

10–15 min. Cell viability was measured at 540 nm using an ELISA reader.

2.4 | Immunofluorescence staining of cultured cells and muscle

Cultured cell and muscle samples were washed twice with 1× DPBS (16777-644, Cytiva) and fixed in paraformaldehyde (4%) for 20 min. After washing twice more with 1× DPBS, the samples were treated with Triton X-100 (0.2%; Sigma-Aldrich) for 20 min and blocked with BSA (1% in 1× DPBS) for 30 min at room temperature. Cells were then incubated overnight at 4°C with the primary antibody (MF20 at 1:500; Myosin Heavy Chain Antibody, MAB4470, Novus Biologicals) dissolved in BSA (1% in 1× DPBS). After incubation, cells were treated with FITC-conjugated secondary antibody for 1 h at room temperature and then stained with Hoechst 33342 (1:1000; 561908, BD Biosciences) and phalloidin (1:1000; A12381, Thermo Fisher Scientific). The differentiated cells or myogenesis images were observed with a fluorescence microscope (KI-3000F, Korea Lab Tech).

2.5 | Western blot analysis of cultured cells and muscle

Western blots were performed on cell lysates obtained by lysing cells directly with Radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor (78420, Thermo Fisher Scientific) and protease inhibitor (87785, Thermo Fisher Scientific). The Bicinchoninic Acid Protein Assay Kit (23227, Thermo Fisher Scientific) was used to quantify the total protein in each sample. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gels) was carried out. After transfer of the proteins to PVDF membranes, the proteins were blocked with BSA (3% in PBS-T) for 1 h and probed overnight with primary antibodies MF20 (1:500; MAB4470, Novus Biologicals) and β -actin (1:2000, ab8227, Abcam). Then, the proteins were incubated with anti-mouse IgG and anti-rabbit IgG as appropriate. Specific signals were detected with an e-BLOT Touch Imager (e-BLOT).

2.6 | Statistical analysis

For the presented results, three to five replicates were used in one experiment, and at least three independent replicates were performed. Statistical analysis was conducted by one-way ANOVA with post hoc Tukey's multi-range

test using the SPSS 22.0 program (IBM). A significance level of $p < 0.05$ was used for all tests. Cell immunofluorescence staining images and Western blot quantification were analyzed using ImageJ (NIH Image).

3 | RESULTS AND DISCUSSION

We studied the possibility of replacing FBS using blood obtained when slaughtering livestock raised for meat. As shown in Table 1, we conducted a comparative analysis of the ingredients of the FBS substitute developed in this study based on a previous study (Lee et al., 2023). In a study comparing the ingredients of 12 types of commercial FBS with bovine serum, porcine serum, and chicken serum, the composition of commercial FBS was almost similar regardless of brand or country of origin, and bovine serum substitute had the most similar composition to FBS (Table 1). Peptides with approximately 2–15 amino acids can enhance cell growth and metabolic activity (Andreassen et al., 2020). FBS substitutes contained amino acids like phosphoserine, phosphorylethanolamine, sarcosine, β -aminoisobutyric acid, γ -amino-*n*-butyric acid, hydroxylysine, 1-methylhistidine, and anserine, but commercial FBS did not (Table 1). However, cystine, which was not present in commercial FBS, was detected in all three FBS substitutes (Table 1). Studies on direct feeding to pigs have shown that threonine deficiency or excess reduces skeletal muscle protein synthesis in pigs (Wang et al., 2007). Compared to commercial FBS, the threonine content of FBS substitutes was lower (bovine serum) or higher (porcine serum and chicken serum) (Table 1) (Wang et al., 2007). These results can serve as a basis for determining the appropriate content of amino acids in the medium to replace FBS.

