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EFFECTS AND MECHANISMS OF DRY NEEDLING AT GV-1 ACUPUNCTURE ON VITAMIN DIGESTION AND ABSORPTION IN DIARRHEAL RABBITS

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ABSTRACT

Acupuncture has emerged as a novel therapeutic approach for diarrheal conditions, gaining increasing attention and utilization in recent studies due to its potential effectiveness. Notably, the GV-1 acupoint is frequently targeted for its efficacy in treating diarrheal conditions. The present study employed proteomics sequencing to disclose the possible mechanisms of rabbit diarrhea induced by *Folium Sennae* (FSAE) and dry needling (DN). A total of 18 rabbits were randomly allocated into three groups (n = 6), healthy control (HC), diarrhea control (DC), and GV-1. The results showed significant alterations in the rabbit colon following FSAE exposure, with 323 proteins showing notable alterations compared to the healthy controls (HC). Dry needling (DN) at GV-1 elicited alterations in the expression of 81 proteins compared with the diarrhea controls (DC). FSAE significantly changed four proteins in the vitamin digestion and absorption signaling pathway, and three of those proteins were altered by DN. Five retinol metabolism signaling pathway proteins changed considerably, caused by FSAE, and DN reversed the expression trends of three proteins. In addition, compared with the HC group, the VB7, VB12, CES2, MTHFR, Hcy, VA, Retinol, Retinal, and RA levels were significantly down-regulated. At the same time, TNF-α, IL-1β, IL-2, INF-γ, RBP4, STRA6, ADH, and RDH were markedly up-regulated in the rabbit colon of the DC group. Also, VB7, VB12, CES2, MTHFR, Hcy, VA, Ret, Retinal, and RA levels were meaningfully increased. Simultaneously, TNF-α, IL-1β, IL-2, INF-γ, RBP4, STRA6, ADH, and RDH were down-regulated in the rabbit colon of the GV-1 group. Applying GV-1 acupoint combined with DN mitigated FSAE-induced diarrhea index by modulating the expression levels of genes and proteins involved in the vitamin digestion/absorption signaling pathway and the retinol metabolism signaling pathway. The present study demonstrated that vitamin digestion, absorption, and retinol metabolism signaling pathways are involved in the underlying mechanism of acupuncture in alleviating diarrhea. This investigation may offer novel insights into the therapeutic management of diarrhea in animals.

Keywords: Proteomics, Acupuncture, GV-1, *Folium Sennae*, Rabbit.

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INTRODUCTION

The domestic rabbit is an herbivorous economic animal that provides humans with high-quality meat, skin, fur, and other products (Siddiqui *et al.,* 2023). They are characterized by high fecundity, short intergenerational intervals, rapid growth and development, and herbivorous habits (Al-Farraj *et al.,* 2024; Rehan *et al.,* 2023). With the development of the market economy, the increasing consumption of rabbit products has made large-scale rabbit farming an inevitable trend (Evangelho *et al.,* 2024). However, as the

scale of rabbit farming continues to expand, there is a growing trend in the prevalence of rabbit diseases (Topal *et al.,* 2023; Younas *et al.,* 2023). Diarrhea in rabbits has a high occurrence rate in some rabbitries, becoming the most significant infectious disease field (Kuehl *et al.,* 2020).

The pathological mechanism of diarrhea in rabbits is highly complex, and any internal or external factors that affect the intestinal flora balance can lead to diarrhea (Scaldaferri *et al.,* 2012). Both pathogenic microbial and non-pathogenic microbial factors contribute to this complexity. Studies indicate that disorders in digestive and absorptive functions can cause diarrhea (Farag *et al.,* 2023). As various nutrients are digested and absorbed in the intestine, diseases that affect intestinal digestion and absorption can lead to diarrhea (Uwaezuoke *et al.,* 2023). Diarrhea can impact the body's absorption of vitamins, leading to vitamin deficiencies (Ford, 2023; El-Sheikh*et al.,* 2024). Malnutrition, anemia, and vitamin deficiencies caused by diarrhea weaken the body's resistance to infectious diseases and various infections, which makes inflammation more likely to spread and diminishes the body's ability to regenerate tissues and heal from injuries (Poeta *et al.,* 2022).

The herb *Folium Sennae* (FSAE) possesses natural laxative properties and is frequently employed to induce diarrhea to establish a diarrhea model. FSAE refers to the dried leaves of the leguminous plants Cassia angustifolia Vahl or Cassia acutifolia Delile. It is known for its efficacy in clearing heat and promoting bowel movements and possesses properties such as inducing diarrhea, stopping bleeding, antimicrobial effects, muscle relaxation, and protection against gastric mucosal damage (Chew *et al.,* 2022). The mechanism of FSAE in diarrhea is to stimulate the intestinal mucosa, causing the blocked absorption or increased secretion of electrolytes and the increased frequency of intestinal peristalsis (Ding *et al.,* 2025). The diarrhea model induced by FSAE is characterized by acute and secretory diarrhea. A previous study demonstrated that fermented wheat bran significantly reduced the intestinal carriage rate and serum contents of IL-6, IL-12, and TNF-α in FSAE-induced diarrhea (Ding *et al.,* 2025; Yi *et al.,* 2020). Another research involved the gastric administration of FSAE extract solution to BALB/c mice, inducing acute diarrhea. The study found that *Malus pumila* leaf flavonoids had an intervention effect on FSAE-induced diarrhea in mice (Yi *et al.,* 2020).

Dry needling (DN) is one of the ancient acupuncture therapies with extensive clinical applications (Nistanak *et al.,* 2023). This therapeutic approach involves the utilization of a round-pointed needle, a filamentous needle, or a small, wide needle to administer appropriate stimulation at specific acupoints for treating livestock diseases (Blackmon and Elson, 2021). The Houhai acupoint is commonly used to treat diarrhea in rabbits. Houhai is a Luo-connecting acupoint of the Du meridian and the crossing acupoint of the Ren and Du Meridians, rich in nerve distribution (Jin *et al.,* 2020). It possesses strong Qi, connects with the Ren meridian, and regulates the intestines and bowel (Reboul *et al.,* 2019). Stimulating the Houhai acupoint can evoke the Qi of the meridians, boost the circulation of Qi and blood in the meridians, nourish the intestines, improve the conduction function of the intestines, and regulate the coordination and adaptability of the internal and external sphincter muscles (Xu and Hu, 2021). Research has indicated that the stimulation of Houhai acupoint had a two-way regulatory effect and was frequently used to treat diarrhea (Jin *et al.,* 2020). To further investigate the antidiarrheal effects of the Houhai acupoint on rabbits, we conducted proteomic sequencing analysis. The present study hypothesized that GV-1 acupoint stimulation combined with DN could alleviate FSAE-induced diarrhea in rabbits by modulating genes and proteins involved in vitamin digestion and absorption, as well as retinol metabolism signaling pathways.

MATERIALS AND METHODS

Animal Model and Experimental Design: For this experiment, a total of 18 male New Zealand rabbits aged 5 months (~2.0-2.5 kg) were obtained from the Experimental Animals Center of Hebei Agricultural University, Hebei, China. The rabbit house temperature was 24 ± 1°C, and the relative humidity was controlled between 50% to 60%. The rabbits were randomly divided into 3 groups: healthy control (HC), diarrhea control (DC), and GV-1 test groups, with 6 rabbits in each group. The rabbits in the HC group were administered sterile saline (8 mL/kg) for two consecutive weeks, whereas those in the DC and GV-1 groups received *Folium Sennae* (FSAE) by gavage at a dose of 8 mL/kg for two consecutive weeks.

On the 7th day of the experiment, the GV-1 group underwent the intervention treatment of DN at Houhai acupoint. After disinfecting the acupoint, the dry needle was inserted vertically into the depression of the dorsal mid-line between the rabbit anus and the ventral side of the tail, about 1 inch deep. By lifting, thrusting, twirling, and rotating the needle, a dull needling sensation is created. Acupuncture treatment was performed for 20 minutes/day for 7 days. The DC group underwent a daily simulated acupuncture procedure without receiving actual acupuncture stimulation. The duration of the experiment, from model construction to sample collection, was two weeks. Acupuncture treatment commences on the 7th day following model induction and continues until the end of the experiment.

At the end of the trial, all animals were euthanized using intravenous injection of pentobarbital

sodium (Ningbo Gino Chemical Co., Ltd.) at a concentration of 100 mg/kg into the auricular vein. Blood samples were collected and serums were obtained by centrifugation (1200 g, 4℃, 10 min) for ELISA analysis. Meanwhile, the colon tissues were rapidly excised and stored at -80 ℃.

