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Effects of the Welfare Housing Systems Based on Environmental Enrichment on Growth Performance, Meat Quality, and Intestinal Health in Rabbits

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ABSTRACT

In recent years, consumers have shown a growing interest in purchasing welfare-friendly livestock products. Environmental enrichment has emerged as an effective approach to enhancing animal welfare. Based on this, this study aims to investigate the effects of welfare housing systems incorporating environmental enrichment on growth performance, meat quality, and intestinal health in rabbits. Rabbits were assigned into three groups: Cage group (Cage), Pen group (Pen), and Pen + environmental enrichment material group (PE). The Pen group exhibited a lower average daily weight gain and a higher feed conversion ratio. However, the meat color and nutrient content of the leg muscles were improved in both the Pen and PE groups to varying extents. Furthermore, fatty acid β-oxidation in the leg muscles was elevated in the Pen group, resulting in reduced lipid deposition. Additionally, both the Pen and PE groups displayed altered cecal microbiota composition and increased short-chain fatty acids content, contributing to enhanced intestinal health through the activation of G protein-coupled receptors. In conclusion, these findings suggest that the two welfare housing systems can improve meat quality and intestinal health. This study offers a theoretical foundation for welfare-oriented rabbit farming and the production of healthier rabbit meat to meet consumer demands.

1 | Introduction

Rabbits are monogastric herbivores, characterized by short gestation periods, high fertility, and efficient feed conversion. They are hindgut fermenters, utilizing cecal fermentation and cecotrophy to enhance nutrient absorption [1]. In short, the caecum of rabbits is critical for maintaining their health. In addition, rabbit meat is healthy due to its high content of

Abbreviations: AAA, aromatic amino acids; AB-PAS, alcian blue-periodic acid Schiff; ADG, average daily weight gain; ALA, alanine; ARG, arginine; ASP, aspartic acid; ATGL, adipose triglyceride lipase; BCA, bicinchoninic acid; CPT1β, carnitine palmitoyltransferase1β; CPT2, carnitine palmitoyltransferase2; CYS, cysteine; EAA, essential amino acids; F/B, Firmicutes to Bacteroidetes; FAA, flavorful amino acids; FCR, feed conversion ratio; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLU, glutamic acid; GLY, glycine; GPCRs, G protein-coupled receptors; GPR41, G protein-coupled receptor 41; GPR43, G protein-coupled receptor 43; HIS, histidine; IL-1β, interleukin-1β; ILE, isoleucine; IMF, intramuscular fat; LEU, leucine; LIPE, lipase E, hormone sensitive type; LPL, lipoprotein lipase; LYS, lysine; MET, methionine; MUFAs, monounsaturated fatty acids; PHE, phenylalanine; PPARα, peroxisome proliferator-activated receptor α; PRO, proline; PUFA, polyunsaturated fatty acid; RIPA, radio immunoprecipitation assay; SAA, sweetening amino acids; SCFAs, short-chain fatty acids; SER, serine; SFAs, saturated fatty acids; TAA, total amino acid; TEAA, total EAAs; TGF-β1, transforming growth factor-β1; THR, threonine; TMLHE, trimethyllysine hydroxylase, epsilon; TNAA, total non-EAA; TNF-α, tumor necrosis factor-α; TYR, tyrosine; VAL, valine; ZO-1, zona occludens-1; β-actin, beta-actin.

polyunsaturated fatty acids (PUFAs) and essential amino acids (EAA), thus meeting consumer demand for a healthy diet [2]. As global demand for livestock products continues rising, the scale of animal farming is expanding annually. This growth has heightened public concerns about animal welfare, particularly in intensive farming systems [3–5]. Public attention to animal welfare has been growing in recent years. Particularly for food production, there is a growing concern about the living conditions during livestock farming [6, 7]. In developed countries, a proportion of consumers condemn the use of cages, high stocking density, and farming systems that restrict the natural behavior of animals [8]. Animal welfare is increasingly becoming a decisive factor in consumers' purchases of different animal-based products [9]. Rabbits are herd animals, and as such, rabbits exhibit numerous positive social behaviors, but in the current breeding system, two to six fattening rabbits live in small, barren cages from weaning to slaughter [10]. The living conditions of rabbits are a cause for concern. These welfare issues directly impact consumer attitudes and purchasing behavior.

Environmental enrichment (EE) refers to incorporating novel objects and promoting group social activities to enhance the living conditions of animals [11–13]. The purpose of EE is to encourage behaviors that are appropriate to a particular species and that satisfy an animal's physical and psychological needs [14]. It is well known that the housing system is a very important factor influencing animal welfare [15]. Therefore, EE may be an effective way to address animal welfare issues in livestock farming. Studies have shown that the addition of EE materials, such as sticks to rabbit cages can improve behavior, growth performance, serum hormones, and even hair follicle development [16, 17]. Not only that, but some studies have also shown that the meat quality of pen-housed rabbits is significantly altered compared to cage-housed rabbits [18] In addition, EE has been applied to improve animal welfare in a wide range of livestock, such as cattle [19], broiler chickens [20], laying hens [21], and pigs [22].

In recent years, there has been a growing concern about rabbit welfare and sustainability. Therefore, we designed two housing systems: an EE housing system based on increasing the socialization activities of rabbits, and another EE housing system that increases the socialization activities of rabbits and adds novelty items. We named these two housing systems Pen (Pen) and Pen + EE materials (PE). These systems were designed in this study to increase rabbits' specific behaviors and social interactions, thereby improving their animal welfare. By evaluating the effects of these housing systems on growth performance, meat quality, and gut health, the results of this study will provide a theoretical basis for the subsequent welfare breeding, contributing to the subsequent improvement of the welfare housing system, aligning with consumer demands for ethically produced, high-quality products.

2 | Materials and Methods

2.1 | Experimental Procedure

All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (No. 11-00850 of the College of Animal Science and Technology, Henan Agricultural University, China).

A total of 72 rabbits of the commercial Hyplus cross-bred line (male, 35 days old) with similar body weights (1.07 ± 0.09 kg) provided by Jiyuan Sunshine Rabbit Technology Co. Ltd. were randomly divided into three groups: cage group (Cage) (Figure 1A); pen group (Pen) (Figure 1B); and pen + EE material (PE) (Figure 1C). Cage group ($n = 12$): two rabbits kept in a cage (50 × 40 × 40 cm; 0.1 m^2 per rabbit); Pen group ($n = 30$): 15 rabbits kept in a pen (150 × 100 × 60 cm; 0.1 m^2 per rabbit); PE group ($n = 30$): 15 rabbits kept in a pen (150 × 100 × 60 cm; 0.1 m^2 per rabbit), adding apple wood ($n = 10$), tin cans with stones ($n = 10$), and straw mats ($n = 9$; 20 × 15 cm) (Figure 1D) to the pen. The facilities were temperature-controlled (18°C–24°C) and the daily light period used was 12 h. Rabbits were fed ad libitum with a pelleted diet and had ad libitum access to water. Record the weight of feed added and remaining each day for calculation of the feed conversion ratio. The pretrial period was 5 days and the experimental period was 5 weeks.

2.2 | Slaughter Performance and Physical Analyses

On day 74, the rabbits were subjected to 24 h of fasting and water deprivation. Then, eight rabbits were randomly selected from each group, electrically stunned and killed by severing the carotid artery and jugular vein. We slaughter rabbits according to a method described in an article published by World Rabbit Science [23]. The general procedure was as follows: the rabbit was bled and skinned, followed by dissecting the chest and abdomen of the rabbit and removing the internal organs, and the distal segment of the legs was removed. Carcass weight is the weight after removing the head, liver, kidneys, fat, trachea, esophagus, and other excess tissue. Subsequent separation of the biceps femoris muscle was used to detect the pH (Ph-STAR, MATTHAEUS, Germany) of the muscle as well as the color (OPTO-STAR, MATTHAEUS, Germany) of the cut surface. Three measurements of each muscle color as well as pH were averaged. The pieces of meat were trimmed to a uniform size (1 × 1 × 1 cm) and weighed. It was then placed in a self-sealing bag and placed in a water bath (100°C, 30 min). The meat samples were then removed to cool naturally to room temperature, dried on the surface, and weighed again. Meat samples were placed on a muscle tenderizer (C-LM3B, Northeast Agricultural University, China) and the shear force was measured.

2.3 | Behavioral Observations

We conducted a 15-day behavioral observation experiment in rabbits from day 60 to day 74 of age. We referred to published studies [24, 25] and developed the judgment criteria for this experiment (Table S1). Behavioral observations were carried out twice a day for 20 min before feeding, and observers were familiar with the criteria before the experiment and did not participate in other experiments. There was a total of three observers in the experiment. Calculation: Percentage of each behavior = number of occurrences of each behavior ÷ total number of occurrences.

