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Original Research Article

Essential oils improve nursery pigs' performance and appetite via modulation of intestinal health and microbiota

Bi-Chen Zhao [a], Tian-Hao Wang [a], Jian Chen [a], Bai-Hao Qiu [a], Ya-Ru Xu [a], Jin-Long Li [a, b, c, *]

[a] College of Veterinary Medicine, Northeast Agricultural University, Harbin, 150030, China
[b] Heilongjiang Key Laboratory for Laboratory Animals and Comparative Medicine, Northeast Agricultural University, Harbin, 150030, China
[c] Key Laboratory of the Provincial Education Department of Heilongjiang for Common Animal Disease Prevention and Treatment, Northeast Agricultural University, Harbin, 150030, China

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ABSTRACT

Optimal intestinal health and functionality are essential for animal health and performance, and simultaneously intestinal nutrient transporters and intestinal peptides are also involved in appetite and feed intake control mechanisms. Given the potential of essential oil (EO) in improving animal performance and improving feed palatability, we hypothesized that dietary supplementation of cinnamaldehyde and carvacrol could improve performance and appetite of nursery pigs by modulating intestinal health and microbiota. Cinnamaldehyde (100 mg/kg), carvacrol (100 mg/kg), and their mixtures (including 50 mg/kg cinnamaldehyde and 50 mg/kg carvacrol) were supplemented into the diets of 240 nursery pigs for 42 d, and data related to performance were measured. Thereafter, the influence of EO on intestinal health, appetite and gut microbiota and their correlations were explored. EO supplementation increased ($P < 0.05$) the body weight, average daily gain (ADG) and average daily feed intake (ADFI) of piglets, and reduced ($P < 0.05$) diarrhea rates in nursery pigs. Furthermore, EO increased ($P < 0.05$) the intestinal absorption area and the abundance of tight junction proteins, and decreased ($P < 0.05$) intestinal permeability and local inflammation. In terms of intestinal development and the mucus barrier, EO promoted intestinal development and increased ($P < 0.05$) the number of goblet cells. Additionally, we found that piglets in the EO-supplemented group had upregulated ($P < 0.05$) levels of transporters and digestive enzymes in the intestine, which were significantly associated with daily gain and feed utilization. In addition, EO supplementation somewhat improved appetite in nursery pigs, increased the diversity of the gut microbiome and the abundance of beneficial bacteria, and there was a correlation between altered bacterial structure and appetite-related hormones. These findings indicate that EO is effective in promoting growth performance and nutrient absorption as well as in regulating appetite by improving intestinal health and bacterial structure.

1. Introduction

The nursery period is considered to be one of the most critical periods in pig management, during which piglets experience changes such as weaning, separation from sows, hierarchy stress, new housing environment, and a sudden change of diets from sow milk to solid feeds (Helm et al., 2019). Providing nursery pigs with optimal nutrition and an ideal growth environment can ensure their health and achieve maximum lifelong performance. Upon weaning, pigs with a poor transition from milk to dry feed may compromise their intestinal health and fall victim to disease challenges (Tang et al., 2022). The intestine is the principal organ for the digestion and absorption of nutrients. Optimal intestinal health and functionality are essential for animal health and performance since it has direct repercussions on both the digestibility and absorption of nutrients (Kogut and Arsenault, 2016). Furthermore, the

* Corresponding author.
E-mail address: Jinlongli@neau.edu.cn (J.-L. Li).
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intestine serves as an innate defense barrier against most of the intestinal pathogens that strongly affect the maintenance of systemic health. Consequently, the intestinal tract is considered a central site for optimizing the health and production of pigs.

Establishing a healthy microbiota in the intestine of nursery pigs is extremely important for intestinal barrier function, immune system development, and utilization rate of feed, and promotes pig growth (Chen et al., 2018; Kabat et al., 2014; Li et al., 2018a). The intestinal microbial community may further interact with the intestinal epithelium and ultimately regulate intestinal absorption and production performance (Gresse et al., 2017). In connection, the gut microbiota generates and releases enormous quantities of compounds that may influence how the host tissues absorb and store energy, as well as energy expenditure and intestinal motility. Moreover, intestines and microbiota can participate in appetite and food intake control mechanisms through a complex multidirectional crosstalk system between gut bacteria, the enteric nervous system and the brain (Han et al., 2021). Notably, several physiological processes, including signals from the stomach and intestines and metabolic hormones, are used to control this regulation (including leptin [LEP], ghrelin and insulin) (Mantzoros et al., 2011). In healthy pigs, intestinally derived hormone improves intestinal development, digestion, absorption, barrier function, and blood flow (Roura et al., 2016). It also prevents intestine damage and speeds up recovery in models of intestinal inflammation (Thymann, 2016). Moreover, the primary site for nutrient absorption is the small intestine, which recognizes the components of the intestinal chyme to communicate nutritional information to the rest of the body. Absorptive epithelial cells selectively absorb nutrition from the gut lumen by regulating the expression of key nutrient transporters in the brush-like border membrane (Chaudhry et al., 2012; Gorboulev et al., 2012). Also, energy intake, expenditure and homeostasis are jointly controlled by the expression of nutrient transporters and intestinal peptide production (Mace and Marshall, 2013). In pig production, the multiple stressors experienced by nursery pigs result in metabolic problems, lack of appetite, sluggish growth and weakened immune function, which seriously affect the growth performance and economic benefits of pigs. Hence, the regulation of the intestinal microbiota, metabolic hormones, and intestinal nutrient transporters can be the key to promoting enhanced responses to nutritional interventions.

Antibiotics are effective in improving feed utilization, maintaining livestock health and promoting growth, but excessive use of antibiotics gradually exposes many drawbacks, such as enhanced resistance to disease-causing bacteria, disturbed gut microbiome composition, and antibiotic residues in livestock. In this context, identifying alternative nutritional strategies for the management of nursery pigs is a critical topic around the world. Essential oils (EO) can not only inhibit the growth of harmful bacteria without developing drug resistance but also improve their productive capacity by increasing feed characteristics, boosting performance and intestinal health (Roura et al., 2016). Because of their antibacterial, anti-inflammatory, and antioxidant qualities, carvacrol and cinnamaldehyde are commonly used in the pig farming industry to boost growth performance and cure postweaning diarrhea (Omonijo et al., 2018). Numerous studies demonstrated that combining multiple EO compounds may lead to more effective treatment than using each component separately. Wei et al. (2017) showed that feeding a combination of carvacrol and thymol to weaned pigs reduced intestinal oxidative stress. Ma et al. (2022) reported that diets supplemented with EO improved intestinal barrier function and regulated the structure of the cecal and colonic microbial community. Some studies reported that carvacrol could enhance digestive enzyme activity and absorption capacity (Hashemipour et al., 2013). Furthermore, carvacrol and cinnamaldehyde were shown to stimulate appetite to diminish health challenges and stress (Froehlich et al., 2017; Ogawa and Ito, 2016). In light of prior studies, we hypothesize that introducing EO into the immature gastrointestinal tract of nursery pigs would stimulate morphological and functional development and further promote appetite. The specific objectives of the current study were to investigate the effects of the addition of cinnamaldehyde, carvacrol, and their mixtures to diets on performance, intestinal villi morphology, intestinal barrier function, digestive capacity, colonic microbiota and appetite-related hormones in nursery pigs, and in particular to screen for the optimal EO additive.

2. Materials and methods

2.1. Animal ethics

The experiment followed animal protection law (Ethic Approval Code: NEAUEC20220825) and was performed in accordance with the Guide for the Care and Use of Laboratory Animals approved by the Northeast Agricultural University Institutional Animal Ethics Committee.

2.2. EO product

The EO combination product was supplied by Weiyuan Animal Pharmaceutical Co., Ltd. (Hebei, China). Briefly, the carvacrol combination product mainly contained 10% carvacrol; the cinnamaldehyde combination product mainly contained 10% cinnamaldehyde; and the carvacrol + cinnamaldehyde combination product mainly contained 5% carvacrol and 5% cinnamaldehyde. EO in all products were microencapsulated in a triglyceride matrix of hydrogenated vegetable oils. The product was added to the diet at approximately 0.1%.

2.3. Animals and experimental design

A total of 240 cross-bred (Landrace × Large White) weaned pigs (age, 28 ± 2 d; initial body weight [BW], 8.08 ± 0.34 kg; 96 females and 144 castrated males) were assigned randomly into four dietary treatments in a randomized whole block design based on BW and gender: (1) corn-soybean meal basal diet (Con group); (2) basal diet with cinnamaldehyde (effective content 100 mg/kg) (Cin group); (3) basal diet with carvacrol (effective content 100 mg/kg) (Car group); and (4) basal diet with cinnamaldehyde (effective content 50 mg/kg) and carvacrol (effective content 50 mg/kg) (Cin + Car group). The feed additive dose was selected according to previous studies of Mo et al. (2021) and Ma et al. (2022). The EO product and the basal diet were mixed well and then fed as a powder. All piglets were healthy and ear-tagged for identification and vaccinations before weaning. For the 42-d trial, each group involved 60 piglets divided into 6 pens with 10 piglets per pen.

The levels of the nutrition met or exceeded NRC (2012) standards (Zhao et al., 2023a) and the ingredients of the basal diet are shown in Table S1. The diets were fed to the piglets in three phases for an average of 6 weeks. The main nutrients (including dry matter, ash, crude fat, crude fiber, crude protein, micronutrients, etc.) in feeds were determined by applying near-infrared spectroscopy (NIRS) using a MATRIX I FT-NIR instrument (Bruker, Germany) (Arce et al., 2009). The NE of the experimental diets and test ingredients were calculated according to the equations established by Noblet et al. (1994). The calculation formula was as follows: NE = 2875 + (4.38 × crude fat) + (7.459 × crude protein) − (5.50 × ash) − [2.01 × (neutral detergent fiber − acid detergent fiber)] − (4.02 × acid detergent fiber).

The experimental piglets were housed in 3.0 m × 2.0 m × 0.7 m fenced pens with a leaky sprayed plastic floor. Each pen was equipped with a rectangular plastic adjustable slot and a duckbill waterer. An automatic monitoring system maintained a suitable temperature (28 °C for 0 to 7 d, then lowered by 1 °C per week until 23 °C), humidity (60% to 70%) and CO_2 concentration (below 0.15%). The weaned piglets were also periodically dewormed and inoculated on d 7, 14, and 21 (attenuated highly pathogenic porcine reproductive and respiratory syndrome virus vaccine, pseudorabies vaccine, and freeze-dried attenuated classical swine fever virus vaccine, respectively). The surveillance system kept track of the feeding and health of the piglets. Feed was replenished in sufficient quantities at 07:30 and 18:30 daily to ensure ad libitum access. Overall, the piglets were fed powdered feed and allowed to eat and drink as they pleased. There was no mortality in the piglets during the experimental period.

2.4. Growth performance and diarrhea rate

On d 0, 14, 28, and 42 postweaning, all the experimental pigs were weighed separately. On the same day, pen feed consumption was calculated by subtracting the weight of any residual feed in the feeder from the total amount of feed supplied to the feeder during each phase. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were measured per pen during each phase and the total feeding period using the data of BW and feed consumption. For diarrhea rate, pigs were observed for diarrhea at the same time each morning throughout the experiment. The fecal consistency of each pig was graded on a scale of 0 to 3 (0 for normal feces, 1 for pasty feces, 2 for semi-liquid feces, and 3 for liquid feces). A fecal score of 2 or above was considered diarrhea. The rate of diarrhea (percent) was computed as the number of diarrheal piglets divided by the number of animals observed and days of inspection. For cough rate, the occurrence of cough was observed by only one veterinarian at the same time each morning throughout the experiment. Pigs in each pen were forced to get up and the number of coughs was recorded for more than 2 min. Piglets with obvious and more than 5 consecutive sneezes or coughs were defined as having piglet asthma. The cough rate (percent) was computed as the number of piglets with cough divided by the number of animals observed and days of inspection.