Proteome Sequencing: Rabbit colon tissue samples were preprocessed using the iST sample preparation kit (PreOmics, Germany). Then, it was ground with liquid nitrogen. An appropriate amount of sample was taken with 50µl of lysis solution added and heated for 10 min (95°C and 1000 rpm). After the sample was cooled to room temperature, trypsin digestion buffer was added, and the sample was incubated at 37°C with shaking at 500 rpm for 2 h. Then stop buffer was added to terminate the enzymatic hydrolysis reaction. The iST cartridge in the kit was used to desalt the peptides, and 2 × 100 µl elution buffer was used for elution. The eluted peptides were dried and stored at -80°C on the condition of vacuum. Afterwards, a spectral database was established qualitatively through high pH reversed phase separation, low pH nano-HPLC-MS/MS analysis, and DDA (Russell *et al.,* 2022). The original data were merged and analyzed by Spectronaut X (Biognosys AG), and the database was searched using Uniprot or the provided database. In addition, the contaminated sequence library was searched at the same time to determine whether the sample was contaminated, and Trypsin digestion was set. Finally, DIA data collection was performed.

Drugs and Reagents: *Folium Sennae* (FSAE) was purchased from Beijing Tong Ren Tang Co., Ltd. (Beijing, China). For every 100 g, the herbal medicine was soaked with 1 L of water in a beaker for 30 min, which was then put into the water bath and heated to 100℃ for 25 min, and then the filtrate was obtained after filtering with gauze. These steps were repeated 80 times to produce about 49 L of FSAE aqueous extract. Subsequently, the aqueous extract was concentrated to 8 L using a multi-functional miniature extractor concentrator under a vacuum of 55 °C, which is equal to 1 g/mL of FSAE aqueous extract, which was then stored at 4 °C.

qPCR Analysis: Total RNA of rabbit colon samples was extracted and reverse transcription assays were performed using the Eastep Super Total RNA extraction kit (Promega, China) and Prime Script TM RT reagent Kit with gDNA Eraser (Promega, China). The relative gene expressions were analyzed by qPCR analysis, and the primers used are shown in Table 1.

Detection of Vitamins, Inflammatory Factors and Proteins: The contents of VA, VB7, VB12, TNF-α, IL-1β, IL-2, INF-γ, and proteins (CES2, MTHFR, Ret, RBP4, RDH, STRA6, Retinal, and RA) in colonic tissues were determined by ELISA kits from Enzyme-linked Biotechnology Co., Ltd (Shanghai, China).

Statistical Analysis: The data were analyzed using SPSS (version 22, IBM Corporation, Armonk, NY, USA) software. Statistical analysis was performed using one-way ANOVA followed by the multiple comparisons test (Tukey test).The differences among groups were considered statistically significant at p-value < 0.05.

RESULTS

Differentially Expressed Proteins: The principal component analysis diagram showed that the HC group, DC group, and GV-1 group exhibited clear separability. The contribution values of PC1, PC2, and PC3 were 64.8%, 21.5% and 8.7%, respectively (Fig. 1a). The correlation heat map showed that the correlation coefficient of the two expression levels among the three biological replicates was above 0.9 (Fig. 1b). The sample cluster diagram showed that HC group, DC group and GV-1 group had a high correlation (Fig. 1c). The repeated scatter plots revealed extremely high Pearson correlation coefficients (> 0.99) between any sample pairs within the same group (Fig.1d-l). In addition, the violin plot showed that in the colon samples of rabbits, a substantial majority of genes exhibit moderate expression, with a small fraction representing low and high-expression genes in the overall gene pool (Fig. 1m). These analyses indicated that the rabbit colon samples exhibited high correlation, reliability, and stability. The high similarity between the three biological replicates of rabbit colon samples suggests good repeatability of the experimental results (i.e., small sample size required with desired power), and the sequencing outcomes are suitable for various subsequent analyses.

Significant Differentially Expressed Proteins: DESeq analysis showed that compared with the HC group, a total of 323 different proteins (90 were up-regulated and 233 were down-regulated) were screened. Compared with the DC animals, almost 81 differentially expressed proteins (55 were up-regulated and 26 were down-regulated) were detected in GV1 rabbits. The corresponding volcano diagram and Venn diagram can be seen in Fig. 2.

Function Analysis: Compared with the HC animals, 4191 and 1961 GO different items were found in the DC and GV-1 groups, respectively. *Folium Sennae* exposure mainly affected the defense response, immune system process, and signaling receptor regulator activity. The treatment of DN could affect the defense response, response to external stimulus, and immune response (Fig. 3). Compared with the HC group, 235 and 111 meaningfully enriched signal pathways were identified in the DC and GV-1 rabbits, respectively (Fig. 4).

Differentially Expressed Proteins Related to Vitamin Digestion and Absorption, and Retinol Metabolism: Proteomic results showed that the expression levels of BTD, TCN2, APOA1, and APOA4 in the vitamin digestion and absorption signaling pathway of *Folium Sennae*-treated rabbits were significantly ($P < 0.05$) down-regulated. BTD, TCN2, and APOA1 expression levels from vitamin digestion and absorption signaling pathways in the rabbit colon treated with DN were up-regulated significantly ($P < 0.05$) compared with those in the GV-1 group (Fig. 5a).

Compared with the HC group, the expression levels of ADH5, RDH11, CYP1A1, CYP3A4, and CYP26A1 proteins were significantly down-regulated in the DC group, while they were significantly ($P < 0.05$) up-regulated in the GV-1 group (Fig. 6a).

qPCR Analysis: Compared with the HC group, BTD, TCN2, APOA1, and APOA4 were significantly decreased in the DC group, whereas BTD, TCN2, and APOA1 were significantly increased in the GV1 group compared to the DC group (Fig. 5b-e). Compared with the HC group, ADH5, RDH11, CYP1A1, CYP3A4, and CYP26A1 were significantly down-regulated in the DC group. In contrast, ADH5, RDH11, and CYP3A4 were significantly ($P < 0.05$) up-regulated in the GV1 group compared to the DC group (Fig. 6b-f).

Detection of Protein Contents and Inflammatory Factors: Compared with HC rabbits, VB7, VB12, CES2, MTHFR, and Hcy levels were significantly lower, while TNF-α, IL-1β, IL-2, and INF-γ were considerably higherin DC animals.Compared with the DC group, the levels of VB7, VB12, CES2, MTHFR, and Hcy were significantly ($P < 0.05$) up-regulated, while TNF-α, IL-1β, IL-2, and INF-γ were significantly ($P < 0.05$) down-regulated in the GV-1 group (Fig. 5f-n).

Compared with the HC group, VA, Ret, Retinal, and RA were significantly down-regulated, while RBP4, STRA6, ADH, and RDH were significantly up-regulated in the DC group ($P < 0.05$).Compared with the DC group, the VA, Ret, Retinal, and RA levels were significantly up-regulated, while RBP4, STRA6, ADH, and RDH were down-regulated considerably ($P < 0.05$) in rabbit colons of the GV-1 group (Fig. 6g-n).

Table 1. Gene-specific primers used in qPCR

Gene ID	mRNA sequence number	Primer sequence (5'-3')	Product length (bp)
BTD	100341187	F: TCTACGAGCAGCAAGTGATGACG R: GCATGAAGTCCAGAAACGGGTA	123
TCN2	100358905	F: ACTTGTCTGGAACGCCTCAACTTG R: GGTGCTGTAGACATTCCCGAAGTG	129
APOA1	100009253	F: GGATTTCGCCACCGTGTATG R: TGCCACTTCTTCTGGAATTCGT	291
APOA4	100328797	F: CCTACGGCGAGACCTACAAC R: GAGCTGACCTTGTCCCTCAG	133
ADH5	100009307	F: TGAAGGTGCTGGAATTGTGGAA R: CAAAGGCGCTGAAGGGTCTATT	298
RDH11	100353716	F: AGCCTGTACTGCGCCTTGA R: CCTTCCTATCGTCTCGTTTCG	111
CYP1A1	100328613	F: TCGTGAACCAGTGGCAGAACAAC R: AGAGCAGCACCTTCTCCGTCAG	126
CYP4A5	100328576	F: CTGCTCCTGCTGCTGCTCAAG R: CATCTGGAACTCTCGGCTGTGC	126
CYP26A1	408183	F: GCTGTCCGCTGCCGTTGC R: CGCCCAAAGAGGTGAGTCTTGTAG	142
β-action	396526	F: CGCAGAAACGAGACGAGATTG R: GATGCTCGCTCCAACGACTG	146

