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Up-to-Date Research on the Composition of Blood Slaughterhouse By-Products Derived FBS Substitutes and Their Applicability to Animal Cell Culture

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ABSTRACT

Fetal bovine serum (FBS) is a critical media component in cell culture, but it is also expensive and highly controversial from an animal welfare level, being of fetal blood origin. Therefore, our study aimed to develop FBS substitutes from slaughterhouse waste blood. We pooled the blood from slaughterhouse by-products (BSBPs) of three major livestock species (cattle, pigs, and chickens) and performed nutritional and hematological analyses of serum, plasma, whole blood, and cruro. Our substitutes were cultured with C2C12 cells, and the serum of each livestock species showed a cell proliferation effect. Nonetheless, the nutritional and hematological profiles of BSBPs from this study can provide basic component information for using livestock blood as a raw material for various preparations. Furthermore, the cell culture results using C2C12 cells indicate that using BSBPs can solve expensive cell culture costs and animal ethics issues by replacing FBS.

1 | Introduction

As traditional livestock farming continues, slaughter by-products pollute the environment and incur greater disposal costs than profits, due to lower demands compared to the main product. As the world population increases, meat consumption also increases, and in 2022 alone, approximately 82.6 billion terrestrial animals were slaughtered, including approximately 310 million cattle, 1.5 billion pigs, 75.2 billion chickens, and 640 million sheep (Orzechowski 2024). By-products such as bones, leather, blood, brains, and various organs account for 34–80% of the generated livestock, depending on the species, and solving this excessive

livestock by-product problem is a task for humankind (Ahn et al. 2019; Limeneh et al. 2022).

Blood by-products are one of the most discarded high-nutrition, high-protein by-products from livestock slaughter, despite accounting for only 3–7% of the carcass weight, depending on the livestock (Bah et al. 2013; Chiroque et al. 2023; Jeon 2013). The most accessible way to utilize blood by-products is in the form of food, such as sausages, ham, black pudding, surimi, sundaes, and stews (Bah et al. 2013; Choi 2013). Blood proteins and hemoglobin can be excellent additives to animal feed, and pharmaceuticals such as immunoglobulins (Igs),

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albumin (ALB), and thrombin/prothrombin that can be obtained through fractionation (Bah et al. 2013; Kim and Burnouf 2017). In addition, their value as excellent protein sources is increasing, as many bioactive peptides that possess antibacterial, antioxidant, and antihypertensive activities have been discovered (Bah et al. 2013; Chiroque et al. 2023; Toldrá et al. 2016).

FBS is an essential component in almost all types of cell culture (Lee et al. 2022; Lee et al. 2024a). However, the high cost, ethical concerns regarding the slaughter of pregnant cows and their fetuses, and an unstable supply system have led to ongoing advocacy for FBS substitute. Animal-derived components such as albumin (ALB), growth factors, and coating agents (e.g., Matrigel, gelatin, and collagen) are still commonly used as FBS substitutes due to their demonstrated efficacy (Ahmad et al. 2023; Lee et al. 2024a, Lee et al. 2024b). Consequently, blood by-products obtained during livestock slaughter present a valuable resource for the continued development and production of FBS substitutes.

In our previous study, we confirmed the possibility that FBS substitutes obtained from three livestock species (cattle, pigs, and chickens) can be used for the production of cultured meat (Lee et al. 2023). The FBS substitutes could reduce the cost of cell culture media by about 60% and were found appropriate for the proliferation and differentiation of bovine muscle satellite cells (Lee et al. 2023). In this study, we pooled a larger amount of slaughterhouse by-products (BSBP) than in previous studies to produce an FBS substitute, and confirmed the possibility of replacing FBS in general cell lines. In addition, we analyzed the characteristics of various forms of BSBPs, such as whole blood, cruor, plasma, and serum, to provide useful information for cell culture and for fields that can utilize blood by-products.

2 | Materials and Methods

2.1 | Preparation of FBS and BSBPs

FBS from Corning (35-015-CV, NY, USA) was used as the control group, and for the FBS treatment group, 15 different lot numbers of FBS, purchased from Biowest, Corning, Gibco, Hyclone, and Sigma were divided into three groups and mixed (FBS 1–3). Bovine blood was collected from female (31–59 months old) and male (27–30 months old) Hanwoo at slaughter, and at least 10 animals were sampled. Porcine blood was collected from female and male pigs (Landrace × Yorkshire dam × Duroc sire or LYD crossbreed) slaughtered at 180–190 days of age, and at least 10 animals were sampled. For bovine and porcine serum samples, 2–3 individuals were grouped according to sex (S1 and S2). Chicken blood was collected from female (42–43 days) and male (38–41 days) broilers (Ross 308 breed), and at least 60 animals were sampled at each slaughter. Hereafter, when referring to sample names, they are abbreviated as bovine (Bo), porcine (Po), chicken (C), female (F), male (M), whole blood (W), cruor (C), serum (S), and Plasma (P) (e.g., BoF-S1 is the first serum group of bovine females).

The pretreatment of BSBPs was performed as shown in Figure 1. Blood samples were sufficiently coagulated at room temperature (25°C). The liquid layer of the blood sample was classified as whole blood (Figure 1(1)), and the coagulated matter was

classified as cruor (Figure 1(2)). Cruor samples were homogenized in water at a ratio of 1:9 (w/w), and the supernatant was collected by centrifugation (1,000 × g, 5 min) for subsequent analysis. The coagulated blood was centrifuged at 1,977 × g for 10–15 min to obtain serum (Figure 1(3)). In the other group, 8% ethylenediaminetetraacetic acid (EDTA)-2Na dihydrate (4002-4405, Daejung Chemicals, Siheung, Korea) was added to 1 L of blood to perform anticoagulation. The anticoagulated blood was centrifuged at 1,500 × g for 10–15 min to obtain plasma (Combi 514R, Hanil Scientific, Gimpo, Korea) (Figure 1(4)). The collected samples were stored frozen until analysis. Serum and plasma obtained from livestock blood by-products were filtered through a membrane filter (0.1 µm PVDF; Millipore, Billerica, MA, USA). Serum samples used in all analyses and cell experiments were inactivated at 55°C, as was the FBS used in the control (Lee et al. 2023). All procedures involving animals were approved by the Animal Experiment Ethics Committee of Chung-Ang University (Approval number: 202301020084).

2.2 | Analysis of Characteristics by BSBPs Composition

2.2.1 | Analysis of Blood Components

Analysis of blood components was performed only on FBS and serum samples from each livestock species, including FBS. A clinical chemistry analyzer (CA-270; Furuno Electric Co. Ltd., Nishinomiya, Japan) was used for the analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and cholesterol (CHO) in serum samples.

2.2.2 | Protein Quantification and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The bicinchoninic acid (BCA) assay was performed to measure the protein content of FBS. Blood by-product standard (bovine serum albumin) and samples were reacted with reagents in a 96-well plate at 37°C for 30 min (Pierce BCA Protein Assay Kits; 23227, Thermo Scientific, Waltham, MA, USA). The amount of protein was measured at 562 nm using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).

SDS-PAGE was performed using a 10% separating gel and 5% stacking gel with a thickness of 1.0 mm (a 15% separating gel was used for the cruor sample). The running tank buffer was a mixture of 100 mL of 10X tank buffer (250 mM Tris + 1.92 M glycine + 1 L of distilled water) + 10 mL of 10% SDS + 890 mL of distilled water. Following electrophoresis at 80 V for 10 min and 100 V for 60 min, the loaded gel was stained overnight using a staining buffer (methanol:distilled water:acetic acid, 45:45:10, v/v/v, with 1 g of Coomassie blue R250 [B7920; Sigma]). Subsequently, destaining was performed using a destaining buffer (methanol:distilled water:acetic acid, 40:50:10, v/v/v). As a protein marker, the Enhanced 3-color High Range Protein Marker (PM2610, Smobio, Hsinchu City, Taiwan) was used.