2.5. Sample collection

On d 42, after weighing all the piglets, 1 pig from each pen with the average weight was selected. A total of 6 piglets were selected and sacrificed after rapid intracardial injection of sodium pentobarbital (50 mg/kg BW) anesthesia (Sun et al., 2020). A 2 to 3 cm segment of the middle section with more vessels of the jejunum (middle jejunum) and 2 to 3 cm segment of the end of the ileum near the cecum (distal ileum) were obtained, flushed in ice-cold physiological saline or PBS and stored in 4% paraformaldehyde or 2.5% glutaraldehyde for histological analysis and scanning electron microscopy. The contents of the jejunum and ileum from other segments of the intestine were collected and kept at −80 °C. The intestinal segment was dissected lengthwise, washed with PBS, and the mucosal layer on the surface was scraped with a glass slide and collected. Colonic mucosa was obtained and kept at −80 °C. Blood samples were collected by the vena jugularis into vacuum blood tubes, then centrifuged at 3000 × *g* for 30 min, and the serum was extracted and kept at −80 °C.

2.6. Cell culture, treatment, transepithelial electrical resistance (TEER) and FD4 flux detection

IPEC-J2 cells were pre-incubated with different concentrations of carvacrol (50 μM) or cinnamaldehyde (25 μM) for 24 h, followed by co-treatment with lipopolysaccharide (LPS; 10 μg/mL) for another 24 h or co-treatment with H_2O_2 (600 μM) for another 8 h. Specifically, the cells were cultured and processed as described in the supplementary material.

2.7. Histological and morphometric analysis

Paraffin wax-embedded sections were cut into 4-μm thick serial sections and stained with haematoxylin and eosin (H&E). In addition, the number of goblet cells and glycogen-positive regions were observed via Alcian blue-PAS-stain and PAS-stain, and selected for evaluation in five independent fields. The crypt depth (CD) was calculated from the bottom of the crypt to the crypt-villus junction. The villus height (VH) was measured from the crypt-villus junction to the tip of the villus. For each group, at least 8 villi were measured from each sample.

2.8. Scanning electron microscope

The tissues were immersed in 2.5% glutaraldehyde for 24 h at 4 °C, then flushed in PBS and treated for 1 h in sodium cacodylate buffer with 1% osmium tetroxide. Next, the samples were sequentially dried to the critical point in alcohol solvent and liquid CO_2 under pressure. Subsequently, the samples were glued to the stubs with carbon tape and covered with gold. The images were captured by a scanning electron microscope (EVO MA 15, Carl Zeiss AG, Jena, Germany).

2.9. RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extraction and qRT-PCR were performed according to previously published methods (Zhao et al., 2022, 2023b). Briefly, the total RNA of the samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocol. The RNA concentration and quality were analyzed using a Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and all samples had optical density (OD) values between 1.9 and 2.1. The reverse transcription (cDNA) was synthesized from 1 μg of total RNA with All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (AT341-01; TransGen Biotech, Beijing, China). Quantitative Real-time PCR was performed using Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) using 1 μL first-strand cDNA in a volume of 10 μL, and was run in the Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Scientific, Wilmington, MA, USA). The conditions were as follows: initial denaturation at 94 °C for 30 s, 30 cycles of amplification (denaturation at 94 °C for 5 s, annealing at 56 °C for 15 s, and extension at 72 °C for 10 s), and extension at 72 °C for 10 min. All samples were run in triplicate. β-Actin was used as an endogenous control. The sequence of primers is listed in Table S2. Eventually, the $2^{-\Delta\Delta Ct}$ method was used to analyze the relative fold changes.

2.10. Biochemical analysis

Using the manufacturer's instructions, serum diamine oxidase (DAO), D-lactic acid (D-LA), endothelin (ET), cholecystokinin (CCK), growth hormone (GH), ghrelin, orexin, gastric inhibitory polypeptide (GIP), insulin, glucagon-like peptide-1 (GLP-1), LEP, and

peptide YY (PYY) were quantified using an available enzyme-linked immunosorbent assay (ELISA) kit (ChengLinBio, Beijing, China). Trypsin (ultraviolet colorimetry), lipase (microplate method) and amylase (starch-iodine colorimetry) from intestinal content were quantified by ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and chymotrypsin from intestinal content was also assayed by ELISA kit (ChengLinBio, Beijing, China). The IL-β, tumor necrosis factor alpha (TNF-α) and IL-6 levels in colonic mucosa samples were quantified according to the ELISA method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.11. Immunofluorescence staining

Immunofluorescence staining was performed according to previously published methods (Zhao et al., 2020, 2021). Briefly, slices were incubated with primary antibody (Table S3) overnight at 4 °C after blocking in 5% bovine serum albumin for 1.5 h at 25 °C. Later, the slices were washed in PBS. Subsequently, slices were incubated with fluorescent dye conjugated secondary antibodies (1:50; SA00003-2, Proteintech, Chicago, USA) for 1 h and kept in dark place. In the end, the nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 10 min at 25 °C. Slices were observed by a DM5000 B fluorescence microscope (Leica, Wetzlar, Germany). Quantization was performed using Image-Pro Plus software.

2.12. Western blotting

Protein lysates were incubated with the primary antibody (Table S3) overnight at 4 °C. The membranes were treated with secondary antibodies for 1 h after being rinsed with PBS with Tween 20. Proteins were visualized using an Ultra High Sensitivity ECL Kit (HY-K1005, MedChemExpress, Shanghai, China). Western blot procedures were performed according to previously published methods (Zhao et al., 2022).

2.13. 16S rRNA sequencing and data analysis

Total genomic DNA was divided from the content of colon samples by the QIAamp DNA Fecal Mini Kit (Qiagen, Hilden, Germany). The purity and integrity of the DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis. The V3–V4 region of the bacterial 16S rDNA was amplified with primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′) using the following program: 95 °C predenaturation for 5 min, 30 cycles of 95 °C denaturation for 30 s, 50 °C annealing for 30 s, 72 °C extension for 45 s, and a final extension at 72 °C for 8 min. Following the concentration of the acquired PCR products, the PCR products were combined in equal quantities, re-electrophoresed on 2% agarose gels, and the target product bands were recovered using a QIAquick Gel Extraction Kit (Axygen, Santa Clara Valley, USA).

Sequencing was performed on the Illumina NovaSeq platform (Wekemo Bioincloud, Shenzhen, China). All raw sequences of all samples were filtered, denoised (sequences corrected for sequencing errors), merged, and dechimericed (non-chimeric) to form operational taxonomic units (OTU) by the DADA2 plugin in Qiime2 software. To collect species annotation information, representative OTU sequences were chosen and compared to a database (Greengenes Database version 13.8) (DeSantis et al., 2006). Based on the absolute abundance and annotation data of OTU, it is possible to calculate the ratio of the number of sequences at the phylum and genus level to the total number of sequences in each sample, which can effectively assess the species annotation resolution of the sample. The determination of alpha-diversity and LEfSe were also conducted in Qiime2 software. The 16S marker sequence function was predicted using the PICRUSt tool.

2.14. Bacterial growth assays of EO in vitro

Prevotella copri, *Lactobacillus acidophilus* (ATCC4356) and *Streptococcus suis* were cultured as described previously (Chen et al., 2021a; Liang et al., 2022; Wu et al., 2020b). The three bacteria mentioned above were grown in the presence or absence of EO in LB medium for 2, 4, 8, 12 and 24 h. The concentration of bacteria within the broth was estimated by measuring OD at 600 nm in a spectrophotometer (Thermo Scientific, Wilmington, MA, USA). OD_{600} measurements are directly correlated with the concentration of bacteria in a liquid culture.

2.15. Statistical analysis

All data were initially organized by Excel software and then statistically analyzed using the GLM model data in SAS software (V9.2, 2008). The dietary treatment was treated as a fixed effect and the blocks were treated as a random effect. Individual pens were used as statistical units to analyze data related to growth performance, diarrhea rate and cough rate of piglets using chi-squared test. The data on piglet serum, digestive enzymes, intestinal morphology and so on were analyzed based on individuals as a unit. Multiple comparisons of group differences and significance were performed using the Turkish method, and the means and standard errors were calculated using the LSMEANS method. A significant difference was defined as $P < 0.05$, and a trend of difference was defined as $0.05 < P < 0.10$.

3. Results

3.1. Dietary cinnamaldehyde and carvacrol improved growth performance and reduced diarrhea rate and cough rates of nursery pigs

EO feeding could promote the growth and development of nursery pigs. For this purpose, cinnamaldehyde, carvacrol and their mixtures were added to the diet for 42 d until the end of the nursery period (Fig. 1A). As presented in Table 1, piglets fed the EO diet had higher BW at d 14 ($P < 0.05$), d 28 ($P < 0.01$) and d 42 ($P < 0.05$) relative to piglets in the Con group. Besides, piglets in the Cin + Car group had higher BW ($P < 0.05$) at the end of the nursery period (d 42), when compared with piglets in Con group. Combined supplementation with carvacrol and cinnamaldehyde increased ADG during 14 to 28 d ($P < 0.05$) and 28 to 42 d ($P < 0.05$) relative to piglets in the Con group. However, for the whole trial period and 28 to 42 d, nutritional supplementation with EO had higher ADG ($P < 0.05$). Piglets fed carvacrol and cinnamaldehyde diet improved ADFI during 14 to 28 d ($P < 0.05$) when compared to the CON diet. Dietary EO supplementation was also able to increase ADFI ($P < 0.01$) of piglets during 14 to 28 d. EO supplementation markedly decreased the rate of diarrhea during 0 to 14 d, 14 to 28 d, 28 to 42 d and the whole experimental period ($P < 0.05$). Dietary supplementation of carvacrol significantly reduced diarrhea rates during 14 to 28 d ($P < 0.05$), and cinnamaldehyde and carvacrol co-feeding also decreased the rate of diarrhea during 0 to 14 d ($P < 0.05$). In addition, compared with piglets in the Con group, EO supplementation dramatically reduced cough rate during 0 to 14 d and 14 to 28 d. The rate of cough was lower in the Car group and Cin + Car group during the weaning period (0 to 14 d) in piglets ($P < 0.05$).

3.2. Dietary cinnamaldehyde and carvacrol increased intestinal absorptive surface and improved villus morphology of nursery pigs

EO supplementation could increase BW of nursery pigs. To determine if the length of the small intestines correlated with BW, we compared small intestine length in the Con group with several EO groups of nursery pigs at d 42. Pigs in the EO-fed group had longer small intestine length, and the length of the small intestines correlated with the BW ($P < 0.01$; Fig. 1B). ADFI and small intestine length strongly matched in the Con, Cin, Car, Cin + Car groups of nursery pigs (Fig. 1C). As shown in Fig. 1D–G, there is a significant increase in the height of the jejunal villi in the nursery pigs fed a mixture of cinnamaldehyde and carvacrol ($P < 0.05$), but not in those fed cinnamaldehyde alone at d 42. In addition, pigs in the Car group had higher VH in the middle jejunum and distal ileum than those in the Con group ($P < 0.05$), but the Car group had a better effect on the VH than the Cin group ($P < 0.05$). The ratio of VH to CD in the distal ileum section was similarly dramatically increased by dietary carvacrol administration ($P < 0.05$). The CD did not differ between the groups. Scanning electron microscopy images revealed that the jejunal and ileal villi in the EO-fed group were more neatly arranged and more compact ($P < 0.05$; Fig. 1H). Moreover, there is a positive relationship between ADG and average villus length in the middle jejunum and distal ileum parts (Fig. 1I), suggesting that increased intestinal absorptive surface can influence daily gain.

3.3. Dietary cinnamaldehyde and carvacrol enhanced intestinal tight junctions of nursery pigs

To further investigate the effect of EO on tight junction structure, we measured the abundance of tight junction protein by immunofluorescence and Western blotting. Thin cross-sections of the middle jejunum and distal ileum parts labeled with tight junction proteins zonula occludens-1 (ZO-1) and occludin revealed that dietary supplementation with EO increased levels of tight junction proteins compared to nursery pigs in the Con group (Fig. 2A). Similarly, the protein abundances of ZO-1, E-cadherin, occludin, claudin-1 and claudin-5 in the jejunum and ileum showed an increasing trend in both the Car group and the Cin + Car group ($P < 0.05$; Fig. 2B and C). Important markers for determining the effectiveness of the intestinal epithelial barrier include serum concentrations of DAO, ET, and D-LA. Feed supplementation with carvacrol sharply reduced serum levels of DAO ($P = 0.022$) and ET ($P = 0.002$), while D-LA ($P = 0.028$) levels were significantly lower in the Cin + Car group (Fig. 2D). Better tight junctions could reduce the impact of pathogenic bacteria on local inflammation in the intestine; we also examined the levels of immune markers in the colonic mucosa. As expected, the level of IL-6 ($P = 0.016$) was markedly decreased in the Car group, and the level of IL-1β ($P = 0.035$) was dramatically decreased in the Cin + Car group (Fig. 2E). In addition, to exclude the effect of approximately 90% of the carriers in the additive, IPEC-J2 cells were cultured in vitro to determine the effect of carvacrol and cinnamaldehyde on intestinal epithelial barrier function. Carvacrol or cinnamaldehyde showed a protective effect on intestinal barrier function, as indicated by preventing the decreased TEER ($P < 0.05$; Figs. S1A and S2A) and increased FD4 flux ($P < 0.05$; Figs. S1B and S2B) induced in LPS or H_2O_2 treated cells. Accordingly, we found that carvacrol and cinnamaldehyde increased the expression of tight junction proteins (ZO-1, E-cadherin and occludin) in IPEC-J2 cells challenged with LPS or H_2O_2 ($P < 0.05$; Figs. S1C and S2C), which was consistent with the immunofluorescence of occludin (Figs. S1D and S2D).

