

## Original Article

# Effect of emodin on intestinal flora in the treatment of iodine-induced thyroiditis in NOD mice

Zhipeng Ye, Keren Wu, Fa Jin, Tao Xu, Ning Li, Jie Huang

Department of Hepatobiliary Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

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**Abstract:** Objective: This study aimed to explore the effect of emodin on intestinal flora in the treatment of iodine-induced thyroiditis in NOD mice. Methods: NOD mice were divided into three groups: control group, model group and emodin group with 10 mice in each group. EAT (experimental autoimmune thyroiditis) animal model was established by inducing NOD mice with iodine in the model group. EAT mice were treated with emodin in the emodin group. The levels of TgAb and T4 in plasma of mice and the changes of intestinal flora measured from feces of mice in each group were detected by ELISA and high throughput sequencing. Results: 1. Compared with the control group, the expression of TgAb in serum was clearly increased, and the expression of T4 in serum was clearly decreased in the model group. Compared with the model group, the expression of TgAb in serum was clearly decreased, and the expression of T4 in serum was clearly increased in the emodin group. 2. The expressions of Dorea, Bacteroides and Prevotella in the model group were higher than those in the control group, and the expressions of Dorea, Bacteroides and Prevotella in the emodin group were lower than those in the model group with statistical differences. Conclusion: 1. Emodin has certain immunosuppressive effects on thyroiditis in NOD mice. 2. Emodin can affect the expression of intestinal flora such as Dorea, Bacteroides and Prevotella, thus inhibiting thyroiditis in NOD mice.

**Keywords:** Hashimoto's thyroiditis, intestinal flora, emodin

## Introduction

Autoimmune thyroid disease (AITD) is one of the most common autoimmune diseases [1]. The incidence of AITD in the population is about 1.5%, and it is most commonly found in females [2]. Most autoimmune diseases are associated with specific variants of the human leukocyte antigen (HLA) genes. Several other genetic polymorphisms have been associated with autoimmune diseases, particularly some located at the genes encoding cytotoxic T lymphocyte-associated 4 (CTLA-4) and the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) [3]. Each of these loci encodes molecules involved in the regulation of T cells. In particular, two rare syndromes illustrate the importance of T cells in maintaining self-tolerance: the first, autoimmune polyglandular syndrome type I (APS 1, also called APECED), is caused by defects in the autoimmune regulator (AIRE) gene that mediates the induction of T-cell self-tolerance in the thymus [4]. In pati-

ents with APS 1, multiple endocrine glands are dysfunctional. The second, FOXP3 deficiency, is associated with the IPEX syndrome, as mentioned above. Whereas APS 1 and IPEX syndrome are caused by mutation of a single gene, most cases of autoimmunity are likely the result of a broad range of genetic predispositions and environmental factors resulting in an imbalance of the peripheral self-tolerance mechanisms sustained by Tregs and Bregs [5]. Thus, genetic polymorphisms in self-antigens, cytokines, estrogen receptors, and adhesion molecules have also been linked to the development of autoimmune disease [6-8], as have genes coding for apoptotic processes.

Hashimoto's thyroiditis (HT) is one of the most common AITD found in clinic. The imbalance of intestinal flora is the abnormal change of intestinal flora composition, which destroys the balance between intestinal flora and host, and is related to many autoimmune diseases. HT is characterized by a direct T-cell attack on the

thyroid gland, leading to thyroiditis and subsequent exposure of thyroid antigens (thyroid peroxidase and thyroglobulin) against which antibodies are then produced. Thyroglobulin antibodies (TgAbs) and thyroid peroxidase antibodies (TPOAbs) are commonly associated with HT, with a destructive pattern, and are considered diagnostic for this disease. In any iodine-sufficient population, however, the prevalence of TPOAbs and TgAbs is much higher than that found in clinical disease, amounting for approximately 15-25%, with the highest prevalence in females and increasing with age [9].

This study explored the relationship between the treatment of NOD mice with thyroiditis by emodin and the change of intestinal flora to provide a theoretical basis for clinical diagnosis and therapy of AITD.

### Materials and methods

#### Materials

*Experimental animals and grouping:* Thirty female non-obese diabetic (NOD) mice aged 6-8 weeks old were randomly divided into three groups: control group, model group and emodin group, with 10 mice in each group. The temperature and humidity of the feeding room were about 20°C and 60%, respectively. The daily lighting time was 12 hours. The mice had access to food and water freely.