2.2.3 | Analysis of Free Amino Acids (AAs)

A 1 mL sample was mixed with an equal volume of 5% trichloroacetic acid by vortexing for 4 min, then centrifuged

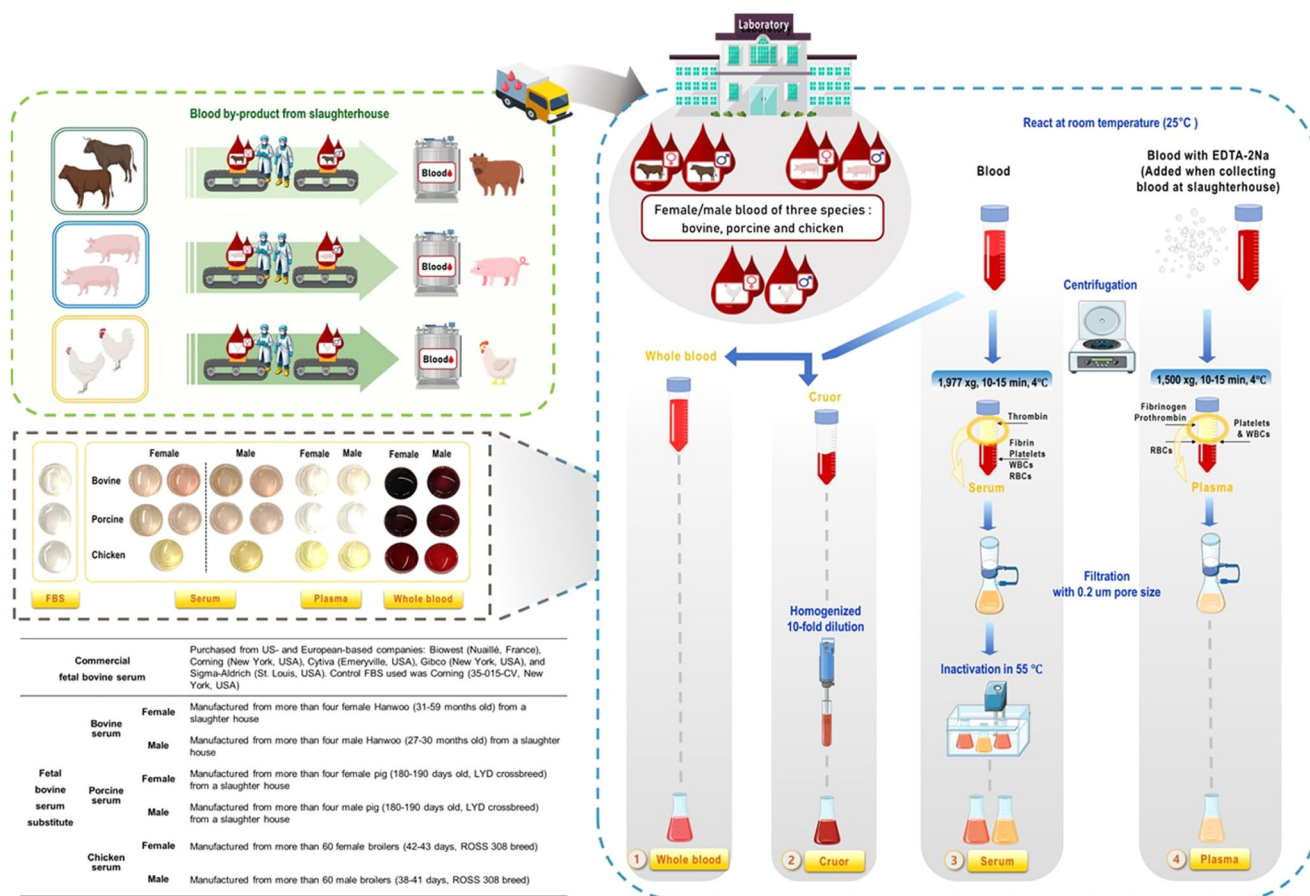


FIGURE 1 | Schematic diagram of the pretreatment process for blood by-products.

(M15R, Hanil Scientific) at 12,000 rpm and 20°C for 20 min to remove precipitated proteins. The protein-free supernatant was combined with 2 mL of *n*-hexane by shaking for 10 min and then centrifuged (Combi 514R, Hanil Scientific) at 3000 rpm and 20°C for 10 min. To ensure that all *n*-hexane was removed, only the lower layer was collected, and this process was repeated once more. The amino acid composition of samples was analyzed using an amino acid analyzer (L-8900, Hitachi High Technologies Corp., Tokyo, Japan). All sample analyses were performed using a Hitachi HPLC ion exchange column (#2622PF, Hitachi High Technologies Corp.). The column temperature was 30–70°C, the reaction coil temperature was 135°C, and chromatograms were recorded at 440 and 570 nm.

2.2.4 | Analysis of Fatty Acids (FAs)

After vortexing a mixture of 1 mL of Folch (chloroform:methanol, 2:1, v/v) and 100 µL of sample, the solution was centrifuged to collect the lower layer containing the dissolved lipids and dried. The dried sample was re-dissolved in 1 mL of *n*-hexane, and 2 mL of boron trifluoride in methanol (10–14%) was subsequently added. The solution was reacted in a constant temperature water bath at 80°C for 5 min, taking care not to evaporate. One milliliter of distilled water was added to the completely cooled solution, the layers were separated, and the hexane layer was collected. Afterward, a small amount of Na₂SO₄ can be optionally added to completely remove moisture. The extracted FAs were separated

using a gas chromatograph mass spectrometer (MS 2400 SQ, Perkin-Elmer, Waltham, MA, USA) with an Elite-5 ms Capillary Column (60 m × 0.25 mm i.d. × 0.25 µm; N9316286, Perkin-Elmer). F.A.M.E. Mix (CRM18920, MilliporeSigma Supelco, Bellefonte, PA, USA) was used as a fatty acid standard.

2.2.5 | Analysis of Minerals

Samples were digested using nitric acid and hydrogen peroxide (5:3, v/v). Digests were mineralized at 150°C for 1 h using a Microwave Digestion System (START D, Milestone, Sorisole, Italy). Mineral contents, including calcium (Ca), iron (Fe), sodium (Na), potassium (K), zinc (Zn), magnesium (Mg), and selenium (Se), were measured by inductively coupled plasma optical emission spectrometry (OPTIMA 7300 DV, Perkin-Elmer).

2.3 | Cell Culture Using FBS and BSBPs

2.3.1 | pH Measurement

pH was monitored using a pH meter (SevenCompact pH/Ion S220; Mettler Toledo, Columbus, OH, USA).

2.3.2 | C2C12 cell Culture

The cell culture medium was prepared by adding 10% FBS and 1% penicillin-streptomycin (15140122, Thermo Fisher Scientific,

Waltham, MA, USA) to Dulbecco's modified Eagle's medium (DMEM). For other treatment groups, FBS was replaced with an equivalent amount of blood by-product-derived components (serum or plasma). Serum or plasma was added at 10% to all treatment groups for C2C12 cell culture.

2.3.3 | Cell Growth and Viability

C2C12 cultured on plates for three days were counted by staining with trypan blue (Gibco). To measure cell viability, 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT; M2128, Sigma-Aldrich, St. Louis, MO, USA) reagent was added to cells cultured for 3 days and reacted for 4 h. DMSO was added back to the plate from which the MTT solution had been removed. All processes were performed in the dark, and then the optical density of each well was measured at 540 nm using a microplate reader (Spectramax190, Molecular Devices).

2.3.4 | Live Cell Analysis

In subsequent cell experiments, each of BSBPs group was combined into one treatment group, representing species and sex, and commercial FBS was also combined into one group. For analysis of live cells, C2C12 cells grown on 48-well cell culture plates for 3 days were first washed with 1X PBS. Fluorescein diacetate (FDA, Sigma-Aldrich) pre-diluted in acetone was adjusted to a final concentration of 50 µg/mL using basal medium. The FDA-containing medium was added, along with Hoechst, to the wells containing the washed cells and reacted for less than 5 min. The stained cells were visualized with a fluorescence microscope (KI-3000F, Korea Lab Tech, Namyangju, Korea).

2.3.5 | Evaluation of FBS Substitutes Contamination for Cell Culture

Microbiological contamination in commercial FBS and FBS substitutes was assessed prior to cell culture using Petrifilm plates. Three types of Petrifilm (3 M, Saint Paul, MN, USA) were employed: aerobic count (AC), *Escherichia coli* (EC), and *Staphylococcus aureus* (STX). Samples were diluted 1:10 in sterile distilled water, applied to the Petrifilm, and incubated at 35°C for 24–48 h.