3.4. Dietary cinnamaldehyde and carvacrol modulated intestinal development and mucus barrier of nursery pigs

The intestine has the fastest turnover rate of any organ and Ki67 is a marker of cellular proliferation. As shown in Fig. 2H, positive Ki67 signals were observed as green spots in the intestine, and far more Ki67 signals were observed in the jejunum and ileum of nursery pigs from the Car group and the Cin + Car group. Dietary EO supplementation increased the protein expression of mucin-2 and lysozyme (Lyz) in the jejunum ($P < 0.01$), and carvacrol supplementation had a more significant effect on expression (Fig. 2F and G). Furthermore, in the ileum, the protein abundance of Ki67 and leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) was significantly increased in the Cin + Car group ($P < 0.05$; Fig. 2F and G). The distribution of mucous and goblet cells in the jejunum and ileum of each group was noticeably different in the jejunum and ileum among each group, according to the sections stained with PAS and AB-PAS (Fig. 2I–L). An increasing number of granules of PAS-positive protein were found in the jejunum and ileum of the Cin group and the Car group.

3.5. Dietary cinnamaldehyde and carvacrol promoted intestinal digestion and absorption in nursery pigs

The small intestinal fluid contains almost all three major categories of digestive enzymes of food (sugar, fat, protein), and the digestion effect of digesta in the small intestine directly affects the absorption of nutrients, which in turn affects the ADG and FCR of nursery pigs. The results revealed that the concentration of trypsin and lipase in jejunal contents was higher in the Car group and the Cin + Car group ($P < 0.05$; Fig. 3A–D). In addition, trypsin levels were higher in the jejunum of piglets from the Cin group and in the ileum of piglets from the Cin + Car group ($P < 0.01$). In addition, Spearman correlation analysis revealed that the activities of digestive enzymes in the jejunum presented a highly significant positive correlation with ADG and FCR ($P < 0.05$; Fig. 3I). Transporters responsible for glucose, fructose, amino acids, and oligopeptides in the jejunum and ileum were also significantly altered upon EO treatment. The mRNA abundance of solute carrier family 1 member 1 (*SLC1A1*), glucose transporter type 2 (*GLUT2*), glucose transporter type 5 (*GLUT5*), B(0,+)-type amino acid transport protein (*RBAT*), cationic amino acid transporter 1 (*CAT1*), sodium-coupled neutral amino acid transporter 2 (*SNAT2*), alanine-serine-cysteine transporter 2 (*ASCT2*), and peptide transporter 1 (*PepT1*) in the jejunum of piglets supplemented with EO in the diet was significantly higher than that of control piglets ($P < 0.01$; Fig. 3E). Dietary EO supplementation also increased the mRNA abundance of *SLC1A1*, *GLUT5*, γ^+ system L-type amino acid transporter 2 (γ^+*LAT2*), $\gamma+$ system L-type amino acid transporter 1 (γ^+*LAT1*), *RBAT*, *SNAT2*, and *PepT1* in the ileum of piglets ($P < 0.01$; Fig. 3F). Furthermore, EO treatment sharply increased the protein abundance of SLC1A1 in the ileum, as well as solute carrier family 5 member 1 (SLC5A1), GLUT2, GLUT5 in the jejunum and ileum ($P < 0.01$; Fig. 3G and H). Spearman correlation analysis demonstrated that the expression levels of amino acid transporter (*SLC1A1*, *ASCT2*), glucose transporter (*GLUT2*), fructose transporter (*GLUT5*), oligopeptide transporter (*SNAT2*) and *PepT1* in jejunum and ileum were significantly positively correlated with ADG and FCR ($P < 0.05$; Fig. 3I).

3.6. Dietary cinnamaldehyde and carvacrol regulated appetite and altered gut microbiota in nursery pigs

To confirm the effect of EO on appetite in nursery pigs, serum levels of orexigenic and appetite-suppressive hormones were

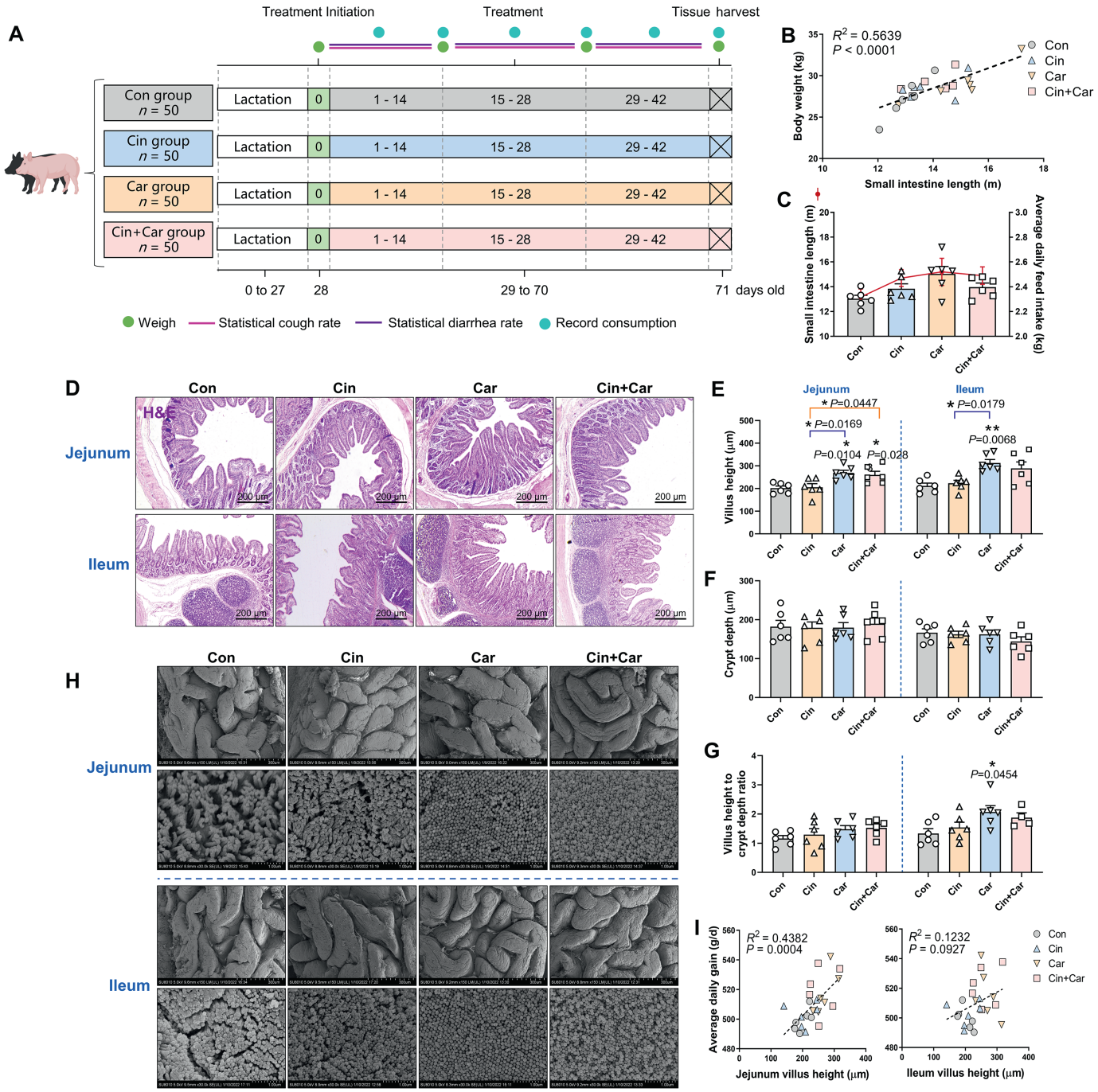

Fig. 1. Effects of cinnamaldehyde and carvacrol supplementation on intestinal morphology in nursery pigs. (A) Nursery pigs were administered Con (basal diet), Cin (basal diet with cinnamaldehyde), Car (basal diet with carvacrol) or Cin + Car (cinnamaldehyde and carvacrol) for 42 d. (B) Linear regression of body weight vs. small intestine length. (C) Small intestine length of nursery pigs and their average daily feed intake throughout the trial. (D) Representative images of the jejunum and ileum stained with H&E (scale bar, 200 μm). (E–G) The CD, VH and VH to CD ratio of jejunum and ileum. (H) Intestinal morphology of jejunum and ileum shown by scanning electronic microscope. (I) Linear regression of average daily gain vs. villus height of jejunum and ileum. CD = crypt depth; VH = villus height. Con means piglets fed basal diet ($n = 6$); Cin means CON diet with 50 mg/kg cinnamaldehyde ($n = 6$); Car means CON diet with 50 mg/kg carvacrol ($n = 6$); Cin + Car means CON diet with 25 mg/kg cinnamaldehyde and 25 mg/kg carvacrol ($n = 6$). Values for R^2 and P are indicated in each graph. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

measured. The results revealed that the ghrelin level in serum was significantly increased in the Car group ($P = 0.033$); the content of orexin in the Car group and the Car + Cin group was significantly up-regulated ($P < 0.05$); and the content of CCK of the Car + Cin group was significantly down-regulated compared with the Con group ($P = 0.016$; Fig. 4A). High-throughput sequencing of 16S rRNA in colonic material was performed to determine the impact of EO on gut microbiota. Alpha diversity was examined by calculating the Chao1, Shannon and Simpson indices (Fig. 4B–D). The Chao1 and Shannon index were higher for the Car + Cin group than for the Con group ($P < 0.05$), and the effect of the Car + Cin group on the diversity of microbiota was greater than that of Cin and Car groups ($P < 0.05$). The overall composition of gut microbiota was further analyzed at phylum level, where Firmicutes and

Table 1
Growth performance of piglets as affected by dietary Cin, Car and Cin + Car supplementation.[1]

Item	Con	Cin	Car	Cin + Car	SEM	*P*-value
BW, kg						
Initial	8.20	8.32	8.12	8.12	0.047	0.91
d 14	11.93^{b}	12.14^{ab}	12.28^{a}	12.15^{ab}	0.073	0.02
d 28	19.43^{b}	20.17^{a}	20.29^{a}	20.12^{ab}	0.195	<0.01
d 42	29.16^{b}	29.38^{ab}	29.85^{a}	30.06^{a}	0.206	0.02
ADG, g/d						
d 0 to 14	266	272	297	288	6.9	0.10
d 14 to 28	536	574	572	570	9.1	0.05
d 28 to 42	662^{b}	658^{b}	683^{ab}	710^{a}	11.8	<0.01
Overall	499^{b}	505^{b}	517^{ab}	522^{a}	5.4	0.02
ADFI, g/d						
d 0 to 14	414	413	435	430	5.7	0.33
d 14 to 28	686^{b}	724^{a}	751^{a}	751^{a}	15.4	<0.01
d 28 to 42	1218	1333	1334	1309	27.4	0.22
Overall	772	823	840	830	15.0	0.05
FCR						
d 0 to 14	1.55	1.36	1.47	1.50	0.040	0.07
d 14 to 28	1.32	1.28	1.26	1.31	0.013	0.12
d 28 to 42	2.03	1.95	1.85	1.75	0.060	0.19
Overall	1.64	1.62	1.55	1.59	0.021	0.35
Diarrhea rate, %						
d 0 to 14	24.54^{a}	19.69^{ab}	18.64^{ab}	18.23^{b}	1.454	<0.01
d 14 to 28	16.83^{a}	11.76^{a}	10.66^{b}	10.97^{b}	1.444	<0.01
d 28 to 42	10.52^{a}	8.20^{b}	8.45^{ab}	8.17^{b}	0.564	0.04
Overall	17.30^{a}	13.22^{b}	12.59^{b}	12.46^{b}	1.148	<0.01
Cough rate, %						
d 0 to 14	13.61^{a}	11.73^{ab}	10.24^{b}	10.51^{b}	0.767	<0.01
d 14 to 28	6.32^{a}	5.44^{ab}	5.227^{ab}	4.42^{b}	0.391	0.03
d 28 to 42	3.71	3.16	2.65	2.83	0.232	0.17
Overall	7.88	6.77	6.04	5.92	0.450	0.41

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.
[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[1] Control (Con): a based diet; Cin: Con + 50 mg/kg cinnamaldehyde; Car: Con + 50 mg/kg carvacrol; Cin + Car: Con + 25 mg/kg cinnamaldehyde + 25 mg/kg carvacrol. $N = 240$ total, $n = 60$ per supplementation.

