

RESEARCH ARTICLE

Integrating morphological embryo quality scoring and untargeted metabolomics identifies candidate biomarkers in Bali cattle embryos



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ABSTRACT

Background and Aim: Embryo quality is a major determinant of the efficiency of *in vitro* embryo production in cattle. Although morphological assessment is routinely used for embryo selection, it may not fully reflect the underlying metabolic state. Metabolomics may complement morphological evaluation by identifying biochemical characteristics associated with embryo quality. This study aimed to evaluate Bali cattle embryo quality and identify candidate metabolite biomarkers by integrating multi-parameter morphological scoring with liquid chromatography–high-resolution mass spectrometry (LC–HRMS)-based untargeted metabolomics.

Materials and Methods: Thirty Bali cattle embryos produced through *in vitro* fertilization were evaluated using six morphological parameters: general embryo structure, blastomere uniformity, fragmentation, cellular compaction, zona pellucida condition, and cellular degeneration. Embryos were classified as good- or poor-quality according to their composite quality scores. Three embryos from the same quality group were pooled per metabolomic sample, generating 10 analytical pools (five/group). Untargeted LC–HRMS metabolomics, differential metabolite analysis, multivariate analysis, Kyoto Encyclopedia of Genes and Genomes-based functional analysis, receiver operating characteristic analysis, and correlation analysis were performed. Candidate biomarkers were prioritized using differential patterns, variable importance in projection scores >1, pathway involvement, area under the curve values >0.8, and significant associations with embryo quality parameters.

Results: Good quality embryos had significantly higher general embryo structure, blastomere uniformity, cellular compaction, zona pellucida condition, and total quality scores. Metabolomic profiles differed between quality groups. Nine metabolites were prioritized as candidate biomarkers. Pyruvate, citrate, (S)-malate, 2-oxoglutarate, nicotinamide adenine dinucleotide phosphate, and L-glutamate were higher in good quality embryos, whereas succinate, oxidized glutathione, and L-tyrosine were higher in poor-quality embryos. These metabolites were principally associated with energy metabolism, amino acid metabolism, and redox regulation and showed significant associations with morphological embryo quality parameters.

Conclusion: Integrating morphological scoring with untargeted metabolomic profiling revealed coordinated metabolic differences associated with Bali cattle embryo quality. The nine-metabolite panel represents a set of candidates, rather than validated, biomarkers because the metabolomic analysis was based on pooled embryos and a small discovery dataset. Individual embryo analysis and validation in larger independent cohorts are required before predictive or practical application.

Keywords: Bali cattle, candidate biomarkers, embryo quality, energy metabolism, *in vitro* fertilization, metabolomics, oxidative balance, untargeted metabolomics.

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INTRODUCTION

Embryo quality is a key determinant of the success of *in vitro* embryo production and subsequent reproductive applications in cattle. *In vitro* fertilization has become increasingly important in livestock reproductive biotechnology because it enables embryo production from oocytes recovered from slaughterhouse-derived ovaries, thereby facilitating the use of female genetic material that might otherwise be discarded [1]. This approach is particularly relevant to Bali cattle, an important Indonesian indigenous genetic resource characterized by adaptation to tropical environments and considerable potential for beef production [2]. Therefore, *in vitro* embryo production may contribute to both genetic improvement and conservation programs for Bali cattle. However, the quality of embryos produced under *in vitro* conditions remains variable because embryo development is influenced by multiple factors, including oocyte quality, fertilization efficiency, culture conditions, and the intrinsic biological characteristics of the developing embryo [3].

Improving embryo quality assessment is important because conventional embryo selection in *in vitro* production systems has inherent limitations. Embryos produced under *in vitro* conditions may differ from those produced *in vivo* in developmental competence, cellular physiology, and metabolic regulation [4]. Furthermore, *in vitro* production systems can influence embryo metabolism and developmental outcomes. Consequently, embryo quality depends not only on external morphology but also on the embryo's underlying physiological and metabolic state [5]. Reliance solely on morphological appearance may therefore provide an incomplete assessment of embryo quality and may limit the efficiency of subsequent reproductive applications.

Embryo quality is commonly assessed using morphological characteristics, including general embryo structure, blastomere uniformity, degree of fragmentation, cellular compaction, zona pellucida condition, and cellular degeneration. These parameters provide practical information on the structural characteristics of embryos following *in vitro* culture and remain widely used because morphological assessment is simple, rapid, and applicable to routine embryo evaluation [6]. However, visual assessment may not fully represent the physiological and metabolic status of an embryo. Embryos with similar morphological scores may differ in metabolic activity, cellular stress responses, and developmental capacity [7]. Therefore, complementary molecular approaches are needed to provide additional insight into the biological differences between embryos of different morphological quality.

Metabolomics has emerged as a powerful approach for investigating small molecules involved in embryo metabolism and developmental regulation. During early embryonic development, dynamic metabolic changes occur in pathways related to energy production, amino acid and lipid metabolism, nucleotide biosynthesis, redox regulation, and cellular signaling [8]. These processes contribute to cell division, compaction, blastocyst formation, and embryo survival under culture conditions. Metabolomic profiling can therefore provide information on the biochemical characteristics associated with embryo quality. Differences in metabolite abundance between embryo quality groups may reflect differences in metabolic states and help identify candidate metabolites associated with morphological characteristics. Integrating metabolomic data with morphological quality scores may consequently provide a more comprehensive assessment of embryo biological status than either approach alone.

Recent advances in liquid chromatography–high-resolution mass spectrometry (LC–HRMS) have enabled broad, untargeted characterization of small molecules. This approach enables detection of diverse metabolite classes and helps identify metabolic differences that targeted biochemical assays may miss [9]. In bovine embryo research, metabolomic approaches have increasingly been applied to investigate embryo physiology, culture-related metabolic alterations, developmental competence, and potential biomarkers for embryo evaluation [10]. Metabolomic profiling may also provide biological information beyond conventional morphological grading and help develop more informative embryo assessment strategies [8]. However, bovine embryo metabolomic studies have largely focused on commonly studied cattle populations and embryo production systems, while information on tropical indigenous cattle breeds remains limited.

Despite advances in bovine embryo metabolomics, information on the metabolic characteristics of Bali cattle embryos remains limited. Previous studies in Bali cattle have primarily investigated oocyte sources, embryo development, apoptosis, and other reproductive biotechnology parameters, whereas studies integrating morphological embryo quality assessment with untargeted metabolomic profiling remain scarce [5]. Slaughterhouse-derived Bali cattle ovaries have shown potential as oocyte sources for *in vitro* embryo production, supporting their relevance to the utilization and conservation of native Indonesian animal germplasm [11]. Nevertheless, integrated characterization linking multi-parameter morphological quality scores with untargeted

metabolomic profiles of good- and poor-quality Bali cattle embryos remains limited. Consequently, the metabolites and metabolic pathways associated with morphological embryo quality have not been adequately characterized. This knowledge gap limits the molecular characterization of embryo quality variation and the identification of candidate molecular indicators that could complement conventional morphological assessment.

Therefore, this study aimed to evaluate the morphological quality of Bali cattle embryos produced through *in vitro* fertilization and to characterize differences in the metabolomic profiles of good- and poor-quality embryo groups using LC–HRMS-based untargeted metabolomics. Specifically, the study integrated multi-parameter morphological embryo quality scoring with metabolomic profiling to identify differential metabolites and metabolic pathways associated with embryo quality and to prioritize candidate metabolite biomarkers. This integrated approach was intended to provide preliminary molecular insight into the metabolic characteristics associated with Bali cattle embryo quality and to establish a foundation for future validation of metabolite-based indicators that may complement conventional morphological embryo evaluation.

MATERIALS AND METHODS

Ethical approval

All procedures involving animal-derived biological materials were reviewed and approved by the Animal Research Ethics Commission, Department of Animal Science, Faculty of Agriculture, Lambung Mangkurat University, Banjarbaru, Indonesia (Approval No. 381/UN8.1.23.5/PG/2026). The study was conducted in accordance with applicable institutional and national ethical standards governing research involving animal biological materials and followed the principles of the 3Rs (replacement, reduction, and refinement) and the Five Freedoms of animal welfare. The ovaries used in this study were obtained from Bali cattle slaughtered for routine commercial purposes at a local slaughterhouse. No animals were purchased, maintained, experimentally treated, or slaughtered specifically for this study, and the researchers did not alter routine commercial slaughter procedures.

Study period and location

The study was conducted from April to June 2026 at the Laboratory of Animal Production, Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara, Indonesia. Embryo production and morphological evaluation were performed at this laboratory. LC–HRMS analysis was performed at Biomarker Technologies (Hong Kong) Company Limited.