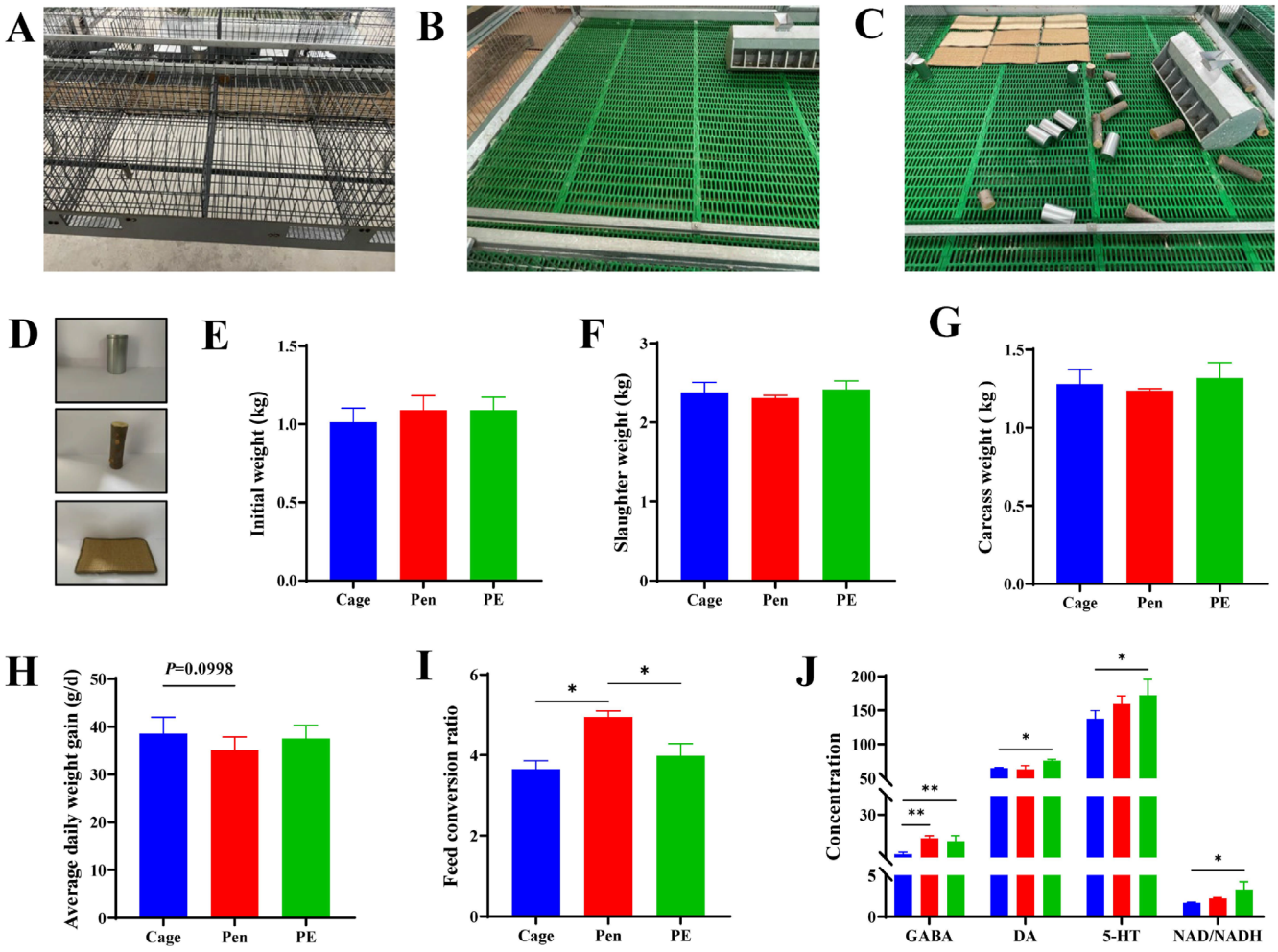

FIGURE 1 | The effects of welfare housing systems based on environmental enrichment on the growth phenotype and cognitive behavior of rabbits. Cage model (Cage) (A), pen model (Pen) (B), pen add environmental enrichment materials model (PE) (C), and environmental enrichment materials (D). The initial weight (E), slaughter weight (F), carcass weight (G), average daily weight gain (H), and feed conversion ratio (I) of three groups. The content of GABA, Dopamine (DA), 5-HT, and NAD/NADH in brain tissue (J). (Cage = Cage models, Pen = Pen models, PE = Pen + environmental enrichment materials models; $^{*}p < 0.05$; $^{**}p < 0.01$).

2.4 | T-Maze Test

T-maze experiments were performed in rabbits from 68 to 74 days of age (the start-arm: 180 × 30 × 60 cm; the goal-arms: 120 × 30 × 60 cm). The experimental methodology was similar to previously published studies [26, 27]. In brief, the rabbits were housed individually in cages and restricted from eating until they lost 15% of their body weight to start the experiment. Open both side arms of the T-maze and evenly sprinkle in the daily fed pellets. Rabbits were placed into the T-maze for 10 min, three times a day for 2 days to ensure that they could adapt. Close one side arm of the T-maze and place pellets in the goal-arm, then place the rabbit in the start-arm and make sure it goes into the goal-arm, eight times a day for 2 days. On the following 2 days, we opened the two side arms of the T-maze, placed the rabbits in the start-arm, and rewarded them if they were able to select the goal-arm. If the choice was incorrect, the rabbit was restricted from movement for 30 s. The rabbit was given a reward for choosing the goal-arm. On the last day, we rotated the T-maze 180° and opened the side arms, performed 10 tests per rabbit, and recorded the number of times the goal-arm was correctly selected. During this period, we also recorded the time it took for the rabbits to reach the goal-arm.

2.5 | Sucrose Preference Test

Rabbits were housed individually in cages and were deprived of both food and water for 24 h. Each rabbit was given a bottle of sucrose water (1% [wt/vol]) as well as a bottle of plain water. The position of the two bottles of water was switched every 3 h, and the consumption of the two bottles of water was recorded every 12 h. Sucrose preference = Sugar water consumption/(sugar water consumption + plain water consumption) × 100%.

2.6 | Sample Collection

Blood was collected from the marginal ear vein of rabbits on day 75. The blood was centrifuged (3000 rpm/min, 10 min) at

4°C to separate the serum. The serum was stored at −80°C until further experiments. The rabbits were then slaughtered. Brain, the hind leg muscles, caecum tissue, and caecum contents were collected and stored at −80°C for molecular biology experiments. In addition, muscle as well as caecum tissues were collected and fixed in 4% paraformaldehyde for making tissue sections.

2.7 | Determination of Fatty Acid Contents

The biceps femoris muscle was removed from fascia, blood vessels, and so forth, and dried in a thermostat at 65°C until constant weight. The dried meat samples were crushed using a food mill, weighed 0.3–0.5 g of the dried meat samples, placed in a test tube, and added 4 mL of isooctane. It was then shaken and mixed for 30 s, placed at 37°C, and shaken overnight. Add 4 mL of 2 mol/L potassium hydroxide-methanol solution, vortex for 30 s, and leave to stratify for 30 min, followed by the addition of about 1 g of sodium bisulfate and leave until clarified. It was then filtered through a 0.45 μm microporous filter membrane and left to be measured.

Gas chromatographic was performed using GC-2010Plus (SHIMADZU, Japan) with a 30 m SH-Rtx-wax capillary column and a flame-ionization detector. Total of 1 μL of sample was injected at a 1:100 split ratio. The separation was achieved using the following temperature program: initially, the column was operated at 100°C for 13 min; then the temperature was programmed at 10°C/min to 180°C, held for 6 min; programmed at 1°C/min to 200°C, held for 20 min; programmed at 4°C/min to 230°C, held for 10.5 min. Fatty acids were identified based on retention times corresponding to standards. Values are expressed as milligrams of fatty acids per 100 g of dried muscle.

2.8 | Determination of Amino Acid Contents

The content of amino acids in the biceps femoris muscle was described by Chai et al. [28]. Briefly, 0.4 g of dried meat sample and 10 mL of HCL (6 mol/L) were mixed thoroughly. After the hydrolysis tubes were frozen, evacuated, and filled with nitrogen (repeat the evacuation and filling with nitrogen three times), in the nitrogen-filled state, the sealed hydrolysis tubes were placed in a hydrolysis furnace at 110°C and hydrolyzed for 22 h. The hydrolysate was filtered into a 50 mL volumetric flask and fixed to 50 mL. Then, 1 mL of the filtrate was pipetted, dried under reduced pressure, and then added with 2 mL of sodium citrate buffer solution. After filtration, it was transferred to the injection bottle for detection. The supernatant was filtered and analyzed using a liquid chromatograph (LC5090, FULI instruments, China) with ultraviolet absorption detector (detection wavelength: 360 nm). A total of 17 amino acids were determined: aspartic acid (ASP), glutamic acid (GLU), serine (SER), arginine (ARG), glycine (GLY), threonine (THR), proline (PRO), alanine (ALA), valine (VAL), methionine (MET), cysteine (CYS), isoleucine (ILE), leucine (LEU), phenylalanine (PHE), histidine (HIS), lysine (LYS), and tyrosine (TYR).

2.9 | Biochemical Analysis

Triglyceride (TG) assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used for the determination of TGs in muscle tissue, and the procedure was carried out according to the instructions.

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the expression of G protein-coupled receptors (GPCRs) (GPR41 and GPR43) in caecum (Jiangsu Meimian Industrial Co. Ltd., China) as well as to determine the content of L-carnitine in the biceps femoris muscle (Shanghai Jianglai Industrial Co. Ltd., China).