Bacteroidetes were the dominant phyla, accounting for more than 90% (Fig. 4E). The population of Firmicutes in the Con, Cin, Car and Cin + Car groups was 57.74%, 72.93%, 72.26% and 76.01%, respectively. Bacteroidetes in the Con, Cin, Car and Cin + Car groups accounted for 37.99%, 20.52%, 18.14% and 20.88%, respectively. Besides, Firmicutes in the Cin, Car and Cin + Car groups increased by 26.31%, 25.14% and 31.64%, respectively, compared to the Con group. At genus level (Fig. 4F), the microorganisms that dominated the top 5 in the Con group were *Lactobacillus* (33.28%), *Streptococcus* (15.79%), *Prevotella* (13.04%), *_Prevotella_* (7.27%) and *Dialister* (6.34%). In the Cin group, it was dominated by *Lactobacillus* (43.11%), *Prevotella* (20.04%), *Megamonas* (5.69%), *_Prevotella_* (4.45%) and *Megasphaera* (3.80%). In the Car group, it was primarily dominated by *Prevotella* (33.65%), *Lactobacillus* (32.723%), *Streptococcus* (5.00%), *Megasphaera* (4.50%) and *_Prevotella_* (3.40%). In the Cin + Car group, it was mainly dominated by *Lactobacillus* (54.88%), *Megasphaera* (7.38%), *Prevotella* (5.88%), *_Prevotella_* (5.86%) and *Blautia* (4.96%). LEfSe analysis revealed significant differences in the taxa found in nursery pigs of the Con, Cin, Car and Cin + Car groups (Fig. 4G and Fig. S3). At the phylum level, in comparison to piglets from other groups, piglets in the Cin + Car group exhibited a higher ($P < 0.05$) abundance of Proteobacteria and Actinobacteria. At the genus level, pigs fed cinnamaldehyde diets demonstrated a higher ($P < 0.05$) abundance of *Prevotella*, *Dialister*, and *Streptococcus*, and compared to other groups, piglets fed carvacrol diets showed greater ($P < 0.05$) abundance of *Oscillospira* and *Ruminococcus*. In addition, pigs in the Cin + Car group demonstrated a higher ($P < 0.05$) abundance of *Lactobacillus*, *Bacteroides*, *Collinsella*, *Parabacteroides* and *Campylobacter* than those fed the other three diets. The results of the in vitro bacterial growth assay showed that EO did not significantly affect the growth of *P. copri* ($P > 0.05$), while carvacrol and cinnamaldehyde co-treatment with carvacrol reduced the growth of *L. acidophilus* after 24 h of incubation ($P < 0.05$; Fig. S4). For *S. suis*, carvacrol and cinnamaldehyde treatments significantly reduced bacterial growth at 8, 12, and 24 h ($P < 0.05$).

Next, Spearman correlation analysis was performed to identify the bacteria potentially responsible for the amelioration of appetite and performance phenotypes in both semi-literate and carvacrol-mediated nursery pigs. We looked for associations between bacterial genera and appetite-related hormones and ADFI in nursery piglets by selecting the top 20 bacterial genera in each group at the genus level (Fig. 4H). Among these bacterial genera, *Lactobacillus*, *Prevotella*, *Ruminococcus*, *Blautia*, *CF231* and *Roseburia* showed strong positive correlations with most of the appetite-related hormonal parameters and ADFI. Moreover, *Streptococcus*, *Campylobacter* and *Dialister* displayed a strong negative association with appetite-related hormonal parameters. We further determined whether these associated bacteria were also linked to the integrity of the gut barrier and mucus barrier, and found a significant inverse relationship between *Lactobacillus* and intestinal permeability, and a positive correlation between this bacterial genus and the expression of occludin and mucin-2 ($P < 0.05$; Fig. 4I). *Prevotella* was found to be positively correlated with the expression of occludin ($P < 0.05$; Fig. 4J). A correlation network approach revealed correlations of differential microbiota induced by dietary EO supplementation (Fig. 5A). A predictive metagenomic analysis by PICRUSt2 showed that 13 pathways were altered after EO supplementation, involving protein export, alanine, aspartate and glutamate metabolism, thiamine metabolism, and ribosome pathways (Fig. 5B).

4. Discussion

The nursery period, as the critical period connecting the suckling stage with the finishing stage of growth, should not only be followed by weaning, but should also lay a good healthy foundation for the need of rapid fattening during the fattening period. In the pig industry, cost-effective antibiotic substitutes, particularly EO, are used in nursery pig feed to improve performance by enhancing feed palatability, affecting digestion and gastrointestinal health. EO, which is derived from plant materials, is a fragrant, volatile, and oily liquid with antibacterial, antioxidant, and improved digestive properties (Brenes and Roura, 2010). Studies have demonstrated that EO improve digestibility and immunity, promote intestinal health by minimizing the impact of pathogens, and control odor and ammonia emissions (Brenes and Roura, 2010; Chitprasert and Sutaphanit, 2014; Varel, 2002). Phenylpropenes (including cinnamaldehyde, safrole and eugenol) and terpenes (including carvacrol, thymol and limonene) are the two main families of chemicals found in EO. Of these, carvacrol and cinnamaldehyde are the most widely used and well-studied EO in animals. Although some studies have confirmed the prominent role of cinnamaldehyde and carvacrol in improving performance (Gholami-Ahangaran et al., 2022; Ma et al., 2022; Omonijo et al., 2018), the relationship between cinnamaldehyde and carvacrol intake and intestinal health, appetite and intestinal bacteria remains unclear. In light of this, the present study, coupled with the findings of earlier studies, then comprehensively evaluated the effects of cinnamaldehyde and carvacrol on nursery pigs, and tentatively revealed the mechanism by which EO improve performance.

During the nursery period, piglets are exposed to environmental and behavioral stresses that predispose them to diarrhea, which

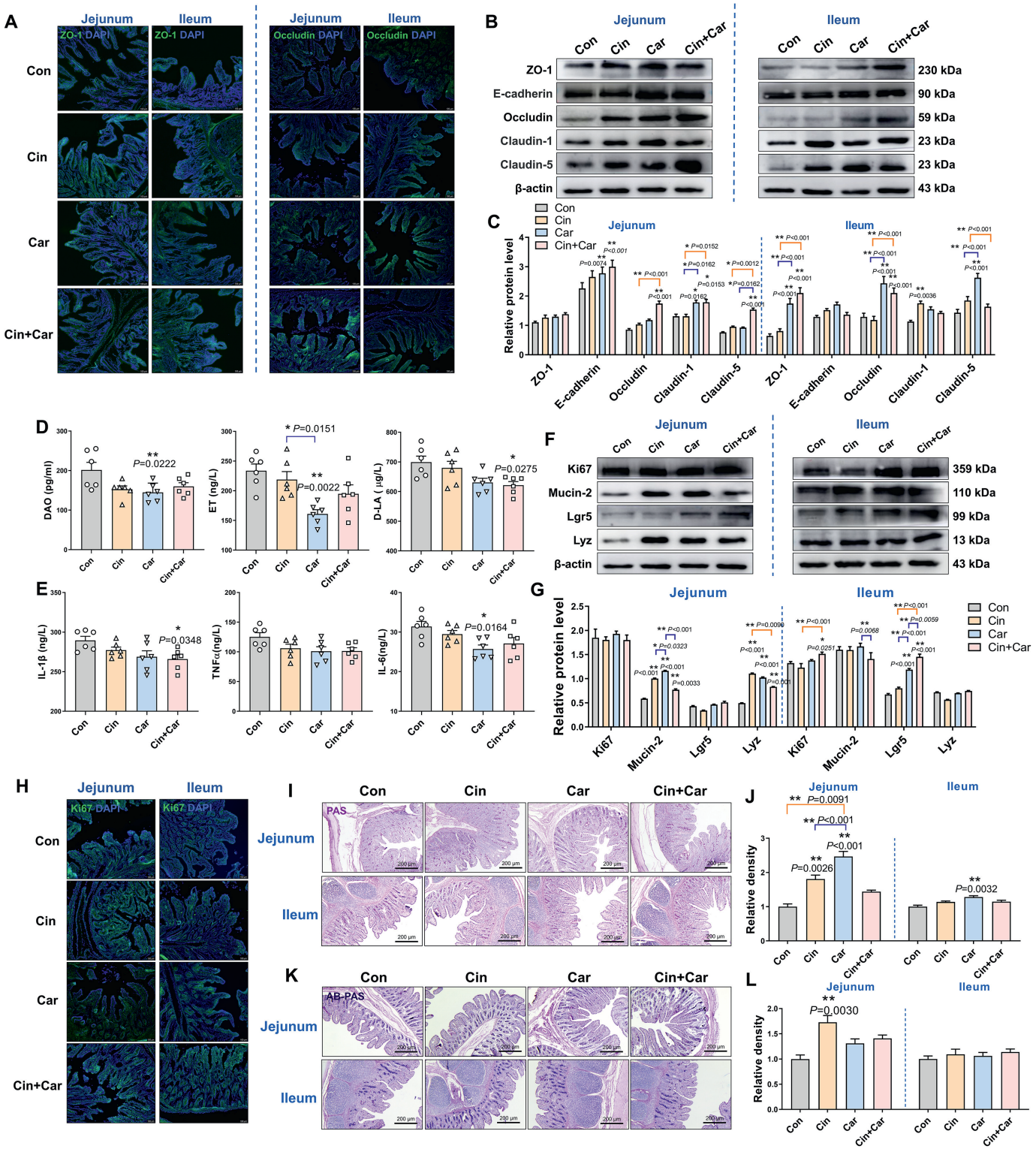

Fig. 2. Effects of cinnamaldehyde and carvacrol supplementation on intestinal tight junction, development and mucus barrier in nursery pigs. (A) Immunofluorescence staining for ZO-1 and occludin in jejunum and ileum (scale bar, 100 μm). (B, C) Protein abundance of tight junction proteins (including ZO-1, E-cadherin, occludin, claudin-1 and claudin-5) of jejunum and ileum and their quantification. (D) Serum levels of DAO, ET and D-LA. (E) IL-1β, TNF-α and IL-6 levels in colonic mucosa. (F, G) Protein abundance of Ki67, mucin-2, Lgr5 and Lyz of jejunum and ileum and their quantification. (H) Immunofluorescence staining for Ki67 in jejunum and ileum (scale bar, 100 μm). (I, K) PAS and AB-PAS staining of jejunum and ileum (scale bar, 200 μm). (J, L) Quantitative analysis of glycogen-positive and goblet cell relative densities. ZO-1 = zonula occludens-1; DAO = diamine oxidase; ET = endothelin; D-LA = D-lactic acid; TNF-α = tumor necrosis factor alpha; Lgr5 = leucine-rich repeat containing G protein-coupled receptor 5; Lyz = lysozyme. Con means piglets fed basal diet ($n = 6$); Cin means CON diet with 50 mg/kg cinnamaldehyde ($n = 6$); Car means CON diet with 50 mg/kg carvacrol ($n = 6$); Cin + Car means CON diet with 25 mg/kg cinnamaldehyde and 25 mg/kg carvacrol ($n = 6$). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