Study design

This experimental study integrated morphological embryo quality assessment with untargeted metabolomic profiling to characterize metabolic differences associated with Bali cattle embryo quality. Embryos were produced *in vitro* from oocytes recovered from slaughterhouse-derived ovaries. A total of 30 embryos were individually evaluated using predefined morphological quality characteristics and classified into good- and poor-quality groups according to their composite quality scores. After morphological classification, embryos within each quality group were pooled for LC–HRMS-based untargeted metabolomic analysis. Three embryos were included in each metabolomic pool, resulting in 10 pooled analytical samples, comprising five pools from each quality group. The pooled sample, rather than the individual embryo, therefore represented the analytical unit for metabolomic analyses. Morphological and metabolomic data were integrated to characterize metabolic differences between embryo quality groups and prioritize candidate metabolite biomarkers associated with embryo quality.

Embryo collection, quality assessment, and group classification

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in sterile physiological saline maintained at 30–35°C. On arrival, the ovaries were washed with sterile physiological saline and processed for oocyte recovery. Cumulus–oocyte complexes (COCs) were recovered by aspiration of visible ovarian follicles and selected based on the presence of at least three compact layers of cumulus cells and a homogeneous, evenly granulated ooplasm. Selected COCs were washed three times in maturation medium and subjected to *in vitro* maturation in TCM-199 supplemented with 10% fetal bovine serum, follicle-stimulating hormone, luteinizing hormone, and antibiotics. Maturation was performed for 22–24 h at 38.5°C in a humidified atmosphere containing 5% CO₂.

Following maturation, frozen–thawed semen was processed using a 45%/90% discontinuous Percoll density gradient to separate motile spermatozoa. The sperm concentration was adjusted to approximately 1×10^6 spermatozoa/mL in fertilization Tyrode's albumin–lactate–pyruvate (Fert-TALP) medium supplemented with

heparin. Matured COCs were co-incubated with spermatozoa for approximately 18 h at 38.5°C in a humidified atmosphere containing 5% CO₂.

After fertilization, presumptive zygotes were denuded of remaining cumulus cells and spermatozoa by gentle pipetting, washed, and transferred into synthetic oviduct fluid (SOF) culture medium. Embryos were cultured at 38.5°C in a humidified atmosphere containing 5% CO₂, 5% O₂, and 90% N₂ until day 7. These experimental conditions were based on the general bovine *in vitro* embryo production procedures described by Ferré *et al.* [12], with adaptations in COC selection, maturation conditions, sperm preparation, fertilization conditions, and embryo culture. Bali cattle embryos produced through *in vitro* fertilization were evaluated on day 7 of *in vitro* culture using embryo handling medium (Minitube, Tiefenbach, Germany). Each embryo was individually identified using an embryo code, donor identification, and culture date. Embryos were transferred to embryo handling medium (Minitube) and evaluated under a stereomicroscope (Olympus, Tokyo, Japan) at an appropriate magnification. A warmed stage (Tokai Hit, Fujinomiya, Japan) was used to maintain embryo temperature during evaluation. All embryos were assessed using identical scoring criteria and observation conditions to minimize variation during morphological evaluation.

Embryo quality was visually assessed using six morphological parameters: general embryo structure, blastomere uniformity, degree of fragmentation, cellular compaction, zona pellucida condition, and cellular degeneration. The assessment was adapted from the International Embryo Technology Society (IETS) Manual and previously established bovine embryo evaluation criteria. In this study, we scored the six morphological characteristics separately using a three-point scale and then combined them into a composite quality score. A score of 3 represented good quality, 2 represented moderate quality, and 1 represented poor-quality. The complete operational definitions of scores 1, 2, and 3 for each morphological parameter are presented in Table 1.

The total embryo quality score was calculated by summing the scores for the six parameters, yielding a possible score of 6–18 for each embryo. Embryos with total scores of 14–18 were classified as good quality, whereas those with scores of 6–13 were classified as poor-quality. This cutoff represented a study-specific operational classification based on the composite six-parameter scoring framework rather than a direct IETS grading threshold. A total score of ≥14 corresponded to an average score of ≥2.33 across the six parameters, indicating predominantly moderate-to-good morphological characteristics, whereas a score of ≤13 indicated a greater contribution of moderate or poor morphological characteristics. This classification formed the basis for subsequent comparisons of metabolomic profiles between embryo quality groups.

All embryos were morphologically evaluated by a single trained observer using the predefined six-parameter scoring criteria presented in Table 1. Because a single observer performed the evaluation, inter-observer agreement could not be assessed. Intra-observer reliability statistics were not available for the present dataset.

Table 1: Embryo quality scoring criteria for Bali cattle embryos.

Parameter	Score 1	Score 2	Score 3
General embryo structure	Abnormal, collapsed, asymmetric, and poorly organized structure	Slight irregularity in shape	Round, symmetrical, and well-organized structure
Blastomere uniformity	Markedly unequal blastomeres and irregular cellular distribution	Moderately unequal blastomeres	Uniform blastomere size and regular cellular arrangement
Fragmentation	High fragmentation, >25% of embryonic area	Moderate fragmentation, 15–25% of embryonic area	Low fragmentation, <15% of embryonic area
Cellular compaction	Poor compaction, loose blastomeres, and weak cellular adhesion	Partial compaction	Compact, cohesive, and well-integrated cells
Zona pellucida condition	Damaged, cracked, collapsed, and severely abnormal zona pellucida	Slight irregularity, mild thickening, and minor deformation	Intact, clear, and regular zona pellucida
Cellular degeneration	Severe degeneration, extensive darkening, and cellular collapse	Mild to moderate degeneration	Absent to minimal degenerative changes

Each of the six embryo quality parameters is scored from 1 to 3, with higher scores indicating more favorable morphological characteristics. The total embryo quality score is the sum of the six parameter scores, with a possible range of 6–18.

Metabolomic sample pooling and preparation

After embryo quality assessment on day 7 of *in vitro* culture, embryos were grouped by morphological quality classification. Embryos within the same quality group were pooled because the amount of biological material available from individual embryos was insufficient for reliable LC–HRMS-based untargeted metabolomic analysis. Ten pooled metabolomic samples were prepared, comprising five pools from the good quality group and five from

the poor-quality group. Each pooled sample contained three embryos from the same quality group. Consequently, each pooled sample, rather than each individual embryo, constituted the analytical unit for metabolomic analysis. Although pooling provided sufficient biological material for metabolite detection, it precluded assessment of individual embryo metabolic variability and limited inference regarding biomarker performance at the individual embryo level.

Pooled embryo samples were transferred to sterile microcentrifuge tubes, labeled according to quality group and pool code, and stored at -80°C until analysis. Before analysis, samples were thawed at 4°C . Each pooled sample was mixed with cold methanol/acetonitrile (1:1, v/v) for metabolite extraction and protein precipitation and centrifuged at $14,000 \times g$ for 20 min at 4°C to remove proteins and cellular debris. The supernatant was transferred to a clean tube and dried using a vacuum centrifuge. Dried extracts were reconstituted in 200 μL of acetonitrile/water (1:1, v/v) and centrifuged at $14,000 \times g$ for 15 min at 4°C . The resulting supernatant was filtered through a 0.2- μm membrane filter and transferred to LC glass vials for subsequent LC–HRMS analysis.

LC–HRMS-based untargeted metabolomic analysis and data processing

Untargeted metabolomic profiling was performed using electrospray ionization in both positive and negative ionization modes. Chromatographic separation was performed using a Thermo Scientific Vanquish Horizon ultra-high-performance liquid chromatography system equipped with a binary pump (Thermo Scientific, Germering, Germany). Metabolites were separated using a Thermo Scientific Accucore C18 analytical column (100 $\times$ 2.1 mm internal diameter, 2.6- μm particle size; Thermo Scientific, Germering, Germany). The mobile phase consisted of MS-grade water containing 0.1% formic acid (mobile phase A) and MS-grade acetonitrile containing 0.1% formic acid (mobile phase B). The flow rate was 0.3 mL/min, and the total run time was 25 min. The gradient began at 5% B, increased gradually to 90% B over 16 min, was maintained at 90% B for 4 min, and then returned to 5% B for the remainder of the run. The column temperature was maintained at 40°C , and the injection volume was 5 μL .

