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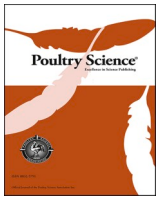

Effects of on-farm hatching versus hatchery hatching on growth performance, gut development, and intestinal health and function in broiler chickens

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ABSTRACT

An alternative hatching system known as hatch on-farm (HOF) provides early access to feed compared to hatch in hatchery (HH) system. Early feeding may promote favorable gut development, potentially improving intestinal health and broiler performance. Previous studies have assessed the effects of HOF on chick quality, welfare and performance, its impacts on gut health remain inconclusive. A total of 560 Ross 308 male chicks were reared until d 38, hatched either in a hatchery (n = 280) or on-farm (n = 280), with 14 replicates per system and 20 birds per pen. Production parameters were periodically monitored. Digestive and immune organ characteristics, intestinal permeability and histomorphology were assessed on d 7, 14, and 38. High-throughput qPCR analyzed 79 ileal genes regarding barrier integrity, immune function, nutrient transporters, gut hormones, metabolism, and oxidation. HOF chicks had higher d1 body weights than HH chicks ($P < 001$), but this advantage disappeared within first week, with no subsequent performance differences. HOF chickens demonstrated increased duodenal villus width on d 7 and 14, and increased ileal crypt depth and submucosal thickness on d 7 ($P < 0.05$). Relative bursal weight was higher on d 14 ($P = 0.018$) and tended to be higher on d 38 in HOF chickens ($P = 0.094$). Intestinal permeability remained unaffected ($P > 0.05$), while HH chicks showed upregulation of gut barrier genes such as *MUC5ac* on d 7 and *CLDN2* and *MUC2* on d 14 ($P < 0.05$). HH chicks also showed upregulation of nutrient transports including *VDR* on d 7 and *SLC30A1* and *SLC5A9* on d 38, and decreased expression of the appetite-suppressing hormone *CCK* on d 7 ($P < 0.05$). HOF chicks upregulated immune-related genes, including *IL-8* on d 7, *IL-6, IFN-γ, AVBD9* on d 14, and *NOS2* on d 38 ($P < 0.05$), and the oxidation gene *HIF1A* on d 38 ($P = 0.039$). In conclusion, although the HOF showed only transient growth advantages, it enhanced mucosal morphology and modulated immunity, indicating improved intestinal health.

Introduction

Optimal early-life conditions have become critical for maintaining efficient and healthy broiler production (Hollemans et al., 2018). A significant challenge in modern broiler production is the delayed access to feed and water for hatchlings due to hatchery procedures and transport logistics, often reaching up to 48-72 h post-hatch (Dieryck et al., 2022). This extended fasting period can significantly impact growth trajectories and may compromise bird health and performance. A delay of 36 to 48 h in access to feed has been associated with increased mortality, impaired growth, and an unfavorable feed-to-gain ratio (Careghi et al., 2005; Shira et al., 2005; de Jong et al., 2017). It also negatively affects gastrointestinal development, delaying the structural and functional maturation of the intestine, reducing nutrient absorption, and impairing gut health by disrupting gut barrier function and immune system development (Panda et al., 2015; Liu et al., 2020; Proszkowiec-Weglarz et al., 2020).

Recent advancements in hatching practices aim to mitigate these challenges by addressing delayed feeding and transportation. Two notable approaches currently implemented in commercial poultry

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production are hatchery feeding and hatch on-farm (HOF) systems. Hatchery feeding involves hatching chicks in the hatchery with immediate access to feed, although it does not eliminate the need for transportation to broiler farms (Giersberg et al., 2021). In contrast, the HOF system allows 18-day incubated eggs to be transported directly to the barn, facilitating hatching on the farm with immediate access to feed and water (Dieryck et al., 2022; Molenaar et al., 2023). The HOF system has demonstrated several welfare and performance benefits, including a reduced incidence of footpad dermatitis and better litter quality (de Jong et al., 2020). It has also been associated with transient advantages in body weight and intestinal development until 21 days of age, with compensatory growth observed in hatchery-hatched (HH) chicks, allowing them to attain similar weights at slaughter age (de Jong et al., 2019, 2020). Importantly, chickens from young breeder flocks appear to benefit more from the HOF system due to their smaller size and higher sensitivity to suboptimal conditions (de Jong et al., 2019; Souza da Silva et al., 2021; Molenaar et al., 2023). Nonetheless, the implementation of the HOF system requires careful consideration of potential limitations, including logistical complexities, costs, and the need for specialized equipment and training.

Despite extensive research on the effects of HOF systems on broiler welfare and performance (de Jong et al., 2019, 2020; Giersberg et al., 2021; Souza da Silva et al., 2021), there is a significant gap in understanding their comprehensive impact on gut health and related physiological processes. Early feeding is crucial for the development of the immune system and increased disease resistance in comparison to delayed-fed chickens (Mulder and Rashidi, 2017). The timing of the first feed intake significantly impacts gut microbiota colonization (Simon et al., 2014), which directly influences the enteric immune system (Kogut et al., 2020). Differences in the intestinal microbiota between HOF and HH chickens have been reported (Akram et al., 2024b), which may cause differential immune responses in birds with early compared to delayed feeding. Immediate post-hatch feeding accelerates the development of immune organs (Madej et al., 2024) and can prevent reduced bursa weight, poor vaccination responses, and decreased disease resistance associated with delayed feeding (Dibner et al., 1998; Shira et al., 2005). Feeding stimulates the digestive system, including the stomach, liver, pancreas, and small intestine to secrete compounds that support the growth of the intestinal mucosa (Rao and Wang, 2010). Early luminal stimulation by feed may positively affect intestinal morphology and nutrient uptake. Luminal nutrients also stimulate structural and functional regulations in the intestine through a process involving different gut hormones (Nelson et al., 2008). These hormonal responses are crucial for appetite regulation, metabolic efficiency, and overall growth performance. Moreover, the timing of feed intake impacts the integrity of tight junctions in the intestinal epithelium, which is essential for maintaining gut barrier function and preventing pathogen invasion (Proszkowiec-Weglarz et al., 2020). On the other hand, delayed feeding in chickens has been shown to impair intestinal structure, reduce nutrient absorption and compromise gut integrity (Richards et al., 2010; Lamot et al., 2014; Proszkowiec-Weglarz et al., 2019, 2020).