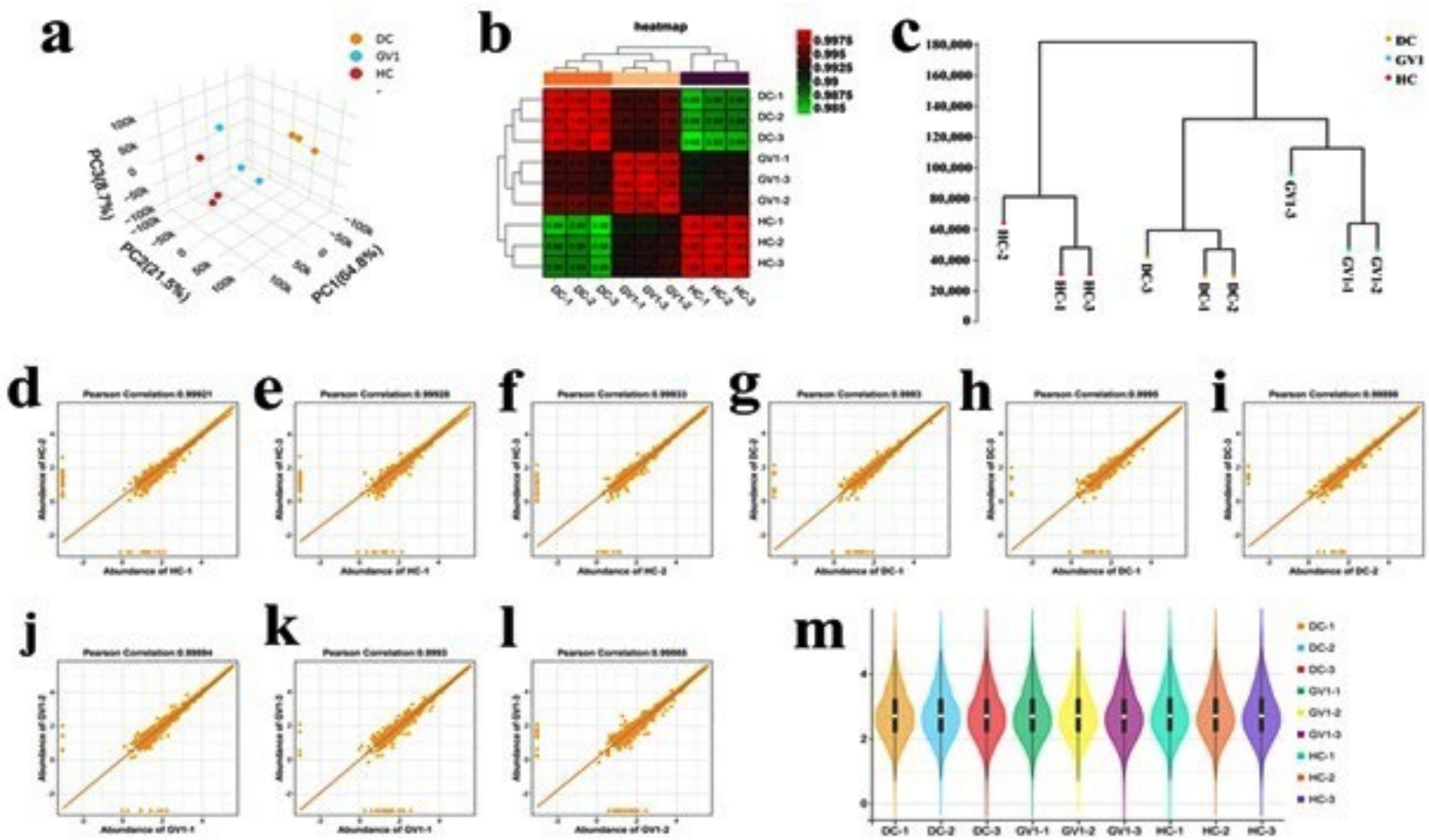

Figure 1: Detection of colonic correlation in the rabbit among each group. (a) The principal component analysis diagram for each group. (b) The correlation heat mapamong each group. (c) The sample cluster diagramfor each group. (d) The repeated scatter plot between the HC-1 and HC-2 groups. (e) The repeated scatter plot between the HC-1 and HC-3 groups. (f) The repeated scatter plot between the HC-2 and HC-3 groups. (g) The repeated scatter plot between the DC-1 and DC-2 groups. (h) The repeated scatter plot between the DC-1 and DC-3 groups. (i) The repeated scatter plot between the DC-2 and DC-3 groups. (j) The repeated scatter plot between the GV1-1 and GV1-2 groups. (k) The repeated scatter plot between the GV1-1 and GV1-3 groups. (l) The repeated scatter plot between the GV1-2 and GV1-3 groups. (m) The violin diagramfor each group.

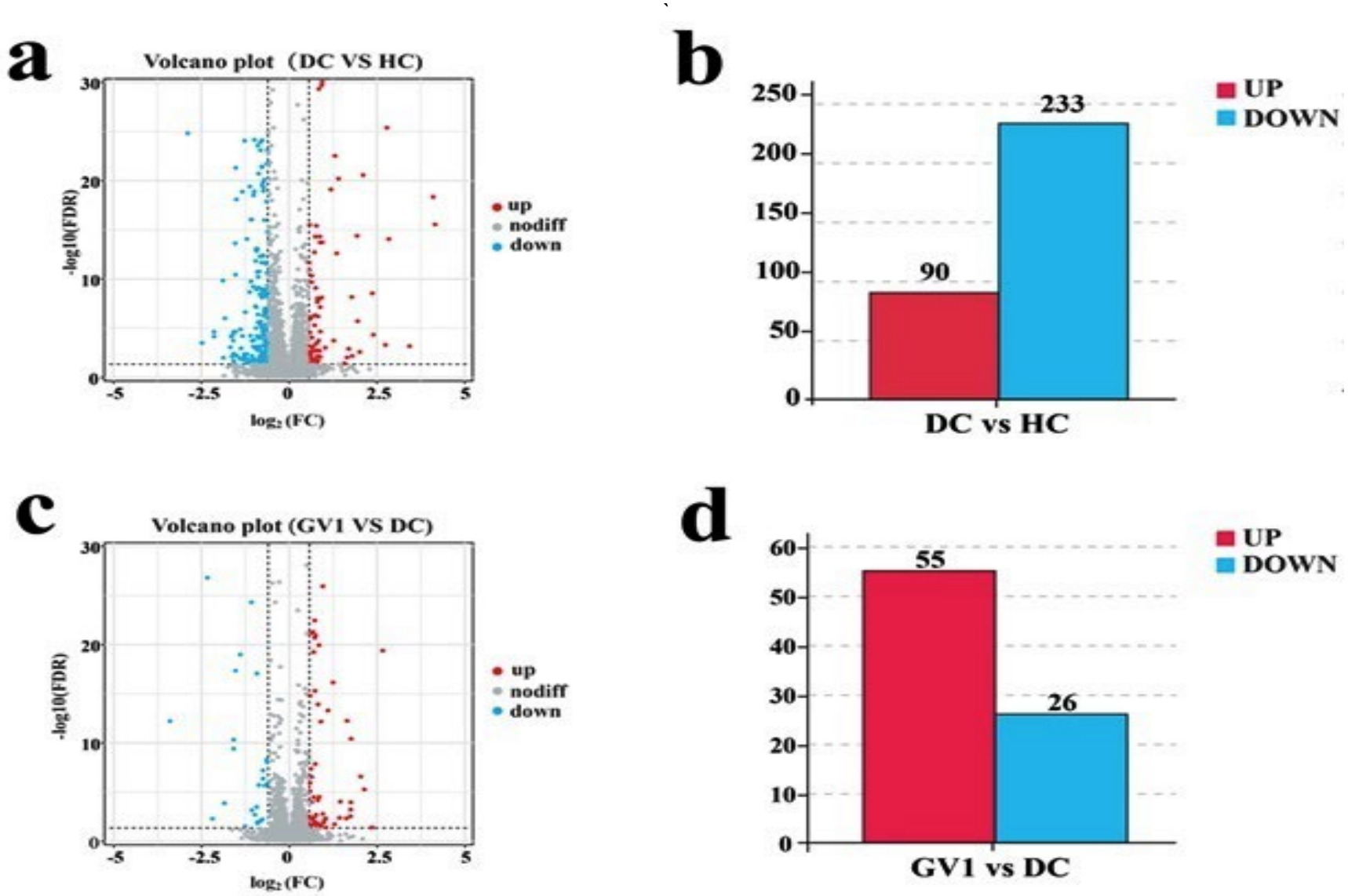

Figure 2:The significantly differentially expressed proteins in the rabbit colon. (a) Effects of *Folium Sennae* exposure on proteins of the rabbit colon. (b) Differentially expressed proteins between the DC group and the HC group. (c) Effects of DN on the proteins of the rabbit colon. (d) Expressed proteins between the GV1 group and the DC group (Red dots: high expression levels; green dots: low expression levels).