High-resolution mass spectrometry was performed using a Thermo Scientific Orbitrap Exploris 240 HRMS (Thermo Scientific). Data were acquired in full MS/dd-MS² mode with polarity switching between positive and negative ionization modes. Full MS spectra were acquired at a resolution of 60,000 full width at half maximum (FWHM) over an m/z range of 67–1000, with a maximum injection time of 100 ms, intensity threshold of 5000, charge state of 1, and mass tolerance of 5 ppm. Fragmentation spectra were acquired at a dd-MS² resolution of 30,000 FWHM using normalized collision energies of 30, 50, and 70. Nitrogen was used as the collision gas [13].

Ionization was performed using an OptaMax NG heated electrospray ionization source (Thermo Scientific, San Jose, USA). Spray voltages were set at 3500 V in positive mode and 2500 V in negative mode. Sheath, auxiliary, and sweep gases were set at 35, 7, and 1 arbitrary units, respectively. The ion transfer tube temperature was maintained at 300°C , and the vaporizer temperature was 320°C .

Raw LC–HRMS data were processed using Compound Discoverer version 3.5 (Thermo Scientific, San Jose, USA). Processing included peak detection, retention-time alignment, feature grouping, compound prediction, and compound annotation. Acquired MS and Tandem mass spectrometry data were searched against mzCloud spectral libraries, ChemSpider databases, and mass-list databases to support metabolite identification. No pooled quality control (QC) samples, solvent blanks, or internal standards were included in the LC–HRMS analytical sequence. Consequently, QC-based metrics could not independently evaluate analytical stability across the sequence, which should be considered when interpreting the untargeted metabolomic findings.

Compound annotation was performed using mzCloud libraries covering endogenous metabolites, natural products, small-molecule chemicals, lipids, steroids, vitamins, hormones, therapeutics, pesticides, and related compound classes. Database searching was further supported by ChemSpider sources, including the Bovine Metabolome Database, Kyoto Encyclopedia of Genes and Genomes (KEGG), LIPID MAPS, BioCyc, FooDB, Golm Metabolome Database, Serum Metabolome Database, and other metabolite-related databases. Additional mass-list searches were conducted using the Extractables and Leachables HRAM Compound Database, Human Metabolome Database (HMDB) All Metabolites, HMDB Serum Metabolites, and PubChemLite 2024.

Bioinformatic analysis

The metabolite peak area matrix generated from LC–HRMS analysis was used for bioinformatic and statistical analyses [14]. Compound annotations were filtered according to the availability of KEGG identifiers. Only metabolites with valid KEGG IDs were included in pathway-related interpretation and downstream analyses.

Metabolomic data processing, visualization, and biomarker evaluation were performed using MetaboAnalyst

6.0. The Statistical analysis module was used for data preprocessing, differential analysis, principal component analysis (PCA), hierarchical clustering, volcano plot visualization, and partial least squares–discriminant analysis (PLS-DA). Pathway-related interpretation was performed using the Pathway Analysis module, and receiver operating characteristic (ROC)-based biomarker evaluation was performed using the Biomarker Analysis module. Before analysis, the metabolite peak-area matrix was assessed for missing values. No additional sample-wise normalization was applied. The data were log₁₀-transformed to reduce data skewness and heteroscedasticity and were subsequently mean-centered by subtracting the mean value of each metabolite from the corresponding observations. The preprocessed data were then used for PCA, PLS-DA, hierarchical clustering, differential analysis, and biomarker evaluation.

Overall differences in metabolomic profiles between embryo quality groups were assessed using PCA and hierarchical clustering heatmap analysis. Differential metabolite patterns were visualized using volcano plots. Differential abundance between the good- and poor-quality embryo groups was assessed using an unpaired two-sample t-test combined with fold-change analysis. Metabolites showing a fold change ≥ 2.0 or ≤ 0.5 together with $p < 0.05$ were considered differentially abundant. To account for multiple comparisons, the procedure was applied to estimate the false discovery rate (FDR), and the corresponding FDR-adjusted p-values were also reported. PLS-DA was performed to assess separation between the good- and poor-quality embryo groups. Metabolites with variable importance in projection (VIP) scores >1 were considered important contributors to group discrimination. Pathway-related interpretation was performed in MetaboAnalyst 6.0 using KEGG ID-based metabolite mapping. The pathway analysis was conducted using the corresponding KEGG pathway library available in MetaboAnalyst. Pathway enrichment and topology results were interpreted according to the software-generated Total, Expected, Hits, and Impact values. Differential metabolites were mapped to metabolic pathways to identify biological pathways potentially associated with embryo quality. Pathway interpretation considered enrichment significance, pathway impact values, and the contribution of differential metabolites to individual pathways.

Correlation analysis was performed to assess associations between pooled embryo quality scores and metabolite peak areas. For each metabolomic pool, the morphological quality score was calculated as the arithmetic mean of the total quality scores of the three embryos comprising that pool. The pooled sample was therefore treated as the analytical unit, yielding 10 paired observations for correlation analysis. Associations between pooled embryo quality scores and differential metabolite peak areas were assessed using Pearson correlation analysis.

Candidate metabolite biomarkers were sequentially prioritized according to five complementary criteria: (1) significant differential patterns between embryo quality groups, (2) VIP scores >1 in the PLS-DA model, (3) involvement in KEGG-based functional pathways, (4) area under the curve (AUC) values >0.8 in ROC analysis, and (5) significant correlations with embryo quality parameters. This integrated strategy was used to prioritize metabolites demonstrating statistical discrimination, biological relevance, and consistent associations with morphological embryo quality.

Raw LC–HRMS data and the processed metabolite peak area matrix will be deposited in a public metabolomics repository, such as MetaboLights or Metabolomics Workbench. The repository name and accession number will be provided after deposition and before final publication.

Statistical analysis

Embryo quality score data were analyzed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY USA). Data distributions were evaluated for normality. Normally distributed data were compared using an independent-samples *t*-test, whereas non-normally distributed data were analyzed using the Mann–Whitney U test. Results were expressed as mean $\pm$ standard deviation (SD), and statistical significance was established at $p < 0.05$.

RESULTS

Embryo quality parameter scores and group classification

Embryo quality parameter scores and group classifications are presented in Table 2. Significant differences between good- and poor-quality embryos were observed for general embryo structure ($p < 0.001$), blastomere uniformity ($p = 0.024$), cellular compaction ($p = 0.004$), and zona pellucida condition ($p = 0.023$). In contrast, fragmentation and cellular degeneration did not differ significantly between the groups ($p > 0.05$). The total embryo quality score was significantly higher in good-quality embryos than in poor-quality embryos ($p < 0.001$).

Thus, differences between the quality groups were primarily evident in general embryo structure, blastomere uniformity, cellular compaction, zona pellucida condition, and the overall embryo quality score.

Table 2: Comparison of embryo quality parameter scores between good-quality and poor-quality Bali cattle embryos.

Parameter	Good quality embryos (mean $\pm$ SD)	Poor quality embryos (mean $\pm$ SD)	p-value
General embryo structure	2.87 $\pm$ 0.35	1.93 $\pm$ 0.70	<0.001
Blastomere uniformity	2.53 $\pm$ 0.52	2.13 $\pm$ 0.35	0.024
Fragmentation	2.53 $\pm$ 0.52	2.27 $\pm$ 0.46	0.150
Cellular compaction	2.80 $\pm$ 0.41	2.20 $\pm$ 0.56	0.004
Zona pellucida condition	2.73 $\pm$ 0.46	2.20 $\pm$ 0.68	0.023
Cellular degeneration	2.40 $\pm$ 0.51	2.07 $\pm$ 0.70	0.189
Total embryo quality score	15.87 $\pm$ 1.19	12.80 $\pm$ 0.41	<0.001

Values are presented as mean $\pm$ standard deviation (SD). Each morphological parameter was scored from 1 to 3, and the total embryo quality score was calculated by summing the six parameter scores. Between-group differences were analyzed using the Mann–Whitney U test. Statistical significance was set at $p < 0.05$.

Differential metabolites between embryo quality groups

Differential metabolite profiles between good- and poor-quality embryos are presented in Figure 1 and summarized in Table 3. A total of 38 differential metabolites were identified, of which 25 showed higher peak areas in the good quality group and 13 showed higher peak areas in the poor-quality group. The volcano plot demonstrated distinct metabolite peak area patterns between the groups, with differential metabolites distributed on both sides of the fold-change threshold.