The aim of the current study was to investigate the impact of HOF compared to the HH system on growth performance and gut health-related parameters in broiler chickens. This includes measurements of growth performance, digestive and immune organ characteristics, intestinal permeability and morphology, and gene expression patterns associated with various intestinal functions, including gut integrity, immune function, nutrient transport and receptors, gut hormones, metabolism, and oxidative processes across various growth stages. It was hypothesized that on-farm hatching, which eliminates transportation stress and enables early access to feed, would result in better growth performance and improved intestinal development and health in HOF chickens compared to conventionally hatched chickens, up to slaughter age.

Materials and methods

The experiment was carried out after approval of the Ethical Committee for Animal Experimentation of KU Leuven (project number P045/2022).

Animals and housing

For this study, Ross 308 male broiler chicks were sourced from the same 40-week-old breeder eggs and were hatched either in a commercial hatchery or on-farm. For the HOF system, 18th day incubated eggs were transported, after candling, to the broiler house, carefully placed on litter material, and provided with feed, with optimal hatching conditions being maintained. In brief, the ambient temperature of the broiler house was regulated to maintain the eggshell temperature within the range of 36.1°C and 37.2°C with relative humidity of 40-50 %. Chicks began hatching on embryonic d 19, two days earlier than the commercial age. As hatching progressed, the temperature regulation was shifted to maintain the chick's body temperature between 39.5°C to 40.5°C. Male chicks hatched slightly later than females, as a larger proportion of female chicks emerged earlier during the hatching process. Continuous light was provided to ensure prompt access to feed and water to chicks after hatch. HOF birds were manually picked up, graded, and sexed. Deformed HOF chicks were promptly culled by decapitation. The shells of the hatched eggs were shredded into the pens. In contrast, HH chicks were incubated at a commercial hatchery (Belgabroed NV, Merksplas, Belgium), with a hatch window of 24-36 h. After hatching, chicks were subjected to standard hatchery procedures including grading, sexing, and vaccination before they were transported to the broiler farm. The broiler farm was 108 km from the hatchery, and transportation took around 2-3 h, which led to it taking more than 40 h before most of the chicks received feed and water.

Following standard commercial practices, the day on which the HH chicks arrived at the broiler farm was designated as day 1 for both hatching system (HS). From that day, standard broiler house settings were implemented, with the ambient temperature gradually reduced from 33.5°C to 21.5°C by d 21, and then maintained at 21.5°C until d 38. On d 1, one hour of darkness was provided, which increased to six hours from d 7 onward. All chicks received vaccinations against Newcastle disease virus on d 1 and 16, and for Gumboro on d 16. All birds received feed and water *ad-libitum*, with three-phase commercial diets (starter diet: d 1 to 14, grower diet: d 15 to 28, and finisher diet: d 29 to 38). Specific details of the ingredients and chemical composition of the diets fed to the birds are given in Table S1.

Experimental design

A total of 560-day-old Ross 308 male chicks were used, 280 from each of the two HS. Birds were housed in 28 pens (1.3 m^2/pen and 14 replicate pens per HS) with 20 chicks each. By d 38, the study concluded with 14 to 15 birds per pen, corresponding to a stocking density of 33 kg/m^2.

Growth performance measurements and sampling

Individual animals were weighed on d 1, 7, 14, 28, and 38. Performance parameters including average daily gain (ADG), average daily feed intake (ADFI), and mortality-corrected feed conversion ratio (FCR) were calculated per pen for each diet phase. The coefficient of variation (CV, %) in body weight of both HS was also calculated on d 7, 14, 28, and 38. Twenty birds per HS were randomly selected (1-2 broilers per pen) for sample collection on d 7, 14, and 38, and euthanized by a trained person using electronarcosis followed by decapitation. For histomorphological examination, duodenum and ileum sections (ileum: small intestine starts after Meckel's diverticulum) from the midpoint were obtained. Additionally, ileal tissues were snap-frozen and stored at

−80°C for high-throughput qPCR gene expression analysis.

Relative weights of digestive and immune organs

Digestive viscera (heart, liver, pancreas, stomach – both proventriculus and ventriculus – and small intestine), as well as immune organs (spleen and bursa), were carefully removed ($n = 20$/group), and their weights were recorded on a scale with a precision of ± 0.01 g. The small intestine weight was recorded without removing the digesta. Afterward, their relative weights as grams per 100 grams of live body weight were calculated. The small intestine length was measured and its relative length was expressed as centimeters per 100 grams of live body weight.

Intestinal permeability

To evaluate intestinal permeability, one bird was randomly selected from each pen for each HS group ($n = 14$). Fluorescein isothiocyanate dextran (FITC-d, 4000 kDa; Sigma-Aldrich, St. Louis, MO, USA) was administered orally via gavage at a concentration of 2.2 mg/mL per bird. Following a 2.5-hour administration period, blood samples (1 mL) were obtained from the jugular vein, which were centrifuged at 3000 × g for 15 min at 4°C to isolate plasma. Subsequently, 1:5 dilutions of the plasma samples and standard solutions were prepared using phosphate buffer solution. Duplicate aliquots were transferred to 96-well microplates for fluorescence measurements. Spectrophotometric analysis was carried out on Victor3 instrument (PerkinElmer Inc., Waltham, MA, USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm. FITC-d concentrations in plasma samples, expressed in ng/mL, were derived from a standard curve generated during the analysis.

Intestinal histomorphology

The duodenum and ileum samples ($n = 20$/group) were fixed in a 4 % formaldehyde solution for 48 h, after which the formaldehyde was replaced with 70 % ethanol, following the standard procedure used by the GIGA Immunohistology Platform (ULiège, Belgium). One slide per sample was prepared, and histological sections were stained with Alcian Blue-Periodic Acid Schiff. Microscopy images of the slides were captured at 20x magnification and examined using specialized software (NDP.view2, Hamamatsu, Japan). Measurements were taken from 20-well-oriented villus-crypt units and morphometric parameters analyzed included villus height (VH), depth of crypts (CD), the ratio between VH and CD (VH:CD), villus width, and the thickness of both the submucosal layer and the tunica muscularis, as described by Masum et al. (2013).