Mature bovine blood was found to contain higher concentrations of total protein, albumin, glucose, and cholesterol (CHO) than commercial FBS, and this trend is very similar to the results of our previous comparative analysis of the components of FBS substitutes (FBS, bovine serum, porcine serum, and chicken serum) (Table 1) (Lee et al., 2022; Yu et al., 2013). Among blood components, chicken serum specifically contains the highest amount of alkaline phosphatase (Table 1). There were also differences in fat and fatty acid content of mature-derived FBS substitutes due to abundant nutrient supply and animal growth (Lee et al., 2022; Velasco et al., 2010). Except for commercial FBS, all FBS substitutes had high total CHO, CHO-HDL, and CHO-LDL values (Table 1). Common differences between the commercial FBS and three substitutes were that all substitutes did not contain myristoleic acid and had a very high total fatty acid content (Table 1).

By analyzing the components of three FBS substitutes and commercial FBS in previous studies, we attempted to predict the similarity and substitutability of the substitutes' components. In addition to the components mentioned, growth factors, cytokines, and other unknown serum components may affect cell growth. Our previous study (Lee et al., 2023) also attempted to analyze these components, but compared to the great progress in research on human cytokines and growth factors, research on methods for analyzing livestock blood (serum) is very insufficient. In particular, it was impossible for us to set up the same analytical method to compare the blood components of three livestock species (cattle, pigs, and chickens). In addition, because it is a very difficult process to remove specific components (e.g., cytokines) that can negatively affect cell culture, future studies will be conducted in the direction of identifying and supplementing the missing components. To conduct such studies, it will be necessary to pool the blood of more individuals from different species, in order to confirm the characteristics of the average serum components.

3.1 | Bovine myosatellite cell proliferation

3.1.1 | Analysis threshold amount of three FBS substitutes

We developed three types of FBS substitutes obtained from slaughterhouse blood waste to replace FBS (Figure 2a). Commercial FBS and the developed FBS substitutes were applied to bovine myosatellite cell culture under the same conditions with the same additives, including basal medium (Figure 2b). All three FBS substitutes showed maximum efficiency in cell proliferation when added at 20%, which is the same amount as FBS. In the present study, the treatment group to which the FBS substitute was applied showed a smaller amount of cells than cells treated with commercial FBS, but myoblasts were formed without significant damage to the cells (Figure 2c,d). Among them, groups treated with the FBS substitute from bovine and porcine serums showed cell growth similar enough to treatment with commercial FBS when added at the same amount (20%) as commercial FBS. Conversely, chicken serum did not promote cell growth sufficiently, even when added at 40% (Figure 2c). This difference may be attributed to the differences in the contents of components essential for cell growth between mammalian- and poultry-derived substitutes (Shahdadfar et al., 2005). From another point of view, the addition of a large amount of FBS rather inhibited the growth of cells, and bovine serum obtained from older individuals

TABLE 1 Summary of common ingredient differences between commercial fetal bovine serum (FBS) and FBS substitutes.