Mycoplasma testing in C2C12 cultures was performed using the Myco-Read mycoplasma detection kit (SMD0172, Guri, Korea) according to the manufacturer's protocol. A positive control group was provided in the kit, and samples were prepared by collecting culture fluid from C2C12 cells cultured for 72 h. Amplified products were run on a 2% agarose gel.

2.4 | Statistical Analysis

Statistical analysis of the experimental data involved one-way ANOVA using the SPSS 22.0 program (IBM Corp., Armonk, NY, USA). Statistical significance was assessed using Tukey's multi-range test, and the significance level of all data was evaluated based on $p < 0.05$. The results are presented as the average of

values obtained from the analysis of separate more than triplicate experiments.

3 | Results and Discussion

3.1 | Pretreatment for Utilization of BSBPs

Blood contains ALB, fibronectin, Igs (and globulins), glycoproteins, transferrin, and various enzyme proteins, and can be separated and used in the form of whole blood, serum, and plasma, depending on the purpose (Bah et al. 2013; Lee et al. 2022). In this study, whole blood and cruor represent the liquid and solid portions of coagulated blood, respectively (Figure 1). Serum is the yellow liquid obtained when coagulated blood is centrifuged. It is generally referred to as a liquid from plasma without fibrinogen, a plasma protein. Serum does not contain white blood cells, red blood cells (RBCs), platelets, or other material that has been coagulated by fibrin, the hydrolysis product of fibrinogen (Figure 1). Plasma is a liquid that contains proteins, such as ALB, globulin, bilirubin, and fibrinogen, and can be used as a raw material for pharmaceuticals, such as ALB preparations, Ig preparations, and blood clotting factor preparations (Kim and Burnouf 2017).

3.1.1 | Analysis of Blood Components

Similar to our previous study, the commercial FBS group (FBS1–3) showed similar blood component compositions to other serum components, suggesting that commercial FBS have similar components and have little variation, regardless of brand or batch number (Lee et al. 2023). Chicken serum showed a significantly different trend in blood components. Chicken serum contained a much higher level of alkaline phosphatase (ALP) compared to FBS, which is consistent with our previous study (Lee et al. 2023). Meanwhile, blood urea nitrogen (BUN), creatinine (CREA), glutamate-pyruvate transaminase (GPT), and inorganic phosphorus (IP) in chicken serum were significantly lower than FBS, in contrast to bovine and porcine sera, which showed similar or higher levels than FBS. Ca was higher in FBS than in sera from other livestock (16.8–17.1 mg/dL, Table 1). This result showed the same trend as the Ca content in BSBPs through mineral analysis (Figure 3c). Among the blood components, the content of total protein (TP) was the highest in all treatment groups, and the concentrations in FBS (3.75–4.06 g/dL), chicken serum (3.50–3.73 g/dL), bovine serum (7.82–9.57 g/dL), and porcine serum (7.31–8.97 g/dL) were similar across all four sera (Table 1). In addition, the Bo-S group (adult animals) exhibited a higher albumin content compared to the FBS group (young animals). The proportion of ALB compared to TP was the highest at 67–73% in the commercial FBS group (Table 1). It is highly likely that sera derived from other livestock BSBPs contain other proteins in larger proportions besides ALB. The total CHO content of all livestock serum was at least about times times (porcine) and up to 5–6 times (bovine and chicken) higher than FBS (Table 1). Not only CHO but also cholesterol-high density lipoprotein (CHO-HDL) and cholesterol-low density lipoprotein (CHO-LDL) levels were higher in BSBPs than in FBS, whereas the triglyceride (TG) content was relatively higher in FBS (Table 1).

TABLE 1 | Blood components composition of blood by-products.

Unit	CHO-																
	ALB	ALP	BIL-D	BIL-T	BUN	Ca	CHO	CHO-HDL	LDL	CREA	CRP	GLU	GOT/AST	GPT/ALT	IP	LDH	TG
FBS 1	g/dL	IU/L	mg/dL	mg/dL	IU/L	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	IU/L	IU/L	mg/dL	IU/L	mg/dL
	2.69	130.22	0.03	0.09	13.79	16.93	33.06	2.37	15.00	2.75	0.01	117.99	36.29	5.91	10.40	770.58	121.32
	± 0.05 ^f	± 1.70 ^{ab}	± 0.00 ^g	± 0.00 ^e	± 0.34 ^{efg}	± 0.62 ^a	± 1.27 ^f	± 0.06 ^f	± 1.35 ^f	± 0.07 ^b	± 0.00 ^e	± 0.87 ^{cd}	± 0.88 ^h	± 0.08 ^h	± 0.02 ^b	± 6.47 ^e	± 1.22 ^a
FBS 2	2.91	133.45	0.03	0.11	16.93	17.37	34.39	8.50	16.10	3.08	0.01	108.00	35.74	5.03	10.30	735.92	110.73
	± 0.05 ^f	± 3.41 ^{de}	± 0.00 ^g	± 0.00 ^c	± 0.47 ^a	± 0.31 ^a	± 0.56 ^f	± 0.03 ^f	± 1.11 ^f	± 0.07 ^a	± 0.48 ^b	± 4.63 ^{def}	± 1.12 ⁱ	± 0.19 ^h	± 0.48 ^b	± 11.28 ^{ef}	± 3.22 ^b
FBS 3	2.73	129.50	0.05	0.10	15.46	16.51	34.91	8.60	15.10	2.75	0.01	102.51	40.50	6.14	10.00	766.49	111.10
	± 0.02 ^f	± 0.46 ^{ab}	± 0.00 ^f	± 0.00 ^d	± 0.32 ^{bc}	± 0.37 ^a	± 0.13 ^f	± 0.81 ^f	± 1.42 ^f	± 0.07 ^b	± 0.55 ^b	± 1.59 ^{efg}	± 0.94 ⁱ	± 0.12 ^h	± 0.55 ^b	± 14.77 ^e	± 1.95 ^b
BoF-S1	4.55	37.49	0.06	0.12	13.59	11.81	168.92	112.80	49.50	1.78	0.02	92.62	110.69	32.88	7.80	901.67	67.25
	± 0.06 ^{bc}	± 0.88 ^e	± 0.00 ^e	± 0.00 ^b	± 0.16 ^{efg}	± 0.29 ^{bc}	± 6.46 ^b	± 2.13 ^b	± 1.75 ^{ab}	± 0.05 ^{de}	± 0.63 ^{cd}	± 2.19 ^g	± 2.31 ^g	± 0.53 ^d	± 0.63 ^{cd}	± 12.98 ^d	± 0.84 ^h
BoF-S2	3.93	57.55	0.08	0.10	15.55	8.68	180.26	132.20	40.50	1.91	0.02	141.52	151.46	26.36	6.30	1,010.27	58.30
	± 0.07 ^e	± 1.28 ^{cd}	± 0.00 ^d	± 0.00 ^d	± 0.32 ^b	± 0.22 ^e	± 2.37 ^a	± 2.19 ^a	± 3.44 ^{cd}	± 0.04 ^d	± 0.27 ^{efg}	± 6.03 ^b	± 3.55 ^d	± 0.54 ^f	± 0.27 ^{efg}	± 14.59 ^c	± 1.24 ⁱ
BoM-S1	4.85	21.53	0.02	0.06	15.25	11.98	151.73	92.40	51.70	2.16	0.02	99.66	126.18	28.48	10.30	1,303.24	90.13
	± 0.04 ^d	± 0.56 ^f	± 0.00 ^h	± 0.00 ^f	± 0.61 ^{bc}	± 0.41 ^b	± 1.48 ^c	± 1.21 ^d	± 1.73 ^a	± 0.05 ^c	± 0.8 ^b	± 0.57 ^{fg}	± 1.27 ^f	± 0.44 ^e	± 0.8 ^b	± 26.13 ^a	± 0.97 ^{cd}
BoM-S2	4.35	35.14	0.12	0.19	14.46	9.64	149.49	106.50	31.60	1.92	0.01	69.72	180.10	22.36	6.50	694.27	62.82
	± 0.14 ^{cd}	± 0.13 ^{bc}	± 0.00 ^b	± 0.01 ^a	± 0.25 ^{cde}	± 0.22 ^d	± 2.22 ^c	± 0.22 ^{bbc}	± 1.47 ^e	± 0.02 ^d	± 0.15 ^{def}	± 1.5 ^h	± 3.79 ^c	± 0.56 ^g	± 0.15 ^{def}	± 3.07 ^f	± 1.02 ^{hi}
PoF-S1	4.22	16.26	0.08	0.05	15.06	11.05	100.92	42.30	36.50	1.59	0.03	123.64	116.85	32.09	7.60	1,021.40	75.01
	± 0.18 ^d	± 0.03 ^f	± 0.00 ^d	± 0.00 ^g	± 0.35 ^{bcd}	± 0.30 ^c	± 1.18 ^e	± 0.7 ^e	± 0.04 ^{de}	± 0.05 ^f	± 0.12 ^{cde}	± 2.77 ^c	± 2.25 ^{fg}	± 1.24 ^d	± 0.12 ^{cde}	± 16.44 ^c	± 0.87 ^g
PoF-S2	4.64	63.72	0.00	0.04	13.25	10.10	110.90	47.60	44.40	1.62	0.04	112.38	94.27	46.66	9.60	903.35	77.64
	± 0.15 ^{ab}	± 2.33 ^d	± 0.00 ⁱ	± 0.00 ^h	± 0.52 ^{fg}	± 0.17 ^d	± 2.74 ^d	± 3.53 ^e	± 0.34 ^{bc}	± 0.06 ^f	± 0.16 ^b	± 2.56 ^{cde}	± 1.13 ^h	± 0.58 ^b	± 0.16 ^b	± 15.02 ^d	± 2.07 ^{fg}
PoM-S1	4.71	19.10	0.00	0.02	14.13	11.22	105.31	42.10	41.90	1.89	0.03	120.56	115.32	36.71	12.20	757.61	107.98
	± 0.10 ^{ab}	± 0.54 ^f	± 0.00 ⁱ	± 0.00 ⁱ	± 0.15 ^{def}	± 0.24 ^{bc}	± 1.18 ^{de}	± 2.16 ^e	± 2.53 ^{cd}	± 0.04 ^d	± 0.61 ^a	± 0.56 ^c	± 2.01 ^g	± 0.82 ^c	± 0.61 ^a	± 5.75 ^e	± 0.16 ^b
PoM-S2	4.14	57.06	0.02	0.06	12.88	9.26	104.61	45.10	41.40	1.73	0.05	106.10	141.46	50.95	9.00	1,041.75	82.29
	± 0.10 ^{de}	± 0.86 ^a	± 0.00 ^h	± 0.00 ^f	± 0.35 ^g	± 0.17 ^{de}	± 2.51 ^{de}	± 3.01 ^e	± 2.47 ^{cd}	± 0.05 ^{ef}	± 0.74 ^{bc}	± 1.82 ^{ef}	± 4.75 ^e	± 0.96 ^h	± 0.74 ^{bc}	± 24.33 ^c	± 1.38 ^{ef}
ChF-S	1.68	1,451.49	0.11	0.09	4.18	7.07	154.83	86.70	45.80	0.22	0.02	297.90	289.57	1.99	5.00	1,130.04	85.39
	± 0.04 ^g	± 25.68 ^g	± 0.00 ^c	± 0.00 ^e	± 0.07 ^h	± 0.12 ^f	± 1.94 ^c	± 0.4 ^d	± 0.07 ^{bc}	± 0.00 ^g	± 0.44 ^g	± 4.31 ^a	± 6.19 ^a	± 0.04 ⁱ	± 0.44 ^g	± 4.46 ^b	± 2.18 ^{de}
ChM-S	1.70	2,409.85	0.15	0.11	4.90	7.66	171.84	102.10	45.70	0.16	0.02	291.76	271.71	0.99	5.60	1,339.14	90.77
	± 0.04 ^g	± 41.77 ^g	± 0.00 ^a	± 0.00 ^c	± 0.10 ^h	± 0.25 ^f	± 5.66 ^{ab}	± 9.3 ^c	± 3.37 ^{bc}	± 0.01 ^g	± 0.15 ^{fg}	± 9.84 ^a	± 6.11 ^b	± 0.01 ⁱ	± 0.51 ^{fg}	± 4.73 ^a	± 2.07 ^c