Gene expression through high throughput qPCR

Primer design and validation

A list of 92 genes (13 housekeeping genes and 79 target genes) associated with different ileal physiological functions was selected based on a thorough literature review. Genes used for high-throughput qPCR and brief description of their main functions is presented in Supplementary File 1, Table S2. Validated primer sequences were adapted from Criado-Mesas et al. (2021), which established a robust ileum gene expression panel for evaluating intestinal health in broiler chickens under various environmental conditions. Additionally, validated primers from other studies were incorporated into our ileum gene panel to ensure comprehensive coverage of important genes relevant to the intestinal health and function of broilers (Supplementary File 1, Table S2).

Primers were cross-checked using the NCBI Primer-Blast tool to span exon-exon junctions, preventing genomic DNA amplification. Target specificity was confirmed using in silico analysis through NCBI databases, ensuring no significant cross-reactivity with non-target sequences. Experimental validation involved melting curve analysis following qPCR amplification. All melting curves showed a single peak, indicating specific amplification without side products or primer dimers. Single-product amplification was further verified through agarose gel electrophoresis, where single sharply defined bands appeared at the expected molecular weight for each amplicon. The primers were optimized for efficiency between 90 and 110 %, with R^2 values >0.99 using three-fold serial dilutions of a pooled cDNA derived from all samples on a QuantStudio 6 Real-Time PCR System (Thermo Fisher Scientific). No-template and no-RT controls confirmed the absence of contamination, and consistent Ct values across biological replicates (CV < 5 %) demonstrated reliable performance.

RNA isolation, reverse transcription and preamplification

RNA was isolated from ileal tissue samples ($n = 20$/group) using a commercial kit (ReliaPrep™ RNA Miniprep Systems, Promega) according to the provided protocol. The quantity and quality of the isolated mRNA were assessed using spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific), and RNA integrity was verified through 1 % agarose gel electrophoresis. cDNA was prepared from extracted RNA using RT MasterMix (Standard BioTools). A preamplification step was carried out in a 96-well qPCR plate using a primer mixture with PreAmp Mastermix (Standard BioTools). The thermal cycling conditions for this step were: initial denaturation at 95°C for 2 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min. Next, a clean-up step was performed using Exonuclease I under specific thermal conditions to eliminate unincorporated primers. The Exonuclease I-treated PreAmp reactions were diluted 10-fold and stored at −20°C until the next step.

High-throughput qPCR

The BioMark™ HD system (Standard BioTools) was used for high-throughput qPCR, following a protocol described in our previous study (Akram et al., 2024c). Three 96.96 Integrated Fluid Circuits (IFCs) were run, each corresponding to the samples obtained on d 7, 14, and 38, respectively. Before qPCR, the sample mix was prepared by combining 2.25 μL pre-amplified Exo-I treated cDNA samples with 2.5 μL of 2x SSoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) and 0.25 μL of 20x DNA Binding Dye (Standard BioTools). The assay mix was prepared by combining 0.5 μL of each forward and reverse primer (100 μM) with 2.5 μL of 2x Assay Loading Reagent (Standard BioTools) and 2.25 μL of low EDTA DNA suspension buffer. The sample and assay mixtures were then transferred into the IFC. The qPCR was performed using a fast program with an initial denaturation at 95°C for 60 s, followed by 30 cycles of denaturation: 96°C for 5 s and 60°C for 20 s. The standard curve based on pooled pre-amplified cDNA samples was used to calculate relative mRNA concentrations. Four reference genes (TBP, B2M, NDUFA, and B-ACTIN) were identified as the most stable across experimental conditions via the NormFinder algorithm (Andersen et al., 2004). The Pfaffl method (Pfaffl, 2001) was used to calculate the relative expression of all genes, with normalization of target genes achieved by using the geometric mean of the reference genes' expression.

Statistical analysis

Prior to the statistical analysis, the normality of data distribution was confirmed using the Shapiro–Wilk test in R (v4.2.3). The effects of HS on investigated variables were analyzed using a linear mixed-effects model for each sampling time. The pen was used as a random factor to account for any confounding effect caused by pen location and different number of animals in pens, while HS was used as a fixed effect. A significance threshold was set at $P < 0.05$, and a trend was considered for values between 0.05 and 0.10. P values for ileal gene expression data were adjusted using the Benjamini-Hochberg approach (Benjamini and Hochberg, 1995) to correct for multiple testing, with $P < 0.05$ set as the significance threshold. Principal component analysis (PCA) was performed to visualize sample clustering between HS based on gene expression data using factoextra package (v1.0.7) in R. Furthermore, Permutational Multivariate Analysis of Variance (PERMANOVA) was performed in R using adonis2 (v2.6.4) to test for multivariate effects of

HS on sample clustering in PCA. Heatmaps were generated that show sample variability and gene expression levels using the pheatmap package (v1.0.12) in R. The heatmaps for two-way hierarchical clustering analysis were based on Pearson's correlation distance and Ward's clustering method, with gene expression levels scaled per gene.

Results

Growth performance parameters

The percentages of non-hatched eggs and culled deformed chicks were only recorded for the HOF system, which were 1.82 % and 1.19 %, respectively. On d 1, the body weight of HOF chicks was significantly higher than that of HH chicks (45.7 ± 3.14 g vs. 42.2 ± 2.89 g) ($P < 0.001$, Table 1). The difference in body weight between HS disappeared by d 7 and remained statistically similar thereafter. There was no point at which the ADG, ADFI, or FCR differed significantly between the HS ($P > 0.05$). The HOF system showed numerically higher CV in body weight than the chicks in HH system, however, these differences did not reach statistical significance ($P > 0.05$).