2.10 | RT-qPCR

Total mRNA was extracted from biceps femoris muscles and caecum tissues of rabbits using TransZol Up (TransGen Biotech, Beijing, China) reagent and liquid nitrogen. After the total mRNA was extracted, the mRNA quality was measured by NanoDrop One (Thermo Fisher Scientific, USA) spectrophotometer. The cDNA was synthesized by HiScript II Reverse Transcriptase (Vazyme Biotech Co. Ltd., Nanjing, China). Subsequently, ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co. Ltd., Nanjing, China) was used to perform real-time qPCR by CFX96 Real-Time System (Bio-Rad, USA). We measured mRNA expression levels by the calculation method of $2^{-\Delta\Delta CT}$. The level of *GAPDH* mRNA was used as an internal reference for caecum tissue, and the level of *β-actin* mRNA was used as an internal reference for muscle tissue. The primer sequences used in this study were displayed in Table S3.

2.11 | Western Blotting

Tissue proteins were first extracted using a RIPA lysate (Beyotime, China) containing a protease inhibitors cocktail (Yamei, Shanghai, China) and a phosphatase inhibitors cocktail (Yamei). Protein concentrations were measured using a BCA kit (Yamei). The protein content of all samples was normalized to 4 μg/μL. The same amount of protein (20 μg) was electrophoresed on a 4%–12% SDS-PAGE gel and transferred to a 0.22 μm PVDF membrane. The membranes were blocked using 5% skimmed milk powder, and after blocking, the membranes were incubated overnight at 4°C using primary antibodies. Subsequently, the membrane was washed using TBST (45 min) and a room temperature incubation of secondary antibody (1 h) was performed. Finally, the membrane was washed again using TBST (45 min), followed by image capture using an imaging system (Monad, China) and quantification using Image J. Antibody information is displayed in Table S4.

2.12 | Histological and Immunofluorescent Staining

Histological and immunofluorescence staining was determined on behalf of the organisms by Pinofei Biological Co. Ltd. (Wuhan, China) according to previously reported methods, and

the experimental steps were broadly as follows. After dehydration and transparency, the tissue was embedded into wax blocks. Then, 5 μm thick paraffin sections were dewaxed to water and used for alcian blue-periodic acid Schiff.

After antigen repair, endogenous enzyme blocking, and blocking, the tissue sections were incubated with primary antibody overnight at 4°C. Subsequently, the secondary antibody was incubated at room temperature for 1 h. Finally, DAP1 was used to stain the nucleus for 5 min and washed three times with PBS. These slices were scanned and observed by PANNORAMIC MIDI (3DHISTECH, Hungary).

2.13 | Serum UHPLC-QE-MS Untargeted Metabolomics

Serum untargeted metabolomic assays were performed by Biotree Biotech (Shanghai, China). Total of 100 μL of serum was transferred to an EP tube. After the addition of 400 μL of extract solution (acetonitrile: methanol = 1:1, containing isotopically labeled internal standard mixture), the samples were vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at −40°C to precipitate proteins. Then, the sample was centrifuged at 12 000 rpm (RCF = 13 800(×*g*), *R* = 8.6 cm) for 15 min at 4°C. The resulting supernatant was transferred to a fresh glass vial for analysis. The quality control sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

LC–MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm) coupled to a Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH 9.75) (A) and acetonitrile (B). The autosampler temperature was 4°C, and the injection volume was 2 μL.

2.14 | 16S rRNA Gene Sequencing

16S rRNA gene sequencing was performed by Personalbio (Shanghai, China). The experimental methodology for 16S rRNA gene sequencing is consistent with that described in one of our previous publications [29]. In short, total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, USA). PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). After purification, quantification, and equal mixing of PCR amplicons, the Illumina NovaSeq platform was used for sequencing.

2.15 | Short-Chain Fatty Acids Analysis

Determination of the content of short-chain fatty acids (SCFAs) in the contents of the caecum was done by Bioprofile (China, Shanghai). Six SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid) were prepared as 100 mg/mL aqueous working standard solutions. Hexanoic acid standard was dissolved in diethyl ether to prepare a 100 mg/mL working solution. The internal standard (4-methylvaleric acid) solution was prepared at 375 μg/mL in diethyl ether. Serial standard solutions spanning 0.02–500 μg/mL (10 calibration points) were prepared by mixing: 200 μL of the six SCFAs working standard solutions; 100 μL 15% (v/v) phosphoric acid; 20 μL hexanoic acid working solution; 20 μL internal standard solution; and 260 μL diethyl ether.

Metabolite extraction starts with weighing a 50 mg sample. The samples were homogenized for 1 min using 500 μL deionized water and 100 mg glass beads, followed by centrifugation at 12 000 rpm (4°C) for 10 min. Subsequently, 200 μL of the resulting supernatant was mixed with 100 μL 15% (v/v) phosphoric acid, 20 μL of 375 μg/mL 4-methylvaleric acid internal standard solution, and 280 μL diethyl ether. After vigorous vortex mixing for 1 min, the mixture was centrifuged again under identical conditions (12 000 rpm, 4°C, 10 min). The upper organic layer was carefully collected and transferred to GC vials for subsequent analysis.

Chromatographic separation was achieved using a TRACE 1300 gas chromatograph (Thermo Fisher Scientific) equipped with an Agilent HP-INNOWAX capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). High-purity helium carrier gas was maintained at a constant flow rate of 1.0 mL/min. The injection port operated in split mode (10:1 ratio) at 250°C with a 1 μL injection volume. The program was set as follows: Initial hold at 90°C for 1 min; 10°C/min to 120°C; 5°C/min to 150°C; 25°C/min to 250°C (held for 2 min). Mass spectra were performed on ISQ 7000 (Thermo Fisher Scientific) with an electron bombardment ionization source, SIM scanning mode, with an electron energy of 70 eV.

2.16 | Statistical Analysis

Statistical analysis system was used to perform a one-way ANOVA by SPSS 24.0 (*$p < 0.05$; **$p < 0.01$; $0.05 \leq p < 0.1$ indicates a tendency to show significant differences). Statistics and plotting were done using GraphPad Prism 8 software. Heatmap was plotted using heatmap tools in the genescloud platform (https://www.genescloud.cn).

3 | Results

3.1 | Effects of Welfare Housing Systems Based on EE on the Growth Performance and Cognitive Behavior of Rabbits

In the process of feeding experiments, we carried out rabbit behavioral tests (Behavioral observations, T-maze test, and sucrose preference test). As demonstrated in Figure S1A, we found that the proportion of exploratory behavior in the PE group was significantly higher than that in the other two groups ($p < 0.01$). The proportion of stereotypic behavior in the Pen and PE groups was significantly lower than that in the Cage group (Figure S1A, $p < 0.01$). In addition, the proportion of subordinate behavior in the PE group was significantly lower than that in the Pen group

TABLE 1 | Physical properties of the biceps femoris muscle of the Cage, Pen, and PE groups.

Parameter	Treatment		
	Cage	Pen	PE
Cooking loss (%)	22.63 ± 1.30	24.34 ± 1.60	23.54 ± 2.58
WBSF(N)	53.01 ± 7.65	52.09 ± 9.23	51.44 ± 4.28
Color *L** (lightness)	55.43 ± 2.82[b]	58.44 ± 1.15[ab]	60.41 ± 2.08[a]
Color *a** (redness)	3.88 ± 0.37[b]	5.19 ± 1.03[a]	3.98 ± 0.49[b]
Color *b** (yellowness)	4.47 ± 0.79	4.68 ± 0.69	5.72 ± 1.01
pH	6.68 ± 0.15	6.68 ± 0.14	6.47 ± 0.27

Note: All data are presented as means ± SD; $n = 6$.
[a]and [b]mean in the same row with differ significantly at 0.05; [ab]means in the same row with no obvious difference.

(Figure S1A, $p < 0.05$). Through the sucrose preference test, we found that the percentage of sucrose intake of rabbits in the Pen group (Figure S1B,C, $p < 0.05$) and the PE group ($p < 0.01$) was higher than that in the Cage group. The subsequent T-maze test indicated that there was no significant difference in the accuracy rate of selecting the correct arm among the three groups of rabbits (Figure S1D, $p > 0.05$). Interestingly, we found that the time required for the rabbits in the Pen group (Figure S1E, $p < 0.05$) and the PE group (Figure S1E, $p < 0.01$) to reach the correct arm was significantly shorter than that in the Cage group. The above results indicated that the Pen group and PE group can improve the anhedonia performance and spatial memory ability of rabbits. In other words, the cognitive behavior of rabbits in those two groups was significantly improved. According to the results of behavioral tests, Pen and PE groups can be identified as welfare housing systems.

As demonstrated in Figure 1E,F, no significant differences were observed in initial weight, slaughter weight, and carcass weight. However, the slaughter weight and carcass weight of the Pen group tended to decrease compared with the other two groups (Figure 1F). In addition, the average daily weight gain (Figure 1H) of the Pen group was lower than that of the other two groups, but not significantly. Subsequent feed conversion ratio (Figure 1I) results illustrated that the feed conversion ratio of the Pen group was higher than that of the other two groups. Based on the results of behavioral tests in rabbits, we also examined the content of neurotransmitters (GABA, Dopamine, 5-HT, and NAD/NADP) in the brain tissue of rabbits. The content of GABA in the Pen group and PE group was significantly higher than that in the Cage group (Figure 1J, $p < 0.01$). In addition, we also found that the content of DA, 5-HT, and NAD/NADP in the PE group was significantly higher than that in the Cage group (Figure 1J, $p < 0.05$).