Abbreviations: FBS: Fetal bovine serum, Bo: Bovine, Po: Porcine, Ch: Chicken, F: Female, M: Male, S: Serum. ALB: Albumin, ALP: Alkaline phosphatase, BIL-D: Direct bilirubin, BIL-T: Total bilirubin, BUN: Blood urea nitrogen, Ca: Calcium, CHO: Total cholesterol, CHO-HDL: Cholesterol-High density lipoprotein, CHO-LDL: Cholesterol-Low density lipoprotein, CREA: Creatinine, CRP: C-reactive protein, GLU: Glucose, GOT/AST: Aspartate aminotransferase, GPT/ALT: Alanine aminotransferase, IP: Inorganic phosphorus, LDH: Lactate dehydrogenase, TG: Triglyceride, TP: Total protein, UA: Uric acid, γ -GTP: γ -glutamyl transpeptidase, -: Not detected.

In our previous study, we expected that the calcification process that occurs during eggshell formation in birds would affect the ALP level in chicken serum; however, very high levels of ALP were also detected in male chicken serum (Table 1) (Lee et al. 2023). The detection of blood ALP can be affected by diet and age, and by eggshell formation as previously mentioned (Lee et al. 2023; Odunitan-Wayas et al. 2018). According to various studies, albumin and globulin account for the largest proportion of protein in total protein (TP) of sera. Therefore, the lower proportion of albumin relative to TP in BSBP is likely due to a greater increase in globulin content (Chiroque et al. 2023; Tóthová et al. 2013). As livestock mature, the total protein level in the blood increases, accompanied by a significant elevation in both albumin and globulin concentrations, including α - and β -globulins as well as Igs (Cincović et al. 2020; Tóthová et al. 2013). Furthermore, studies have shown that blood TP increases with age in young cattle and pigs, and the increase is greater when high-efficiency feed is fed (Fang et al. 2018; Knowles et al. 2000; Mohri et al. 2007; Song et al. 2021; Tóthová et al. 2013). This trend was also confirmed in blood CHO levels. Sufficient nutrition supplied to livestock raised for fattening can induce lipid deposition in muscles and blood vessels, thereby increasing blood CHO (Antunović et al. 2009; Lee et al. 2023; Özdoğan and Akşit 2003). Although some studies have confirmed the effect of high-fat feed for fattening on CHO levels, the effect on neutral fat is not clear (Özdoğan and Akşit 2003).

3.1.2 | Protein Quantification and Electrophoresis

The commercial FBS group (FBS1–3) showed protein contents of about 43 mg/mL without significant differences (Figure 2). Bovine and porcine sera showed relatively high values of 89–97 and 82–95 mg/mL, respectively. In contrast, chicken serum showed a protein content of approximately 37 mg/mL, which was not significantly different from that of commercial FBS group (FBS1–3). Protein levels in plasma were comparable to those of the corresponding serum (Figure 2). Whole blood and crur samples showed protein levels of 160–200 mg/mL, 115–127 mg/mL, and 62–84 mg/mL for bovine, porcine, and chicken, respectively (Figure 2).

Despite the larger number of pooled samples in this study, the tendency for bovine and porcine serum to show higher protein contents than FBS was consistent with our previous serum analysis results (Lee et al. 2023). As alluded to above, whole blood and crur were confirmed to have higher protein levels than those in serum or plasma for all livestock species. However, while whole blood and crur from bovine and chicken showed about 2 times higher protein levels than serum/plasma, the porcine group showed only 1.5 times higher. It was predicted that whole blood would show similar trends to crur, but the proteins cleaved during the preprocessing of crur did not form bands at the same level of protein concentration. Even in samples with a high protein concentration (200 μ g/mL), relatively faint bands were formed, particularly thick bands below 10 kDa (Figure 2e).

The FBS groups showed a similar protein band distribution pattern, which is consistent with previous studies (Figure 2a) (Lee et al. 2023). Compared with the FBS group, the distribution trends

of bands between 20 and 25 kDa, and 45 and 60 kDa in bovine serum were different (Figure 2a). Unlike bovine serum, the sera of pigs and chickens displayed more prominent bands between 20 and 25 kDa (Figure 2b). In comparison to FBS, the bands over 140 kDa and between 35 and 75 kDa were more diverse and clearer in serum from BSBPs (Figure 2).