Metabolites with higher peak areas in the good quality group included (S)-malate, L-isoleucine, nicotinamide adenine dinucleotide phosphate (NADPH), L-carnitine, D-ribose, citrate, pyruvate, L-glutamate, O-acetylcarnitine, L-leucine, nicotinamide adenine dinucleotide (NADH), 2-oxoglutarate, L-glutamine, L-valine, L-serine, D-glucose, adenosine triphosphate (ATP), glycine, reduced glutathione, cerebrosterol, linoleoyl- Coenzyme A (CoA), acetic acid, 6-phospho-D-gluconate, decanoic acid, and sapienoyl-CoA. In contrast, succinate, oxidized glutathione, L-tyrosine, uric acid, L-palmitoylcarnitine, adenosine monophosphate, (9Z,12Z,15Z)-octadecatrienoic acid, acetyl-CoA, oxaloacetate, ribose 5-phosphate, docosanoic acid, butanoic acid, and cytidine showed higher peak areas in the poor-quality group. Collectively, these findings demonstrated distinct metabolite peak area profiles between good- and poor-quality embryos.

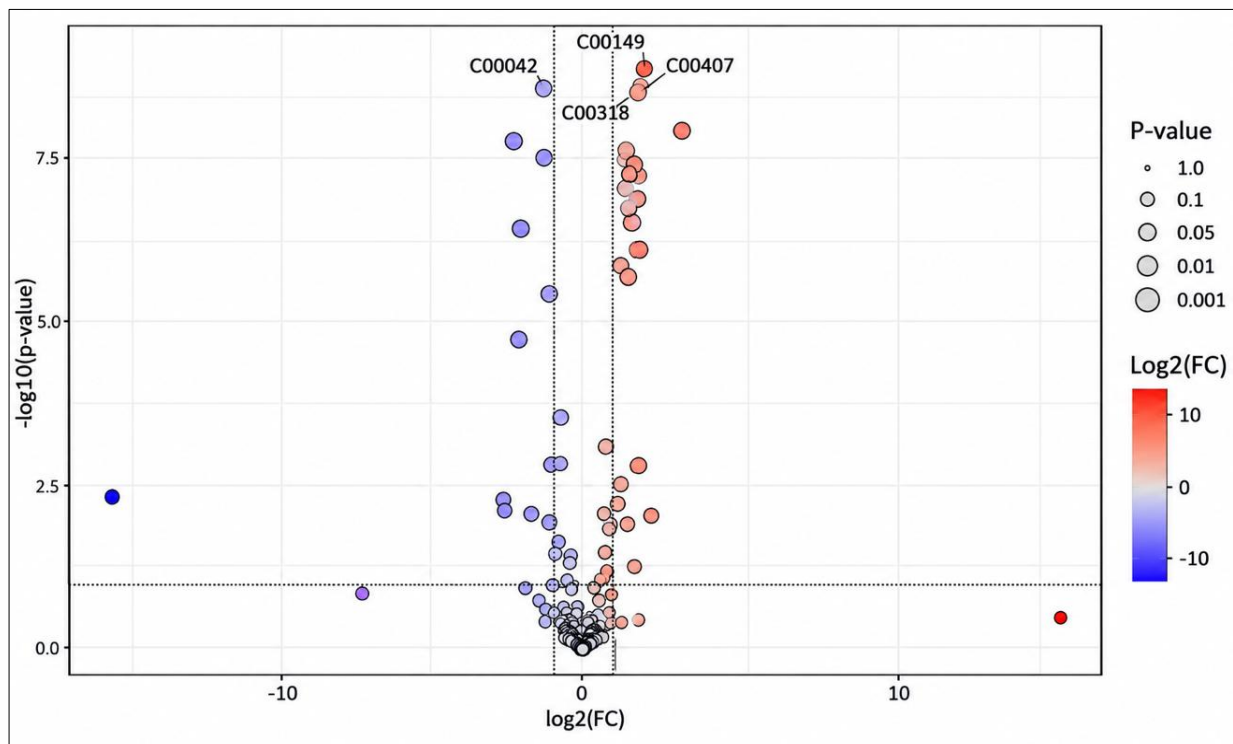


Figure 1: Volcano plot of differential metabolites between good-quality and poor-quality Bali cattle embryos. The plot shows metabolite fold changes and statistical significance, illustrating differences in metabolite abundance between the two embryo-quality groups.

Table 3: Differential metabolites between good-quality and poor-quality Bali cattle embryos.

KEGG ID	Metabolite	FC	log ₂ FC	Raw p-value	−log ₁₀ (p)	Higher group
C00149	(S)-Malate	3.6947	1.8855	5.10E−10	9.2927	Good quality
C00042	Succinate	0.3286	−1.6056	6.33E−10	9.1987	Poor quality
C00407	L-Isoleucine	3.8971	1.9624	1.05E−09	8.9806	Good quality
C00005	NADPH	3.3814	1.7576	1.50E−09	8.8247	Good quality
C00318	L-Carnitine	2.1986	1.1366	1.85E−09	8.7333	Good quality
C00121	D-Ribose	11.4230	3.5139	5.91E−09	8.2284	Good quality
C00127	Oxidized glutathione	0.2264	−2.1429	8.51E−09	8.0702	Poor quality
C00158	Citrate	2.7163	1.4417	1.04E−08	7.9824	Good quality
C00082	L-Tyrosine	0.4462	−1.1642	1.37E−08	7.8641	Poor quality
C00022	Pyruvate	2.6037	1.3806	1.61E−08	7.7935	Good quality
C00025	L-Glutamate	3.3433	1.7413	1.83E−08	7.7387	Good quality
C02571	O-Acetylcarnitine	3.7255	1.8974	2.69E−08	7.5703	Good quality
C00123	L-Leucine	3.0293	1.5990	2.72E−08	7.5649	Good quality
C00004	NADH	2.6532	1.4078	4.33E−08	7.3631	Good quality
C00026	2-Oxoglutarate	3.5518	1.8285	5.97E−08	7.2243	Good quality
C00064	L-Glutamine	3.4337	1.7798	6.26E−08	7.2033	Good quality
C00183	L-Valine	2.8573	1.5147	8.66E−08	7.0625	Good quality
C00065	L-Serine	3.1197	1.6414	1.46E−07	6.8370	Good quality
C00366	Uric acid	0.2404	−2.0566	1.68E−07	6.7743	Poor quality
C00031	D-Glucose	3.5198	1.8155	3.72E−07	6.4294	Good quality
C00002	ATP	3.9888	1.9960	3.82E−07	6.4177	Good quality
C00037	Glycine	2.3205	1.2144	6.50E−07	6.1872	Good quality
C00051	Reduced glutathione	2.7748	1.4724	8.77E−07	6.0571	Good quality
C02990	L-Palmitoylcarnitine	0.4742	−1.0765	1.86E−06	5.7303	Poor quality
C00020	AMP	0.2432	−2.0401	9.93E−06	5.0031	Poor quality
C06427	(9Z,12Z,15Z)-Octadecatrienoic acid	0.4790	−1.0619	1.40E−03	2.8508	Poor quality
C13550	Cerebrosterol	3.4549	1.7886	1.50E−03	2.8173	Good quality
C02050	Linoleoyl-CoA	2.3126	1.2095	2.80E−03	2.5500	Good quality
C00024	Acetyl-CoA	0.1704	−2.5529	4.70E−03	2.3323	Poor quality
C00036	Oxaloacetate	1.78 × 10 ^{−5}	−15.7740	4.90E−03	2.3117	Poor quality
C00033	Acetic acid	2.1805	1.1246	5.50E−03	2.2607	Good quality
C00117	Ribose 5-phosphate	0.1773	−2.4954	6.80E−03	2.1659	Poor quality
C08281	Docosanoic acid	0.3170	−1.6573	7.90E−03	2.1026	Poor quality
C00345	6-Phospho-D-gluconate	4.9360	2.3033	8.60E−03	2.0655	Good quality
C00246	Butanoic acid	0.4556	−1.1341	1.03E−02	1.9885	Poor quality
C00475	Cytidine	0.4609	−1.1174	1.10E−02	1.9570	Poor quality
C01571	Decanoic acid	2.7347	1.4514	1.15E−02	1.9403	Good quality

KEGG = Kyoto Encyclopedia of Genes and Genomes; ID = Identifier; FC = Fold change; log₂FC = Base-2 logarithm of fold change; −log₁₀(p) = Negative base-10 logarithm of the raw p-value; NADPH = Reduced nicotinamide adenine dinucleotide phosphate; NADH = Reduced nicotinamide adenine dinucleotide; ATP = Adenosine triphosphate; AMP = Adenosine monophosphate; CoA = Coenzyme A. The higher group identifies the embryo quality group with the greater reported metabolite abundance. Raw p-values are presented as supplied, without adjustment for multiple comparisons.