Relative weights of digestive and immune organs

HH chicks demonstrated higher relative heart weights on d 7 ($P = 0.041$), and HOF chicks demonstrated higher relative weight of the bursa of Fabricius on d 14 ($P = 0.018$, Table 2). In addition, HOF chicks tended to have increased relative liver and small intestine weights on d 14 ($P = 0.060$ and $P = 0.059$, respectively) and higher relative bursa weights on d 38 ($P = 0.094$) as compared to HH chicks.

Intestinal permeability

FITC-d levels were not significantly different between HH and HOF chickens at any of the time points ($P > 0.05$; Fig. 1).

Table 1
Performance indicators of chickens hatched in the hatchery (HH) or on-farm (HOF).

Indicator	Days of age	HH	HOF	SD	*P* value
Body weight (g)	1	42.2	45.7	3.60	**<0.001**
	7	187.1	191.1	23.70	0.929
	14	507.0	511.7	62.90	0.892
	28	1863.7	1857.1	159.79	0.906
	38	3115.5	3098.6	179.66	0.897
ADG (g/g)	1-14	32.9	33.1	4.50	0.831
	15-28	96.9	96.2	7.10	0.917
	29-38	125.1	124.2	9.39	0.876
	1-38	85.0	84.5	4.67	0.825
ADFI (g)	1-14	41.9	41.02	3.70	0.513
	15-28	127.1	126.7	11.18	0.921
	29-38	191.8	195.1	13.03	0.521
	1-38	112.8	113.1	7.60	0.905
FCR (g/g)	1-14	1.19	1.18	0.080	0.892
	15-28	1.35	1.37	0.869	0.466
	29-38	1.60	1.58	0.128	0.743
	1-38	1.33	1.34	0.038	0.729
CV (%)	7	4.85	4.95	0.28	0.679
	14	6.05	6.55	1.72	0.868
	28	7.75	8.15	1.37	0.321
	38	10.4	11.01	3.09	0.472

Note: The bolded P-values in the table indicate statistical significance at $P < 0.05$, as mentioned in the table.
Abbreviations: ADG = average daily gain, ADFI = average daily feed intake, FCR = feed conversion ratio, CV = coefficient of variation.
Body weight was recorded from individual birds, while a pen was considered the experimental unit for all other measurements.
Data are presented as mean and pooled standard deviation (SD).

Table 2
Relative visceral organ weights (g/100 g body weight) and small intestine length (cm/100 g body weight) of chickens hatched in the hatchery (HH, $n = 20$) or on-farm (HOF, $n = 20$).

Organ	Days of age	HH	HOF	SD	*P* value
Heart	7	0.78	0.71	0.199	**0.041**
	14	0.79	0.82	0.081	0.106
	38	0.47	0.50	0.065	0.164
Liver	7	4.38	4.27	0.420	0.731
	14	3.22	3.46	0.399	**0.060**
	38	2.01	2.01	0.279	0.977
Spleen	7	0.07	0.08	0.030	0.549
	14	0.09	0.08	0.021	0.477
	38	0.11	0.12	0.032	0.855
Pancreas	7	0.43	0.44	0.080	0.682
	14	0.38	0.39	0.077	0.749
	38	0.16	0.16	0.039	0.931
Bursa of fabricius	7	0.17	0.17	0.056	0.121
	14	0.21	0.26	0.070	**0.018**
	38	0.14	0.16	0.131	**0.094**
Stomach	7	6.59	6.31	0.700	0.178
	14	4.51	4.61	0.535	0.522
	38	1.79	1.83	0.398	0.707
Small intestine weight	7	18.40	18.35	1.393	0.889
	14	15.25	15.80	1.601	**0.059**
	38	7.68	7.70	1.265	0.970
Small intestine length	7	55.94	55.84	7.903	0.954
	14	27.46	28.40	2.713	0.194
	38	6.68	6.80	0.924	0.619

Data are presented as mean and pooled standard deviation (SD).

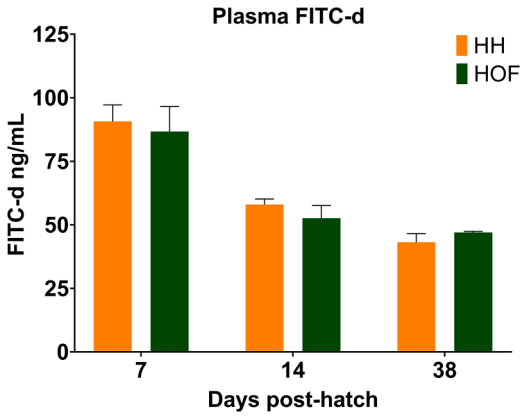

Fig. 1. Plasma fluorescein isothiocyanate dextran (FITC-d, ng/mL) levels 2.5 h after oral administration to chickens hatched in the hatchery (HH, $n = 14$) or on-farm (HOF, $n = 14$) on 7, 14 and 38 days post-hatch. The data is provided as mean ± SD and analyzed with Student's t-test.

Intestinal histomorphology

Compared with HH chicks, HOF chicks demonstrated an increased duodenal villus width on d 7 and 14 ($P = 0.031$ and 0.030, respectively) and a thicker submucosal layer ($P = 0.045$) on d 7 (Table 3). In addition, HOF chicks had deeper ileal crypts ($P = 0.018$) and tended to have a lower VH:CD ratio on d 7 ($P = 0.099$) than HH chicks (Table 4).

Ileal gene expression

During the high-throughput qPCR, three samples from the HH group on d 7 and one sample from the HOF group on d 38 had to be excluded because of technical problem. No expression readouts were obtained from some genes due to technical issues and these were withdrawn from the statistical analysis (Supplementary file 1, Table S3).

Table 3
Duodenal histomorphological characteristics of chickens hatched in the hatchery (HH, $n = 20$) or on-farm (HOF, $n = 20$).