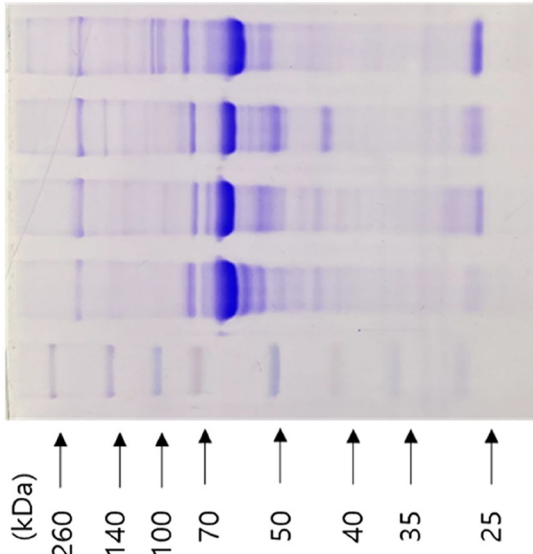
Amino acids	FBS	BS	PS	CS	Blood component	FBS	BS	PS	CS	Unit	Fatty acid profile (mg/L)	FBS	BS	PS	CS
Phosphoserine	-	-	-	-	ALB (albumin)	2.6	4.5	5.0	1.4	g/dL	C12:0	2.5	2.2	2.5	2.4
Taurine (mg/L)	10.0	7.3	18.4	19.2	ALP (alkaline phosphatase)	208.6	92.7	116.0	3091.1	IU/L	C14:0	2.4	3.3	3.0	2.4
Phosphoryl-ethanolamine	-	-	-	-	BIL-T (total bilirubin)	1.0	0.9	0.7	1.0	mg/L	C14:1	3.1	-	-	-
Urea (mg/L)	148.7	140.5	174.8	2.2	BIL-D (direct bilirubin)	3.7	0.7	0.1	1.4	mg/L	C16:0	78.3	200.5	174.4	278.0
Aspartic acid (µg/dL)	582.5	46.6	170.3	1871.8	BUN (blood urea nitrogen)	15.6	14.2	17.3	2.1	IU/L	C16:1	1.5	4.8	2.9	14.3
Threonine (µg/dL)	756.6	300.7	1054.5	4842.2	Ca (calcium)	15.8	11.1	13.1	8.4	mg/dL	C17:0	1.8	7.4	5.7	-
Serine (mg/L)	15.4	4.8	8.9	39.9	CHO-HDL	8.9	183.1	52.0	67.4	mg/dL	C18:0	62.2	327.0	166.3	318.9
Asparagine (µg/dL)	156.3	117.7	174.9	8.6	CHO-LDL	23.7	87.6	75.6	30.6	mg/dL	C18:1n9	23.8	110.3	95.6	105.8
Glutamic acid (mg/L)	62.1	10.4	27.9	32.0	CHO (total cholesterol)	35.5	212.2	116.2	104.2	mg/dL	C18:1n7	6.7	5.0	7.1	8.7
Glutamine (mg/L)	39.3	13.9	18.2	71.4	CREA (creatinine)	30.4	19.4	19.9	2.5	mg/L	C18:2n6	4.8	1.7	97.7	216.9
Sarcosine	-	-	-	-	GLU (glucose)	133.4	105.6	110.9	390.4	mg/dL	C18:3n3	-	-	2.1	4.8
Glycine (mg/L)	22.9	10.9	32.4	34.7	GOT/AST (aspartate aminotransferase)	34.9	80.6	74.4	312.9	IU/L	C20:0	-	-	-	-
Alanine (mg/L)	43.3	17.1	39.3	42.9	GPT/ALT (alanine aminotransferase)	5.7	14.9	39.6	1.0	IU/L	C20:2n6	-	-	3.7	12.1
Citrulline (µg/dL)	669.9	437.2	628.0	90.5	LDH (lactate dehydrogenase)	936.4	1635.0	1585.0	1828.5	IU/L	C20:3n6	-	-	-	3.9
α-Amino-n-butyric acid (µg/dL)	167.6	13.1	44.4	132.1	IP (inorganic phosphorus)	10.2	7.2	13.5	8.4	mg/dL	C20:4n6	3.7	-	12.5	31.7
Valine (mg/L)	19.7	15.6	21.5	14.5	TG (triglyceride)	131.1	76.8	96.4	132.3	mg/dL					
Cystine (µg/dL)	-	4.3	23.4	1188.6	TP (total protein)	3.6	7.4	8.5	2.5	g/dL					
Methionine (µg/dL)	251.2	150.5	296.2	1169.8	UA (uric acid)	16.8	6.0	1.0	81.3	mg/L					

(Continues)

TABLE 1 (Continued)