Multivariate analysis of embryo metabolomic profiles

Multivariate analysis of the metabolomic data is presented in Figure 2. The PCA score plot (Figure 2A) showed separation between the good and poor-quality embryo groups, indicating differences in their overall metabolomic profiles. Principal component 1 (PC1) explained 32.1% of the variation, whereas PC2 explained 11.7%.

The PLS-DA biplot (Figure 2B) further demonstrated separation between the embryo quality groups and identified metabolites contributing to this separation. (S)-Malate, L-isoleucine, NADPH, L-carnitine, and D-ribose were oriented toward the good quality group, whereas succinate, oxidized glutathione, L-tyrosine, and uric acid were oriented toward the poor-quality group. Model performance evaluation (Figure 2C), based on accuracy, R², and Q² values, showed high classification performance across the tested components, with the highest Q² value observed for the two-component model.

Important discriminant metabolites and hierarchical clustering analysis are shown in Figure 3. The VIP score plot identified the principal metabolites contributing to discrimination between the good and poor-quality groups. These included (S)-malate, succinate, L-isoleucine, NADPH, L-carnitine, D-ribose, oxidized glutathione, citrate, L-tyrosine, pyruvate, L-glutamate, L-leucine, O-acetylcarnitine, NADH, and 2-oxoglutarate. Hierarchical clustering showed distinct clustering of the good- and poor-quality embryo groups. Most energy- and amino acid-related

metabolites showed higher peak areas in the good quality group, whereas succinate, oxidized glutathione, and L-tyrosine showed higher peak areas in the poor-quality group.

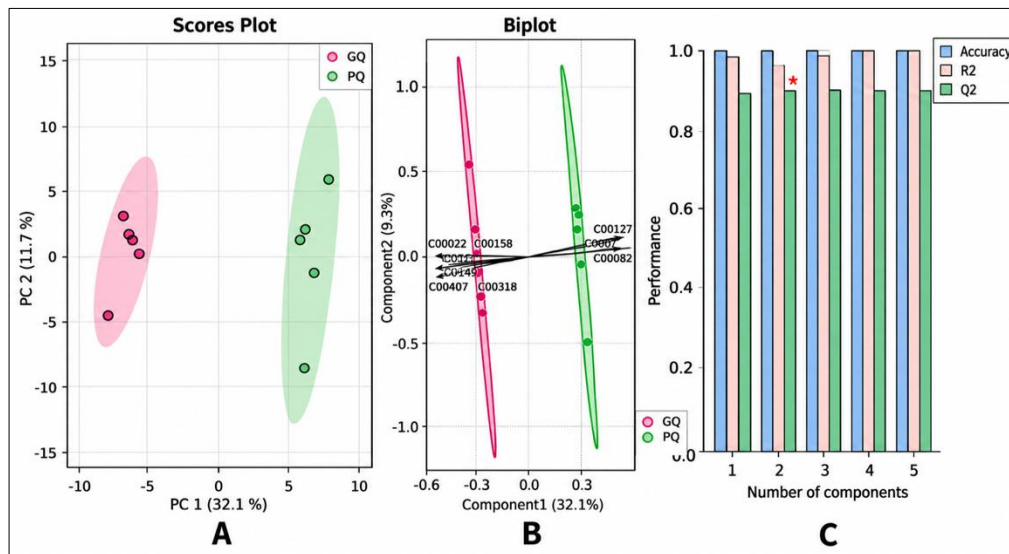


Figure 2: Multivariate analysis of metabolomic profiles in good-quality and poor-quality Bali cattle embryos. (A) Principal component analysis score plot showing the distribution of pooled metabolomic samples by embryo quality group. (B) Partial least squares–discriminant analysis biplot illustrating group separation and the contribution of individual metabolites to discrimination between groups. (C) Partial least squares–discriminant analysis model performance across the tested components, assessed using classification accuracy, R^2 , and Q^2 . GQ = Good quality embryos; PQ = Poor quality embryos; R^2 = Coefficient of determination; Q^2 = Cross-validated predictive ability.

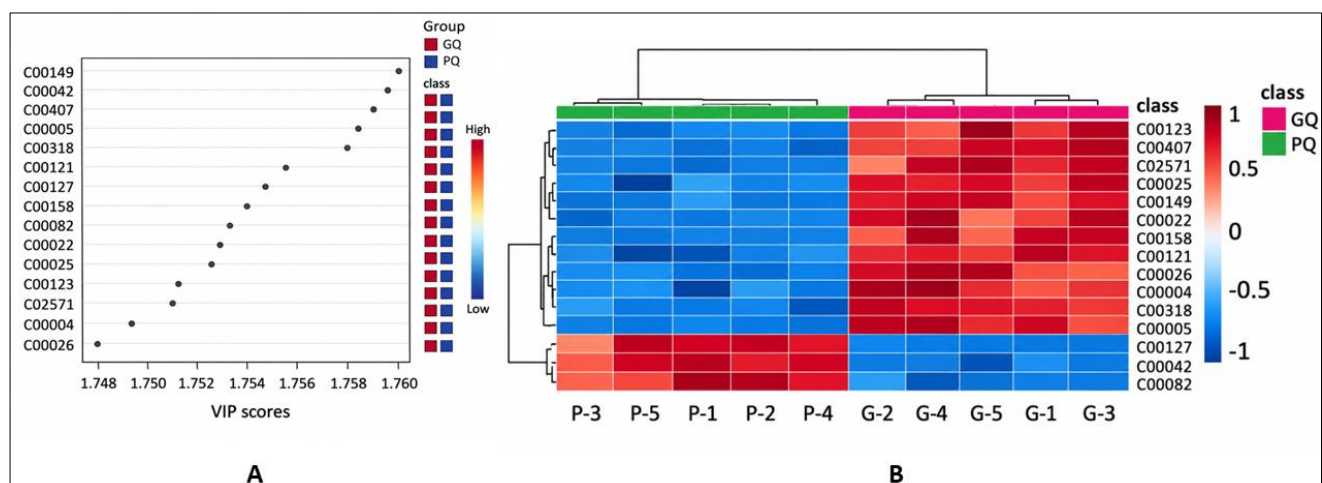


Figure 3: Important discriminant metabolites and hierarchical clustering of metabolomic profiles in good-quality and poor-quality Bali cattle embryos. (A) VIP scores of the top discriminant metabolites derived from the partial least squares–discriminant analysis model, indicating their relative contributions to group discrimination. (B) Hierarchical clustering heatmap showing the relative abundance patterns of important discriminant metabolites across pooled embryo samples. VIP = Variable importance in projection; PLS-DA = Partial least squares–discriminant analysis; GQ = Good quality embryos; PQ = Poor quality embryos.

Functional analysis of differential metabolites

Functional analysis of the differential metabolites is presented in Figure 4 and summarized in Table 4. Nine pathways had non-zero pathway impact values and were included in the functional interpretation. The most significant pathways included the citrate cycle (TCA cycle); alanine, aspartate, and glutamate metabolism; glyoxylate and dicarboxylate metabolism; and glutathione metabolism. Among these, the citrate cycle showed the strongest enrichment significance, with five matched metabolites: 2-oxoglutarate, succinate, (S)-malate, citrate, and pyruvate. Alanine, aspartate, and glutamate metabolism also included five matched metabolites: L-glutamate, citrate, pyruvate, succinate, and 2-oxoglutarate.

Based on pathway impact, phenylalanine, tyrosine, and tryptophan biosynthesis had the highest impact value, followed by the citrate cycle; alanine, aspartate, and glutamate metabolism; pyruvate metabolism; and

tyrosine metabolism. Glutathione metabolism was represented by NADPH, oxidized glutathione, and L-glutamate. Overall, the differential metabolites were predominantly mapped to pathways related to energy metabolism, amino acid metabolism, carbon metabolism, and redox regulation.

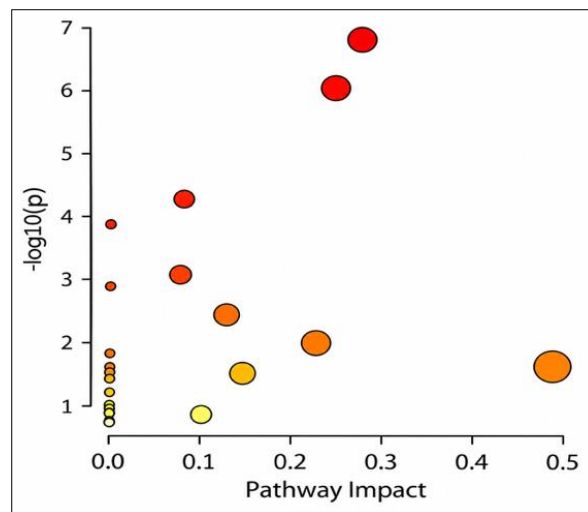


Figure 4: Functional pathway analysis of differential metabolites between good-quality and poor-quality Bali cattle embryos. The analysis illustrates the metabolic pathways associated with the identified differential metabolites.