Indicator	Days of age	HH	HOF	SD	*P* value
VH (μm)	7	1418	1431	120.7	0.690
	14	1917	1933	160.2	0.773
	38	2077	2068	218.5	0.899
CD (μm)	7	132	140	27.4	0.366
	14	200	211	40.7	0.421
	38	188	205	65.4	0.447
VH:CD	7	11.1	10.6	2.51	0.536
	14	10.1	9.3	2.13	0.323
	38	12.3	11.5	3.95	0.351
Villus width (μm)	7	148	160	18.5	**0.031**
	14	175	193	26.1	**0.030**
	38	191	195	27.8	0.612
Submucosa (μm)	7	21.6	23.8	3.90	**0.045**
	14	21.7	23.0	3.62	0.309
	38	29.0	28.3	4.75	0.623
Tunica muscularis (μm)	7	133	132	16.7	0.830
	14	151	149	23.1	0.972
	38	190	195	33.5	0.608

Abbreviations: VH = villus height, CD = crypt depth, VH:CD = ratio of VH to CD. Data are presented as mean and pooled standard deviation (SD).

Table 4
Ileal histomorphological characteristics of chickens hatched in the hatchery (HH, $n = 20$) or on-farm (HOF, $n = 20$).

Indicator	Days of age	HH	HOF	SD	*P* value
VH (μm)	7	536	547	67.1	0.517
	14	646	648	86.8	0.944
	38	1075	1019	147	0.235
CD (μm)	7	118	131	17.9	**0.018**
	14	180	182	28.8	0.774
	38	162	158	27.2	0.590
VH:CD	7	4.6	4.3	0.72	0.099
	14	3.5	3.6	0.75	0.652
	38	6.8	6.6	1.21	0.551
Villus width (μm)	7	152	144	18.2	0.222
	14	177	184	15.5	0.180
	38	170	149	34.8	0.217
Sub mucosa (μm)	7	20.0	21.0	2.13	0.456
	14	27.2	27.9	3.91	0.545
	38	34.1	38.2	9.24	0.359
Tunica muscularis (μm)	7	111	116	21.7	0.579
	14	153	150	21.1	0.697
	38	189	225	53.8	0.146

Abbreviations: VH = villus height, CD = crypt depth, VH:CD = ratio of VH to *CD*. Data are presented as mean and pooled standard deviation (SD).

Principal component analysis

On d 7, the first two principal components (PCs) combined accounted for 37.5 % of the total variability. However, the variability of these components did not effectively separate the samples into distinct clusters based on HS treatment ($P = 0.362$; Fig. 2A). PERMANOVA further showed no statistically significant relationship between HS groups and the variability in gene expression as captured by PCs. On d 14 and 38, the PCA results showed that PC1 explained 26 % and 30.7 % of the total variance, respectively. However, at these later growth stages, the separation of samples based on the HS was also not distinctly observable. PERMANOVA further confirmed the absence of substantial differences in gene expression profiles between the HH and HOF treatments ($P = 0.255$ for d 14 and $P = 0.427$ for d 38; Fig. 2B and 2 C).

Heatmap clustering

The heatmaps provide a visual representation of the gene expression variability across samples from both HS groups (Fig. 3, S1, and S2). Two-way hierarchical analysis on d 7, 14, and 38 revealed no clear clustering of samples or genes according to HS conditions or biological functions. However, three distinct gene expression clusters were identified at each age. On d 7, the first-row cluster had genes that tended to be co-expressed and were associated with gut barrier function, immunological response, nutrition transport, gut hormone, metabolism, and oxidation. On d 14, the first row of cells showed co-expression of genes related to gut barrier function. The second-row cluster consisted primarily of nutrient transport genes, while the third-row cluster contained mostly immune response genes. On d 38, the first-row cluster showed co-expression of genes primarily from the immune response category. The second-row cluster contained mostly nutrient transport genes and gut barrier function genes. The third-row cluster contained genes associated with both gut barrier function and immune response.

Differential gene expression analysis

On d 7, HH chicks showed higher expression of *MUC5ac* ($P = 0.048$) and *VDR* ($P = 0.015$), genes associated with gut barrier function and nutrient transport, respectively (Fig. 4A). In contrast, HOF chicks had higher expression of *CCK* ($P = 0.041$) and *IL-8* ($P = 0.009$) genes associated with gut hormones and the immune response, respectively. On d 14, HH chicks demonstrated higher expression of *CLDN2* ($P = 0.029$) and *MUC2* ($P = 0.046$), with a tendency toward increased *ZO-2* ($P = 0.062$; Fig. 4B), genes related to gut-barrier function, compared to HOF chicks. HOF chicks had higher expression of the immune-related genes *AVBD9* ($P = 0.047$), *IFN-γ* ($P = 0.048$), and *IL-6* ($P = 0.040$). However, the expression of the other immune-related genes *IL-18* ($P = 0.052$) and

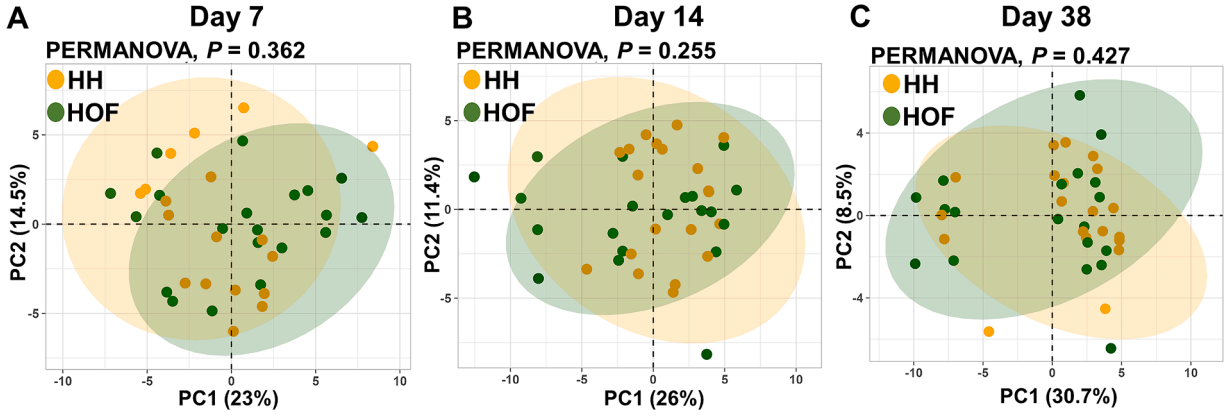

Fig. 2. Principal component analysis of gene expression data obtained from the ileum of chickens hatched in the hatchery (HH) or on-farm (HOF) on days 7 (A), 14 (B), and 38 (C).