Amino acids	FBS	BS	PS	CS	Blood component	FBS	BS	PS	CS	Unit	Fatty acid profile (mg/L)	FBS	BS	PS	CS
Cystathionine (μg/dL)	87.5	37.5	62.7	326.7	γ-GTP (r-Glutamyl transpeptidase)	6.2	18.1	60.6	13.4	IU/L					
Isoleucine (μg/dL)	904.1	608.0	945.4	905.5	CRP (C-reactive protein)	4.0	9.77	80.00	–	μg/dL					
Leucine (mg/L)	15.7	13.1	18.6	22.7											
Tyrosine (μg/dL)	849.0	495.8	767.2	3338.4											
Phenylalanine (mg/L)	12.0	5.3	10.0	16.5											
β-Alanine (μg/dL)	254.6	7.7	65.8	323.7											
β-Amino isobutyric acid	–	–	–	–											
γ-Amino-n-butyric acid	–	–	–	–											
Tryptophan (μg/dL)	419.3	512.2	544.9	600.1											
Ethanol amine (μg/dL)	42.7	–	–	114.7											
Ammonia (μg/dL)	537.7	366.2	511.1	384.5											
Hydroxylysine	–	–	–	–											
Ornithine (mg/L)	14.9	6.3	15.9	2.1											
Lysine (mg/L)	13.3	8.6	20.7	22.0											
1-Methylhistidine	–	–	–	–											
Histidine (μg/dL)	703.4	528.3	798.5	546.5											
3-Methylhistidine (μg/dL)	345.1	66.4	92.9	76.9											
Anserine (μg/dL)	–	–	–	356.5											
Carnosine (μg/dL)	447.3	218.6	255.3	202.6											
Arginine (μg/dL)	336.8	1262.8	1092.1	4765.5											
Hydroxyproline (μg/dL)	754.3	119.9	370.7	1302.9											
Proline (mg/L)	10.9	4.4	15.7	29.1											

Note: This table was modified from our previous study. Reproduced with permission from Lee et al. (2023). Figure in the table is the analysis of SDS–PAGE. FBS is the average of 12 commercial FBS assays. Amino acid, blood component, and fatty acid analysis values were written as average values only. Abbreviations: BS, bovine serum; CS, chicken serum; PS, porcine serum.



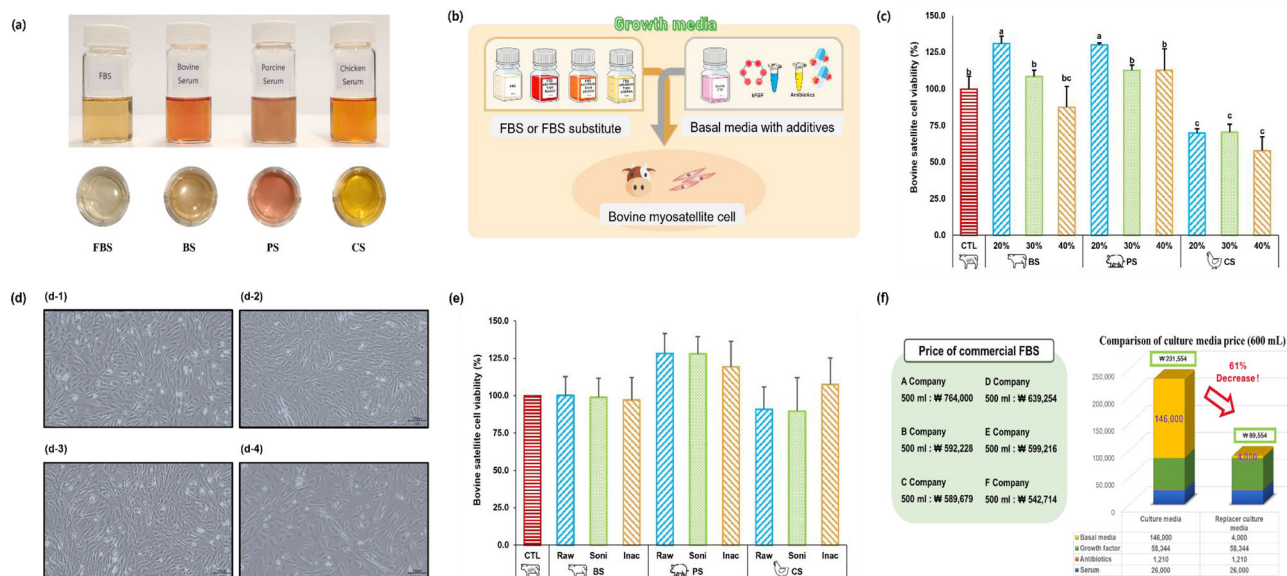


FIGURE 2 Cell viability analysis between commercial fetal bovine serum (FBS) and FBS substitutes derived from livestock slaughterhouse blood in cultured bovine myosatellite cells. (a) FBS and FBS substitutes. (b) Schematic of three types of cell culture with additives. (c) Analysis of maximum cell viability (WST) requirements of FBS substitutes. (d) Brightfield images of best cell proliferated treatment of (b). d-1: CTL, d-2: 20% bovine serum (BS), d-3: 20% porcine serum (PS), d-4: 20% chicken serum (CS). (e) Cell viability (thiazolyl blue tetrazolium bromide [MTT]) analysis based on pretreatment between commercial FBS and FBS substitutes. (f) Illustration of developed FBS substitutes price competitiveness comparison with commercial FBS. Data are expressed as the means $\pm$ SEM. ^{a-c} Means with different superscripts within the same row are significantly different ($p < 0.05$).