Table 4: Functional analysis of differential metabolites between good-quality and poor-quality Bali cattle embryos.

Pathway	Total	Expected	Hits	Raw p	$-\log_{10}(p)$	Holm adjust	FDR	Impact	KEGG ID
Citrate cycle (TCA cycle)	20	0.20	5	1.06E-07	6.80	2.06E-05	2.06E-05	0.27	C00026; C00042; C00149; C00158; C00022
Alanine, aspartate and glutamate metabolism	28	0.22	5	9.66E-07	6.02	7.63E-05	3.86E-05	0.25	C00025; C00158; C00022; C00042; C00026
Glyoxylate and dicarboxylate metabolism	32	0.25	4	6.63E-05	4.18	5.17E-03	1.77E-03	0.08	C00149; C00025; C00022; C00158
Glutathione metabolism	28	0.22	3	1.04E-03	2.98	7.94E-02	1.67E-02	0.07	C00005; C00127; C00025
Arginine biosynthesis	14	0.11	2	4.76E-03	2.32	3.62E-01	5.44E-02	0.12	C00025; C00026
Pyruvate metabolism	23	0.18	2	1.27E-02	1.90	9.29E-01	1.27E-01	0.22	C00022; C00149
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.03	1	3.07E-02	1.51	1.00E+00	2.28E-01	0.50	C00082
Tyrosine metabolism	42	0.33	2	3.90E-02	1.40	1.00E+00	2.48E-01	0.14	C00022; C00082
Glycolysis or gluconeogenesis	26	0.20	1	1.84E-01	0.73	1.00E+00	7.38E-01	0.10	C00022

TCA = Tricarboxylic acid; FDR = False discovery rate; KEGG = Kyoto Encyclopedia of Genes and Genomes; ID = Identifier; $-\log_{10}(p)$ = negative base-10 logarithm of the raw p-value. Total = total metabolites associated with the corresponding pathway in the pathway reference library; Expected = expected number of pathway hits; Hits = number of identified differential metabolites mapped to the pathway; Impact = pathway topology impact value reported by MetaboAnalyst 6.0. Numerical values have been retained as supplied, with decimal commas standardized to decimal points.

Candidate metabolite biomarkers for embryo quality

Candidate metabolite biomarkers associated with embryo quality are presented in Figure 5 and summarized in Table 5. Candidate metabolites were prioritized using an integrated multi-criteria framework comprising significant differential patterns between embryo quality groups, VIP scores >1 in the PLS-DA model, involvement in KEGG-based functional pathways, AUC values >0.8 in ROC analysis, and significant associations with morphological embryo quality parameters.

The integrated analysis identified a nine-metabolite candidate panel comprising NADPH, pyruvate, L-glutamate, 2-oxoglutarate, succinate, L-tyrosine, oxidized glutathione, (S)-malate, and citrate. Six metabolites, NADPH, pyruvate, L-glutamate, 2-oxoglutarate, (S)-malate, and citrate, showed higher normalized peak areas in good quality embryos, whereas succinate, L-tyrosine, and oxidized glutathione showed higher normalized peak areas in poor-quality embryos. These metabolites represented coordinated differences involving energy metabolism, amino acid metabolism, and redox regulation.

All nine candidate metabolites had AUC values of 1.00 in the present dataset. The ROC analysis was based on five pooled analytical samples per group, and the reported AUC values therefore represent apparent within-dataset discriminatory performance.

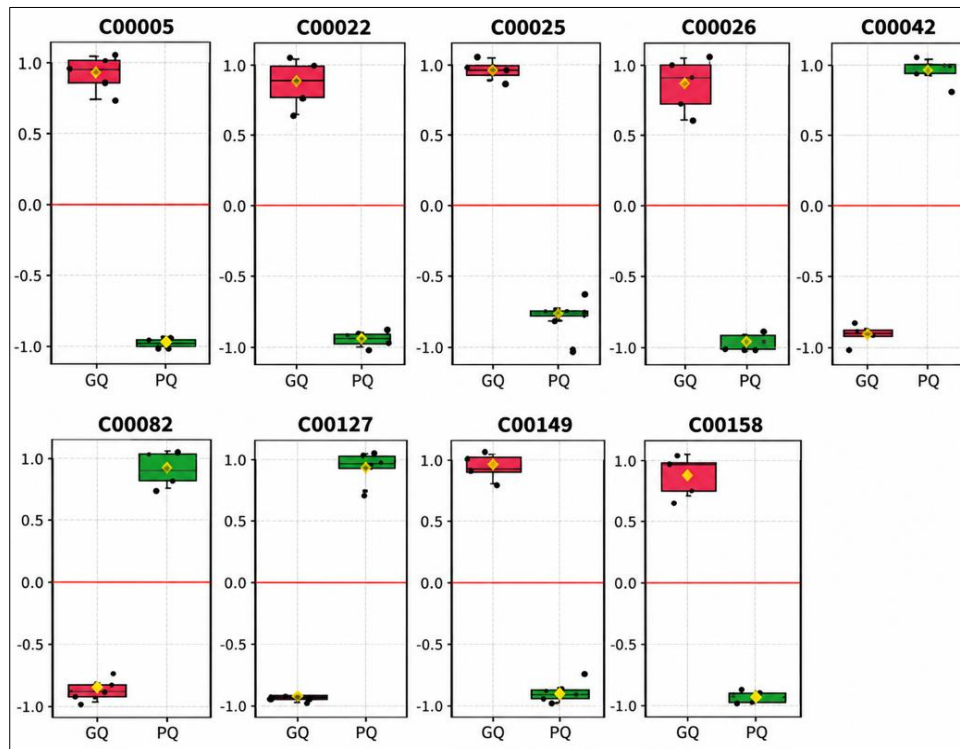


Figure 5: Distribution of candidate metabolite biomarkers between good-quality and poor-quality Bali cattle embryos. The figure illustrates differences in the distribution of candidate biomarkers between the two embryo quality groups.

Table 5: Candidate metabolite biomarkers associated with embryo quality.

KEGG ID	Metabolite	Direction	VIP	Pathway	AUC
C00149	(S)-malate	GQ	1.76107	Citrate cycle; Glyoxylate and dicarboxylate metabolism; Pyruvate metabolism	1
C00042	Succinate	PQ	1.76073	Citrate cycle; Alanine, aspartate and glutamate metabolism	1
C00005	NADPH	GQ	1.75926	Glutathione metabolism	1
C00127	Oxidized glutathione	PQ	1.75520	Glutathione metabolism	1
C00158	Citrate	GQ	1.75442	Citrate cycle; Alanine, aspartate and glutamate metabolism; Glyoxylate and dicarboxylate metabolism	1
C00082	L-tyrosine	PQ	1.75355	Phenylalanine, tyrosine and tryptophan biosynthesis; Tyrosine metabolism	1
C00022	Pyruvate	GQ	1.75295	Citrate cycle; Alanine, aspartate and glutamate metabolism; Glyoxylate and dicarboxylate metabolism; Pyruvate metabolism; Tyrosine metabolism; Glycolysis or gluconeogenesis	1
C00025	L-glutamate	GQ	1.75272	Alanine, aspartate and glutamate metabolism; Glyoxylate and dicarboxylate metabolism; Glutathione metabolism; Arginine biosynthesis	1
C00026	2-oxoglutarate	GQ	1.74762	Citrate cycle; Alanine, aspartate and glutamate metabolism; Arginine biosynthesis	1

KEGG = Kyoto Encyclopedia of Genes and Genomes; ID = Identifier; GQ = Good quality embryos; PQ = Poor quality embryos; VIP = Variable importance in projection; AUC = Area under the receiver operating characteristic curve. Direction indicates the embryo quality group associated with the candidate metabolite. AUC values are reproduced as reported.

Correlation analysis between candidate metabolites and embryo quality scores

Correlations between candidate metabolite biomarkers and embryo quality parameters are presented in Figure 6. Candidate metabolites with higher peak areas in the good-quality group, including (S)-malate (C00149), NADPH (C00005), citrate (C00158), pyruvate (C00022), L-glutamate (C00025), and 2-oxoglutarate (C00026), generally showed positive correlations with morphological embryo quality parameters. In contrast, candidate metabolites with higher peak areas in the poor-quality group, including succinate (C00042), oxidized glutathione (C00127), and L-tyrosine (C00082), showed negative correlations with these parameters.