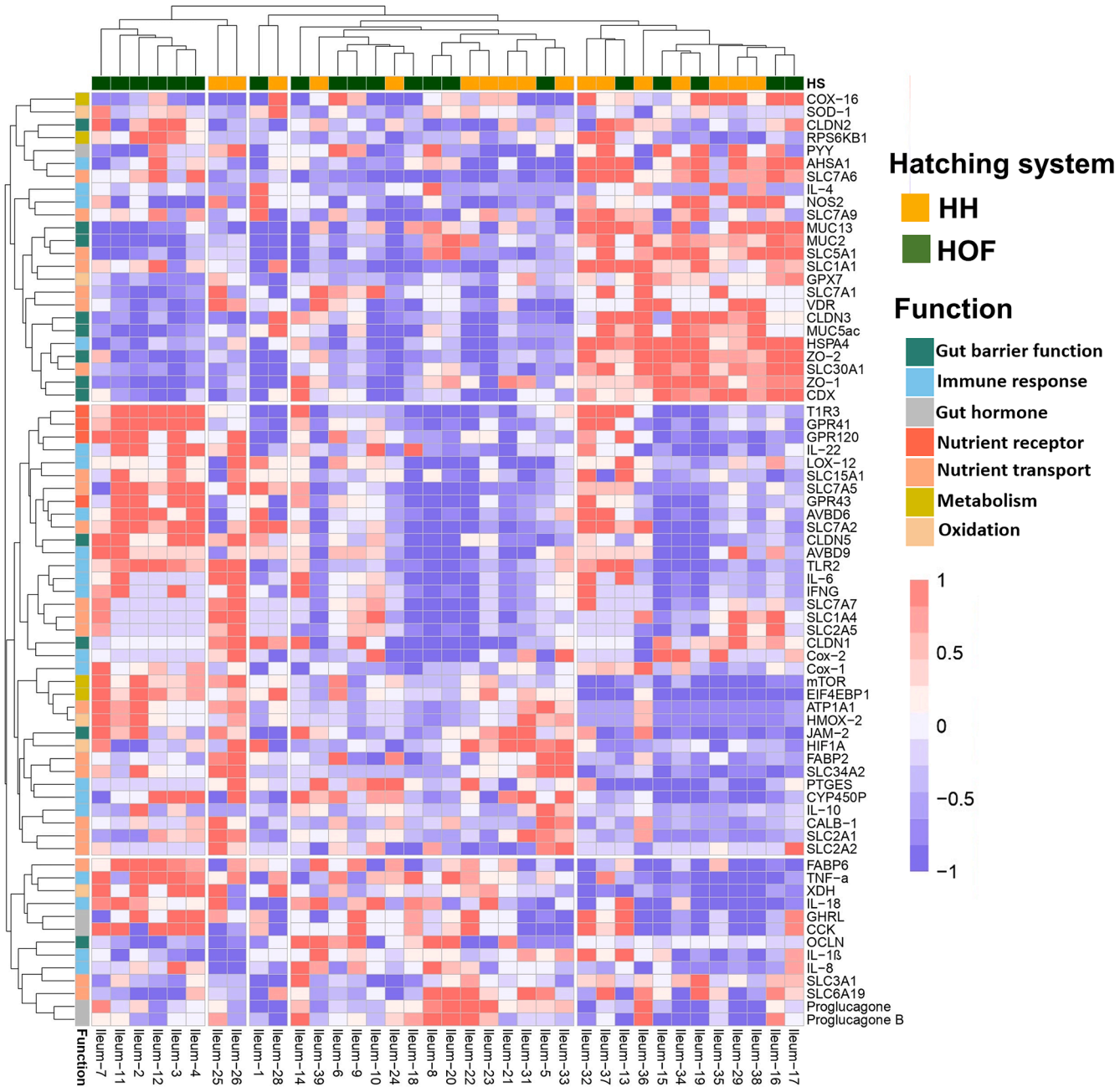

Fig. 3. Heatmap of ileal gene expression levels on day 7 of chickens hatched in hatchery (HH, $n = 17$) or on-farm (HOF, $n = 20$). The x-axis represents individual samples, while the y-axis shows the genes. Expression levels are color-coded, with red corresponding to high expression and blue indicating low. Gene functions are denoted by different colors on the y-axis. The left dendrogram clusters genes with similar expression patterns, and the top dendrogram groups samples with similar gene expression profiles.

COX-1 ($P = 0.093$) tended to decrease in HOF chicks. Additionally, HOF chicks had a tendency toward higher expression of *T1R3* ($P = 0.084$), a nutrient receptor-related gene. On d 38, HOF chicks showed upregulation of *ZO-1* ($P = 0.018$), *HIF1A* ($P = 0.039$), and *NOS2* ($P = 0.035$), genes related to barrier function, oxidation, and the immune response, respectively (Fig. 4C). In contrast, HH chickens showed upregulation of nutrient transport-related genes *SLC5A9* ($P = 0.045$) and *SLC30A1* ($P = 0.002$), with a tendency toward higher *SLC6A19* ($P = 0.068$) expression.