had a negative effect on cell proliferation when added in higher amounts (30% or 40%) (Figure 2b). This result may have been caused by components in the blood of livestock with already-formed immune factors that are not in fetal serum (Andreassen et al., 2020). Therefore, the amount of FBS replacement for the follow-up study was set at 20% to achieve maximum effect with the minimum amount.

3.1.2 | Establishment of pretreatment methods for suitable FBS substitutes

Subsequent experiments were conducted to enhance the substitution effect of mixed FBS substitutes obtained from various subjects. Even though bovine and porcine sera were subjected to separate pretreatments, no particular growth superiority in cell proliferation was confirmed (Figure 2e). Conversely, chicken serum showed that the effect of Inactivation treatment was slightly higher than the other two treatments (Raw and Soni), but there was no significant difference (Figure 2e). Commercial FBS, our control, was inactivated as described in Section 2.2.1. Although there was no significant difference, all subsequent FBS substitutes were manufactured by applying an inactivation pretreatment method to prevent any possible

infection risk and to ensure the highest efficiency of all substitutes.

Currently, the average selling price of FBS from major companies is more than \$600–800 for 500 mL (based on Korean purchases), the reason being that almost all FBS products used in Korea are manufactured in the USA or Australia (Figure 2f). The price of the growth media for muscle satellite cell proliferation (basal media + 20% FBS + 1% antibiotic + additives) using the FBS substitute produced by our method can be produced at a price that is about 61% lower than the price of commercial FBS (Figure 2f). This figure could be even higher when considering the impact of reduced disposal costs and added value creation from slaughterhouse blood products. FBS substitutes could be a key solution for cultured meat production, considering not only the reduced production cost but also the effect of reducing the cost of slaughter byproduct disposal, which is a chronic problem for many livestock farms (Etemadzadeh & Emtiazi, 2021; Lee et al., 2020; Limeneh et al., 2022).

We predicted that the use of fetal serum would be significantly efficient for cell growth because it is free from the formation of immune factors due to environment factors after birth. However, the FBS substitute promoted superior or similar cell proliferation to commercial FBS. During the muscle recovery process, cytokines

secreted from immune cells infiltrating the damaged area have important roles in muscle recovery and regeneration (Ma et al., 2018; Waldemer-Streyer et al., 2022). Some cytokines, such as IL-6, TGF- β , IGF, TNF- α , and CCL2, can regulate the proliferation, differentiation, and fusion of myosatellite cells (Waldemer-Streyer et al., 2022). Therefore, additional research is needed to confirm the positive/negative effects of blood components on the growth of myosatellite cells depending on the growth period of livestock.

3.2 | Cell differentiation into muscle

3.2.1 | Microscopic observation of bovine myosatellite cell proliferation and differentiation during muscle development

After the cells were proliferated using commercially available FBS or FBS substitute, differentiation was induced in all treatments using the same differentiation medium (Section 2.2.4). In a serum-poor environment, myosatellite cells undergo cell cycle arrest and differentiation, and their nuclei fuse to form myotubes (Bajaj et al., 2011; Messmer et al., 2022; Pirkmajer & Chibalin, 2011). The morphology of small cells on the third day of proliferation and the fourth day of differentiation in the commercial FBS, bovine serum, porcine serum, and chicken serum treatments were analyzed.