3.2 | Effects of Welfare Housing Systems Based on EE on the Meat Quality of Rabbits

Differences among the three groups of rabbits were found for the physical attributes of the biceps femoris muscle (Table 1). Specifically, a higher *a** color value was found in the Pen group compared with other groups ($p < 0.05$). In addition, the *L** color value in the PE group was significantly higher than that in the Cage group ($p < 0.05$). Nevertheless, there was no significant difference in cooking loss, WBSF, color *b**, and pH among the three groups. Subsequent experiments showed that the increase in redness of rabbit leg muscles in the Pen group may be due to the increase in the number of slow muscle fibers in rabbit leg muscles (Figure S2).

The fatty acid composition of the biceps femoris muscle is reported in Table 2. In terms of saturated fatty acids (SFAs), the concentrations of C14:0 and C16:0 in the Pen group were notably lower than those in the Cage group. The concentration of C12:0 in the PE group was significantly higher than that in the remaining two groups. For monounsaturated fatty acids (MUFAs), the concentrations of various MUFA (C14:1, C16:1, C18:1, and total MUFA) in the Pen group were significantly lower than those in the Cage group. Compared to the Cage group, the Pen group had an increased concentration of C20:3n3 but a decreased concentration of C18:3n3.

The amino acid composition of the biceps femoris muscle is reported in Table 3. In terms of EAAs, the proportions of THR, VAL, ILE, LEU, HIS, and TEAA were significantly higher in samples from the Pen group than in the remaining two groups ($p < 0.05$). The proportion of LYS was significantly higher in samples from the Pen group than in samples from the Cage group ($p < 0.05$). In addition, the proportion of HIS was significantly higher in the samples from the PE group than in the remaining two groups ($p < 0.05$). As far as non-EAAs are concerned, the proportions of ASP and GLU in the samples from the Pen group were significantly higher than those in the remaining two groups ($p < 0.05$). In addition, the proportions of SER, ALA, ARG, and TNAA in the samples from the Pen group were significantly higher than those from the Cage group ($p < 0.05$). Overall, the amino acid composition of the Pen group was significantly different from that of the remaining two groups. Moreover, the FAA and SAA ratios of the Pen group were significantly higher than those of the Cage group ($p < 0.05$).

3.3 | The Pen Group Reduced Fat Deposition in the Leg Muscle by Increasing the Fatty Acid β-Oxidation

After dissecting the rabbit, we collected the biceps femoris muscle and stained it with Oil red O. The lipid deposition status of the

TABLE 2 | Fatty acids profile (mg/100 g) of the biceps femoris muscle in the Cage, Pen, and PE groups.

	Treatment		
Fatty acid (mg/100 g)	**Cage**	**Pen**	**PE**
C12:0	4.01 ± 1.29^{b}	3.81 ± 0.98^{b}	6.95 ± 3.60^{a}
C14:0	60.39 ± 26.98^{a}	27.33 ± 7.21^{b}	44.70 ± 20.47^{ab}
C15:0	15.77 ± 3.40	11.14 ± 1.73	15.19 ± 6.22
C16:0	937.57 ± 285.94^{a}	630.36 ± 85.05^{b}	827.02 ± 274.95^{ab}
C17:0	17.18 ± 3.13	14.09 ± 2.74	17.01 ± 6.34
C18:0	300.71 ± 49.45	255.58 ± 41.59	291.84 ± 75.04
Other SFA	9.92 ± 2.92	9.18 ± 1.08	12.29 ± 4.63
Total SFA	1345.55 ± 150.01	951.49 ± 54.03	1215.00 ± 158.61
C14:1	3.85 ± 1.19^{a}	2.05 ± 0.28^{b}	2.84 ± 0.77^{ab}
C16:1	60.08 ± 28.53^{a}	19.33 ± 6.43^{b}	28.92 ± 14.77^{b}
C18:1	589.58 ± 184.53^{a}	355.36 ± 73.31^{b}	467.14 ± 176.69^{ab}
C20:1	7.81 ± 2.77	4.92 ± 1.24	6.68 ± 3.33
Other MUFA	5.13 ± 1.02^{ab}	3.28 ± 1.09^{b}	7.63 ± 3.42^{a}
Total MUFA	666.46 ± 215.84^{a}	384.95 ± 880.18^{b}	513.21 ± 191.16^{ab}
C18:2	776.44 ± 143.51	651.63 ± 95.78	706.33 ± 152.19
C18:3 n6	5.25 ± 0.76	4.57 ± 0.97	5.35 ± 1.59
C18:3n3	41.66 ± 10.45^{a}	27.90 ± 7.24^{b}	31.75 ± 11.75^{ab}
C20:2	9.30 ± 3.27	6.82 ± 1.82	6.91 ± 1.68
C20:3n6	5.50 ± 0.86	5.59 ± 0.74	5.34 ± 0.51
C20:3n3	105.34 ± 15.25^{b}	126.60 ± 11.81^{a}	115.17 ± 19.44^{ab}
C20:4	2.39 ± 1.39^{a}	1.43 ± 0.65^{ab}	1.19 ± 0.19^{b}
C22:6	1.45 ± 0.25	1.42 ± 0.63	1.22 ± 0.36
Other PUFA	3.45 ± 1.45	2.22 ± 0.41	3.89 ± 2.43
Total PUFA	950.78 ± 155.67	828.18 ± 109.55	877.16 ± 175.76
n-3	146.99 ± 16.14	154.50 ± 14.22	146.92 ± 25.63
n-6	10.75 ± 1.22	10.16 ± 1.22	10.69 ± 1.85
Total fatty acids	2962.79 ± 713.98^{a}	2164.61 ± 312.61^{b}	2605.37 ± 732.14^{ab}

Note: All data are presented as means ± SD; $n = 6$.
[a]and [b]mean in the same row with differ significantly at 0.05; [ab]means in the same row with no obvious difference.

hind leg muscle was evaluated by oil red O staining (Figure 2A). After quantifying the stained red positive part, we found that the lipid deposition in the Pen group was significantly lower than that in the Cage group (Figure 2A,B, $p < 0.01$) and the PE group (Figure 2A,B, $p < 0.05$). In addition, the content of TG (Figure 2C) was determined, and the results were consistent with the results of oil red O staining. That is, the rabbits of the Pen group reduced hind leg muscle lipid deposition.

To investigate the cause of this phenomenon, the metabolites in the serum samples of the three groups were investigated using UHPLC-QE-MS. As demonstrated in Figure 2D, PCA revealed that the Cage group and the PE group were significantly separated from the Pen group. The KEGG pathways enriched by differential metabolites between the Cage and PE groups are shown in Figure 2E. Subsequently, we identified two different metabolites associated with fatty acid β-oxidation by comparing serum metabolites between the Cage group and the Pen group, which were L-carnitine and L-acetylcarnitine. The relative content of L-carnitine in the Pen group was significantly higher than that in the Cage group (Figure 2F, $p < 0.01$) and the PE group (Figure 2F, $p = 0.0523$). In addition, there was a significant difference in Acetylcarnitine between the Pen versus Cage and Pen versus PE (Figure 2F, $p < 0.01$). Subsequently, we observed that the expression of *TMLHE* related to carnitine synthesis was significantly upregulated in the Pen group compared with the Cage group (Figure 2H, $p < 0.05$).

TABLE 3 | Amino acids profile of the biceps femoris muscle in the Cage, Pen, and PE groups.

	Treatment		
Amino acids (%)	**Cage**	**Pen**	**PE**
Essential amino acids			
LYS	8.81 ± 0.39^{b}	9.40 ± 0.35^{a}	9.07 ± 0.20^{ab}
MET	1.45 ± 0.14	1.50 ± 0.33	1.57 ± 0.27
THR	4.07 ± 0.11^{b}	4.33 ± 0.13^{a}	4.18 ± 0.09^{b}
PHE	3.67 ± 0.10	3.85 ± 0.13	3.47 ± 0.53
VAL	4.84 ± 0.13^{b}	5.05 ± 0.15^{a}	4.85 ± 0.10^{b}
ILE	4.29 ± 0.12^{b}	4.56 ± 0.14^{a}	4.37 ± 0.08^{b}
LEU	7.10 ± 0.22^{b}	7.45 ± 0.22^{a}	7.17 ± 0.15^{b}
HIS	3.44 ± 0.18^{b}	3.45 ± 0.25^{b}	3.79 ± 0.19^{a}
Total EAA (TEAA)	32.78 ± 1.04^{b}	34.65 ± 1.08^{a}	33.12 ± 0.62^{b}
Nonessential amino acid			
ASP	8.47 ± 0.27^{b}	8.84 ± 0.27^{a}	8.53 ± 0.18^{b}
SER	3.70 ± 0.13^{b}	3.89 ± 0.13^{a}	3.77 ± 0.10^{ab}
GLU	13.88 ± 0.54^{b}	14.64 ± 0.41^{a}	13.98 ± 0.28^{b}
GLY	4.43 ± 0.11	4.53 ± 0.23	4.54 ± 0.28
ALA	4.90 ± 0.13^{b}	5.12 ± 0.18^{a}	4.96 ± 0.14^{ab}
TYR	2.58 ± 0.15	2.76 ± 0.15	2.70 ± 0.16
ARG	5.95 ± 0.18^{b}	6.22 ± 0.19^{a}	6.05 ± 0.14^{ab}
PRO	3.37 ± 0.10	3.46 ± 0.11	3.45 ± 0.17
CYS	0.47 ± 0.05	0.45 ± 0.03	0.44 ± 0.03
Total NAA (TNAA)	52.65 ± 1.37^{b}	54.86 ± 1.48^{a}	53.75 ± 1.18^{ab}
Total amino acid (TAA)	85.43 ± 2.40^{b}	89.51 ± 2.53^{a}	86.87 ± 1.60^{ab}
FAA	33.51 ± 1.09^{b}	35.20 ± 0.97^{a}	33.64 ± 0.71^{b}
SAA	16.58 ± 0.63^{b}	17.63 ± 0.59^{a}	16.99 ± 0.37^{ab}
AAA	6.26 ± 0.20	6.60 ± 0.18	6.17 ± 0.58

Note: All data are presented as means ± SD; $n = 6$.
Abbreviations: AAA, aromatic amino acids = TYR + PHE; FAA, flavorful amino acids = ASP + GLU + PHE + ALA + TYR; SAA, sweetening amino acids = SER + THR + LYS.
[a]and [b]mean in the same row with differ significantly at 0.05; [ab]means in the same row with no obvious difference.