General embryo structure and cellular compaction showed the most consistent correlations with the candidate metabolite panel. General embryo structure was significantly correlated with all candidate metabolites ($p < 0.01$). Positive correlations were observed for (S)-malate (C00149), NADPH (C00005), citrate (C00158), pyruvate (C00022), L-glutamate (C00025), and 2-oxoglutarate (C00026), whereas negative correlations were observed for succinate (C00042), oxidized glutathione (C00127), and L-tyrosine (C00082). Cellular compaction was

also significantly correlated with all candidate metabolites ($p < 0.01$), with positive correlations for metabolites associated with good quality embryos and negative correlations for those associated with poor-quality embryos.

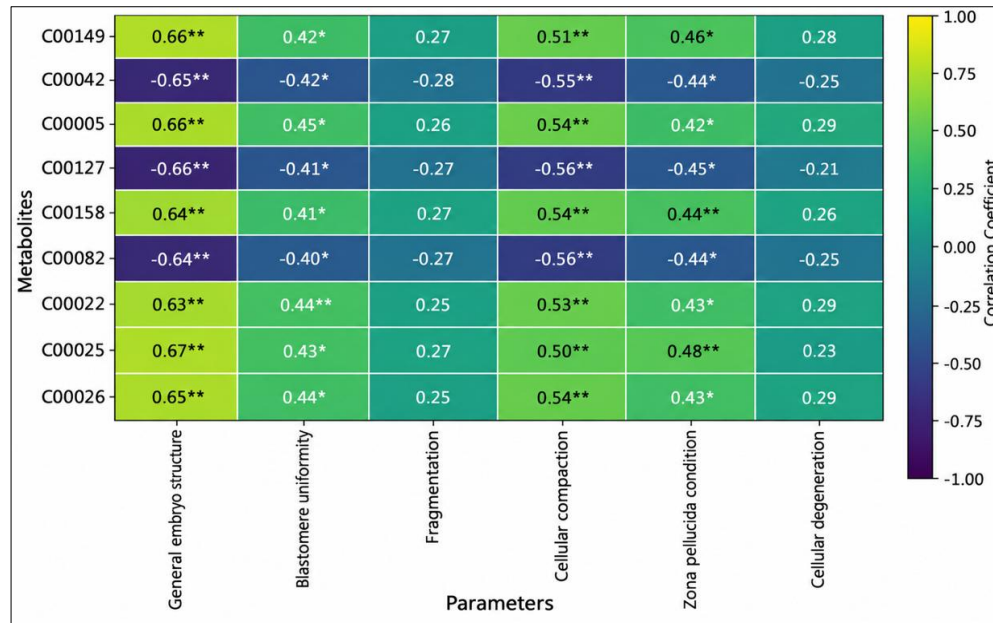


Figure 6: Correlation heatmap between candidate metabolite biomarkers and embryo quality parameters. The heatmap illustrates the direction and strength of correlations between candidate metabolite biomarkers and morphological embryo quality parameters.

Blastomere uniformity was significantly correlated with all candidate metabolites ($p < 0.05$), with (S)-malate (C00149) showing a stronger level of significance ($p < 0.01$). The zona pellucida condition was significantly correlated with all candidate metabolites; L-glutamate (C00025) showed significance at $p < 0.01$, whereas the remaining candidate metabolites showed significance at $p < 0.05$. In contrast, fragmentation and cellular degeneration were not significantly correlated with the candidate metabolites ($p > 0.05$).

Overall, the correlation analysis extended the comparison between good- and poor-quality groups by demonstrating associations between members of the candidate metabolite panel and specific morphological characteristics, particularly general embryo structure and cellular compaction. The good quality-associated and poor-quality-associated metabolites showed opposite directions of correlation with these morphological parameters.

DISCUSSION

Morphological characteristics associated with embryo quality

Embryo quality evaluation in this study was based on a visual scoring approach adapted from standard principles used for bovine embryo grading. Routine embryo assessment relies on observable morphological characteristics, including general embryo structure, blastomere uniformity, fragmentation, cellular compaction, zona pellucida condition, and cellular degeneration. Although visual assessment has an inherent subjective component, it remains widely used because it is practical, rapid, and directly applicable to embryo selection. Moreover, these morphological characteristics are biologically relevant because they reflect cellular organization, synchrony of blastomere division, structural integrity, and developmental coordination during early embryogenesis [15, 16].

In the present study, good quality embryos had higher scores than poor-quality embryos for general embryo structure, blastomere uniformity, cellular compaction, zona pellucida condition, and total embryo quality score (Table 2). Embryos with better structural organization, greater blastomere uniformity, and stronger compaction may exhibit more coordinated cleavage and cellular organization. Compaction is particularly important because it marks the transition from loosely associated blastomeres to a more integrated embryonic structure and involves cell adhesion, cytoskeletal organization, membrane remodeling, and cellular energy requirements [17, 18]. Therefore, the observed morphological differences may reflect variation in developmental coordination rather than differences in external appearance alone.

Nevertheless, morphological scoring cannot fully characterize the physiological state of an embryo. Embryos

with similar visual characteristics may differ in mitochondrial function, redox balance, amino acid utilization, lipid metabolism, and biosynthetic processes. This limitation supports the use of metabolomics as a complementary approach because metabolomic profiling provides information on small molecules reflecting the biochemical state of biological systems. Early embryonic development is highly dependent on energy production, substrate utilization, and redox homeostasis; therefore, metabolomic profiling can provide additional insight into the biological characteristics associated with embryo quality [7, 19].

Metabolic characteristics associated with embryo quality

The metabolomic analysis demonstrated differences between good and poor-quality embryos. Good quality embryos showed higher peak areas of pyruvate, citrate, (S)-malate, 2-oxoglutarate, NADPH, and L-glutamate, whereas poor-quality embryos showed higher peak areas of succinate, oxidized glutathione, and L-tyrosine. These metabolites were primarily associated with the citrate cycle, pyruvate metabolism, amino acid metabolism, and glutathione metabolism (Figures 2–4; Tables 3–4). Rather than representing isolated changes in individual metabolites, these patterns indicate coordinated differences involving mitochondrial carbon metabolism and intracellular redox balance [20].

Energy metabolism was prominently associated with embryo quality. Pyruvate is an important carbon substrate for mitochondrial ATP production during early embryonic development, whereas citrate, (S)-malate, and 2-oxoglutarate link the citrate cycle to biosynthetic and redox-related pathways. Their higher peak areas in good quality embryos may reflect greater availability or retention of intermediates associated with coordinated mitochondrial metabolism, consistent with the energy requirements of cleavage, membrane remodeling, cytoskeletal reorganization, and compaction [21, 22]. However, because the present study measured relative metabolite peak areas rather than metabolic flux, these differences cannot be interpreted as direct evidence of increased citrate cycle activity.

In contrast, succinate showed a higher peak area in poor-quality embryos. Although succinate is a normal citrate cycle intermediate, its accumulation may occur in association with alterations in mitochondrial electron transport and redox regulation. Thus, the combination of higher succinate and oxidized glutathione in poor-quality embryos may be consistent with altered mitochondrial and redox states. This interpretation is further supported by the contrasting pattern of NADPH, which showed a higher peak area in good quality embryos.

Redox regulation and embryo quality

Redox regulation may contribute to the metabolic differences observed between embryo quality groups. NADPH showed a higher peak area in good quality embryos, whereas oxidized glutathione was higher in poor-quality embryos. NADPH provides cellular reducing power and supports antioxidant systems, including glutathione recycling, whereas an increase in oxidized glutathione may be associated with a shift toward a more oxidized intracellular environment. *In vitro*-produced bovine embryos are particularly sensitive to oxidative disturbances, and experimental heat stress has been reported to increase reactive oxygen species and impair bovine embryo development [23]. Thus, the contrasting patterns of NADPH and oxidized glutathione observed in the present study may reflect differences in redox homeostasis between embryo quality groups.

This aspect may also be relevant to future investigations of embryo production in cattle adapted to tropical environments. However, embryos in the present study were cultured under controlled laboratory conditions and were not experimentally exposed to heat stress. Consequently, the observed metabolic differences cannot be directly attributed to tropical heat exposure. Instead, these findings provide a basis for investigating whether redox-related metabolic responses of Bali cattle embryos differ under controlled thermal or oxidative challenges.