After cell proliferation using cell medium containing 20% of FBS and substitutes, commercial FBS and bovine serum treatments showed very similar cell proliferation trends. In the porcine serum and chicken serum treatment groups, it was confirmed that fusion and differentiation of some cells progressed even under proliferation medium conditions (Figure 3a). However, differentiation proceeds simultaneously during the proliferation process, and relatively more empty spaces where cells are not attached were identified (Figure 3a). This is consistent with previous results in chicken serum that showed the cells did not proliferate sufficiently (Figure 2).

On the third day after inducing differentiation by applying differentiation medium, clear differentiation patterns were confirmed in all treatments (Figure 3b). In particular, a noticeable number of myotube branches were identified in commercial FBS and bovine serum that were not differentiated at the end of proliferation (Figure 3b). In the other two treatments, a number of myotube branches were also observed, but despite this, the overall amount of myotubes was lower than that of FBS and bovine serum (Figure 3b). All treatment groups had empty areas due to differentiation, and the porcine serum and chicken serum treatment

groups had particularly large areas (Figure 3b). The formed myotubes in all treatment groups have muscle contract, and [Supporting Information video files](#) can demonstrate that movement.

Based on these results, it was confirmed that myoblast cells to which FBS derived from slaughterhouse blood waste were applied had differences in early proliferation rate or speed but did not affect the degree of cell differentiation. That is, if a sufficient amount of FBS substitute is applied, the possibility that cell differentiation can occur normally while exhibiting the same cell growth-promoting effect as commercial FBS was confirmed. We conducted a follow-up study for a quantitative comparison of myotube formation.

3.2.2 | Quantification of myotube differentiation (muscle) by fluorescent staining and western blot analysis

The entire differentiation period of bovine myosatellite cells into muscles lasted 4–5 days. To confirm myotube formation in bovine myosatellite cells, fluorescent staining of myosin heavy chain was performed using MF20 (green, Figure 3c) (Lee et al., 2024; Stout et al., 2022). Myotube differentiation was quantified through the fusion index (%), which was determined by dividing the number of nuclei in the area stained with MF20 (green) by the total number of nuclei (Figure 3d) (Ben-Arye & Levenberg, 2019). In Figure 3d, the cell fusion index of the bovine serum and chicken serum treatments was similar to that of commercial FBS (Figure 3d). The cell fusion index of the porcine serum treatment group was found to be relatively low, which means that of the total cells that proliferated, only a small amount led to differentiation (Figure 3d, $p < 0.001$).

The amount of cell nuclei in the group grown with bovine serum was similar to that in the group grown with FBS (Figure 3e). As in the previous results after initial proliferation (Figure 3a,b), chicken serum had the smallest amount of cell nuclei after differentiation (Figure 3e). This low number of nuclei can be assumed to be because differentiation was promoted during the cell proliferation process (Figure 3e, $p < 0.001$). Conversely, porcine serum resulted in a small amount of nuclei observed, contrary to having the highest cell proliferation effect (Figure 3e). These results suggest that cell culture using porcine serum may not ultimately result in differentiation into myotubes or may cause differentiated cell loss.

Evaluation of protein levels in actual myotubes was confirmed through Western blot analysis (Figure 3f,g). Similar to the cell fusion index, the myosin heavy chain (MF20)