*Reagents and instruments:* Enzyme-linked Immunosorbent Assay (ELISA) Kit for Mouse anti-thyroglobulin antibody (TAGA/TGAB) was from Wuhan Colorful Gene Biological Technology Co., LTD (JYM0353Mo). Enzyme-linked Immunosorbent Assay (ELISA) Kit for Mouse Thyroxine (T4) was from Bio-Swamp (MU30596), Agarose from Invitrogen (75510-019), Marker from Takara (DL15000 or DL2000), TAE from Invitrogen (AM9870), Q5®High-Fidelity DNA Polymerase from NEB (M0491L), Quant-iTPico Green dsDNA Assay Kit from Invitrogen (P75-89), Microplate Reader from Thermo Scientific (Multiskan MK3), Normal temperature/freezing centrifuge from Eppendorf, Nanodrop from Thermo Scientific (NC2000), Electrophoresis Apparatus from Beijing Liuyi Biological Technology Co., LTD (DYY-6C), Gel Imaging System from Beijing Bay Gene Biological Technology Co., LTD (BG-gdsAUTO (130)), Microplate Reader

from BioTek (FLX800T), PCR Gene Amplification Instrument from ABI (2720).

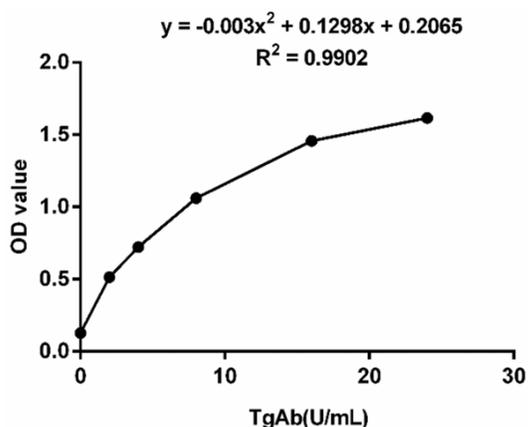
#### Methods

*Establishment of animal model:* Mice in the control group were given deionized water, while mice in the other groups were given 0.05% iodine water (0.64 g/L NaI) for 8 weeks. Mice in the emodin group were given 75 mg/kg of emodin daily from the 6th week on, while mice in the control group and the model group were both given the same amount of normal saline. After 3 weeks of treatment, the mice were killed and their peripheral blood and cecum were taken. Every procedure was approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University.

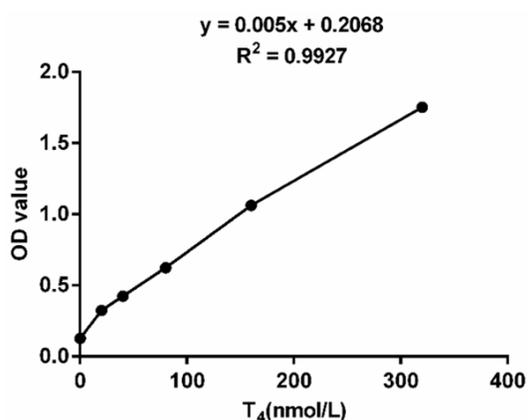
*Specimen collection:* After anesthesia with ether, the blood was collected from the orbital vein of mice, placed at room temperature for 6 hours, and centrifuged for 20 minutes at 3000 r/min to separate the plasma. The cecum was resected about 10 cm through a median abdominal incision. The fecal contents and feces were taken out and placed in a dry sterile test tube. The skin and muscle layers were separated through a median cervical incision. The organs and two lobes of the thyroid in the posterolateral thyroid cartilage were exposed. The trachea and thyroid were removed together. The thyroid was removed quickly, rinsed with cold normal saline, and dried with filter paper.

*Determination of plasma anti-mouse TgAb and T4:* Double antibody sandwich ELISA method was used to determine plasma anti-mouse TgAb and T4. One hundred µL of standard or sample was added into each well, and incubated for 90 minutes at 37°C. One hundred µL of biotinylated antibody working fluid was added, and incubated for 60 minutes at 37°C. The solution was washed three times. One hundred µL of enzyme conjugate working fluid was added, and incubated for 30 minutes at 37°C. The solution was washed five times. Ninety µL of substrate solution was added, and incubated for about 15 minutes at 37°C. Fifty µL of termination solution was added immediately at 450 nm. OD values were immediately measured at 450 nm and calculated (**Figures 1 and 2**).

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**Figure 1.** Curve that measured at wavelength was converted into TgAb (U/ml).



**Figure 2.** Curve that OD values measured at wavelength was converted into TgAb (U/ml) or T<sub>4</sub> (nmol/L).

### *High-throughput sequencing for determination of intestinal flora in mice*

**Pretreatment of fecal samples:** Fresh fecal sample were suspended in sterile PBS solution (pH 4.7) at a ratio of 1:30. The sample was whirled for about 30 min, and 200 g of sample was centrifuged three times for 5 min each time. Coarse particles were removed and the supernatant was collected. Then, 10,000 g of sample was centrifuged for 5 min, the precipitation was collected, and with 30 ml of PBS solution samples were washed four times. Finally, the sample was re-suspended in PBS solution, separately packed and stored at -70°C for subsequent DNA extraction.