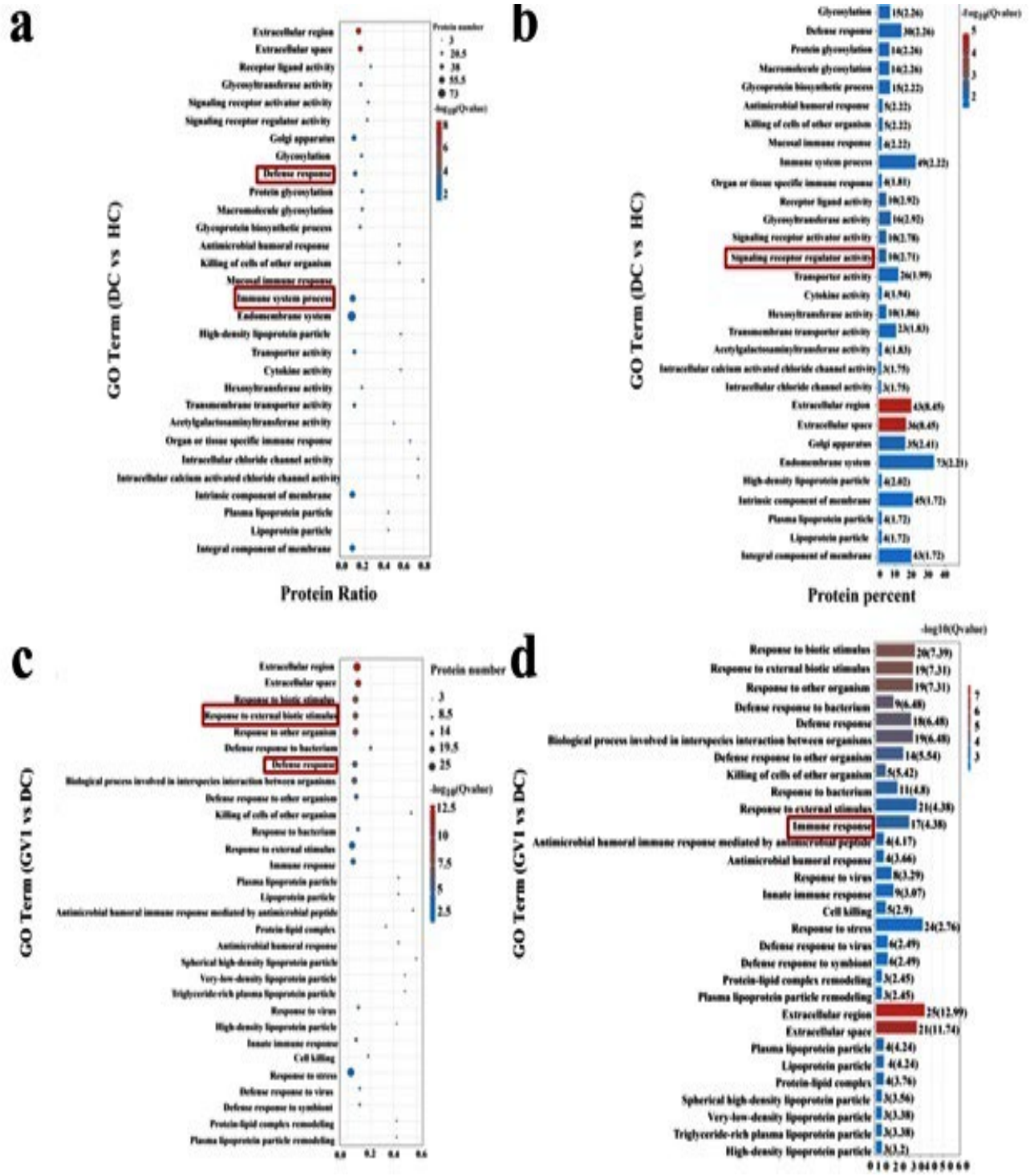

Figure 3: GO functional clustering results of significantly differentially expressed proteins in the rabbit colon. (a) The top 30 proteins among GO terms with the highest level of significant differences in the bubble diagram between the DC group and the HC group. (b) The top 30 proteins among GO terms with the highest level of significant differences in the histogram between the DC group and the HC group. (c) The top 30 proteins among GO terms with the highest level of significant differences in the bubble diagram between the GV1 group and the DC group. (d) The top 30 proteins among GO terms with the highest level of significant differences in the histogram between the GV1 group and the DC group.

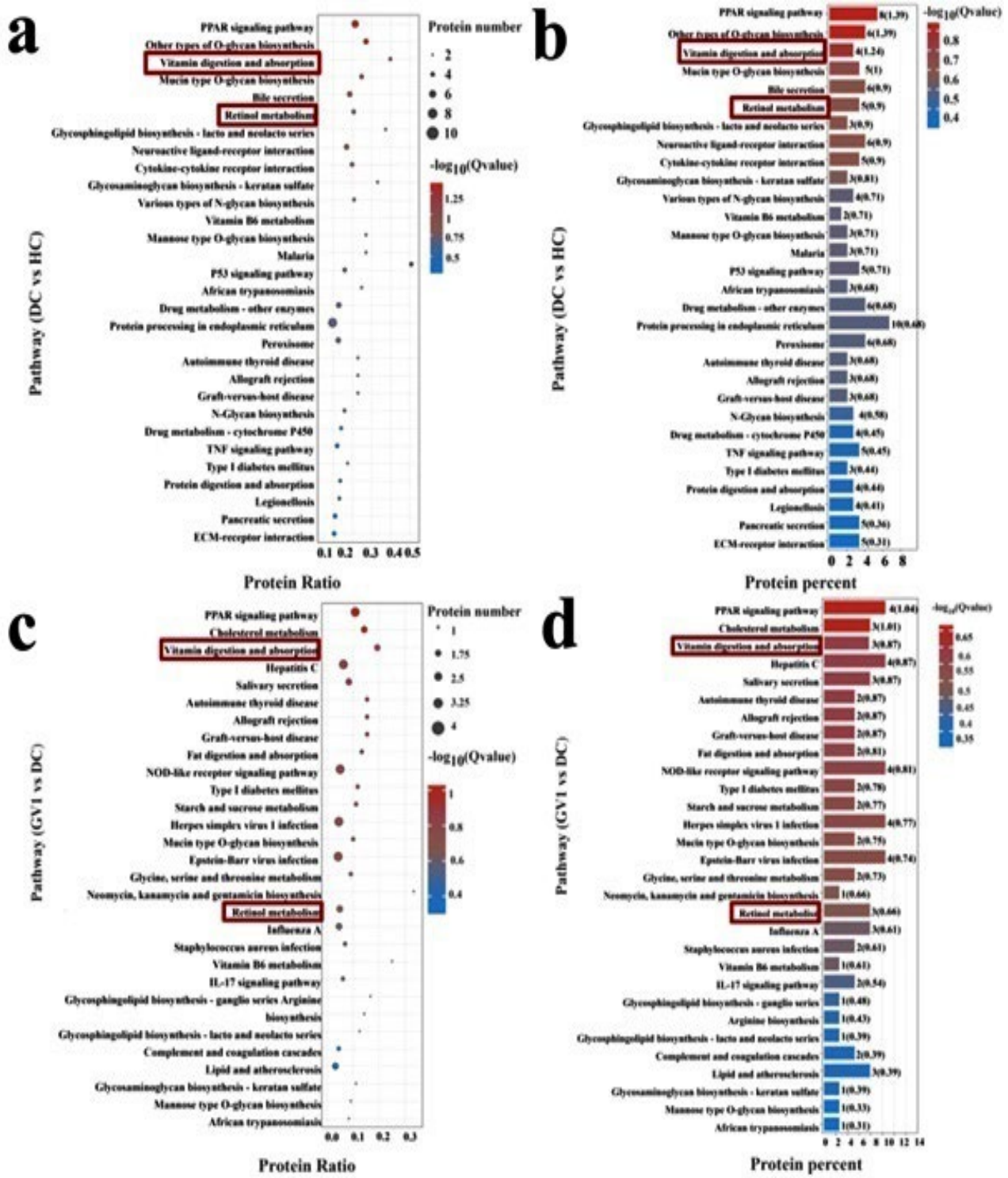

Figure 4: KEGG pathway enrichment analysis results of significantly differentially expressed proteins in the rabbit colon. (a) The top 30 pathways with the highest enrichment ratio of different proteins in the bubble diagram between the DC group and the HC group. (b) The top 30 pathways with the highest enrichment ratio of different proteins in the histogram between the DC group and the HC group. (c) The top 30 pathways with the highest enrichment ratio of different proteins in the bubble diagram between the GV1 group and the DC group. (d) The top 30 pathways among the highest enrichment ratios of different proteins in the histogram between the GV1 group and the DC group.