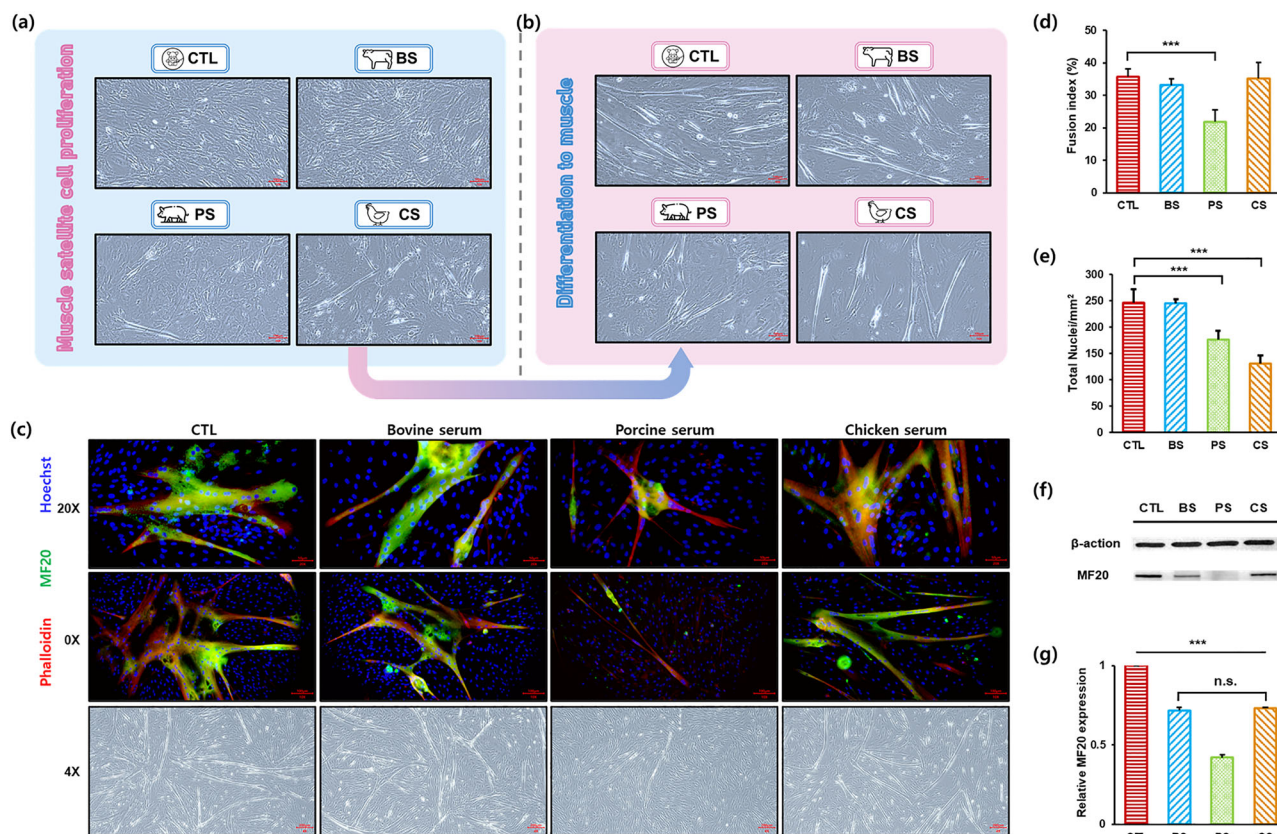


FIGURE 3 Analysis of cell morphology in response to myogenic differentiation in bovine myosatellite cells treated with fetal bovine serum (FBS) or FBS substitutes. (a) Proliferation after 3 days. (b) Differentiation after 4 days. (c) Immunofluorescent staining images of differentiation to muscle in bovine myosatellite cells using FBS and FBS substitutes. (d) Muscle cell differentiation was determined by calculating the fusion index manually. (e) The number of nuclei per unit area (mm^2) was determined manually. (f and g) The effect of FBS substitutes on the expression of myogenic marker genes during culture using Western blot. All treatments contain 20% of CTL. BS, bovine serum—inactivation, CS, chicken serum—inactivation; PS, porcine serum—inactivation. Data are expressed as the means $\pm$ SEM.

*** $p < 0.001$, n.s., not significant.

expression rate in bovine serum and chicken serum treatments was excellent, but the effectiveness of porcine serum was very low, with significantly less differentiation achieved compared to commercial FBS (Figure 3f,g, $p < 0.001$). However, even in the bovine serum and chicken serum treatment groups with relatively excellent differentiation, the myotube formation rate was 70%–75% as compared to FBS (Figure 3f,g).

The three developed FBS substitutes ultimately showed different characteristics when they replaced 100% of FBS. The summary of our research results is as follows:

Tendency of FBS substitute

- **Bovine serum:** The FBS substitute obtained from bovine blood most reliably replaced FBS and resulted in bovine muscle cell proliferation and differentiation.
- **Porcine serum:** The FBS substitute obtained from porcine blood was confirmed to have a cell proliferation effect, but its final differentiation efficiency was poor.