We also measured L-carnitine (Figure 2G) content in muscle by ELISA, and the results were consistent with the previous results. In addition, we observed upregulated expressions of Lipolysis (*ATGL*, *LIPE*, and *PNPLA2*) and fatty acid β-oxidation (*CPT1β*, *CPT2*, and *PPARα*) in the Pen group (Figure 2I–L). In brief, the reduced lipid accumulation in the leg muscles of the Pen group may be due to increased fatty acid β-oxidation.

3.4 | Effects of Welfare Housing Systems Based on EE on the Caecum Microflora of Rabbits

To investigate the effects of welfare housing systems based on EE on the gut microbiota, we performed 16S rRNA gene amplicon sequencing on caecum content from the three groups. For α-diversity, the trends of the Shannon and Simpson indices were consistent among the three groups (Figure 3A). There was no difference in the Good's coverage index between the three groups, and the mean values were all > 0.98. Pielou's evenness index showed a significant difference between the Cage group and the PE group ($p < 0.01$). We observed a clear separation among the three groups using Bray-Curtis distance-based principal coordinate analysis (Figure 3B, ANOSIM: $p = 0.001$, $r = 0.50$). The Firmicutes, Bacteroidetes, and Tenericutes were the most abundant phyla detected in the caecum contents (Figure 3C). The *Ruminococcaceae_UCG-014*, *Muribaculaceae*, and *Ruminococcaceae_NK4A214_group* were the most abundant genera detected in the caecum contents (Figure 3D). The relative abundance of Firmicutes in the PE group was significantly lower than that in the other groups (Figure 3E, $p < 0.01$). However, the results of Bacteroidetes were opposite to

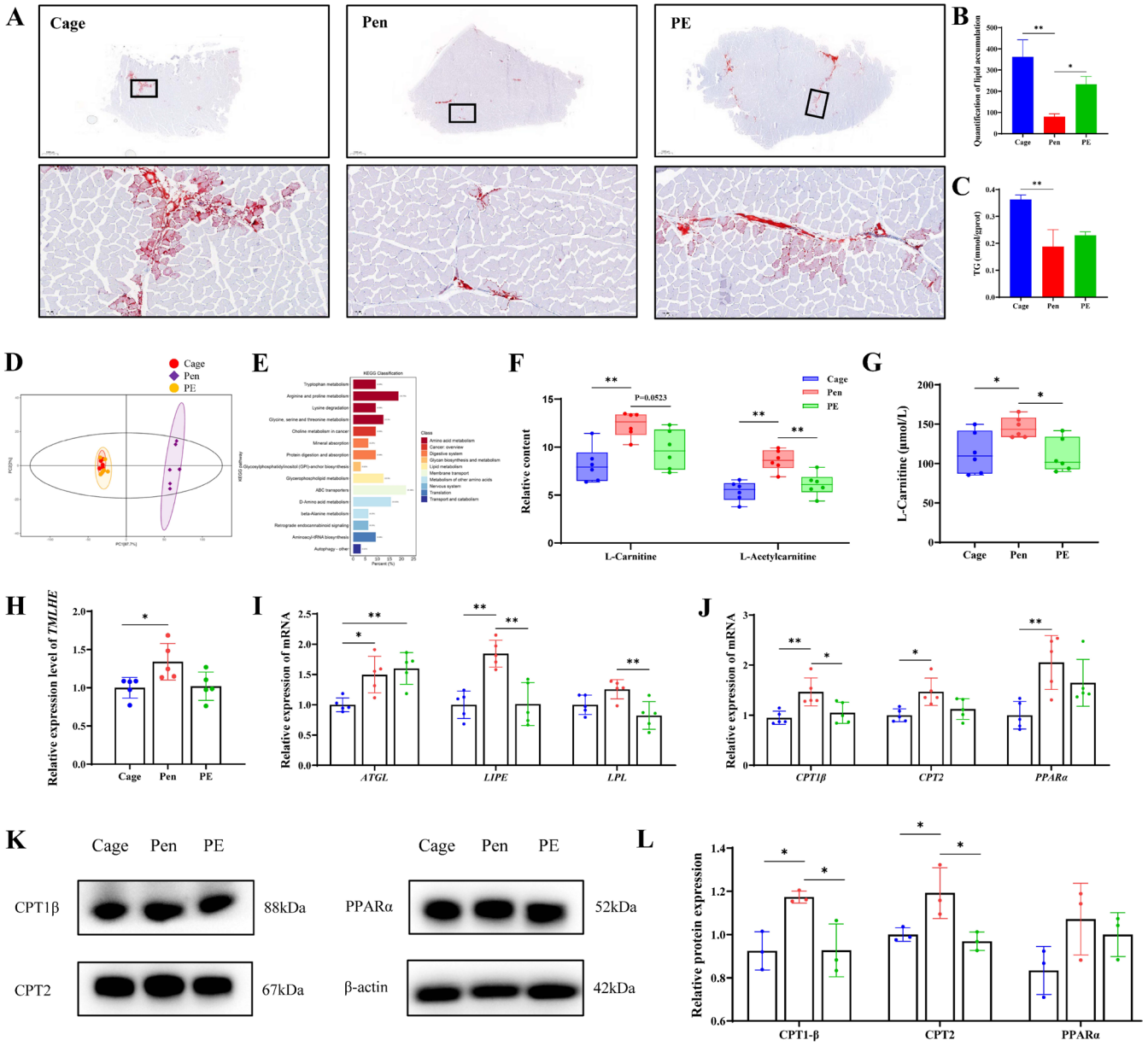

FIGURE 2 | The Pen group reduced fat deposition in the biceps femoris muscle by increasing the fatty acid β-oxidation. Oil red O staining (A) of hind leg muscle. Quantitative results of Oil Red O staining (B). Triglyceride (TG) content in hind leg muscle of rabbits (C). Score plots of PCA (D) of serum metabolome among the three groups. KEGG classification diagram of differential metabolites between Cage group versus Pen group (E). The relative content of L-carnitine and L-acetylcarnitine (F) was detected by serum metabolomics. The content of L-carnitine (G) was detected by ELISA. The RT-qPCR analysis of *TMLHE* (H), *ATGL*, *LIPE*, *LPL*, *CPT1β*, *CPT2*, and *PPARα* in the biceps femoris muscle samples (I, J). Western blots of CPT1β, CPT2, and PPARα (K). Quantitative results of western blots (L). (Cage = Cage models, Pen = Pen models, PE = Pen + environmental enrichment materials models; $^{*}p < 0.05$; $^{**}p < 0.01$).

those of Firmicutes (Figure 3E, $p < 0.01$). In addition, we found that the relative abundance of *Muribaculaceae* in the PE group was significantly higher than in the other two groups, and the relative abundance of *Ruminococcaceae_NK4A214_group* in the Cage group was significantly higher than in the other two groups (Figure 3F, $p < 0.01$). Based on the random forest analysis, the top 20 crucial genera included *Muribaculum*, *Mollicutes_RF39*, *Lachnospiraceae_UCG_010*, and *Muribaculaceae* (Figure 3G). According to the Venn diagram of the top 10 genera in abundance and the top 20 genera in importance, three species were identified, including *Muribaculaceae*, *Clostridiales_vadinBB60_group*, and *Mollicutes_RF39* (Figure 3H). The relative abundance results of primary and secondary metabolic pathways in the MetaCyc database are demonstrated in Figure 3I. Compared with the Cage group, the upregulated metabolic pathways in the PE group included PWY-6876, ORNDEG-PWY, PWY-6629, ARGDEG-PWY, ORNARGDEG-PWY, PWY-5181, METHGLYUT-PWY, THREOCAT-PWY, PWY-5384, PWY-7446, PWY-5430, PWY-5178, and PWY-6562 (Figure 3J, Table S2). It is worth noting that the metabolic pathway of PWY-5384 is related to sucrose degradation IV (sucrose phosphorylase). By linking the pathway to microorganisms, we found that genera such as *Subdoligranulum*, *Roseburia*, and *Tyzzerella_3* are associated with the PWY-5384 pathway (Figure 3K).