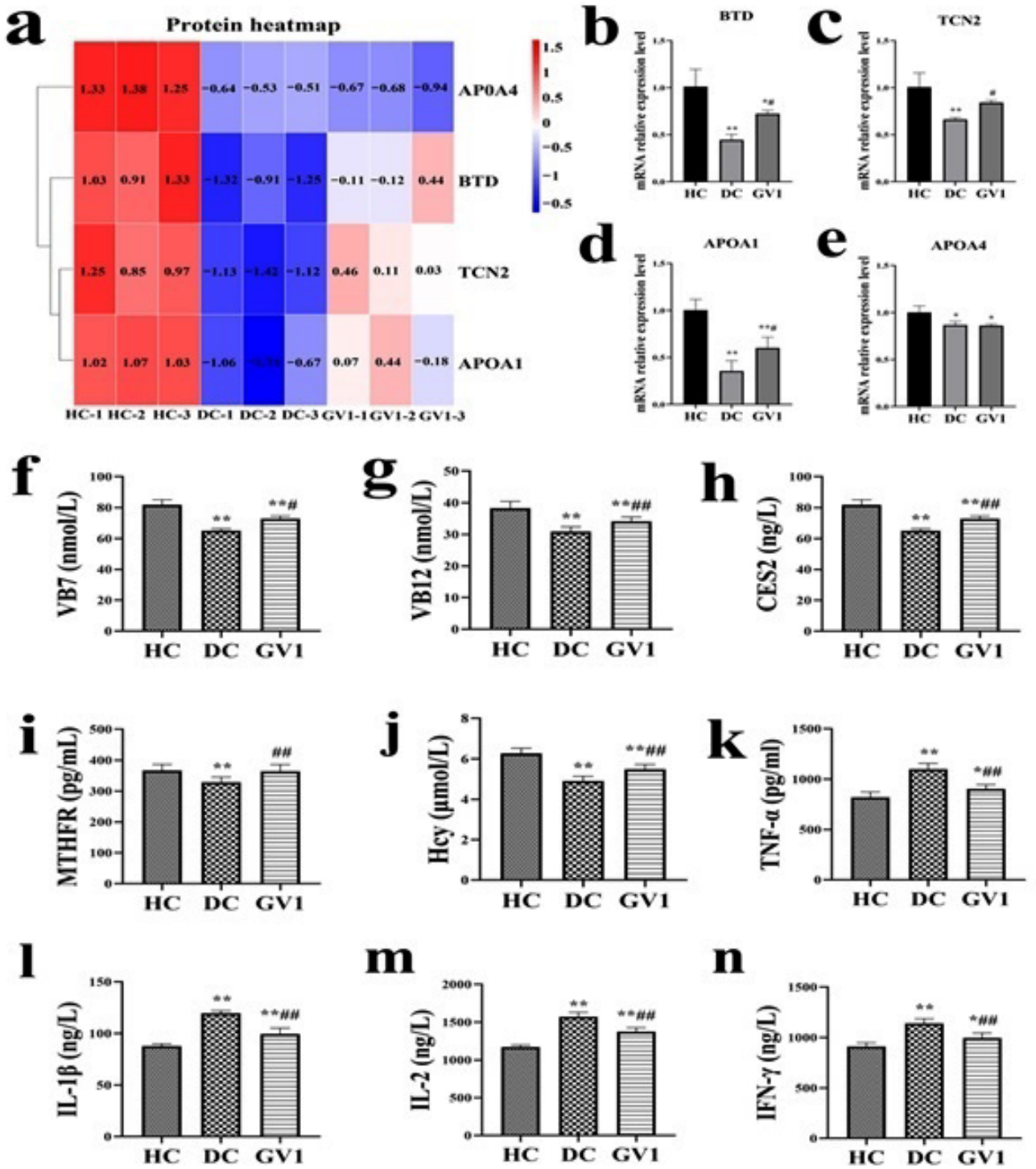

Figure 5: The significantly differentially expressed factors in the retinol metabolism signaling pathway. (a) Heat maps of 5 significantly differentially expressed genes. (b) The mRNA expression level of RDH11 by qPCR. (c) The mRNA expression level of ADH5 by qPCR. (d) The mRNA expression level of CYP3A4 by qPCR. (e) The mRNA expression level of CYP1A1 by qPCR. (f) The mRNA expression level of CYP36A by qPCR. (g) The level of VA by ELISA. (h) The level of Ret by ELISA. (i) The level of RBP4 by ELISA. (j) The level of STRA6 by ELISA. (k) The level of ADH by ELISA. (l) The level of RDH by ELISA. (m) The level of Retinal by ELISA. (n) The level of RA by ELISA.(Compared with the HC group, * means $P < 0.05$, and ** means $P < 0.01$. Compared with the DC group, $^{\#}$ means $P < 0.05$, and $^{\#\#}$ means $P < 0.01$.)

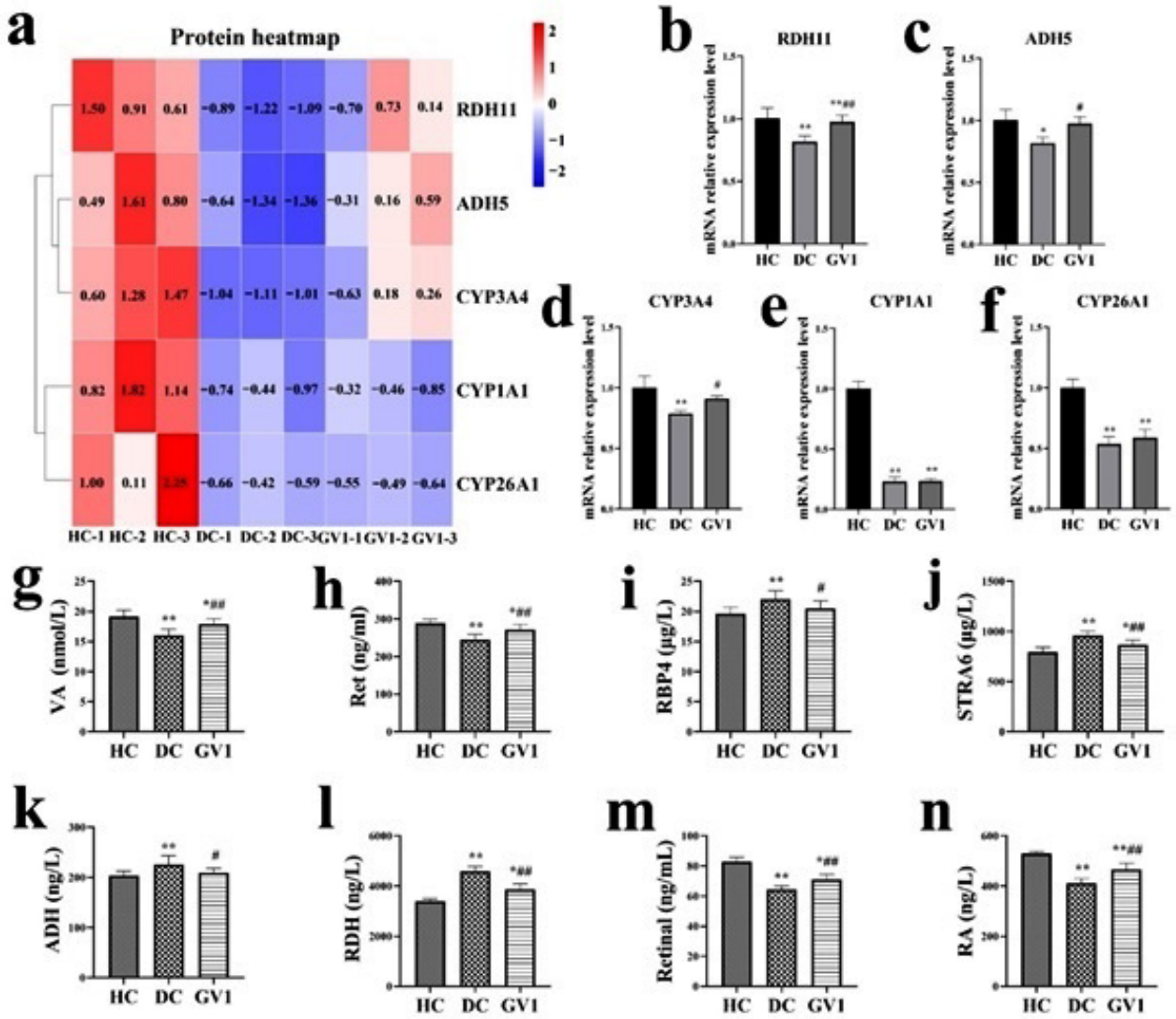

Figure 6: The significantly differentially expressed factors in the retinol metabolism signalling pathway. (a) Heat maps of 5 significantly differentially expressed genes. (b) The mRNA expression level of RDH11 by qPCR. (c) The mRNA expression level of ADH5 by qPCR. (d) The mRNA expression level of CYP3A4 by qPCR. (e) The mRNA expression level of CYP1A1 by qPCR. (f) The mRNA expression level of CYP36A by qPCR. (g) The level of VA by ELISA. (h) The level of Ret by ELISA. (i) The level of RBP4 by ELISA. (j) The level of STRA6 by ELISA. (k) The level of ADH by ELISA. (l) The level of RDH by ELISA. (m) The level of Retinal by ELISA. (n) The level of RA by ELISA. (Compared with the HC group, * means $P < 0.05$, and ** means $P < 0.01$. Compared with the DC group, $^{\#}$ means $P < 0.05$, and $^{\#\#}$ means $P < 0.01$.)