Discussion

Although HOF chicks had a temporary body weight advantage on d 1, it did not persist beyond the first week. This initial body weight advantage is likely due to the favorable start in the HOF system, characterized by immediate feeding and the absence of transportation. HOF chicks had immediate feeding, unlike the 48 – 72 h delay in traditional HH practices due to prolonged hatch windows and hatchery protocols. Early nutritional access is crucial, as previous studies have demonstrated its positive impact on broiler body weight and feed intake by 7 days of age (Lamot et al., 2014). Even brief early fasting, as short as 24 h, negatively impacts weight gain during the starter phase (Zulkifli et al., 2016). Furthermore, the HOF system likely minimized the adverse effects of transportation. Bergoug et al. (2013) reported that transportation negatively impacts body weight in chickens up to 21 days of age, with transported chickens showing lower body weights compared to those that were not transported. The presence of transportation,

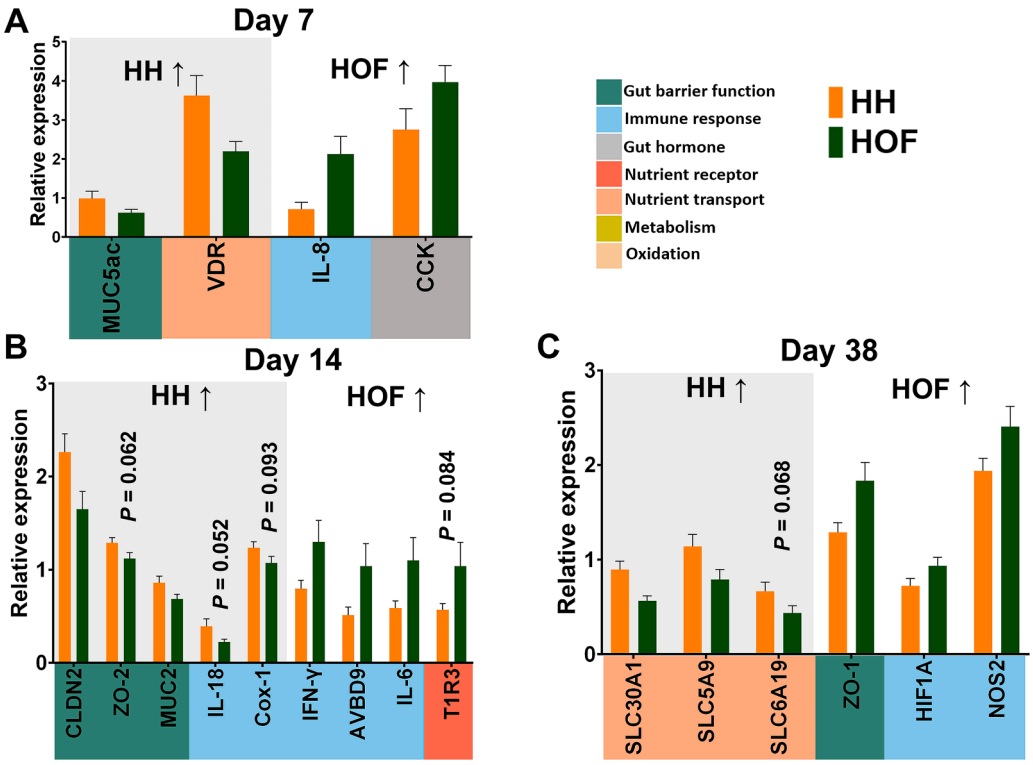

Fig. 4. Differential gene expression (Student's t-test with an FDR-adjusted *P* value < 0.05) between chickens hatched in the hatchery (HH) or on-farm (HOF) chickens on days 7 (A), 14 (B), and 38 (C). The gray background section shows genes upregulated in HH chickens, while white background section shows those upregulated in HOF chickens. Gene functions are annotated by color codes on the x-axis.

combined with initial deprivation of feed and water, might have negatively affected the development of HH chicks and have resulted in their lower body weight at placement (de Jong et al., 2017; Gaweł et al., 2022). Although long-term effects of the HOF system on broiler performance were not observed, implementing this system resulted in immediate improvements in early growth rates as compared to the HH system. HOF chicks exhibited a temporary body weight advantage until first week, which can be attributed to the immediate post-hatch access to feed and water, facilitating nutrient intake and hydration during a critical developmental period. In the current study, HH chickens experienced an average post-hatch feed deprivation of approximately 40 h, which did not appear long enough to cause significant long-term performance differences between the two HS. Boyner (2023) similarly reported that HH chicks can effectively compensate for early-life setbacks, including short-term feed deprivation and transportation stress, minimizing their impact on overall performance. Additionally, male chicks were hatched slightly later than females, which aligns with previous studies (Burke, 1992; Reis et al., 1997). Since our study exclusively used male chicks, their relatively shorter time to access feed may have contributed to the observed short-term body weight advantage in HOF chicks.

The findings showed that duodenal and ileal VH and the VH:CD ratio remained unaffected by the feed deprivation associated with the HH system. This observation aligns with previous studies (de Jong et al., 2017, 2019), which reported no deleterious effects of delayed feeding due to the HH system on intestinal morphology. However, it contrasts with studies showing higher duodenal and ileal villus height and crypt depth in early-fed chickens (Geyra et al., 2001) or decreased villus surface area and height in fasted broilers (Gonzales et al., 2003; Mahmoud and Edens, 2012). Discrepancies across studies may stem from different sampling times. Uni et al. (1998) reported that the duodenal villus surface area in feed-deprived chicks, which was initially reduced, recovered after 4 days. Our first sampling occurred on d 7 post-hatch, which may have been too late from the critical window to capture the full spectrum of transitional effects of HS on intestinal morphology. Future investigations should consider earlier sampling points, specifically on days 1-3 post-hatch, to better understand the effects of feed deprivation on intestinal development. HH chickens had lower duodenal villus width on d 7 and 14, and decreased submucosa thickness and ileal crypt depth on d 7. These findings suggest that while some intestinal features catch up with those of HOF chickens, the persistent reduction in some parameters suggests that feed deprivation may have lasting impacts on certain intestinal traits. The reduction in villus width and crypt depth in HH chicks could be interpreted as a physiological response to the absence of early luminal stimulation by feed. In contrast, the enlargement of histomorphological parameters in HOF chicks may be related to early nutrient availability, which facilitated more rapid intestinal development. This is consistent with a previous study in ducklings, where early feeding resulted in increased villi height, villi width and crypt width compared to delayed feeding (Williams et al., 2021). In contrast to prior studies (Van de Ven et al., 2011; de Jong et al., 2020), our study found a significant difference in relative heart weights between HS, with HH chicks showing higher relative heart weights on day 7 compared to HOF chicks. This suggests that hatching conditions may influence cardiovascular development. Stressors associated with conventional hatchery practices, such as continuous darkness, high noise levels, and handling stress, could have contributed to physiological stress responses in HH chicks. Although stress responses were not measured in this study, perinatal stress is known to activate the hypothalamic-pituitary-adrenal (HPA) axis, potentially triggering adaptive changes in organ development, including the cardiovascular system (Xiong and Zhang, 2013). The increased relative heart weight in HH chicks may reflect a compensatory mechanism to mitigate stress-induced challenges during early post-hatch development.