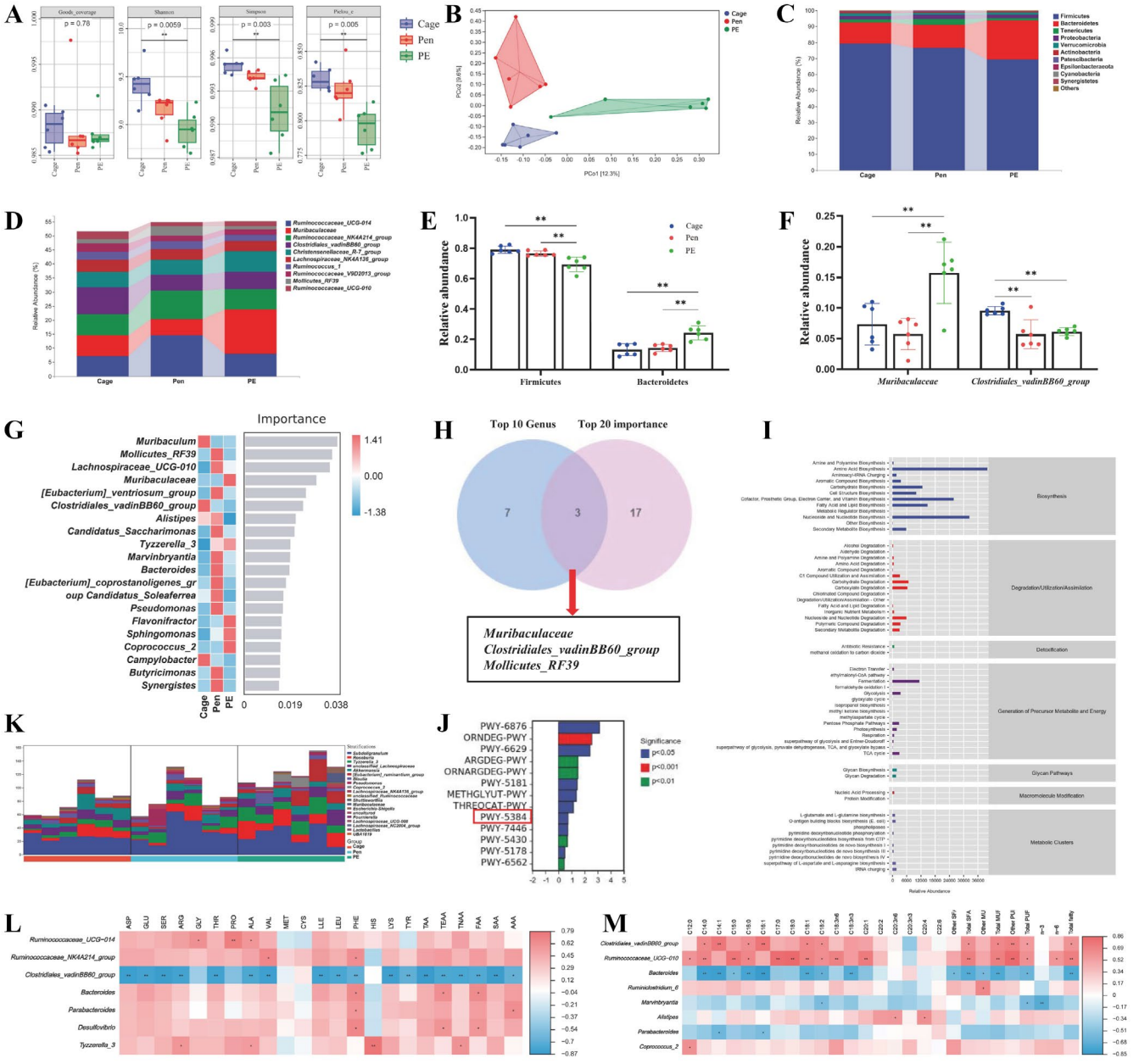

FIGURE 3 | The effects of welfare housing systems based on environmental enrichment on caecum microflora of rabbits. Alpha diversity of microbial communities in Cage, Pen, and PE groups, including Goods coverage, Shannon index, Simpson index, and Pielou evenness (A). Coordination plot of principal coordinate analysis (PCoA) results of microbial communities among Cage, Pen, and PE groups based on Bray_Curtis dissimilarity (B). The top 10 most abundant gut microbiota at the phyla (C) and genus (D) levels. The relative abundance of Firmicutes, Bacteroidetes, *Muribaculaceae*, and *Clostridiales_vadinBB60_group* (E, F). Genus-level analysis of the gut microbiota using the random forest model. The abscissa indicates the importance of species to the classifier model, while the ordinate indicates the taxa name at the genus level. From top to bottom, the importance of species to the model decreases (G). Venn diagram of two groups with top 10 abundance and top 20 importance (H). The analysis results of MetaCyc metabolic pathway including various pathways involved in primary and secondary metabolism (I), differential metabolic pathways (J), and the microbial composition of differential metabolic pathways (K) of Cage, Pen, and PE groups. Spearman's analysis of the correlations between relative abundance of top 50 genera and amino acids (L); spearman's analysis of the correlations between relative abundance of top 50 genera and fatty acids (M). (Cage = Cage models, Pen = Pen models, PE = Pen + environmental enrichment materials models; $^{*}p < 0.05$; $^{**}p < 0.01$).

3.5 | Correlation Analysis

Spearman's correlation analysis was conducted to investigate the correlations between relative abundance of top 50 genera and amino acids (Figure 3L). Our results show that the abundance of *Ruminococcaceae_UCG-014* is positively correlated with the content of GLY ($r = 0.621$, $p = 0.024$), PRO ($r = 0.725$, $p = 0.005$), and ALA ($r = 0.588$, $p = 0.035$). The abundance of *Ruminococcaceae_NK4A214_group* was positively correlated with VAL ($r = 0.681$, $p = 0.010$) and PHE ($r = 0.626$, $p = 0.022$). The abundance of *Bacteroide* and *Desulfovibrio* was positively correlated with PHE ($r = 0.659$, $p = 0.014$; $r = 0.670$, $p = 0.012$),

TEAA ($r=0.626$, $p=0.022$; $r=0.599$, $p=0.031$), and FAA ($r=0.643$, $p=0.018$; $r=0.588$, $p=0.035$). In addition, the abundance of *Clostridiales_vadinBB60_group* was negatively correlated with ASP ($r=-0.830$, $p<0.001$); GLU ($r=-0.791$, $p=0.001$); SER ($r=-0.841$, $p<0.001$); ARG ($r=-0.797$, $p=0.001$); THR ($r=-0.808$, $p<0.001$); ALA ($r=-0.753$, $p=0.002$); VAL ($r=-0.824$, $p<0.001$); ILE ($r=-0.808$, $p<0.001$); LEU ($r=-0.758$, $p=0.003$); PHE ($r=-0.692$, $p=0.009$); LYS ($r=-0.819$, $p<0.001$); TYR ($r=-0.687$, $p=0.010$); TAA ($r=-0.868$, $p<0.001$); TEAA ($r=-0.747$, $p=0.003$); TNAA ($r=-0.764$, $p=0.002$); FAA ($r=-0.731$, $p=0.005$); SAA ($r=-0.841$, $p<0.001$); and AAA ($r=-0.681$, $p=0.010$).

Spearman's correlation analysis was conducted to investigate the correlations between the relative abundance of the top 50 genera and fatty acids (Figure 3M). Our results show that the abundance of *Clostridiales_vadinBB60_group* is positively correlated with C14:0 ($r=0.654$, $p=0.015$); C14:1 ($r=0.769$, $p=0.002$); C16:0 ($r=0.632$, $p=0.021$); C16:1 ($r=0.780$, $p=0.002$); C18:1 ($r=0.670$, $p=0.012$); C18:2 ($r=0.626$, $p=0.022$); total SFA ($r=0.615$, $p=0.025$); total MUFA ($r=0.676$, $p=0.011$); other PUFA ($r=0.725$, $p=0.005$); total PUFA ($r=0.555$, $p=0.049$); and total fatty acid ($r=0.604$, $p=0.027$). The abundance of *Ruminococcaceae_UCG-010* was positively correlated with C12:0 ($r=0.593$, $p=0.033$); C14:0 ($r=0.692$, $p=0.009$); C15:0 ($r=0.786$, $p=0.001$); C16:0 ($r=0.676$, $p=0.011$); C17:0 ($r=0.824$, $p<0.001$); C18:0 ($r=0.841$, $p<0.001$); C18:1 ($r=0.703$, $p=0.007$); C18:2 ($r=0.626$, $p=0.022$); C18:3n6 ($r=0.703$, $p=0.007$); C20:1 ($r=0.736$, $p=0.004$); total SFA ($r=0.720$, $p=0.006$); total MUFA ($r=0.720$, $p=0.006$); other PUFA ($r=0.857$, $p<0.001$); total PUFA ($r=0.610$, $p=0.027$); n-6 ($r=0.577$, $p=0.039$); and total fatty acids ($r=0.720$, $p=0.006$). In addition, the abundance of *Bacteroides* was negatively correlated with C14:0 ($r=-0.797$, $p=0.001$); C14:1 ($r=-0.808$, $p<0.001$); C15:0 ($r=-0.566$, $p=0.044$); C16:0 ($r=-0.764$, $p=0.002$); C16:1 ($r=-0.852$, $p<0.001$); C18:1 ($r=-0.731$, $p=0.005$); C18:2 ($r=-0.676$, $p=0.011$); C18:3n3 ($r=-0.720$, $p=0.006$); other SFA ($r=-0.588$, $p=0.035$); total SFA ($r=-0.714$, $p=0.007$); other MUFA ($r=-0.582$, $p=0.037$); total MUFA ($r=-0.736$, $p=0.004$); total PUFA ($r=-0.643$, $p=0.018$); and total fatty acids ($r=-0.731$, $p=0.005$).