The SDS-PAGE results provide the protein profile based on the molecular weight of all blood used in this study. Presumably, the band around 60 kDa, which was the thickest in serum from FBS, can be attributed to ALB, well-known to have a size of 60–66 kDa and to be the most abundant protein in blood (Balkani et al. 2016; Ofori and Hsieh 2015). We confirmed that more than half of the total protein in all treatment groups of bovine and porcine was albumin, and about 46% of chicken was also albumin (Table 1). Therefore, the thickest protein band, and most abundant, at 60 kDa is most likely albumin.

Compared to FBS, a notable difference in the serum bands of BSBP was that additional protein bands were identified at positions 20–25, 40–60, and 150 kDa (Figure 2). This protein distribution profile is likely attributed to Igs because they are the second most abundant proteins in animal serum, following albumin. Although their levels may surpass those of albumin under certain health conditions (e.g., infection, inflammation, the colostrum-feeding period), albumin and Igs collectively constitute the major protein components of serum (Chiroque et al. 2023; Cincović et al. 2020; LaMotte 1977; Song et al. 2021; Tóthová et al. 2013; Tothova et al. 2016; Tóthová et al. 2019). Depending on the species, there are various classes (IgG, IgY, IgA, IgM, IgE, etc.) of Igs with sizes ranging from 150 to 1000 kDa (Bogahawaththa et al. 2017; Crawley and Wilkie 2003; Muramatsu et al. 2014; Yadav et al. 2015). Typically, the predominant immunoglobulin in blood is IgG (or IgY in avian species), which has a molecular weight exceeding 150 kDa (Crawley and Wilkie 2003; Muramatsu et al. 2014; Yadav et al. 2015). Igs can be reduced during pretreatment electrophoresis processes and separated into a heavy chain (50–80 kDa) and a light chain (22–25 kDa) (Bogahawaththa et al. 2017; Crawley and Wilkie 2003; Muramatsu et al. 2014). Therefore, the additional protein bands observed in BSBP are presumed to be globulins, the concentrations of which increase in association with active immunity or physiological growth in adult animals; however, this interpretation requires further verification. The above trends were similarly confirmed in the plasma of each species (Figure 2c). To clearly support this assertion, additional validation using antibody-based techniques such as Western blotting is required. However, the diversity of source species used in FBS substitute production makes impractical to conduct a direct comparison via Western blot or ELISA, as existing antibodies and commercial kits often lack the cross-species reactivity required to cover all of these species simultaneously. Therefore, further studies are warranted to accurately identify and characterize the protein components within these samples.

3.1.3 | Analysis of Free AAs

The contents of free AAs in the FBS treatment groups (FBS1–3) were statistically comparable, confirming similar quality of protein in terms of amino acid profile (Figure 3a, Supplementary

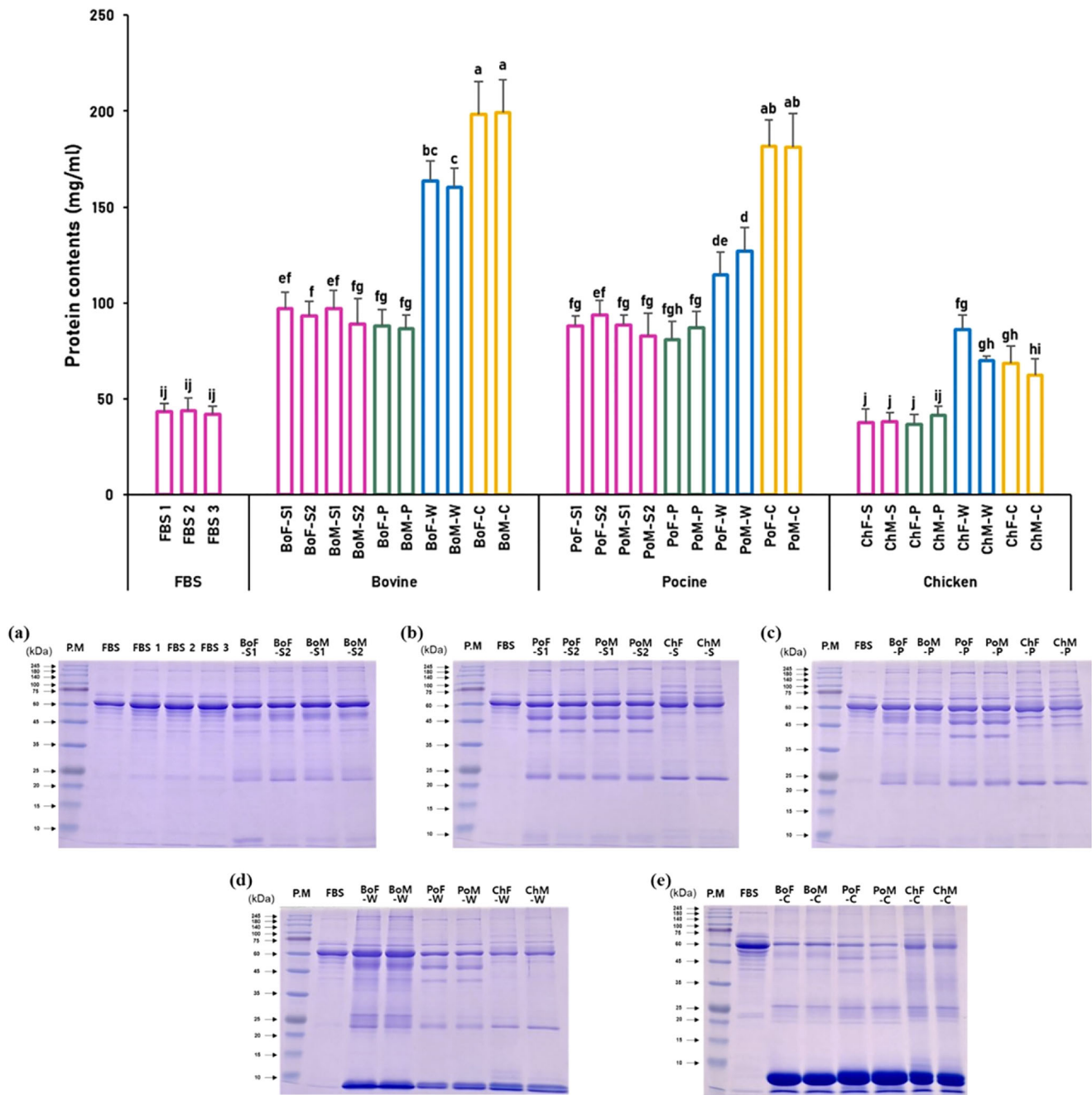


FIGURE 2 | Protein contents as determined by BCA analysis and SDS-PAGE of blood by-product samples. FBS: Fetal bovine serum, Bo: bovine, Po: porcine, Ch: chicken, F: female, M: male, S: serum, P: plasma, W: whole blood, C: cruor. SDS-PAGE sample name (protein amount)—(a) FBSs and bovine sera (7 µg); (b) porcine and chicken sera (7 µg); (c) plasma of bovine, porcine, and chicken (7 µg); (d) whole blood of bovine, porcine, and chicken (7 µg), (e) cruor of bovine, porcine, and chicken (200 µg).

Table 1). AAs such as taurine, threonine, glycine, alanine, valine, cysteine, methionine, isoleucine, tryptophan, ornithine, histidine, and proline were detected in similar amounts among FBS, bovine serum, and porcine serum, but with clear differences in content among individuals (Figure 3a, Supplementary Table 1). Aspartic acid, serine, glutamic acid, glutamine, and 3-methylhistidine were significantly lower in bovine and porcine sera than in FBS (Figure 3a, Supplementary Table 1). Unlike FBS and other sera from BSBPs, asparagine was not detected in chicken serum, and anserine was only detected in chicken serum (Figure 3a). Ethanolamine was detected only in FBS and chicken

blood by-products. However, the content of urea in chicken blood was about 20 times lower than that in FBS and other livestock species, showing a tendency for the AAs in chicken blood and mammalian-derived blood by-products to be different.