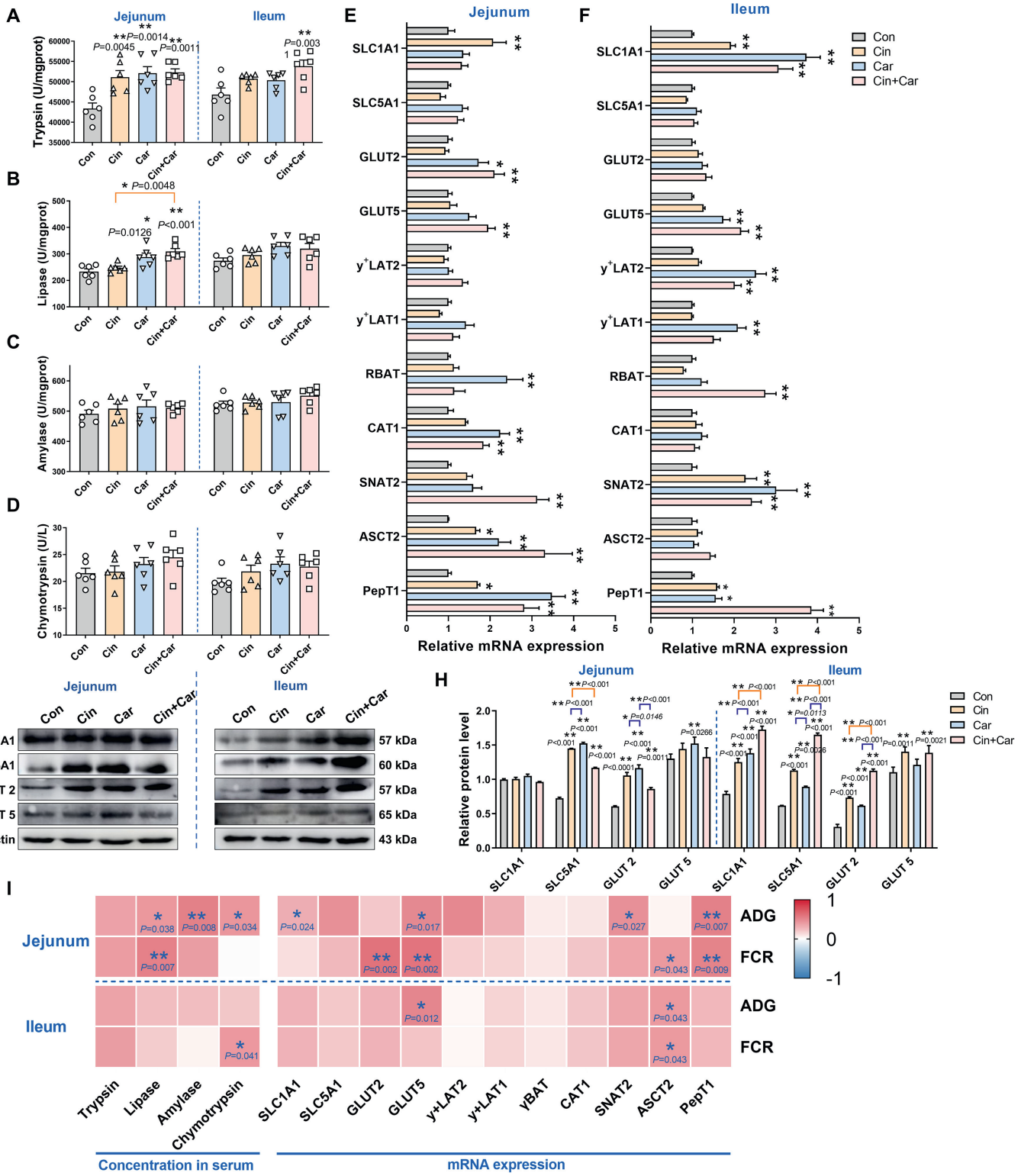

Fig. 3. Effects of cinnamaldehyde and carvacrol supplementation on intestinal digestion and absorption in nursery pigs. (A–D) Levels of trypsin, lipase, amylase and chymotrypsin in jejunal and ileal contents. (E, F) The mRNA expression of nutrient transporters of jejunum and ileum. (G, H) Protein abundance of SLC1A1, SLC5A1, GLUT2 and GLUT5 of jejunum and ileum and their quantification. (I) Correlation analysis of digestive enzymes and nutrient transporters of jejunum and ileum with ADG and FCR. *SLC1A1* = solute carrier family 1 member 1; *SLC5A1* = solute carrier family 5 member 1; *GLUT* = glucose transporter type; *γ⁺LAT* = γ⁺ system L-type amino acid transporter; *RBAT* = B(0,+)-type amino acid transport protein; *CAT1* = cationic amino acid transporter 1; *SNAT2* = sodium-coupled neutral amino acid transporter 2; *ASCT2* = alanine-serine-cysteine transporter 2; *PepT1* = peptide transporter 1; ADG = average daily gain; FCR = feed conversion ratio. Con means piglets fed basal diet ($n = 6$); Cin means CON diet with 50 mg/kg cinnamaldehyde ($n = 6$); Car means CON diet with 50 mg/kg carvacrol ($n = 6$); Cin + Car means CON diet with 25 mg/kg cinnamaldehyde and 25 mg/kg carvacrol ($n = 6$). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

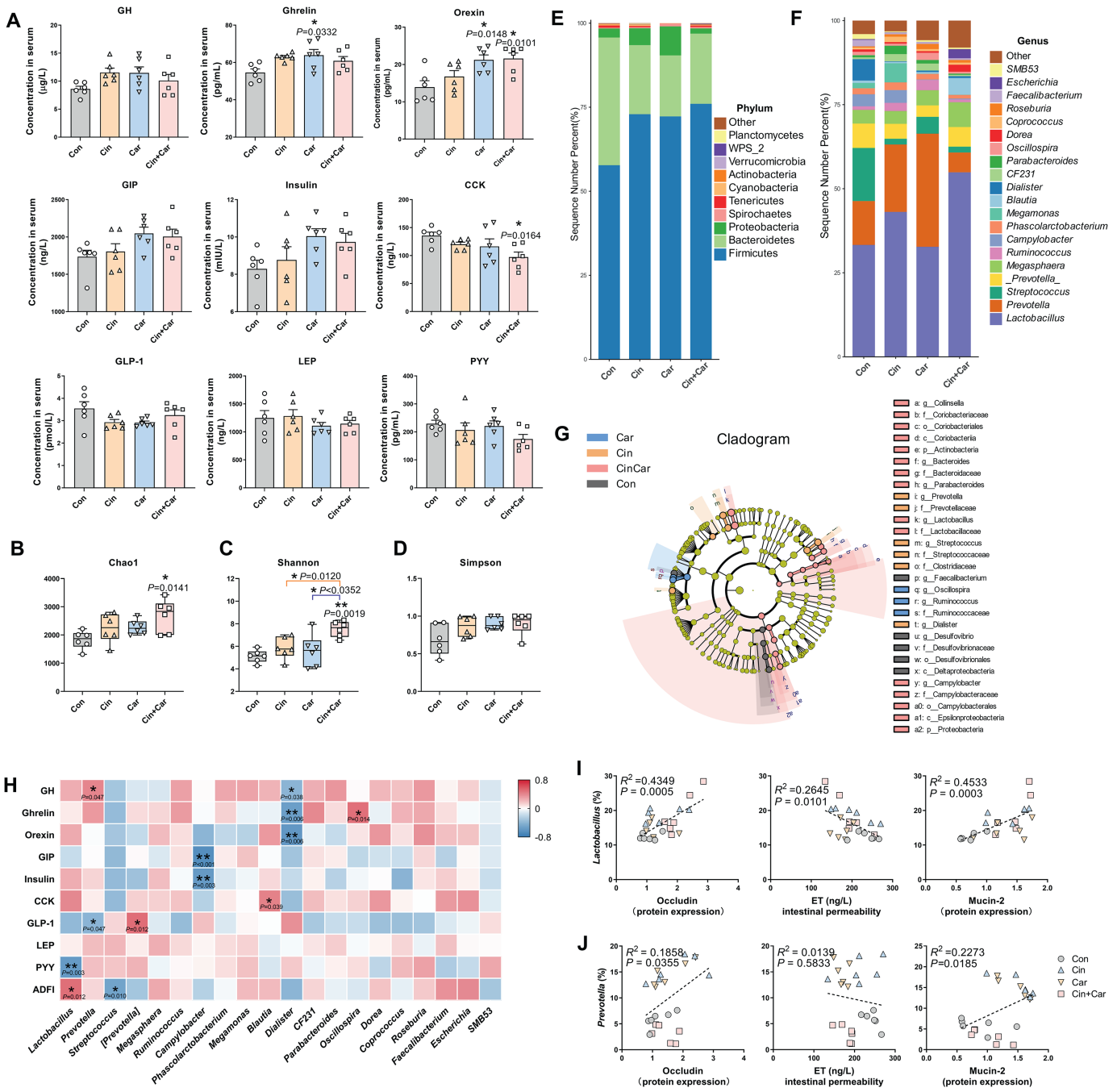

Fig. 4. Effects of cinnamaldehyde and carvacrol supplementation on appetite and gut microbiota in nursery pigs. (A) Expression of appetite-related hormones in serum. (B) Chao 1 index. (C) Shannon index. (D) Simpson index. (E) Relative abundance of microbiota at the phylum level. (F) Relative abundance of microbiota at the genus level. (G) Cladogram generated from LEfSe analysis. (H) Correlation analysis of top 20 microbes with appetite-related hormones and feed intake. Correlation analysis of (I) *Lactobacillus* and (J) *Prevotella* with occludin and mucin-2 protein expression, serum ET level. GH = growth hormone; GIP = gastric inhibitory polypeptide; CCK = cholecystokinin; GLP-1 = glucagon-like peptide-1; LEP = leptin; PYY = peptide YY; ADFI = average daily feed intake; ET = endothelin. Con means piglets fed basal diet ($n = 6$); Cin means CON diet with 50 mg/kg cinnamaldehyde ($n = 6$); Car means CON diet with 50 mg/kg carvacrol ($n = 6$); Cin + Car means CON diet with 25 mg/kg cinnamaldehyde and 25 mg/kg carvacrol ($n = 6$). Values for R^2 and P are indicated in each graph. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

could adversely impact their survival at a very early and most vulnerable stage (Li et al., 2018b). The protective effects of EO on intestinal health have been documented in nursery pigs. Balasubramanian et al. (2016) reported that supplementation of EO increased growth performance (BW, ADFI, FCR) and reduced the diarrhea rate of piglets. In the present study, we found that piglets supplemented with EO had higher BW at d 14, 28, and 42, higher ADG throughout the experimental period, and higher ADFI from 14 to 28 d. These findings were consistent with those of Tian and Piao (2019), who found that dietary supplementation with cinnamaldehyde could lower the diarrhea rate and increase the ADG in piglets. Importantly, in line with the results of Tian and Piao (2019), we also observed that the addition of cinnamaldehyde to the diet reduced the diarrhea rate in nursery pigs. Previous research confirmed that the addition of oregano essential oil also has the potential to improve piglet performance (Tan et al., 2015). Conversely, Yang et al. (2019) found that dietary cinnamaldehyde and thymol supplementation was beneficial in increasing ADG but

not significantly improving diarrhea, contradicting the results of many studies. The reason for this may be related to the environment in which the animal is raised and the composition, proportion and quantity of EO provided.

Intestinal morphology is a significant predictor of intestinal health status, in which VH and CD serve as the main markers of cell maturation rate as well as intestinal digestion and absorption area, respectively (Chen et al., 2021b). Thus, CD, VH and their ratio are commonly used as indicators of intestinal health in piglets, and the height of the intestinal villi is considered a marker of the capacity of the pig to absorb nutrients from the feed. The intestinal morphology results show a significant increase in the length of the villi and a close alignment of the microvillus in the Cin + Car group. Since the VH is positively correlated with the absorptive surface area of the common luminal villi (Taylor et al., 2021), an increase in VH may imply a better FCR; the current study also demonstrated that longer intestinal villus length is also accompanied by higher daily gain. Meanwhile, Michielssup et al. (2010) reported higher ratio of VH to CD in the small intestine of carvacrol-fed piglets.