3.6 | The Two Welfare Housing Systems Can Enhance the Intestinal Barrier and Anti-Inflammatory Effects by Activating SCFAs Receptors

In order to better explore the changes of intestinal microflora among the three groups, we detected the content of SCFAs in the caecum contents using GC–MS (Figure 4A). We found that the content of acetic acid in the caecum contents of the PE group was the highest ($p<0.01$). The contents of propionic, butyric, valeric, and caproic acids were significantly higher in the Pen and PE groups than those in the Cage group ($p<0.05$). We subsequently correlated the top 50 genera in abundance with SCFAs using Spearman's correlation analysis (Figure 4B). The results showed that the abundance of *Ruminococcaceae_NK4A214_group* was positively correlated with acetic acid ($r=0.636$, $p=0.026$) and butyric acid ($r=0.699$, $p=0.011$); the abundance of *Ruminococcaceae_UCG-014* was positively correlated with butyric acid ($r=0.615$, $p=0.033$); the abundance of *Mollicutes_RF39* was positively correlated with acetic acid ($r=0.671$, $p=0.016$); the abundance of *Clostridiales_vadinBB60_group* was negatively correlated with acetic acid ($r=-0.909$; $p<0.001$), propionic acid ($r=-0.804$; $p=0.002$), butyric acid ($r=-0.860$; $p<0.001$), and valeric acid ($r=-0.699$; $p=0.011$); the abundance of *Ruminococcaceae_UCG-010* was negatively correlated with acetic acid ($r=-0.650$; $p=0.022$), propionic acid ($r=-0.657$; $p=0.020$), and butyric acid ($r=-0.720$; $p=0.008$); the abundance of *Ruminococcaceae_UCG-005* ($r=-0.650$; $p=0.022$), *Ruminiclostridium_1* ($r=-0.867$; $p<0.001$), and *Pygmaiobacter* ($r=-0.594$; $p=0.042$) was negatively correlated with acetic acid. We then calculated the total abundance of genera that were positively and negatively correlated with SCFAs among the three groups based on the correlation results. The results showed that the total abundance of genera negatively correlated with SCFAs was significantly lower in the Pen group as well as in the PE group than in the Cage group (Figure 4C, $p<0.01$). In addition, the total abundance of genera positively correlated with SCFAs was significantly higher in the Pen group than in the other two groups (Figure 4D, $p<0.01$).

Since the content of SCFAs and genera closely related to SCFAs varied considerably among the three groups, we examined the expression levels of GPCRs (*GPR41* and *GPR43*) in the caecum tissues of the three groups (Figure 4E,F). Subsequently, we also investigated the expression levels of intestinal tight junction proteins, pro-inflammatory cytokines, and anti-inflammatory factors in the caecum tissues of the three groups (Figure 4G–J). The results showed that the expression of *ZO-1* and *Occludin* in the caecum tissue of the Pen group was significantly higher than that of the Cage group; the expression of *ZO-1* and *Claudin-1* in the caecum tissue of the PE group was significantly higher than that of the Cage group (Figure 4G,I–K). In addition, the expression of *IL-1β* (Figure 4H, $p<0.01$) in the caecum was significantly lower in the Pen group than in the Cage group; *IL-1β* (Figure 4H, $p<0.01$) and *TNF-α* (Figure 4H, $p<0.01$) in the caecum were significantly lower in the PE group than in the Cage group. Not only that, we also found that the expression of mucin and the number of goblet cells in the Pen group and the PE group were significantly higher than those in the Cage group (Figure S3). These results suggest that both welfare housing systems (Pen and PE) increase the content of SCFAs in the caecum contents and enhance the intestinal barrier and anti-inflammatory effects through GPCRs.

4 | Discussion

The increasing consumer demand for high-quality, healthier meat products, combined with the nutritional profile of rabbit meat, which aligns with the preferences for a healthy diet, has enhanced the popularity of rabbit meat. However, growing concerns about livestock welfare are placing significant pressure on the industry to improve animal welfare in rabbit farming. Consequently, there is an urgent need to develop housing systems that enhance rabbit welfare and meet consumer demand for healthy livestock products, thus benefiting both the industry and consumers. To meet this need, we designed two welfare housing systems based on EE (Pen and PE) and analyzed these two welfare housing systems in comparison with the Cage

group. The focus was on the effects of these housing systems on growth performance, meat quality, and gut health.

Behavioral observations revealed that rabbits in the Pen and PE groups exhibited significantly increased exploratory behavior and reduced stereotypic behavior compared to the Cage group. We suggest that the reduction in stereotypic behavior is associated with increased vigorous exercise in the Pen group and increased interaction with environmentally enriched materials in the PE group. Notably, rabbits in the Pen group chased

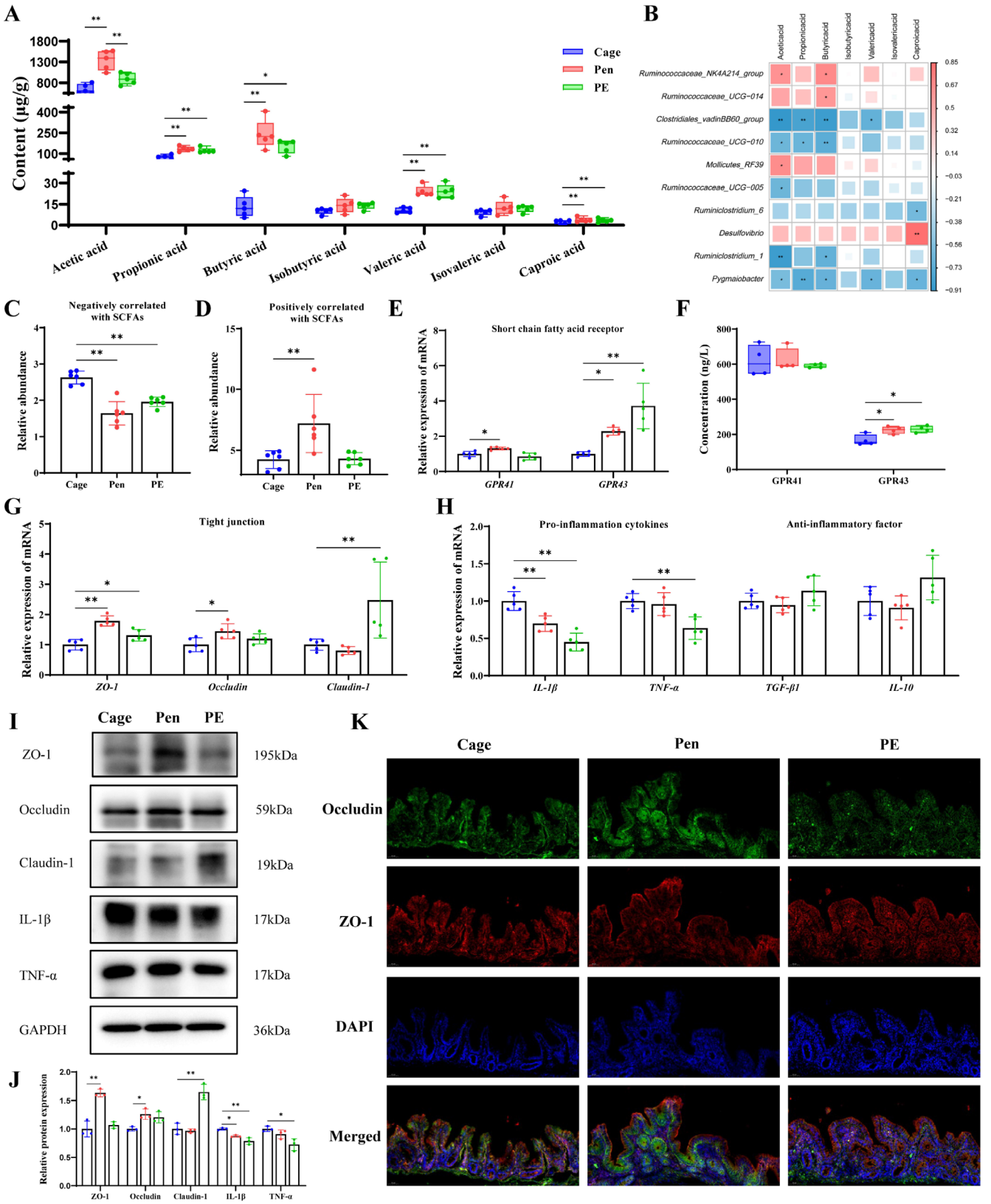

FIGURE 4 | Legend on next page.