DISCUSSION

The primary function of the intestine is to digest and absorb nutrients in food and macromolecules that cannot be decomposed entirely by themselves and to provide the body with energy and nutrients necessary for growth and development (Goodman *et al.,* 2010). Dry needling (DN) at GV-1 may stimulate nerve endings connected to the autonomic nervous system, which in turn modulates intestinal function and protein expression. This neural stimulation can influence pathways involved in vitamin digestion and absorption by altering the expression of key genes and proteins, ultimately improving intestinal health and alleviating diarrhea (Lázaro-Navas *et al.,* 2021). Vitamins are tiny amounts of macromolecule substances from foods related to physiological functions, including growth, metabolism, and development. The colon is responsible for digesting and absorbing the vitamins (Pham *et al.,* 2021). In our study, proteomics sequencing revealed that both *Folium Sennae* (FSAE) exposure and DN can activate the signaling pathway of vitamin digestion and absorption in

rabbit colon tissue, thus affecting the digestion and absorption function of vitamins. The differential factor biotinidase (BTD), screened from the vitamin digestion and absorption signaling pathway, is the key enzyme for biotin synthesis. Biotin is vitamin B7 (VB7), also known as vitamin H or coenzyme R, which belongs to the water-soluble B vitamins (Reininghaus *et al.,* 2020). Biotin is composed of a heterocyclic core and a valeric acid side chain. The imidazolone ring part of the heterocyclic core can carry a carboxyl group, and the side chain of the carboxyl group can be connected to the lysine residue of the carboxylase through an amide bond (Kaczorowska *et al.,* 2021). Biotin is used as a carboxyl carrier in the colon, and its N1 can be carboxylated by carbon dioxide at the expense of energy, and then this carboxyl group is provided to the receptor molecule (Wolf, 2019). Briefly, biotin is a coenzyme of carboxylase. The oral administration of FSAEinduced a significant down-regulation of the expression level of BTD in the rabbit colon. The carboxyl groups in the colon cannot be connected to the corresponding carrier, which may make it difficult to carry out many reactions that require carboxylase (León-Del-Río, 2019). DN increased the expression level of BTD, restored the content of some coenzymes in the colon, and maintained the normal progress of some reactions requiring carboxylase. Carboxylesterase (carboxylesterase 2, CES2) is a common carboxylase in the colon and can participate in the drug metabolism process (Laizure *et al.,* 2022). We further tested the level of CES2 in the rabbit colon and found that the use of FSAE dropped the level of CES2, in that way weakening the metabolic process of FSAE in the colon. In addition, the DN could increase the level of CES2 and enhance the metabolic process of FSAE in the colon, which protects normal function.

Vitamin B12 (VB12), synthesized by natural microorganisms, also called cobalamin, is the only vitamin containing metal elements. The animal body cannot synthesize VB12 (Wang *et al.,* 2018). The colon microflora contains various bacteria and fungi (Peng *et al.,* 2023). VB12 is the only vitamin that requires intestinal secretions to be absorbed. Previous studies have shown that VB12 can serve as a coenzyme and participate in the process of resynthesis of methionine from homocysteine (Hcy) catalyzed by methylenetetrahydrofolate reductase (MTHFR) (Hiraoka and Kagawa, 2017). VB12 absorption and cellular delivery are related to the transporter transcobalamin II (TCN2) (Al-Batayneh *et al.,* 2020), which is a transporter protein located on chromosome 22, with specifical binding to VB12 to form the holo transcobalamin (Holo-T), thereby entering cells and reaching target organs to play its role (Allis *et al.,* 2010). The genetic polymorphism of TCN2 is considered a genetic factor that can affect Hcy metabolism by regulating the bioavailability of VB12 (Li *et al.,* 2017). Sequencing results showed that after rabbits were given FSAE leaves, the VB12 and the expression level of TCN2 in the colon tissue were significantly reduced, so affecting the metabolic level of Hcy. Ultimately, it may lead to a disorder of methionine metabolism in the colon and even difficulty in maintaining the methylation of the body. In rabbit colon tissue, DN increased the VB12 and the expression level of TCN2, restored the metabolic level of Hcy, and allowed Hcy to reach a relatively stable state. Other studies have shown that TCN2 is related to the inflammatory disease of ulcerative colitis (Chen *et al.,* 2008). We found that pro-inflammatory factors of IL-1β, IL-2, TFN-α, and TFN-γ were related to the vitamin digestion and absorption signaling pathway. The increased pro-inflammatory factors in FSAE-treated rabbits may induce the occurrence and development of inflammation. However, DN can reduce the levels of those pro-inflammatory factors, which may alleviate the severe inflammatory response in animals.

Both VB7 and VB12 are water-soluble vitamins (Stevens, 2021), which generally contain three elements: carbon, hydrogen, and oxygen, with absorption in the body closely related to the lipids in the intestines. We found that FSAE induced a lower expression level of APOA1 and APOA4 in rabbits, while DN reversed the expression of APOA1. APOA1 and APOA4 both belong to apolipoproteins (Fan *et al.,* 2021), which bind to lipids and help transport lipids in the blood. Therefore, apolipoproteins are crucial for the absorption process of fat-soluble vitamins in the intestine. Apolipoprotein synthesis occurs mainly in the intestine and liver (Liu *et al.,* 2022). The use of FSAE reduced the amount of apolipoprotein in the rabbit colon tissue, affected the transport process of some lipids, and led to a slowdown in the absorption efficiency of fat-soluble vitamins, which may affect the growth and development in rabbits, while DN reversed this adverse change.

Vitamin A is important for host metabolism, which can be converted into retinol (Hu *et al.,* 2020). Through proteomic sequencing, it was found that FSAE exposure and DN can activate the signaling pathway of retinol metabolism in rabbit colon tissue, thereby affecting retinol's metabolic level and interfering with retinol's digestion and absorption. The metabolic function of retinol is critical for adaptive immunity, as it promotes the recruitment of B and T cells into the intestine (Mars *et al.,* 2020). Myeloid cells convert retinol into retinoic acid, which is then passed on to developing B and T cells. This activates a retinoic acid-dependent gene expression program that directs B and T cells into the intestine and induces B cells to produce immunoglobulin A. The conversion process of retinol to retinoic acid is very complex. Retinol first binds to retinol-binding protein 4 (RBP4) and is transported into colon cells through the signaling receptor and transporter of retinol (STRA6), then it is converted into retinaldehyde under the action of

alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH), and retinal dehydrogenase catalyzes retinal to form retinoic acid (Pasutto *et al.,* 2018; Steinhoff *et al.,* 2021). The significantly different factors ADH5 and RDH11 screened from the retinol metabolism signaling pathway can interfere with the transport process of retinol to retinoic acid. The administration of FSAE induced a significant decrease in the expression levels of ADH5 and RDH11 in rabbit colon tissue. Part of the retinol lacks the enzyme, which combines with it to play a transport role, causing the transport process of retinol to retinaldehyde to be hindered. Finally, the contents of retinaldehyde and retinoic acid are reduced, thus weakening the immune process of intestinal myeloid cells and inducing the occurrence and development of inflammation. DN increased the expression levels of ADH5 and RDH11, restored part of the transport process of retinol to retinaldehyde, and increased the contents of retinaldehyde and retinoic acid. DN also restored intestinal myeloid cells' immune process and alleviated inflammatory response damage to colon tissue (Bang *et al.,* 2021).

CYP450 enzymes play an important role in retinol metabolism. The significantly different CYP1A1, CYP3A4, and CYP26A1 screened out from the sequencing results belong to the CYP450 enzymes. The expression level of CYP1A1 is regulated by the aryl hydrocarbon receptor (AhR). Studies have shown that tetrachlorodibenzo-para-dioxin (TCDD), as an AhR receptor agonist and environmental poison, can activate AhR receptors to induce the expression of the CYP1A1 gene and also reduce the level of retinol in the liver. Changes in the expression level of CYP1A1 are negatively correlated with changes in retinol levels (Chen and Chan, 2018). CYP3A catalyzes various stages of vitamin A metabolism and has high catalytic activity in converting retinol to retinaldehyde (Tabata and Shidoji, 2022). It is reported that CYP26A1 can also effectively catalyze the conversion process of retinal to retinoic acid (Lynch *et al.,* 2011). After being given FSAE, it induced a significant decrease in the expression level of CYP1A1 in rabbit colon tissue. Therefore, the level of retinol was increased to a certain extent; however, the expression levels of CYP3A4 and CYP26A1 in colon tissue were significantly reduced, and the conversion process, including retinol to retinaldehyde and retinaldehyde to retinoic acid, was slowed down. The increased retinol was over-consumed, resulting in a significant decrease in the final retinol content. DN increased the expression level of CYP3A4 in colon tissue and slowed down the conversion process from retinol to retinaldehyde and retinaldehyde to retinoic acid. After a complex series of reactions, DN increased the retinol content in the rabbit colon.