The damage to the intestinal mucosa and increased intestinal permeability by multiple stressors experienced during the nursery period increase the likelihood of pathogen adhesion and invasion

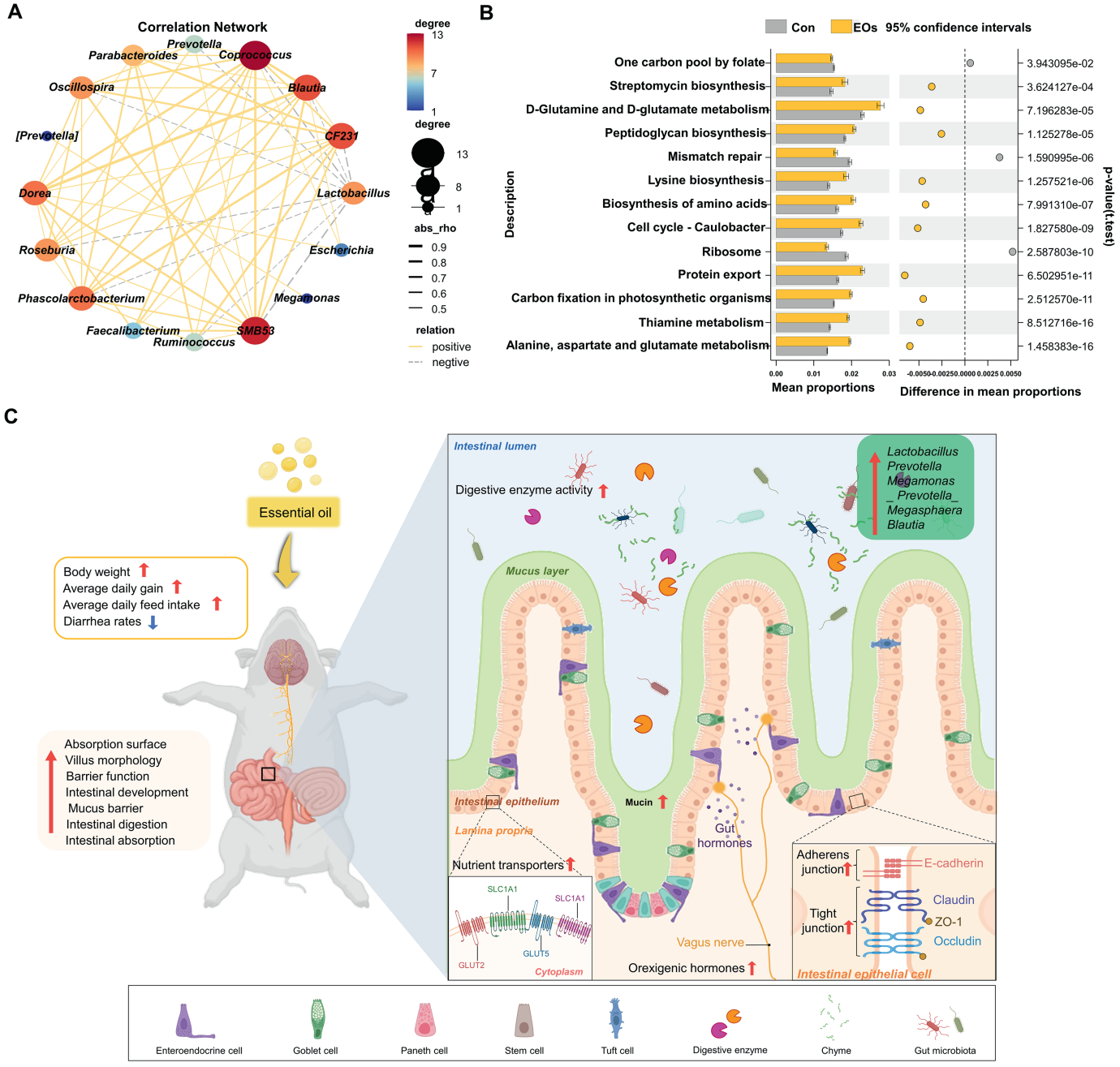

Fig. 5. Analysis of correlation and function of differential intestinal microbiota. (A) Correlation network of the microbiota at the genus level. (B) Predicted functionality of the microbiota. (C) The schematic diagram depicting EO driving improvements in intestine health and microbiota structure to promote performance and appetite in nursery pigs. Obviously, dietary supplementation of EO can improve the performance of nursery pigs and improve the digestion and absorption efficiency of feeds by ensuring the integrity, turnover and development of the intestinal barrier and increasing digestive enzyme activity and nutrient transporter abundance. Furthermore, the increased diversity and structure of intestine microbiota will affect appetite-related hormones, thereby promoting appetite.

(Moeser et al., 2007). In addition, this can also affect nutrient absorption and increase disease susceptibility in nursery pigs (Wijtten et al., 2011). Tight junctions are crucial modulators of the paracellular permeability of water, ions, and macromolecules in adjacent cells, which consist of transmembrane proteins (such as claudin and occludin) and intracellular plaque proteins (such as ZO-1) (Slifer and Blikslager, 2020). Besides, as the intestinal barrier plays key roles in controlling intestinal inflammation, chemical barriers like mucin-2 secreted by goblet cells are utilized to measure the integrity of the barrier (Capaldo et al., 2017; Mao et al., 2011; Moughan et al., 2013). According to the results of Pu et al. (2020) and Ma et al. (2022), who found that dietary supplementation of cinnamaldehyde or carvacrol improved the expression of claudin-1 and ZO-1 in the jejunum and occludin, claudin-1, and mucin-2 in the ileum of piglets, we demonstrated that dietary supplementation of EO improved the expression of tight junction-associated proteins and mucin-2 in the jejunum and ileum of piglets. Similarly, piglets fed carvacrol and the mixture also showed lower intestinal permeability indicators (DAO, ET, and D-LA) and intestinal local inflammatory indicators (IL-1, IL-6) compared to the Con group, suggesting that EO improved intestinal barrier function and allowed for the prevention of penetration of harmful feed additives and bacterial metabolites. In addition, in agreement with the results of increased mucin-2 protein abundance, we also found an increase in the numbers of intestinal glycoproteins and goblet cells after feeding EO. It suggests that the increased intestinal mechanical and chemical barriers may be responsible for the decreased rate of diarrhea and intestinal local inflammation in piglets.

The cornerstone of preserving intestinal epithelial barrier function is intestinal stem cells driving the production of all epithelial cell types (Hou et al., 2018; Zhou et al., 2020). Previous studies found that the stress of weaning has an inhibitory effect on the function of intestinal stem cells in nursery pigs (Verdile et al., 2019). In the present study, dietary EO supplementation significantly elevated the protein expression of Lyz in the jejunum and Lgr5 in the ileum. Lgr5 serves as a marker for intestinal stem cells, and the degree of its expression reflects the ability of the intestine to maintain intestinal epithelial homeostasis and turnover rate (van der Flier and Clevers, 2009). Lyz secreted by Paneth cells lyses bacteria by causing the contents of the cell wall to escape, thereby reducing the amount of endogenous bacteria and their toxins that attack epithelial cells (Smith et al., 2017). Therefore, this evidence suggests that EO enhances intestinal barrier function and improves intestinal homeostasis by promoting intestinal stem cell differentiation. On the other hand, the normal structure and function of the intestinal mucosa rely on homeostasis for rapid self-renewal of the gut (van der Flier and Clevers, 2009; Zhu et al., 2019). The results of this study suggest that EO supplementation may contribute to intestinal development by increasing the number of Ki67-positive cells and stimulating epithelial cell proliferation.

Satisfactory gastrointestinal digestion and absorption efficiency increase feed utilization. Enzyme activity in the intestine is important in the digestion and absorption of nutrients. Therefore, when the enzyme activity is upregulated, it may have a positive effect on nutrient absorption. Several studies have proved that dietary supplementation with active substances extracted from plants modulates the gene expression of nutrient transporters in the intestine and stimulates digestive juice secretion to improve the digestibility of nutrients (Li et al., 2012; Liu et al., 2013). Our results also found that pigs supplemented with cinnamaldehyde and carvacrol had higher levels of trypsin and lipase in their jejunum compared with the Con group, which is consistent with the study by Li et al. (2012). Through chemosensory systems contain many neurons and receptors, the gastrointestinal tract not only absorbs nutrition but also perceives intraluminal nutrients, chemicals, and microbes (Furness et al., 2013; Mace and Marshall, 2013). The system regulates digestive enzymes, intestinal peptide secretion and transporter expression which in turn governs metabolism, digestion and absorption. In order to further influence the expression of nutrient transporters, we proposed that EO in feed may also be sensed by as-yet-undiscovered intestinal chemosensors. Approximately 90% of intestinal epithelial cells are absorptive epithelial cells, which express a variety of nutrient transporters (Omonijo et al., 2018). For instance, facilitatory transporters GLUT2 and GLUT5 in the basolateral membrane allow sugar to enter the systemic circulation in the forms of fructose, galactose and glucose (Koepsell, 2020). Furthermore, employing the transmembrane Na^+ gradient as a driving factor, SLC5A1 transports monosaccharides across the brush border membrane of small intestine enterocytes. Next, membrane potential is created by Na^+-K^+-ATPase and further leaves enterocytes via GLUT2 across the basolateral membrane (Gromova et al., 2021; Lee et al., 1994). Peptides and amino acids are the final by-products of protein digestion, which are subsequently absorbed by amino acid transport carriers in the intestine. For instance, SLC1A1 in the gut absorbs glutamate and aspartate, while PepT1 transports dipeptides and tripeptides (Spanier and Rohm, 2018; Wellington et al., 2021). Interestingly, we also detected higher levels of nutrient transporters at the gene and protein levels in the jejunal and ileal mucosa of EO-fed pigs. Notably, the fact that the correlation exists between the increased digestive enzyme activity and nutrient transporter expression and ADG, feed utilization in EO-supplemented nursery pigs led us to suggest that dietary EO supplementation is an effective measure to improve food intake, intestinal development, piglet health and growth efficiency.

The gastrointestinal tract is the initial point of contact with ingested nutrients and is responsible for informing the brain about the amount and composition of feed ingested. Recent studies have demonstrated that a small subset of enteroendocrine subtypes exists within the intestinal mucosa with the ability to secrete peptides (e.g., CCK, GLP-1, and PYY) that further regulate appetite, energy metabolism, and intestinal health (Bauer et al., 2016). In addition, appetite-related hormones including LEP, GH, ghrelin, orexin, GIP, and insulin also play a role in regulating food cravings and reward-driven eating behaviors (Chao et al., 2017). EO were also exploited for their flavoring properties and for their potential to improve the taste of feed (Bauer et al., 2016; Zeng et al., 2015). Specifically, GLP-1, GIP, PYY, insulin, and LEP act as peripheral intestinal hormones that increase satiety, whereas GH, ghrelin and orexin are able to induce hunger (Schellekens et al., 2012; van de Wouw et al., 2017). Our results suggest that EO supplementation may increase feed intake in nursery pigs, for reasons that may be related to the fact that EO improves food flavor and increases appetite.