The major AAs in serum, whole blood, and cruor were similar in all species except for a few components. In most plasma samples, the content of AAs was found to be lower than in whole blood or serum, while the contents of urea, alanine, ammonia, ornithine, and lysine were reduced (Figure 3a, Supplementary Table 1). In bovine and chicken, phosphor ethanol amine was

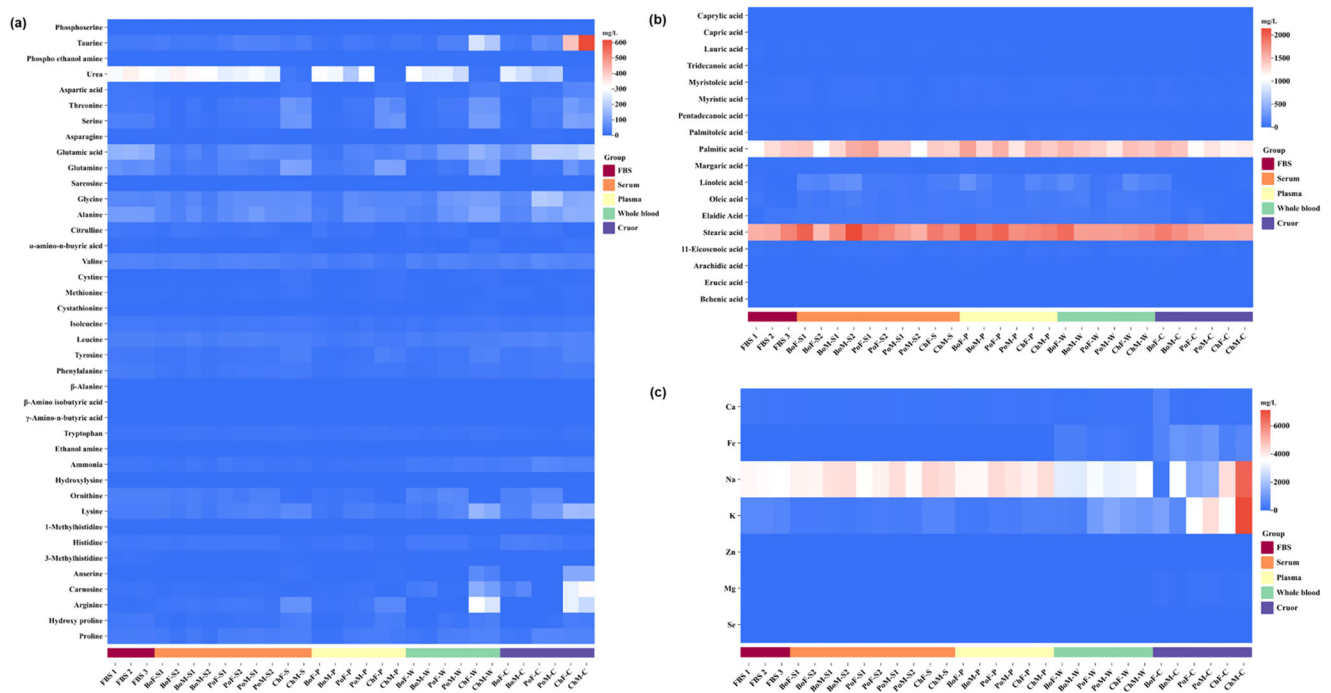


FIGURE 3 | Visualization of nutritional component profiles in FBS and blood by-products (heatmap). (a) free amino acids composition; (b) fatty acids composition; (c) minerals composition (Na data only: mg/dL). FBS: fetal bovine serum, Bo: bovine, Po: porcine, Ch: chicken, F: female, M: male, S: serum, P: plasma, W: whole blood, C: cruor, Ca: calcium, Fe: iron, Na: sodium, K: potassium, Zn: zinc, Mg: magnesium, Se: selenium, -: not detected (content values can be confirmed with Supplementary Tables 1–3).

detected only in plasma, but detected in porcine whole blood and cruor (Figure 3a, Supplementary Table 1). This is a different result from the detected ethanolamine in FBS and all types of chicken blood-derived treatment groups. In addition, carnosine was greatly reduced by about 1/100 in fractionated serum and plasma (Figure 3a, Supplementary Table 1).

Aspartic acid, serine, glutamic acid, and glutamine are AAs that promote cell proliferation, and a lack of these components may increase the possibility of insufficient cell growth (Lee et al. 2024b). According to Xu et al. (2014), when glutamine and asparagine were added alone or together, the pH decrease due to cell growth was prevented, and cell viability and growth rate were improved. Therefore, asparagine supplementation in chicken serum with high glutamine content may improve cell growth. Additionally, anserine can increase the diameter and development of myotubes by promoting gene expression related to skeletal muscle cell differentiation and sarcomeric structure formation (Nagai et al. 2023). Ethanolamine is a substance added to basal media, such as Roswell Park Memorial Institute (RPMI) 1640, DMEM, and Iscove's Modified Dulbecco's Medium (IMDM), and has been mentioned as an essential component for cell growth under serum-free conditions in mammalian cell culture (Kano-Sueoka et al. 2001; Yao and Asayama 2017). Carnosine can help muscle cell proliferation by activating the Akt/mTOR/S6K signaling pathway (Liu et al. 2022). Therefore, additional research is needed on how to maintain effective components for cell culture during the process of collecting and preprocessing BSBPs.

3.1.4 | Analysis of FAs

The most abundant FAs in all BSBPs, not just FBS, are stearic acid and palmitic acid (Figure 3b). Based on serum standards, the total amount of FAs detected in the sera of all livestock by-products was greater than or similar to FBS (Figure 3b, Supplementary Table 2). Additionally, linoleic acid, oleic acid, and elaidic acid were detected in greater amounts in the sera of all livestock by-products than in FBS. Furthermore, serum from bovine BSBPs contained 3–10 times more linoleic acid than other sera, including FBS (Supplementary Table 2). Conversely, lauric acid, myristic acid, palmitic acid, margaric acid, stearic acid, 11-eicosenoic acid, and arachidonic acid were detected at similar amounts across all sera, including FBS (Figure 3b, Supplementary Table 2).

Excessive amounts of stearic acid or palmitic acid can induce the production and proliferation of adipocytes, which may inhibit the proliferation and differentiation of muscle satellite cells and myoblasts (Choi et al. 2015; da Paixão et al. 2021; Lee et al. 2023; Małodobra-Mazur et al. 2020). Linoleic acid and oleic acid can promote the proliferation and differentiation of muscle cells at appropriate concentrations (Belal et al. 2018; Hurley et al. 2006; Lee et al. 2009; Moreno et al. 2024). Fatty acid components, such as lauric acid, myristic acid, and arachidonic acid, are excellent at promoting cell differentiation (Lee et al. 2024b). In three livestock species from which BSBPs were obtained, no significant differences in FA content were found due to pretreatment compared to whole blood (Figure 3b, Supplementary Table 2).

3.1.5 | Analysis of Minerals

Among the seven minerals analyzed (Ca, Fe, Na, K, Zn, Mg, Se), Na was detected the most in all treatment groups, followed by K or Fe (Figure 3c). Ca was detected in FBS at 120–134 mg/L, and in sera from cattle, pigs, and chickens, it was detected at a minimum of 83 mg/L and a maximum of 110 mg/L (Figure 3c, Supplementary Table 3). Fe was detected at only about 2 mg/L in the commercial FBS group but at higher concentrations in the serum from BSBPs (Supplementary Table 3). The Fe content was relatively higher in whole blood and cruor with RBCs. Na was detected in sera from all types of BSBPs at a similar amount to that in FBS (Figure 3c). The K content detected in bovine and porcine sera was about half in FBS. Trace amounts of Zn were detected in the FBS group, and a level equivalent to about half this amount was found in chicken serum (Figure 3c).

The high Fe content of BSBPs is thought to be due to hemolysis during the blood acquisition process. Among minerals, Ca can increase the expression of Myf5, which is associated with myocyte proliferation, and muscle contractions (Porter et al. 2002). An appropriate increase in K concentration can stimulate cell proliferation, while the direct cell growth effect of Na has not been confirmed (Kravchenko et al. 2019; Lee et al. 2024b). The non-detection of Se or Zn in some treatments was likely due to a low concentration below the limit of detection of the device, so additional component analysis is necessary.