Amino acid metabolism and embryo quality

Amino acid metabolism also contributed to the metabolic differences between embryo quality groups. L-glutamate showed a higher peak area in good quality embryos and may link amino acid metabolism with 2-oxoglutarate-associated mitochondrial carbon metabolism and glutathione-related antioxidant pathways. Conversely, the higher L-tyrosine peak area in poor-quality embryos may reflect differences in amino acid utilization or turnover rather than enhanced biosynthesis [24]. Together with the energy- and redox-related metabolites, these findings indicate coordinated differences across several interconnected metabolic processes associated with morphological embryo quality.

The present findings are broadly consistent with previous bovine embryo metabolomic studies showing associations between developmental potential, substrate utilization, and metabolite profiles. Individually cultured

bovine embryos with different developmental rates have shown differences in pyruvate and amino acid utilization, whereas other studies have reported associations among embryo culture conditions, developmental stage, implantation potential, and metabolomic profiles. A LC–HRMS -based study also reported greater citric acid consumption by embryos associated with successful pregnancy, supporting a relationship between citrate cycle-related metabolism and developmental competence [25].

Nevertheless, direct breed-specific comparisons require caution. Most previous bovine studies have analyzed spent culture medium, frequently from individually cultured embryos, and have differed in analytical platforms, metabolite panels, culture systems, and developmental endpoints. Therefore, the metabolic profile identified in the present study cannot yet be considered specific to Bali cattle. The contribution of this study lies in integrating multi-parameter morphological scoring with untargeted intracellular embryo metabolomics in this indigenous cattle population. Comparative studies conducted under standardized culture conditions and analytical platforms across Bali cattle and *Bos taurus* breeds are required to determine whether any of the observed metabolic characteristics are breed-associated.

Candidate metabolite panel and morphological associations

The candidate biomarker selection strategy integrated significant differential patterns, VIP scores >1, involvement in KEGG-based functional pathways, AUC values >0.8, and significant associations with embryo quality parameters (Figure 6; Table 5). This multi-criteria approach reduced reliance on a single analytical criterion and prioritized metabolites demonstrating both discriminatory and biological relevance. Nevertheless, the resulting panel remains exploratory because biomarker performance was evaluated using a small number of pooled analytical samples [26].

The nine prioritized metabolites, pyruvate, citrate, (S)-malate, 2-oxoglutarate, NADPH, L-glutamate, succinate, oxidized glutathione, and L-tyrosine, constituted a candidate metabolic panel associated with morphological embryo quality. Metabolites showing higher peak areas in good quality embryos generally correlated positively with embryo structure and cellular compaction, whereas metabolites showing higher peak areas in poor-quality embryos exhibited the opposite pattern. These associations demonstrate consistency between morphological and metabolic characteristics but do not establish causal relationships (Figure 6). Furthermore, because each metabolomic observation represented a pool of three embryos, these correlations represent pool-level rather than individual embryo associations [27].

Interpretation of discriminatory performance

All nine candidate metabolites had AUC values of 1.00 in the present dataset. However, with only five pooled analytical samples per group, ROC estimates are inherently unstable and may be susceptible to overfitting and optimistic estimates of discriminatory performance. Although cross-validation metrics were used to evaluate the PLS-DA model, they do not constitute independent validation of the ROC performance of individual metabolites. Therefore, the observed AUC values represent apparent discriminatory performance within the present discovery dataset rather than validated predictive accuracy. Larger independent cohorts, preferably comprising individual embryos, are required to obtain robust estimates of AUC, confidence intervals, sensitivity, and specificity.

Strengths and limitations

A principal strength of this study was the integration of a six-parameter morphological scoring framework with untargeted intracellular metabolomic profiling. This approach enabled morphological characteristics to be examined alongside metabolic differences involving energy metabolism, amino acid metabolism, and redox regulation. The use of multiple criteria to prioritize candidate metabolites further provided an integrated framework for identifying metabolite patterns associated with embryo quality.

Several limitations should be considered. Metabolomic analysis was based on only 10 pooled analytical samples, comprising five pools per quality group and three embryos per pool. Although pooling provided sufficient material for analysis, it reduced information on inter-embryo variability and limited the effective number of independent biological units. The small number of analytical units may also reduce statistical power and increase the risk of overfitting, particularly in supervised PLS-DA and ROC analyses. Accordingly, the observed discriminatory performance should be considered exploratory rather than evidence of validated predictive performance. In addition, morphological assessment was conducted by a single observer without formal inter- or intra-observer reliability estimates, limiting assessment of scoring reproducibility.

Future perspectives and practical relevance

Future studies should employ individual embryo metabolomic analysis, preferably incorporating targeted

quantification of the nine prioritized metabolites in larger independent validation cohorts. Morphological evaluation should incorporate formal inter- or intra-observer reliability assessment, and metabolomic profiles should be prospectively linked to developmental competence and embryo transfer outcomes. Such studies are necessary to determine whether the candidate metabolite panel has reproducible predictive value beyond the pooled discovery dataset [8, 28].

From a broader perspective, these findings may contribute to the development of improved embryo evaluation strategies for Bali cattle. As an important indigenous genetic resource, Bali cattle may benefit from *in vitro* embryo production approaches that support genetic improvement and conservation by increasing the availability of embryos from locally adapted animals [29]. Integrating morphological assessment with metabolomic information may ultimately provide a more comprehensive framework for characterizing embryo quality than morphological scoring alone [30, 31]. At present, however, metabolomic profiling should be regarded as a research approach for understanding the biological basis of embryo quality rather than as a routine embryo selection method.

Overall, the candidate metabolites identified in this study demonstrate that morphological embryo quality in Bali cattle is associated with coordinated differences in energy metabolism, amino acid metabolism, and redox regulation. These findings support the concept that morphological appearance represents one dimension of embryo quality and that complementary metabolic information can provide additional insight into the underlying biological state [32]. The present study therefore provides an initial framework for investigating metabolite-assisted embryo quality assessment in Bali cattle, while emphasizing the need for individual embryo and developmental validation before practical application.

CONCLUSION

The integration of morphological embryo quality scoring with LC–HRMS-based untargeted metabolomic profiling demonstrated distinct differences between good and poor-quality Bali cattle embryos. Good quality embryos showed higher scores for general embryo structure, blastomere uniformity, cellular compaction, zona pellucida condition, and total embryo quality. Metabolomic analysis identified nine candidate metabolites associated with embryo quality: pyruvate, citrate, (S)-malate, 2-oxoglutarate, NADPH, L-glutamate, succinate, oxidized glutathione, and L-tyrosine. Pyruvate, citrate, (S)-malate, 2-oxoglutarate, NADPH, and L-glutamate showed higher peak areas in good quality embryos, whereas succinate, oxidized glutathione, and L-tyrosine showed higher peak areas in poor-quality embryos. These metabolites were primarily associated with energy metabolism, amino acid metabolism, and redox regulation.

A major strength of this study was the integration of a six-parameter morphological scoring framework with untargeted metabolomic profiling. The use of multiple complementary criteria, including differential metabolite patterns, VIP scores, KEGG-based pathway involvement, AUC values, and correlations with embryo quality parameters, enabled systematic prioritization of candidate metabolites associated with morphological embryo quality.

This study provides an integrated morphological and metabolomic framework for characterizing Bali cattle embryo quality. The coordinated differences observed in energy metabolism, amino acid metabolism, and redox regulation support the value of combining morphological assessment with metabolic profiling to obtain a broader representation of embryo biological status. The nine-metabolite panel represents a set of candidate biomarkers that requires individual embryo and independent validation before any practical application in embryo selection or reproductive biotechnology.

DATA AVAILABILITY

The raw LC–HRMS data and processed metabolite peak area matrix generated and analyzed during this study will be deposited in a public metabolomics repository, such as MetaboLights or Metabolomics Workbench. The repository name and accession number will be provided once available. Additional supporting data are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

The authors declare that generative artificial intelligence (AI) tools were used solely to improve language, grammar, and readability during manuscript preparation. All scientific content, data analysis, interpretation of results, and conclusions were developed and verified by the authors. The authors take full responsibility for the

accuracy, integrity, and originality of the work presented, and no AI tool was listed as an author.

AUTHORS' CONTRIBUTIONS

FH, DTF, RR, AMD, MFA, and SS: Conceptualization, study design, and coordination. FH, DTF, and RR: Investigation and overall execution of the study. AMD, MFA, SS, and ANS: Development of data collection tools. RR, DTF, AMD, MFA, and ANS: Supervision of sample collection, data collection, laboratory analyses, and data entry. FH, DTF, AMD, RR, MFA, and ANS: Statistical analysis, data interpretation, and writing of the original manuscript. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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