The gut microbiota is also involved in regulating host physiology and health. In general, gut bacteria become increasingly stable as the animal grows, and the abundance and diversity of microorganisms boost the organism's resistance to invasion by foreign infections. Therefore, this explains why young pigs are more vulnerable to diseases than adult pigs (Kim and Isaacson, 2015; Wang et al., 2022). Consistent with the conclusions of Mo et al. (2021), in the present study, EO supplementation enhanced the Chao 1 and Shannon indices of the colonic microbiota. To further explore variations in how EO affects pathogenic and beneficial bacteria at the genus level in the colon, the structure of gut microbiota was analyzed. *Streptococci* and *Campylobacter* are the most common potential pathogens in the colon of piglets, and studies have demonstrated that they are associated with diarrhea, mucosal damage, intestinal inflammation, and inflammatory bowel

disease (Lun et al., 2007; Yang et al., 2020). The bactericidal activity of carvacrol and cinnamaldehyde as the bacterial membrane penetrant has been extensively studied. Particularly, carvacrol leads to the collapse of the proton motive force, alters cell membrane permeability and depletion of the ATP pool, and inhibits bacterial motility by preventing the synthesis of flagellin (Lambert et al., 2001; Nazzaro et al., 2013). Similarly, our study found that EO was able to significantly reduce the abundance of *Streptococci* and *Campylobacter* in the colon of nursery pigs, which may be associated with a reduced rate of diarrhea. The predominant bacterial genera in the intestines of young animals that enhance immune function and sustain intestinal health are *Lactobacillus* and *Megasphaera* (Yoshida et al., 2009). Currently, a few strains from these two genera have been produced into probiotic products to exert beneficial activity and improve the health of the host (Direkvandi et al., 2021; Śliżewska et al., 2021). As a core component of one of the two most common bacterial enterotypes in the swine gut microbiota, *Prevotella*-driven enterotypes have been shown to positively correlate with animal performance, including feed intake, feed effectiveness and weight gain, and can also increase fat accumulation in pigs (Amat et al., 2020; Chen et al., 2021a). *Ruminococcus* is a genus that produces secondary bile acids and maintains intestinal homeostasis (Sinha et al., 2020). *Phascolarctobacterium* has been reported to inhibit the growth of *Portunus* by depleting succinic acid, which in turn alleviates intestinal inflammation (Nagao-Kitamoto et al., 2020). In the colon, dietary EO supplementation markedly increased the relative abundance of beneficial bacteria, indicating that EO encouraged the proliferation of probiotics. More startling is the fact that the gut microbiota has a major impact on the microbiota-gut-brain axis, which is the bidirectional connection between the gastrointestinal system and the brain (Nagao-Kitamoto et al., 2020; van de Wouw et al., 2017). Gut microbiota have been shown to influence bile acid metabolism and are able to make various metabolites, including short-chain fatty acids, neuroactive substances, biomolecules, and toxins (Romaní-Pérez et al., 2021). Subsequently, these metabolite changes exert their effects by directly interacting with enteroendocrine L cells or intestinal receptors on the vagus nerve, or by translocation of the intestinal epithelium into the peripheral circulation (Fetissov, 2017). Specifically, dietary-induced alterations in microbial antigens can affect intestinal mucosal immunity. Circulating IgG may enhance the orexigenic effects of ghrelin by acting as a peptide carrier to protect the highly labile peptide hormone from degradation (Takagi et al., 2013). Furthermore, the gut microbiota influence host metabolism and appetite by regulating the synthesis of primary bile acids in the liver (Joyce et al., 2014). *Lactobacillus* and *Streptococcus* species have been shown to metabolize to produce tryptophan in vitro (O'Mahony et al., 2015). Tryptophan can be fermented to indole, which acts as a ligand for the aryl hydrocarbon receptor to increase GLP-1 secretion (Natividad et al., 2018). Studies have shown that protein restriction in pigs is associated with lower levels of gastrointestinal hormones (including ghrelin, progesterone, and somatostatin), decreased levels of lactobacilli, streptococci, and short-chain fatty acids, and higher levels of *Prevotella* (Yu et al., 2019). In another study, a favorable correlation was found between plasma GH levels and the quantity of lactobacilli (Cheng et al., 2019). By Spearman correlation analysis, it was found that the abundance of most of the beneficial colonic bacteria was positively correlated with GH and ghrelin. The reason may be related to the regulatory effect of metabolites of the genus. Abdelhamid and Luo (2018) found that intestinal barrier function can be enhanced by intestinal colonization with probiotics such as *Lactobacillus* (Abdelhamid and Luo, 2018). Moreover, *Lactobacillus* can repair damaged intestinal mucosa under TNF-induced intestinal mucosal injury (Wu et al., 2020a), which also partially explains the correlation we found between *Lactobacillus* abundance and intestinal integrity. Recent studies have shown that in addition to acting as a physical barrier to keep pathogens out of the digestive tract, the mucus layer also works as a substrate for bacteria like *Prevotella* which can break down mucin (Amat et al., 2020). However, *Prevotella* was positively correlated to some extent with the expression of mucin-2 in our results, but the linear regression model is poorly fitted. Collectively, improvements in the colonic microbial community in nursery pigs can regulate appetite-related hormone levels and improve intestinal health.

5. Conclusion

In conclusion, our results demonstrate that dietary carvacrol and cinnamaldehyde may improve intestinal barrier function, development and mucus barrier; increase digestive enzyme activity and absorption capacity; alter microbial communities and increase appetite, thereby improving performance and alleviating diarrhea in nursery pigs (Fig. 5C). Improvements in absorption and digestion capacity are crucial for increased feed conversion and increased performance. Given the role of the microbiome-gut-brain axis, we also tentatively verified that there is a correlation between gut microbiome and appetite. This study lays the theoretical foundation for the application of carvacrol and cinnamaldehyde as natural plant extracts to promote intestinal health and appetite in young mammals.

Author contributions

Bi-Chen Zhao: conceptualization, investigation, writing-original draft. **Tian-Hao Wang:** methodology, visualization. **Jian Chen:** data curation, investigation, methodology. **Bai-Hao Qiu:** validation, visualization. **Ya-Ru Xu:** data curation, visualization. **Jin-Long Li:** conceptualization, validation, supervision, funding acquisition, writing - review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.10.007.

References

Abdelhamid L, Luo XM. Retinoic acid, leaky gut, and autoimmune diseases. Nutrients 2018;10:1016.

Amat S, Lantz H, Munyaka PM, Willing BP. Prevotella in pigs: the positive and negative associations with production and health. Microorganisms 2020;8:1584.

Arce L, Domínguez-Vidal A, Rodríguez-Estévez V, López-Vidal S, Ayora-Cañada MJ, Valcárcel M. Feasibility study on the use of infrared spectroscopy for the direct authentication of Iberian pig fattening diet. Anal Chim Acta 2009;636:183–9.

Balasubramanian B, Park JW, Kim IH. Evaluation of the effectiveness of supplementing micro-encapsulated organic acids and essential oils in diets for sows and suckling piglets. Ital J Anim Sci 2016;15:1–8.

Bauer PV, Hamr SC, Duca FA. Regulation of energy balance by a gut-brain axis and involvement of the gut microbiota. Cell Mol Life Sci 2016;73:737–55.

Brenes A, Roura E. Essential oils in poultry nutrition: main effects and modes of action. Anim Feed Sci Technol 2010;158:1–14.

Capaldo CT, Powell DN, Kalman D. Layered defense: how mucus and tight junctions seal the intestinal barrier. J Mol Med (Berl) 2017;95:927–34.

Chao AM, Jastreboff AM, White MA, Grilo CM, Sinha R. Stress, cortisol, and other appetite-related hormones: prospective prediction of 6-month changes in food cravings and weight. Obesity (Silver Spring) 2017;25:713–20.

Chaudhry RM, Scow JS, Madhavan S, Duenes JA, Sarr MG. Acute enterocyte adaptation to luminal glucose: a posttranslational mechanism for rapid apical recruitment of the transporter GLUT2. J Gastrointest Surg 2012;16:312–9.

Chen C, Fang S, Wei H, He M, Fu H, Xiong X, Zhou Y, Wu J, Gao J, Yang H, Huang L. Prevotella copri increases fat accumulation in pigs fed with formula diets. Microbiome 2021a;9:175.

Chen X, Xu J, Ren E, Su Y, Zhu W. Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. Anaerobe 2018;49:30–40.

Chen Y, Xie Y, Zhong R, Liu L, Lin C, Xiao L, Chen L, Zhang H, Beckers Y, Everaert N. Effects of xylo-oligosaccharides on growth and gut microbiota as potential replacements for antibiotic in weaning piglets. Front Microbiol 2021b;25:641172.

Cheng CS, Wei HK, Wang P, Yu HC, Zhang XM, Jiang SW, Peng J. Early intervention with faecal microbiota transplantation: an effective means to improve growth performance and the intestinal development of suckling piglets. Animal 2019;13:533–41.

Chitprasert P, Sutaphanit P. Holy basil (Ocimum sanctum Linn.) essential oil delivery to swine gastrointestinal tract using gelatin microcapsules coated with aluminum carboxymethyl cellulose and beeswax. J Agric Food Chem 2014;62:12641–8.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72:5069–72.

Direkvandi E, Mohammadabadi T, Salem AZM. Influence of three microbial feed additives of Megasphaera elsdenii, Saccharomyces cerevisiae and Lactobacillus sp. on ruminal methane and carbon dioxide production, and biofermentation kinetics. J Appl Microbiol 2021;131:623–33.

Fetissov SO. Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. Nat Rev Endocrinol 2017;13:11–25.

Froehlich KA, Abdelsalam KW, Chase C, Koppien-Fox J, Casper DP. Evaluation of essential oils and prebiotics for newborn dairy calves. J Anim Sci 2017;95:3772–82.

Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B. The gut as a sensory organ. Nat Rev Gastroenterol Hepatol 2013;10:729–40.

Gholami-Ahangaran M, Ahmadi-Dastgerdi A, Azizi S, Basiratpour A, Zokaei M, Derakhshan M. Thymol and carvacrol supplementation in poultry health and performance. Vet Med Sci 2022;8:267–88.

Gorboulev V, Schürmann A, Vallon V, Kipp H, Jaschke A, Klessen D, Friedrich A, Scherneck S, Rieg T, Cunard R, Veyhl-Wichmann M, Srinivasan A, Balen D, Breljak D, Rexhepaj R, Parker HE, Gribble FM, Reimann F, Lang F, Wiese S, Sabolic I, Sendtner M, Koepsell H. Na(+)-D-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. Diabetes 2012;61:187–96.

Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol 2017;25:851–73.

Gromova LV, Fetissov SO, Gruzdkov AA. Mechanisms of glucose absorption in the small intestine in health and metabolic diseases and their role in appetite regulation. Nutrients 2021;13:2474.

Han H, Yi B, Zhong R, Wang M, Zhang S, Ma J, Yin Y, Yin J, Chen L, Zhang H. From gut microbiota to host appetite: gut microbiota-derived metabolites as key regulators. Microbiome 2021;9:162.

Hashemipour H, Kermanshahi H, Golian A, Veldkamp T. Effect of thymol and carvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid composition, digestive enzyme activities, and immune response in broiler chickens. Poult Sci 2013;92:2059–69.

Helm ET, Curry S, Trachsel JM, Schroyen M, Gabler NK. Evaluating nursery pig responses to in-feed sub-therapeutic antibiotics. PLoS One 2019;14:e0216070.

Hou Q, Ye L, Liu H, Huang L, Yang Q, Turner JR, Yu Q. Lactobacillus accelerates ISCs regeneration to protect the integrity of intestinal mucosa through activation of STAT3 signaling pathway induced by LPLs secretion of IL-22. Cell Death Differ 2018;25:1657–70.

Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, Hill C, Gahan CG. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proc Natl Acad Sci U S A 2014;111:7421–6.

Kabat AM, Srinivasan N, Maloy KJ. Modulation of immune development and function by intestinal microbiota. Trends Immunol 2014;35:507–17.

Kim HB, Isaacson RE. The pig gut microbial diversity: understanding the pig gut microbial ecology through the next generation high throughput sequencing. Vet Microbiol 2015;177:242–51.

Koepsell H. Glucose transporters in the small intestine in health and disease. Pflugers Arch 2020;472:1207–48.

Kogut MH, Arsenault RJ. Editorial: gut health: the new paradigm in food animal production. Front Vet Sci 2016;3:71.

Lambert RJ, Skandamis PN, Coote PJ, Nychas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol 2001;91:453–62.

Lee WS, Kanai Y, Wells RG, Hediger MA. The high affinity Na+/glucose cotransporter. Re-evaluation of function and distribution of expression. J Biol Chem 1994;269:12032–9.

Li N, Huang S, Jiang L, Wang W, Li T, Zuo B, Li Z, Wang J. Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. Front Microbiol 2018a;9:1798.

Li P, Piao X, Ru Y, Han X, Xue L, Zhang H. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. Asian-Australas J Anim Sci 2012;25:1617–26.

Li Y, Guo Y, Wen Z, Jiang X, Ma X, Han X. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. Sci Rep 2018b;8:18068.

Liang Z, Wu H, Bian C, Chen H, Shen Y, Gao X, Ma J, Yao H, Wang L, Wu Z. The antimicrobial systems of Streptococcus suis promote niche competition in pig tonsils. Virulence 2022;13:781–93.

Liu Y, Song M, Che TM, Almeida JA, Lee JJ, Bravo D, Maddox CW, Pettigrew JE. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. J Anim Sci 2013;91:5294–306.