3.2 | Cell Culture Using FBS and BSBPs

3.2.1 | pH Measurement

In cell culture, the maintenance of appropriate temperature and pH is critical. The pH of commercially available FBS, serum that can be used as a substitute for FBS, and other blood products was compared. The pH of the three commercial FBS groups showed a significant difference between 7.96 and 8.04, but almost no difference was observed within the FBS group (Figure 4a). In contrast, the BSBP serum and plasma were confirmed to have relatively high pH (8.1–8.7). Most whole blood and cruor samples showed pH values between 7.0–7.7 and 7.4–7.8 (Figure 4a). In some treatment groups, the pH was significantly lower (PoF-W and ChM-C).

These results may vary in proportion to the exposure time of the sample to air. This theory is supported by the observation that the average pH of the commercial FBS group (FBS1–3) immediately after opening was 7.46, which was approximately 0.5 lower than the value when all samples were prepared and measured simultaneously (data not shown). This initial value is similar to the normal animal blood pH (7.5) (Chiroque et al. 2023). For serum, plasma, and whole blood samples derived from other BSBPs, the pH increased by 0.3 to 0.9 when sampled immediately after slaughter (data not shown; in the case of cruor, prior measurement is not possible). The obtained sera from BSBPs can be sufficiently used for general cell culture. Nonetheless, in order to effectively utilize BSBP-derived FBS substitutes in pharmaceuticals or food production, it is thought that the development of a processing system that blocks contact with air as much as possible to maintain a certain quality is necessary.

3.2.2 | Cell Proliferation and Viability

It has been reported that FBS substitutes derived from cattle, pigs, and chickens showed excellent cell proliferation and differentiation effects towards bovine muscle cells, although slightly less than FBS (Lee et al. 2024a). In the present study, to determine whether FBS substitutes derived from livestock blood by-products can effectively be used to culture general cell lines, we selected mouse myoblast C2C12 cells. For replacing the FBS, two types of substitutes were separately tested: plasma and serum. In the treatment groups where the same amount of plasma was added to replace FBS, none of the cells grew, and the medium became gelatinous (data not shown). These changes in the media characteristics can be inferred to be due to the additional action of fibrinogen or prothrombin contained in the plasma. Furthermore, the complicated acquisition process and additional cost of plasma obtained by adding EDTA-2Na prove that serum alone cannot replace FBS.

In the cell culture experiment using serum from BSBPs, the effects of bovine and porcine serum on myoblast proliferation were observed, but were less effective compared to FBS (Figure 4). Bovine serum showed a proliferation rate comparable to that of the FBS group, regardless of gender (Figure 4b–c). Porcine serum was less effective than bovine serum but showed about 70% cell proliferation and survival rate compared to FBS. Meanwhile, chicken serum resulted in a significantly lower cell growth and also affected the cell morphology (Figure 4b–d). This trend shows that bovine and porcine serum are more suitable as replacements for FBS in analyses comparing cell growth, although to a different degree. This can also be supported by the results of subsequent experiments.

The low cell proliferation effect of BoF-S2 may be attributed to potential damage during blood transport or the characteristics of the individual from which the serum was obtained. Unlike BoF-S1, which is derived only with serum from heifers, BoF-S2 contains serum from heifers that have given birth to 1–2 calves (Data not shown). Following calving, cows produce colostrum, which is accompanied by a decrease in blood protein levels, including albumin, and an increase in Ig concentrations (Bell et al. 2000; Rowlands et al. 1975). In addition, the levels of insulin-like growth factor-I and insulin also decrease for a certain period after giving birth (Bell et al. 2000). These changes are known to influence cell proliferation and concentrations of several blood components—such as total protein, calcium, sodium, and hemoglobin—undergo marked fluctuations before and after parturition (Bell et al. 2000; Lee et al. 2024b; Rowlands et al. 1975). Therefore, the low cell proliferation effect of BoF-S2 may have been influenced by the serum from multiparous cows. In Korea, porcine and chickens are typically slaughtered prior to parturition, whereas cows are often slaughtered post-parturition. Accordingly, further study is warranted to ensure the stable and reproducible use of adult bovine serum in cell culture applications.

Prior to cell culture, microbiological contamination was assessed using Petrifilm™ plates (3 M, Saint Paul, MN, USA). No contamination was detected on AC, EC, and STX plates (Supplementary Figure 1). Following cell culture, mycoplasma contamination in the culture medium was evaluated using the Myco-Read™

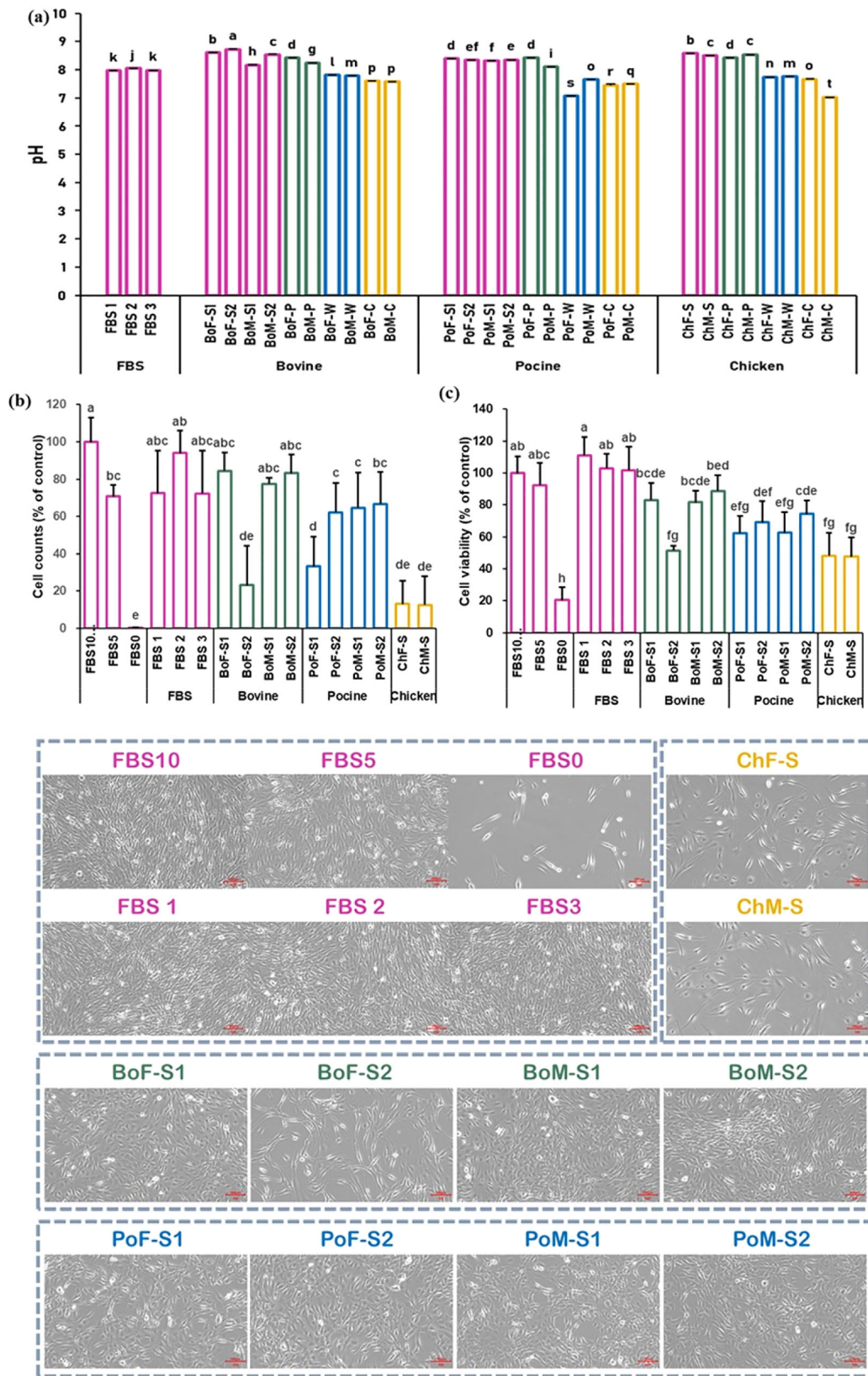


FIGURE 4 | Analysis and application of FBS and blood by-products for C2C12 cell culture. (a) pH of blood by-product-derived samples; (b) percentage of proliferated cells using counting; (c) cell viability analysis using MTT assay, FBS 10: 10% FBS with DMEM (control), FBS 5: 5% FBS with DMEM, FBS 0: DMEM only, FBS: fetal bovine serum, Bo: bovine, Po: porcine, Ch: chicken, F: female, M: male, S: serum, P: plasma, W: whole blood, C: cruor.