Conclusion: Applying GV-1 acupoint combined with DN mitigated FSAE-induced diarrhea in rabbits by significantly modifying the expression levels of genes (BTD, TCN2, APOA1, APOA4, ADH5, RDH11, CYP1A1, CYP3A4, and CYP26A1) and proteins (CES2, MTHFR, Ret, RBP4, RDH, STRA6, Retinal, and RA) involved in vitamin digestion and absorption signaling pathway, as well as retinol metabolism signaling pathway. The study demonstrated that the vitamin digestion, absorption, and retinol metabolism pathways are associated with the underlying mechanism of acupuncture in alleviating diarrhea. This investigation may offer novel insights into the therapeutic management of diarrhea.

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Animal ethics: This experiment was approved by the Laboratory Animal Ethics Committee of Hebei Agricultural University (Approval No. 2021066).

Authors' Contributions: The authors, HD, ML, CL, and AM, designed the experiments. HD, WL, XW, ML, and ZH performed the experiments and data collection. HD, WL, SAR, RAM, HMA, and AM analyzed the data, reviewed, and edited the manuscript. AM and DS provided financial support for the project and wrote the manuscript. All the authors read and approved the final version of the manuscript.

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REFERENCES

Al-Batayneh, K.M., M.S. AL-Zoubi, B. Al-Trad, E. Hussein, W. Alkhateeb, A.A.A. Aljabali, K. Bodoor, M. Shehab, M.A. Al Hamad,G.J. Eaton and C.T. Cornelison (2020). Homologous g776g variant of transcobalamin-ii gene is linked to vitamin b12 deficiency. Int. J. Vitam. Nutr. Re.90(1-2):151-55.https://doi.org/10.1024/0300-9831/a000536

Al-Farraj, D.A., M. Kashif, F. Ullah, H.M. Ali, A. Qayyum, A. Yamin, J., Aslam,S. Bibi, S.M. Eldin, I. Ali, A.E.M.A. Mustafa and M.S. Elshikh (2024). Enhanced wound healing effects of herbal gel formulations in a rabbit model: a comparative study. Asian J. Agric. Biol. 2024(1): 2023190. https://doi.org/10.35495/ajab.2023.190

Allis, D.G., T.J. Fairchild and R.P. Doyle (2010). The binding of vitamin b12 to transcobalamin(ii); Structural considerations for bioconjugate design: a molecular dynamics study. Mol. Biosyst. 6(9):1611-18.https://pubs.rsc.org/en/content/articlelanding/2010/mb/c003476b

Bang, Y.J., Z. Hu, Y. Li, S. Gattu, K.A. Ruhn and P. Raj (2021). Serum amyloid a delivers retinol to intestinal myeloid cells to promote adaptive immunity. Scence. 373(6561): eabf9232.https://doi.org/10.1126/science.abf9232

Blackmon, A.M. and L. Elson (2020). Dry needling and acupuncture in treatment of dance-related injuries, MD, and PT perspectives. Phys. Med. Rehab. Clin. North America 32(1):169-183. 10.1016/j.pmr.2020.08.005.

Chen, M., L. Peyrin-Biroulet, B. Xia, R.M. Guéant-Rodriguez, J.P. Bronowicki, M.A. Bigard and J.L. Guéant (2008). Methionine synthase a2756g polymorphism may predict ulcerative colitis and methylenetetrahydrofolate reductase c677t pancolitis, in central China. BMC Med. Genet. (2008) 9:78.https://doi.org/10.1186/1471-2350-9-78

Chen, Y.Y. and K.M. Chan (2018). Modulations of tcdd-mediated induction of zebrafish cyp1a1 and the ahr pathway by administering cd (2+) in vivo. Chemosphere 210:577-87.https://doi.org/10.1016/j.chemosphere.2018.07.032

Chew, Y.L., M.A. Khor, Z. Xu, S.K. Lee, J.W. Keng, S.H. Sang, G.A. Akowuah, K.W. Goh, K.B. Liew and L.C. Ming(2022). Cassia alata, coriandrum sativum, curcuma longa and azadirachta indica: food ingredients as complementary and alternative therapies for atopic dermatitis-a comprehensive review. Molecules 27(17).https://doi.org/10.3390/molecules27175475

Ding, H., M. Li, N. Ma, S.A. Rajput, M.H. Almutairi, B.O. Almutairi, Z. Han, A. Ma and D. Shiau (2025). Microbiome and metabolomic changes in rabbits induced by Folium sennae. PLoS ONE 20(3):e0320496. https://doi.org/10.1371/journal.pone.0320496

El-Sheikh, E.S.A., I.A. Hamed, M.A. Alduwish, M.A. Momenah, S.J. Melebary, S.A. Alsolmy, M.S. Alghamdi, A.A Alharbi, R.M. Sherif and A.A. Shalaby (2024). The ameliorative effect of vitamin C against subchronic thiamethoxam toxicity in male rats. Pakistan Vet. J. 44(3): 803-811. http://dx.doi.org/10.29261/pakvetj/2024.206

Evangelho, K.D.S., C. Cifuentes-González, W. Rojas-Carabali, C. De Vivero-Arciniegas, C Téllez-Conti, M. Cañas-Arboleda, G. Salguero, C. Ramírez-Santana and A. de-La-Torre (2024). Early detection of functional changes in an intraocular hypertension rabbit model treated with human wharton jelly mesenchymal stem cells (hWJ-MSCs) using chromatic pupillometry. Pakistan Vet. J., 44(1): 29-37. http://dx.doi.org/10.29261/pakvetj/2024.132

Fan, Y., J. Gao, Y. Li, X. Chen, T. Zhang, W. You, Y. Xue and C. Shen (2021). The variants at apoa1 and apoa4 contribute to the susceptibility of schizophrenia with inhibiting mrna expression in peripheral blood leukocytes. Front. Mol. Biosci. 8:785445. https://doi.org/10.3389/fmolb.2021.785445

Farag, E.F., M.A. Hassan, A.A. Basiony and E. Mahrous (2023). Role of Clostridium perfringens and *Escherichia coli* in the occurrence of diarrhea in newborn rabbits. Int. J. Vet. Sci. 12(2): 218-223. https://doi.org/10.47278/journal.ijvs/2022.178

Ford, C.K. (2023). Nutrition considerations in patients with functional diarrhea. Curr. Gastroenterol. Rep. 25(9):198-203. https://doi.org/10.1007/s11894-023-00878-5

Abd-El Ghany, F.T., S.H. Morsy, H.M.A. Hassan and A. Samy (2023). Evaluation of olive leaves and pomace extracts in growing rabbit diets on productive performance, nutrient digestibility, carcass characteristics, antioxidant status, and economic efficiency. Int. J. Vet. Sci. 12(1): 37-44. https://doi.org/10.47278/journal.ijvs/2022.155

Goodman, B.E. (2010). Insights into digestion and absorption of major nutrients in humans. Adv. Physiol. Educ. 34(2):44-53.https://doi.org/10.1152/advan.00094.2009

Hiraoka, M. and Y. Kagawa (2017). Genetic polymorphisms and folate status. Congenit. Anom. (Kyoto) 57(5):142-149.https://doi.org/10.1111/cga.12232

Hu, W., L. Pan, H. Chen and M. Tang (2020). VBA-AMF: a vba program based on the magnified intersections method for quantitative recording of root colonization by arbuscular mycorrhizal fungi. Ind. J. Microbiol. 60(3):374-78.https://doi.org/10.1007/s12088-020-00866-7

Jin, H., Y. Wu, S. Bi, Y. Xu, F. Shi, X. Li, X. Ma and S. Hu (2020). Higher immune response induced by vaccination in houhai acupoint relates to the lymphatic drainage of the injection site. Res. Vet. Sci. 130:230-36. https://doi.org/10.1016/j.rvsc.2020.03.018

Kaczorowska, A., M. Malinga-Drozd, W. Kałas, M. Kopaczyńska, S. Wołowiec and K. Borowska (2021). Biotin-containing third generation glucoheptoamidated polyamidoamine dendrimer for 5-Aminolevulinic acid delivery system. Int. J. Mol. Sci. 22(4):1982.https://doi.org/10.3390/ijms22041982

Kuehl, C.J., J.D. D'Gama, A.R. Warr and M.K. Waldor (2020). An oral inoculation infant rabbit model for shigella infection. Microbiol. 11(1):e03105-19. https://doi.org/10.1128/mBio.03105-19