Lun ZR, Wang QP, Chen XG, Li AX, Zhu XQ. Streptococcus suis: an emerging zoonotic pathogen. Lancet Infect Dis 2007;7:201–9.

Ma J, Long S, Wang J, Gao J, Piao X. Microencapsulated essential oils combined with organic acids improves immune antioxidant capacity and intestinal barrier function as well as modulates the hindgut microbial community in piglets. J Anim Sci Biotechnol 2022;13:16.

Mace OJ, Marshall F. Digestive physiology of the pig symposium: gut chemosensing and the regulation of nutrient absorption and energy supply. J Anim Sci 2013;91:1932–45.

Mantzoros CS, Magkos F, Brinkoetter M, Sienkiewicz E, Dardeno TA, Kim SY, Hamnvik OP, Koniaris A. Leptin in human physiology and pathophysiology. Am J Physiol Endocrinol Metab 2011;301:E567–84.

Mao X, Zeng X, Qiao S, Wu G, Li D. Specific roles of threonine in intestinal mucosal integrity and barrier function. Front Biosci (Elite Ed) 2011;3:1192–200.

Michielssup J, Missotten J, Hoorick AV, Ovyn A, Fremaut D, Smet SD, Dierick N. Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. Arch Anim Nutr 2010;64:136–54.

Mo K, Li J, Liu F, Xu Y, Huang X, Ni H. Superiority of microencapsulated essential oils compared with common essential oils and antibiotics: effects on the intestinal health and gut microbiota of weaning piglet. Front Nutr 2021;8:808106.

Moeser AJ, Klok CV, Ryan KA, Wooten JG, Little D, Cook VL, Blikslager AT. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am J Physiol Gastrointest Liver Physiol 2007;292:G173–81.

Moughan PJ, Rutherfurd SM, Balan P. Kiwifruit, mucins, and the gut barrier. Adv Food Nutr Res 2013;68:169–85.

Nagao-Kitamoto H, Leslie JL, Kitamoto S, Jin C, Thomsson KA, Gillilland 3rd MG, Kuffa P, Goto Y, Jenq RR, Ishii C, Hirayama A, Seekatz AM, Martens EC, Eaton KA, Kao JY, Fukuda S, Higgins PDR, Karlsson NG, Young VB, Kamada N. Interleukin-22-mediated host glycosylation prevents Clostridioides difficile infection by modulating the metabolic activity of the gut microbiota. Nat Med 2020;26:608–17.

Natividad JM, Agus A, Planchais J, Lamas B, Jarry AC, Martin R, et al. Impaired aryl hydrocarbon receptor ligand production by the gut microbiota is a key factor in metabolic syndrome. Cell Metab 2018;28:737–749.e4.

Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V. Effect of essential oils on pathogenic bacteria. Pharmaceuticals (Basel) 2013;6:1451–74.

Noblet J, Fortune H, Shi XS, Dubois S. Prediction of net energy value of feeds for growing pigs. J Anim Sci 1994;72:344–54.

O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 2015;277:32–48.

NRC (National Research Council). Nutrient Requirements of Swine. 11th ed. Washington DC: National Academic Press; 2012.

Ogawa K, Ito M. Appetite-enhancing effects of trans-cinnamaldehyde, benzylacetone and 1-phenyl-2-butanone by inhalation. Planta Med 2016;82:84–8.

Omonijo FA, Ni L, Gong J, Wang Q, Lahaye L, Yang C. Essential oils as alternatives to antibiotics in swine production. Anim Nutr 2018;4:126–36.

Pu J, Chen D, Tian G, He J, Zheng P, Mao X, Yu J, Huang Z, Luo J, Luo Y, Yu B. Effects of benzoic acid, Bacillus coagulans and oregano oil combined supplementation on

growth performance, immune status and intestinal barrier integrity of weaned piglets. Anim Nutr 2020;6:152–9.
Romaní-Pérez M, Bullich-Vilarrubias C, López-Almela I, Liébana-García R, Olivares M, Sanz Y. The microbiota and the gut-brain axis in controlling food intake and energy homeostasis. Int J Mol Sci 2021;22:5830.
Roura E, Koopmans SJ, Lallès JP, Le Huerou-Luron I, de Jager N, Schuurman T, Val-Laillet D. Critical review evaluating the pig as a model for human nutritional physiology. Nutr Res Rev 2016;29:60–90.
Schellekens H, Finger BC, Dinan TG, Cryan JF. Ghrelin signalling and obesity: at the interface of stress, mood and food reward. Pharmacol Ther 2012;135:316–26.
Sinha SR, Haileselassie Y, Nguyen LP, Tropini C, Wang M, Becker LS, et al. Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. Cell Host Microbe 2020;27:659–670.e5.
Slifer ZM, Blikslager AT. The integral role of tight junction proteins in the repair of injured intestinal epithelium. Int J Mol Sci 2020;21:972.
Śliżewska K, Chlebicz-Wójcik A, Nowak A. Probiotic properties of new Lactobacillus strains intended to be used as feed additives for monogastric animals. Probiotics Antimicrob Proteins 2021;13:146–62.
Smith NR, Davies PS, Levin TG, Gallagher AC, Keene DR, Sengupta SK, Wieghard N, El Rassi E, Wong MH. Cell adhesion molecule CD166/ALCAM functions within the crypt to orchestrate murine intestinal stem cell homeostasis. Cell Mol Gastroenterol Hepatol 2017;3:389–409.
Spanier B, Rohm F. Proton coupled oligopeptide transporter 1 (PepT1) function, regulation, and influence on the intestinal homeostasis. Compr Physiol 2018;8:843–69.
Sun Z, Li H, Li Y, Qiao J. Lactobacillus salivarius, a potential probiotic to improve the health of LPS-challenged piglet intestine by alleviating inflammation as well as oxidative stress in a dose-dependent manner during weaning transition. Front Vet Sci 2020;7:547425.
Takagi K, Legrand R, Asakawa A, Amitani H, François M, Tennoune N, Coëffier M, Claeyssens S, do Rego JC, Déchelotte P, Inui A, Fetissov SO. Anti-ghrelin immunoglobulins modulate ghrelin stability and its orexigenic effect in obese mice and humans. Nat Commun 2013;4:2685.
Tan C, Wei H, Sun H, Ao J, Long G, Jiang S, Peng J. Effects of dietary supplementation of oregano essential oil to sows on oxidative stress status, lactation feed intake of sows, and piglet performance. Biomed Res Int 2015;2015:525218.
Tang X, Xiong K, Fang R, Li M. Weaning stress and intestinal health of piglets: a review. Front Immunol 2022;13:1042778.
Taylor SR, Ramsamooj S, Liang RJ, Katti A, Pozovskiy R, Vasan N, Hwang SK, Nahiyaan N, Francoeur NJ, Schatoff EM, Johnson JL, Shah MA, Dannenberg AJ, Sebra RP, Dow LE, Cantley LC, Rhee KY, Goncalves MD. Dietary fructose improves intestinal cell survival and nutrient absorption. Nature 2021;597:263–7.
Thymann T. Endocrine regulation of gut maturation in early life in pigs. Domest Anim Endocrinol 2016;56(Suppl):S90–3.
Tian QY, Piao XS. Essential oil blend could decrease diarrhea prevalence by improving antioxidative capability for weaned pigs. Animals (Basel) 2019;9:847.
van de Wouw M, Schellekens H, Dinan TG, Cryan JF. Microbiota-gut-brain axis: modulator of host metabolism and appetite. J Nutr 2017;147:727–45.
van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 2009;71:241–60.
Varel VH. Carvacrol and thymol reduce swine waste odor and pathogens: stability of oils. Curr Microbiol 2002;44:38–43.
Verdile N, Mirmahmoudi R, Brevini T, Gandolfi F. Evolution of pig intestinal stem cells from birth to weaning. Animal 2019;13:1–10.
Wang C, Wei S, Liu B, Wang F, Lu Z, Jin M, Wang Y. Maternal consumption of a fermented diet protects offspring against intestinal inflammation by regulating the gut microbiota. Gut Microbes 2022;14:2057779.
Wei HK, Xue HX, Zhou ZX, Peng J. A carvacrol-thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets. Animal 2017;11:193–201.
Wellington MO, Rodrigues LA, Li Q, Dong B, Panisson JC, Yang C, Columbus DA. Birth weight and nutrient restriction affect jejunal enzyme activity and gene markers for nutrient transport and intestinal function in piglets. Animals (Basel) 2021;11:2672.
Wijtten PJ, van der Meulen J, Verstegen MW. Intestinal barrier function and absorption in pigs after weaning: a review. Br J Nutr 2011;105:967–81.
Wu H, Xie S, Miao J, Li Y, Wang Z, Wang M, Yu Q. Lactobacillus reuteri maintains intestinal epithelial regeneration and repairs damaged intestinal mucosa. Gut Microbes 2020a;11:997–1014.
Wu Z, Wu J, Lang F, Cai Z, Zeng X, Guo Y, Liu X, Pan D. Characterization of the sortase A from Lactobacillus acidophilus ATCC 4356 involved in adherence to intestinal cells. Future Microbiol 2020b;15:485–96.
Yang C, Zhang L, Cao G, Feng J, Yue M, Xu Y, Dai B, Han Q, Guo X. Effects of dietary supplementation with essential oils and organic acids on the growth performance, immune system, fecal volatile fatty acids, and microflora community in weaned piglets. J Anim Sci 2019;97:133–43.
Yang G, Yan Y, Zhang L, Ruan Z, Hu X, Zhang S, Li X. Porcine circovirus type 2 (PCV2) and Campylobacter infection induce diarrhea in piglets: microbial dysbiosis and intestinal disorder. Anim Nutr 2020;6:362–71.
Yoshida Y, Tsukahara T, Ushida K. Oral administration of Lactobacillus plantarum Lq80 and Megasphaera elsdenii iNP-001 induces efficient recovery from mucosal atrophy in the small and the large intestines of weaning piglets. Anim Sci J 2009;80:709–15.
Yu D, Zhu W, Hang S. Effects of low-protein diet on the intestinal morphology, digestive enzyme activity, blood urea nitrogen, and gut microbiota and metabolites in weaned pigs. Arch Anim Nutr 2019;73:287–305.
Zeng Z, Zhang S, Wang H, Piao X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. J Anim Sci Biotechnol 2015;6:7.
Zhao B, Luo C, Zhang M, Xing F, Luo S, Fu S, Sun X. Knockdown of phosphatase and tensin homolog (PTEN) inhibits fatty acid oxidation and reduces very low density lipoprotein assembly and secretion in calf hepatocytes. J Dairy Sci 2020;103:10728–41.
Zhao BC, Tang YX, Qiu BH, Xu HL, Wang TH, Elsherbeni AIA, Gharib HBA, Li JL. Astragalus polysaccharide mitigates transport stress-induced hepatic metabolic stress via improving hepatic glucolipid metabolism in chicks. J Anim Sci 2022;100:skac244.
Zhao BC, Wang TH, Chen J, Qiu BH, Xu YR, Zhang Q, Li JJ, Wang CJ, Nie QF, Li JL. Effects of dietary supplementation with a carvacrol-cinnamaldehyde-thymol blend on growth performance and intestinal health of nursery pigs. Porcine Health Manag 2023a;9:24.
Zhao Y, Cui JG, Zhang H, Li XN, Li MZ, Talukder M, Li JL. Role of mitochondria-endoplasmic reticulum coupling in lycopene preventing DEHP-induced hepatotoxicity. Food Funct 2021;12:10741–9.
Zhao Y, Zhang H, Cui JG, Wang JX, Chen MS, Wang HR, Li XN, Li JL. Ferroptosis is critical for phthalates driving the blood-testis barrier dysfunction via targeting transferrin receptor. Redox Biol 2023b;59:102584.
Zhou JY, Huang DG, Zhu M, Gao CQ, Yan HC, Li XG, Wang XQ. Wnt/β-catenin-mediated heat exposure inhibits intestinal epithelial cell proliferation and stem cell expansion through endoplasmic reticulum stress. J Cell Physiol 2020;235:5613–27.
Zhu M, Qin YC, Gao CQ, Yan HC, Li XG, Wang XQ. Extracellular glutamate-induced mTORC1 activation via the IR/IRS/PI3K/Akt pathway enhances the expansion of porcine intestinal stem cells. J Agric Food Chem 2019;67:9510–21.