FIGURE 4 | The rabbits of two welfare housing systems based on environmental enrichment can enhance the intestinal barrier and anti-inflammatory effects by activating short-chain fatty acid receptors. Short-chain fatty acid (SCFAs) in cecum contents (A). Correlation analysis of relative abundance of top 20 genera with SCFAs contents (B). The total abundance of microbial at the genus level, which negatively correlated with SCFAs contents (C). The total abundance of microbial at the genus level, which positively correlated with SCFAs contents (D). The RT-qPCR analysis of GPCRs (*GPR41* and *GPR43*) in caecum samples (E). GPR41 and GPR43 in caecum with Elisa (F). The RT-qPCR analysis of tight junction (*ZO-1*, *Occludin*, and *Claudin-1*) and inflammatory factor (pro-inflammation factor: *IL-1β* and *TNF-α*; anti-inflammatory factor: *TGF-β1* and *IL-10*) (G, H). Western blots showing ZO-1, Occludin, Claudin 1, IL-1β, and TNF-α expression in the caecum (I, G). Immunofluorescence staining of Occludin and ZO-1 in caecum tissue samples (K). *$p < 0.05$; **$p < 0.01$.

each other and ran across the pen, whereas those in the PE group showed greater interest in the enrichment materials. Additionally, rabbits in the Pen and PE groups stood on their hind legs more often, a natural behavior associated with predator awareness in the wild. These findings suggest that both systems allowed rabbits to express innate behaviors, enhancing their welfare. However, the Pen group also exhibited increased aggression and skin injuries, a phenomenon not observed in the PE group. Furthermore, greater activity has been shown to have multiple health benefits for rabbits, decreasing the risks of osteoporosis and gastrointestinal stasis [30] Therefore, increasing exploratory behavior can be considered a way to improve animal welfare. The T-maze and sucrose preference tests are widely used to assess cognitive behavior [27, 31, 32]. Therefore, we have also demonstrated through the T-maze and sucrose preference experiments that rabbits in the Pen group and the PE group improved cognitive behavior, thereby enhancing animal welfare. Neurotransmitters play an extremely important role in the cognitive behavior of animals and are involved in several physiological functions, including motor control, regulation of affective and emotional states, reward mechanisms, circadian rhythms, feeding, social interaction, and aggression, to name a few [33–35]. The above results provide further evidence of improved animal welfare in the Pen and PE groups of rabbits.

Growth performance indicators are commonly used to evaluate livestock production. Our study showed a tendency toward lower slaughter weight, carcass weight, and daily weight gain in rabbits from the Pen group compared to the other two groups. Additionally, the feed conversion ratio was higher in the pen group, which may be attributed to the increased exercise in this group. Enhanced physical activity is known to affect carcass traits and meat quality by influencing muscle development and fat deposition [36]. Previous research has demonstrated that increased exercise alters the type and size of muscle fibers, thereby impacting meat color [18]. Mammalian skeletal muscles have been classified into slow-twitch dark red muscles and fast-twitch lighter white muscles [37]. When we analyzed the effect of the Pen group on hind leg muscle color, our findings aligned with those of Gondret, and so forth [38], who reported that increased muscle redness is associated with enhanced oxidative metabolism and the presence of muscle fibers rich in mitochondria and myoglobin, both of which are linked to higher levels of exercise. Our results, as presented in Figure S2, further support this conclusion.

Amino acids are essential building blocks that sustain life. The role of amino acids in protein synthesis is well defined, but they also contribute to many other intracellular metabolic pathways, including ATP production, nucleotide synthesis, and redox homeostasis to support cellular and organismal functions [39]. Adequate consumption of amino acids, particularly EAAs that cannot be synthesized by the body and must be obtained through the diet, is crucial. Our study showed that the ratios of EAA, FAA, and SAA were significantly higher in the Pen group, which means that the nutrient content and taste of rabbit meat in the Pen group would be significantly improved. This aligns with consumer preferences for healthier dietary options. Furthermore, we examined the fatty acids composition in the leg muscles of rabbits and found significantly lower levels of SFA and MUFA in the Pen group, which may be attributed to increased physical activity. These findings are consistent with Fritzen et al., who reported that physical activity enhances fatty acid metabolism, providing an energy source [40]. Fatty acids content is closely linked to intramuscular fat (IMF) levels. Compared to other species, rabbit meat is lean and contains less IMF [41]. However, the IMF content is closely related to the flavor of the meat as well as the organoleptic properties [42]. Using oil red O staining, we observed a significant reduction in lipid deposition in the leg muscles of rabbits in the Pen group. Consequently, we explored the reasons for the reduction in SFA, MUFA, and fat deposition in the hind leg muscle of the Pen group. L-carnitine is a nutrient found in abundance in red meat, and it is one of the key nutrients that maintains the normal function of mitochondria and plays a significant role in fatty acid oxidation [43, 44]. Our results revealed increased L-carnitine content and enhanced fatty acid β-oxidation in the leg muscles of rabbits in the Pen group, explaining the reduced accumulation of fatty acids and lipids in these muscles.

The animal intestine harbors trillions of microorganisms that are critical to animal health, growth performance, and welfare [45]. Moreover, the intestinal microflora is closely related to the quality of food products originating from animals [45]. Gut dysfunction in rabbits, including intestinal inflammation and imbalances in the gut microbiota, poses a major challenge to animal welfare, growth performance, and farm profitability [46]. To address this, we conducted 16S rRNA gene sequencing of the caecum contents from three groups of rabbits. Most studies have correlated the ratio of Firmicutes to Bacteroidetes (F/B) with host weight as well as obesity. For instance, it has been shown that feeding a high-fructose, high-fat diet to rabbits significantly increases the F/B ratio in rabbits [47]. Additionally, studies using culture-free methods suggest that the *Muribaculaceae* specialize in the fermentation of complex polysaccharides and possess the capacity for propionate production [48–51]. Our results show that the F/B was lower in the Pen group as well as in the PE group, which explains the reduced lipid deposition and reduced daily weight gain in the Pen group. The reason for the unaltered phenotype of rabbits in the PE group may

be related to the PWY-5384 pathway (sucrose degradation IV [sucrose phosphorylase]). Further analysis revealed that the abundance of microbiota associated with PWY-5384 pathway activity was significantly higher in the PE group compared to the Cage group, suggesting that microorganisms in the caecum contents of the PE group contributed to improved sucrose breakdown and energy absorption. Moreover, the significantly higher *Muribaculaceae* content in the PE group further supports this conclusion.

It has been shown that PWY-5384 pathway (sucrose degradation IV (sucrose phosphorylase)) is associated with inflammatory bowel disease symptom relief [52, 53]. As metabolites of gut microbes, SCFAs can influence immunity as well as intestinal homeostasis via GPCRs [54]. Previous studies have shown that butyric and acetic acids in the gut play an important role in reducing intestinal inflammation and strengthening the intestinal barrier via GPCRs [55–57]. Our results are consistent with previous studies. Briefly, rabbits in the Pen group as well as in the PE group increased the content of SCFAs in the caecum contents, inhibited intestinal inflammation, and increased the intestinal barrier via GPCRS.

5 | Conclusions

In conclusion, two welfare housing systems can improve the meat quality of rabbits and inhibit intestinal inflammation and strengthen the intestinal barrier by regulating gut microbiota and metabolite SCFAs. The results of the study provide a theoretical basis for the subsequent replacement of caged rabbits and welfare feeding, aiming to produce healthy rabbit meat to the satisfaction of a wide range of consumers.

The limitation of this study is that the role of each EE material on rabbits was not explored separately. Nevertheless, the results of this study provide a reference for future improvements in welfare housing systems. This also provides direction as well as ideas for our subsequent studies, in addition to increasing the number of biological replicates in each group in subsequent related studies.

Author Contributions

Huifen Xu: conceptualization, funding acquisition, resources, supervision, writing – review and editing. **Ming Li:** conceptualization, funding acquisition, and resources. **Zhitong Wang:** writing – original draft, data curation, methodology, software, and visualization. **Ran Zhang:** visualization and investigation. **Guanchen Du:** software and investigation. **Mengke Ni:** visualization and software. **Hui He:** software and formal analysis. **Zhichao Li:** software and formal analysis. **Dongdong Yuan:** visualization and formal analysis. **Mengjuan Chen:** visualization and formal analysis. **Xin Li:** visualization and formal analysis. **Hanfang Cai:** formal analysis. All authors contributed to reading and approving the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and analyzed during the current study are available in the Sequence Read Archive (SRA) under the BioProject: PRJNA1164122.

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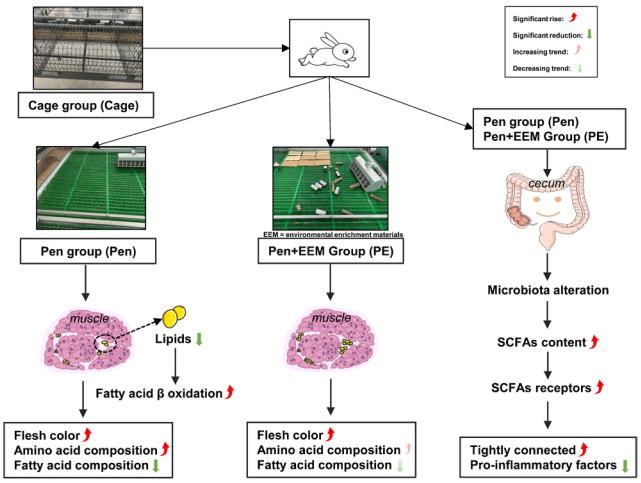

In recent years, consumers have shown a growing interest in purchasing welfare-friendly livestock products. Environmental enrichment has emerged as an effective approach to enhancing animal welfare. Our study showed that two environmental enrichment housing models (Pen and PE) could improve the fatty acid as well as amino acid composition of rabbit meat to different degrees. Moreover, Pen and PE can improve intestinal health by increasing short-chain fatty acids content.