Laizure, S.C., F. Chen, J.E. Farrar, D. Ali, B. Yang and R.B. Parker (2022). Carboxylesterase-2 plays a critical role in dabigatran etexilate active metabolite formation. Drug Metab. Pharmacokinet.47:100479. https://doi.org/10.1016/j.dmpk.2022.100479

Lázaro-Navas, I., C. Lorenzo-Sánchez-Aguilera, D. Pecos-Martín, J.J. Jiménez-Rejano, M.J. Navarro-Santana, J. Fernández-Carnero and T. Gallego-Izquierdo (2021). Immediate effects of dry needling on the autonomic nervous system and mechanical hyperalgesia: A randomized controlled trial. Int. J. Environ. Res. Public Health. 18(11):6018. https://doi.org/10.3390/ijerph18116018

León-Del-Río, A. (2019). Biotin in metabolism, gene expression, and human disease. J. Inherit. Metab. Dis. 42(4): 647-654. https://doi.org/10.1002/jimd.12073

Li, P., L. Huang, Y. Zheng, X. Pan, R. Peng, Y. Jiang, R.H. Finnell, H. Li, B. Qiao and H.Y. Wang (2017). A missense mutation in tcn2 is associated with decreased risk for congenital heart defects and may increase cellular uptake of vitamin b12 via megalin. Oncotarget 8(33):55216-29. https://doi.org/10.18632/oncotarget.19377

Liu, Y., Z. Wu, Y. Zhang, B. Chen, S. Yu and W. Li (2022). Alcohol-dependent downregulation of apolipoprotein h exacerbates fatty liver and gut microbiota dysbiosis in mice. Lipids. Health Dis. 21(1):89.https://doi.org/10.1186/s12944-022-01699-7

Lynch, J., J. McEwan and C.W. Beck (2011). Analysis of the expression of retinoic acid metabolising genes during xenopus laevis organogenesis. Gene Expr. Patterns. 11(1-2):112-17.https://doi.org/10.1186/s12944-022-01699-7

Mars, R.A., Y. Yang, T. Ward, M. Houtti, S. Priya, H.R. Lekatz, X. Tang, Z. Sun, K.R. Kalari, T. Korem and Y. Bhattarai (2020). Longitudinal multi-omics reveals subset-specific mechanisms underlying irritable bowel syndrome. Cell 182(6): 1460-73. https://doi.org/10.1016/j.cell.2020.08.007

Pasutto, F., F. Flinter, A. Rauch and A. Reis (2018). Novel stra6 null mutations in the original family described with matthew-wood syndrome. Am. J. Med. Genet. A. 176(1):134-38.https://doi.org/10.1002/ajmg.a.38529

Peng, S., C. Xu, Q. He, J. Xu, F.A. Kiani, O.P. Choudhary, A. Idrees, M.M. Mares, Y. Wu and K. Li(2024). Fucoidan alleviates intestine damage in mice induced by LPS via regulation of microbiota. Pakistan Vet. J. 44(2): 517-525. http://dx.doi.org/10.29261/pakvetj/2024.190

Pham, V.T., S. Fehlbaum, N. Seifert, N. Richard, M.J. Bruins, W. Sybesma, A. Rehman and R.E. Steinert (2021). Effects of colon-targeted vitamins on the composition and metabolic activity of the human gut microbiome- a pilot study. Gut Microbes. 13(1):1-20. https://doi.org/10.1080/19490976.2021.1875774

Poeta, M., F. Nunziata, M. Del Bene, F. Morlino, A. Salatto, S.M. Scarano, V. Cioffi, M. Amitrano, E. Bruzzese, A. Guarino and A. Lo Vecchio (2022). Diarrhea is a hallmark of inflammation in pediatric covid-19. Viruses. 4(12): 2723. https://doi.org/10.3390/v14122723

Reboul, G., D. Moreira, P. Bertolino, A.M. Hillebrand-Voiculescu and P. Lopez-Garcia (2019). Microbial eukaryotes in the suboxic chemosynthetic ecosystem of movile cave, Romania. Environ. Microbiol. Rep. 11(3):464-73. https://doi.org/10.1111/1758-2229.12756

Reininghaus, E.Z., M. Platzer, A. Kohlhammer-Dohr, C. Hamm, S. Mörkl, S.A. Bengesser, F.T. Fellendorf, T. Lahousen-Luxenberger, B. Leitner-Afschar, H. Schöggl and D. Amberger-Otti (2020). PROVIT: supplementary probiotic treatment and vitamin b7 in depression-a randomized controlled trial. Nutrients 12(11): 3422. https://doi.org/10.3390/nu12113422

Russell, M., A. Currin, W. Rowe, G.Q. Chen, P. Barran and N.S. Scrutton (2022). Baseline proteomics characterisation of the emerging host biomanufacturing organism Halomonas bluephagenesis. Sci. Data. 13;9(1):492. https://doi.org/10.1038/s41597-022-01610-0

Siddiqui, S.A., F. Gerini, A. Ikram, F. Saeed, X. Feng and Y. Chen (2023). Rabbit meat—production, consumption and consumers' attitudes and behavior.Sustainability (2023), *15*, 2008.https://doi.org/10.3390/su15032008

Scaldaferri, F., M. Pizzoferrato, S. Pecere, F. Forte and A. Gasbarrini (2012). Bacterial flora as a cause or treatment of chronic diarrhea. Gastroenterol. Clin. North Am. 41(3): 581-602. https://doi.org/10.1016/j.gtc.2012.06.002

Nistanak, H.S., H. Fattahian, B. Vazir, M. Marjani and A.E.K. Rahimi (2023). Evaluation of GV26 electrical acupuncture stimulation on anesthetic recovery time of spur-thighed tortoise (*Testudo graeca)*. Kafkas Univ. Vet. Fak. Derg. 29(2): 177-182.http://dx.doi.org/10.9775/kvfd.2022.28921

Steinhoff, J.S., A. Lass and M. Schupp (2021). Biological functions of rbp4 and its relevance for human diseases. Front. Physiol. 12:659977. https://doi.org/10.3389/fphys.2021.659977

Stevens, S.L. (2021). Fat-soluble vitamins. Nurs. Clin. North. America. 56(1):33-45.https://doi.org/10.1016/j.cnur.2020.10.003

Tabata, Y. and Y. Shidoji (2022). Hepatic cyp3a4 enzyme compensatively maintains endogenous geranylgeranoic acid levels in maob-knockout human hepatoma cells. Metabolites 12(2): 140. https://doi.org/10.3390/metabo12020140

Topal, A., N.Y. Gül Satar, O. Ates, E. Uckan, O. Yavas and I. Cangül(2023). Comparison of the effects of ketamine-diazepam, tiletamine-zolazepam and propofol infusion anesthesia in rabbits. Kafkas Univ. Vet. Fak. Derg,29(2): 137-143.https://doi.org/10.9775/kvfd.2022.28744

Uwaezuoke, S.N., C.L. Odimegwu, N.R. Mbanefo, C.I. Eneh, I.O. Arodiwe, U.V. Muoneke, F.N. Ogbuka, C.O. Ndiokwelu and A.T. Akwue (2023). Vitamin D(3) supplementation as an adjunct in the management of childhood infectious diarrhea: a systematic review. BMC Infect. Dis. 23(1):159. https://doi.org/10.1186/s12879-023-08077-3

Wang, J.Y., Y.H. Wu, S.J. Liu, Y.S. Lin and P.H. Lu (2018). Vitamin b12 for herpetic neuralgia: a meta-analysis of randomised controlled trials. Complement Ther. Med. 41:277-82. https://doi.org/10.1016/j.ctim.2018.10.014

Wolf, B. (2019). Biotinidase deficiency should be considered in individuals thought to have multiple sclerosis and related disorders. Mult. Scler. Relat. Disord. 28:26-30. https://doi.org/10.1016/j.msard.2018.11.030

Xu, W. and S. Hu (2021). Administration of infectious bursal disease vaccine in Houhai acupoint promotes robust immune responses in chickens. Res. Vet. Sci. 142:149-153. https://doi.org/10.1016/j.rvsc.2021.12.006

Yi, R., Y. Tian, F. Tan, W. Li, J. Mu, X. Long, Y. Pan and X. Zhao (2020). Intervention effect of malus pumila leaf flavonoids on senna-induced acute diarrhea in balb/c mice. Food Sci. Nutr. 8(5):2535-42. https://doi.org/10.1002/fsn3.1549

Younas, M., K. Ashraf, M.I. Rashid, M. Ijaz, M. Suleman and T.A. Chohan (2023). Expression and purification of recombinant multi-epitope protein of Rhipicephalus microplus tick and its antigenicity in rabbits. Pakistan Vet. J., 43(4): 778-784. http://dx.doi.org/10.29261/pakvetj/2023.086.