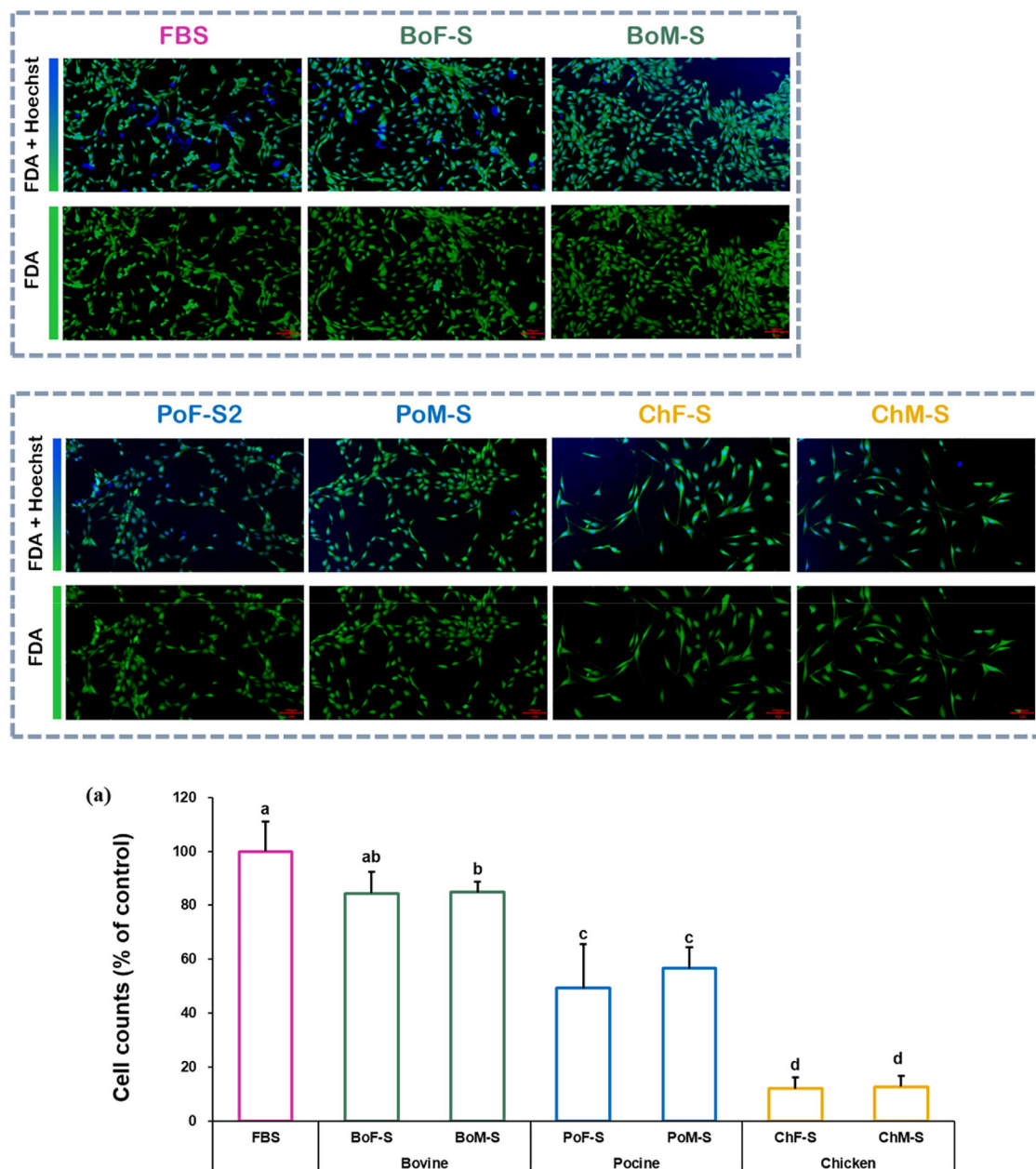


FIGURE 5 | Comparison of cell culture effects of FBS and serum derived from blood by-products using fluorescence analysis in C2C12 cell culture. (a) FDA-positive cell percentage. FBS: fetal bovine serum (control), Bo: bovine, Po: porcine, Ch: chicken, F: female, M: male, S: serum.

Mycoplasma Detection Kit (SMD0172, Guri, Korea) according to the manufacturer's protocol, and no mycoplasma was detected (Supplementary Figure 2).

3.2.3 | Cell Growth and Viability

C2C12 cells cultured for three days were measured for fluorescence based on the Hoechst/FDA reagent (Figure 5). The bovine serum group exhibited a proliferation rate most comparable to that of FBS, whereas the chicken serum group demonstrated the lowest proliferation capacity (Figure 4a). Image analysis revealed that live cells stained with (FDA) and total cells stained with Hoechst dye were nearly completely the same. Furthermore, consistent with the trend observed in the direct cell counting assay

using trypan blue (Figure 4b), superior cell proliferation effects were observed in both bovine and porcine serum groups when sera from the same sex and species were combined (Figure 5).

The lower proliferation rate on cultures with serum from BSBPs compared commercial FBS-treated group may be due to the excessive Fe content in serum from BSBPs. Excessive amounts of Fe can inhibit cell proliferation, becoming an important consideration in the optimization of FBS-free media (Mueller et al. 2006). In general, the older the livestock, the higher the level of Ig in the blood or colostrum, and high Ig levels can inhibit cell growth (Borchardt et al. 2022; Lammers et al. 2010). Unlike bovine or porcine serum, the effect of chicken serum was not significantly better than FBS. This result contradicts the prediction of effective cell growth by AAs (glutamine, anserine, and ethanolamine)

in chicken serum. It is likely that cell growth was inhibited by chicken blood components, which are predicted to have the greatest increase in Ig ratio when predicted by excluding ALB content from TP content or electrophoresis results. Alternatively, it is possible that chicken blood had a negative effect on the growth of mammalian cells like C2C12, but additional studies are needed to verify this hypothesis.

4 | Conclusion

In this study, blood was collected in substantial quantities from major livestock species—cattle, pigs, and chickens—and subjected to nutritional and hematological analyses based on sex and blood pretreatment methods. Furthermore, we evaluated the potential for substituting FBS with plasma or serum through cell culture experiments. The components of commercial FBS are highly uniform, and pooled BSBPs exhibited compositional differences, depending on the species. It is inferred that the levels of immunoglobulins and cholesterol in the blood increase as livestock grow. The composition of whole blood and cruor were found to be very similar, indicating that the remaining cruor after serum recovery can be a valuable resource. BSBPs were observed to have a higher pH and a significantly higher iron (Fe) content compared to FBS. However, in cell culture, plasma alone was not suitable as a substitute for FBS. Bovine and porcine sera, on the other hand, proved to be highly effective substitutes for FBS, particularly in C2C12 cell culture.

The repurposing of BSBPs has the potential to alleviate the cost and environmental challenges associated with traditional livestock farming. Additionally, BSBPs show promise as an alternative material for cell culture, particularly as a substitute for FBS. Future research could accelerate the commercialization of BSBP-derived FBS substitutes through the following approaches: (1) the production of high-quality and stable BSBPs by preventing pH fluctuations and minimizing blood cell destruction; (2) the establishment of standardized quality evaluation and production protocols for blood by-products to minimize individual variability; (3) the enhancement of the FBS substitute efficacy of serum derived from BSBPs; and (4) the development of BSBP-derived serum (FBS substitute) free from cell growth inhibitory factors specific to certain species.

Author Contributions

Da Young Lee: investigation, data curation, writing – review and editing, writing – original draft. **Yeongwoo Choi:** data curation, investigation. **Dahee Han:** data curation, investigation. **Jinmo Park:** data curation, investigation. **Jin Soo Kim:** data curation, investigation. **Ermie Jr. Mariano:** data curation, investigation. **Ji Won Park:** data curation, investigation. **Seok Namkung:** data curation, investigation. **Seung Yun Lee:** writing – review and editing. **Inho Choi:** writing – review and editing. **Seon-Tea Joo:** writing – review and editing. **Sun Jin Hur:** supervision, conceptualization, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Materials: jfds70492-sup-0001-SuppMat.docx