The findings indicated that the HS exerted no significant influence on intestinal permeability, as measured by FITC-d concentrations in plasma. This observation suggests that intestinal barrier function was maintained despite differences in early feeding practices between the

HH and HOF systems. Although early fasting is often linked to altered gene expression related to gut barrier integrity (Proszkowiec-Weglarz et al., 2020; Li et al., 2022), higher mRNA expression of barrier function genes was observed in HH chicks during the early growth stages. Upregulated genes included *MUC5ac* on d 7, and *CLDN2, ZO-2,* and *MUC2* on d 14, all of which are essential for tight junction formation and mucin production, critical to intestinal barrier function (Gilani et al., 2016; Akram et al., 2024a). Their upregulation may indicate an adaptive response to feed deprivation, explaining the intact intestinal integrity in HH chicks.

Early feeding is key to immune maturation, as the timing of the first feed significantly impacts gut microbiota colonization, which directly affects immune development (Turnbaugh et al., 2009; Geuking et al., 2011; Simon et al., 2014; Akram et al., 2024b). Feeding triggers rapid bacterial growth in the intestine (Ballou et al., 2016), suggesting that diet shapes immune function by altering the gut microbiota. Consistent with previous studies (Dibner et al., 1998; Shira et al., 2005), the findings of the current study demonstrated that early feeding, facilitated by the HOF system, led to increased relative bursa weights on d 14 and 38 compared to the HH birds. This immune stimulation was further evidenced by increased expression of immune-related genes in the ileum of the HOF chickens. An enhanced immune response in early-fed chickens has been linked to higher levels of T and B cells in the bursa (Shira et al., 2005). Hollemans et al. (2021) reported that early feeding improves the humoral immune response against infections at young ages and reduces the risk of disease and mortality.

HOF chicks showed higher *IL-8* expression on d 7, a pro-inflammatory cytokine crucial for heterophil recruitment and bacterial clearance (Kogut et al., 2003; Hashem et al., 2022). Furthermore, chicks in the HOF system had higher expression of *IL-6, AVBD9*, and *IFN-γ* on d 14 as compared to those in the HH system. *IL-6*, a pleiotropic cytokine, aids in infection response and tissue repair through *IL-8* activation (Lynagh et al., 2000; Vasalou et al., 2023). AVBD9, an antimicrobial peptide, directly kills microbes and stimulates cytokine production and dendritic cell differentiation (Cuperus et al., 2016). It effectively targets both Gram-positive and Gram-negative bacteria and has strong fungicidal activity (van Dijk et al., 2007; Yacoub et al., 2015). *IFN-γ*, produced by NK cells and T lymphocytes, activates macrophages, enhancing viral inhibition, antigen presentation, and pathogen elimination (Digby and Lowenthal, 1995; Yeh et al., 1999; Santhakumar et al., 2017). On d 38, HOF birds demonstrated elevated *NOS2* expression, boosting pathogen-targeting nitric oxide (Dao et al., 2022), and increased *HIF1A,* linked to anti-inflammatory responses and cellular adaptation to hypoxia (Sun et al., 2019). Despite broilers' typical trade-off between rapid growth and immune support (Dadfar et al., 2023), the upregulation of immune genes in HOF birds did not lead to tissue damage or reduced production. This implicates that the HOF system can promote early immune maturation, particularly in intensive production systems, and support operations transitioning to antibiotic-free production by enhancing disease resilience while maintaining growth performance.

On d 7, a higher *VDR* expression was observed in HH birds compared to HOF chicks. *VDR* regulates genes involved in calcium and phosphorus transport (Proszkowiec-Weglarz et al., 2019), suggesting a compensatory response to optimize nutrient absorption after feed availability in the intestinal lumen. HH birds also showed unexpected upregulation of *SLC5A9* and *SLC30A1* genes related to glucose and zinc transport on d 38, potentially due to delayed feeding effects. The timing of initial feeding after hatch could have significant implications for appetite regulation and possibly feed intake patterns in broiler chickens. Lower *CCK* expression was observed in HH chicks on d 7. CCK is a well-known gut hormone that plays a crucial role in appetite suppression (Tachibana et al., 2012). Reduced *CCK* expression in HH birds during early life may result from increased appetite due to delayed feeding, potentially altering feeding behaviors that led to compensatory growth. This finding is corroborated by a previous study (Kanayama and Liddle, 1991), which reported that plasma CCK levels in rats decreased rapidly in response to feed deprivation for up to five days and returned to control levels after just one day of refeeding. In addition, the hormone CCK plays a role in stimulating gut reflexes and promoting the release of bile acids and pancreatic enzymes, which collectively enhance digestive efficiency (Rehfeld, 2017).

Conclusion

This study explored a new HS, where chicks hatch on-farm, which was compared to the standard hatchery system. The findings demonstrated short-term advantage of the HOF system on chicken growth performance as compared to the HH system. Age-related compensatory growth occurred in HH chicks within the first week, after which both HS groups showed similar growth trajectories. The delayed feeding associated with HH did not fundamentally alter intestinal permeability. Instead, HH birds showed an adaptive upregulation of genes associated with intestinal barrier function, suggesting a mechanism to maintain gut integrity despite early feed deprivation. HOF birds had improved intestinal architecture, higher bursa weight, and higher expression of immune-related genes, suggesting that early feeding facilitated by the HOF system aids in intestinal development and supports the immune system. It is important to note that the present study was performed in healthy and non-challenged conditions; hence, the potential impacts of the HOF system on the immune system may become more apparent in stressful or challenged settings. Overall, the findings of this study demonstrate the potential of the HOF system as an effective management strategy for supporting the immune system and intestinal health in broiler chickens, which may significantly improve overall health and productivity.

Consent for publication

Not applicable.

Availability of data and material

The data analyzed in this study is available in the manuscript and supplementary file.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104770.

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