- **Chicken serum:** For the FBS substitute obtained from chicken blood, the onset of differentiation was faster than other treatments (commercial FBS, bovine serum), and although the amount of cells was not large, myosin heavy chain was produced similarly to the bovine serum substitute in the final product.

These results suggest that among the three FBS substitutes we developed, bovine serum and chicken serum have the potential to be effective in proliferating cells for cultured meat production and differentiating them into muscle. Additionally, Supporting Information imaging data show that bovine muscle tissues cultured using the FBS substitute that we developed contract and relax and that successful muscle differentiation is also possible using the FBS substitute.

FBS is a byproduct of cattle slaughtering and is collected from deceased pregnant cows under government approval. One of the reasons it is difficult to replace FBS,

which comes from bovine fetuses, may be because its exact composition is not known. Although the exact composition of commercial FBS is disclosed, some companies and researchers suggested that FBS is made up of attachment and spreading factors, growth factors, hormones, lipids, sugars, transport proteins, and vitamins (Lee et al., 2022; van der Valk et al., 2018). A variety of media have been postulated as alternatives to FBS, such as serum-free media, protein-free media, animal source-free media, or synthesized media (Kolkman et al., 2020; Lee et al., 2022). Not only have these technologies been extended to various cell species cultures, but there also are some FBS-free cell culture (Jang et al., 2011; Reza & Nicoll, 2010; Wang et al., 2013). Although we developed an FBS substitute that cannot completely replace animal raw materials, FBS substitutes extracted from livestock slaughterhouse blood can effectively grow muscle satellite cells, contributing to reducing the cost of cultured meat production. Nevertheless, the complete chemical profile or mechanism of FBS and mature livestock blood has not been confirmed, and in other studies using blood (serum) byproducts, the specific ingredients that showed effectiveness were not analyzed (Andreassen et al., 2020; Yu et al., 2013). Therefore, further research is needed to optimize and formulate the composition of media utilizing effective FBS substitutes. In addition, in order to effectively use blood byproducts generated from slaughterhouses for domestic cultured meat production, it is additionally necessary to ensure that the effect is uniform as in commercial FBS, regardless of variables, such as sex, farm, and age of livestock.

4 | CONCLUSION

For the first time, we found that FBS for cell cultivation could be replaced by semi-processed serum in bovine, porcine, and chicken slaughterhouse blood. We reduced the price of the medium composition (including FBS) by about 61% through the developed cultured meat. FBS substitutes (from bovine serum and chicken serum) successfully formed bovine myotubes and were found to be the most suitable FBS substitute when considering the production cost. Commercial FBS is highly dependent on specific regions and specific companies, whereas blood byproducts can easily be obtained from slaughterhouses in many countries, so FBS alternatives obtained from adult livestock blood are expected to have high economic value and various applications. Therefore, the FBS substitute obtained from mature livestock slaughterhouse blood could contribute to lowering the production cost of cell culture media and play an important role in the mass production of cultured meats. Moreover, although

FBS substitute made from slaughterhouse blood waste is also an animal-derived substance, it is also expected to contribute to more sustainable practices in the meat production sector and decrease food and drug production costs by permeating a range of applications, such as experimental cell culture technology and vaccine production as well as cultured meat production. Therefore, further research on the development of cell culture media using livestock byproducts will be needed.

AUTHOR CONTRIBUTIONS

Da Young Lee: Investigation; validation; formal analysis; writing—review and editing; writing—original draft. **Seung Yun Lee:** Writing—review and editing. **Seung Hyeon Yun:** Validation; formal analysis; investigation. **Yeongwoo Choi:** Formal analysis; validation. **Dahee Han:** Validation; formal analysis. **Jinmo Park:** Validation; formal analysis. **Jin Soo Kim:** Validation; formal analysis. **Ermie Mariano Jr.:** Validation; formal analysis. **Juhyun Lee:** Formal analysis; validation. **Jung Seok Choi:** Writing—review and editing. **Gap-Don Kim:** Writing—review and editing. **Inho Choi:** Writing—review and editing. **Seon-Tea Joo:** Writing—review and editing. **Sun Jin Hur:** Writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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