**Microbial DNA extraction from feces:** Microbial total DNA was extracted from fecal samples of each group by DNA Kit. Genomic DNA was

extracted by 1% agarose gel electrophoresis. The extracted DNA was stored at -20°C.

**Amplification of 16SrRNA V3-V4 regions:** PCR amplification was performed by using universal primers for amplification in 16SrRNA V3-V4 variable regions. Using the extracted total DNA as a template, the 16S PCR primer consisted of the sequencing linker primer, the Index and the V3 region primer 3 portions. Index is a sequence of random 6 bp nucleotides to mark the source of the PCR product. Amplification conditions: pre-denaturation at 98°C for 30 s, 98°C for 10 s, 50°C for 30 s, 72°C for 30 s, 20 cycles, and finally 72°C for 7 min, stored at 4°C. After the end of the reaction, the entire reaction product was subjected to 1.5% agarose gel electrophoresis (staining in ethidium bromide) to measure the size of the amplified fragment, and the target strip gel was recovered and purified.

**High-throughput sequencing and bioinformatics analysis:** The purified PCR products in 16SrRNA V3-V4 regions were sequenced by high-throughput sequencing. The sequencing results were analyzed by bioinformatics, and the different flora were selected.

### *Statistical analysis*

The experimental data was analyzed by t-test between groups with SPSS software, which were expressed as “mean ± standard deviation”. All the experiments were repeated at least three times. Variance analysis was used for the statistical analysis, and Scheffe’s test was used for multiple comparisons.  $P < 0.05$  indicated statistical significance.

## **Results**

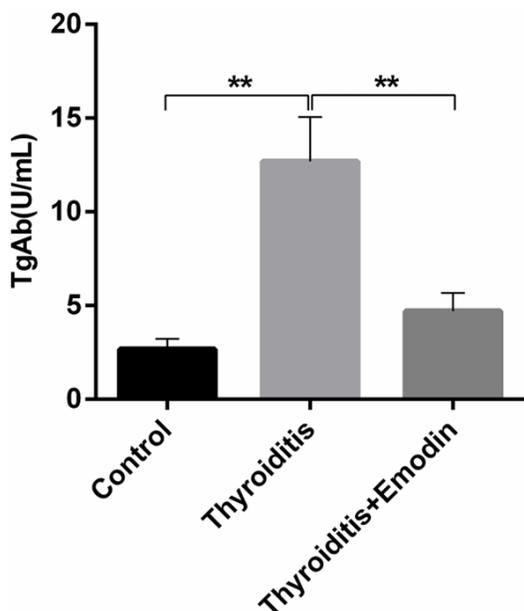
### *Detection of thyroid hormone and autoantibodies in serum*

Compared with the control group, the serum TgAb level in the model group was increased and T<sub>4</sub> level was decreased ( $P < 0.01$ ). Compared with the model group, the serum TgAb level was decreased and T<sub>4</sub> level was increased after emodin treatment ( $P < 0.01$ ) (**Figures 3, 4**).

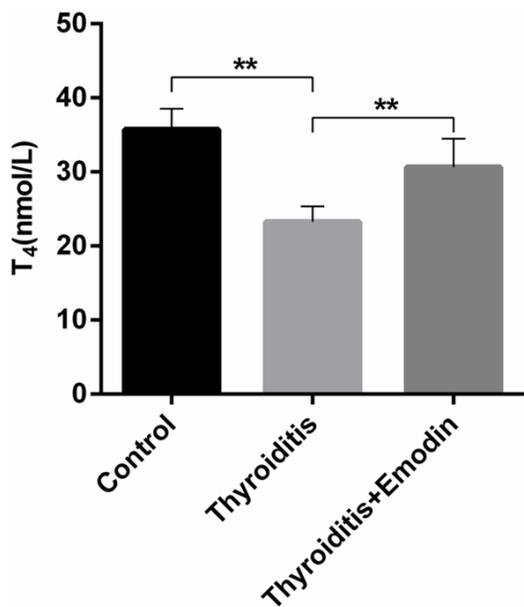
### *High-throughput sequencing for the determination of intestinal flora in mice*

The results showed that the expressions of Unclassified-S24-7, Unclassified-Clostridiales,

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**Figure 3.** Serum TgAb level of each group. “Control” is the control group; “Thyroiditis” is the iodine-induced thyroiditis model group; “Thyroiditis + Emodin” is the emodin group for the treatment of iodine-induced thyroiditis. \*\*There was statistical significance between the two groups, and  $P < 0.01$ .



**Figure 4.** Serum T4 level of each group. “Control” is the control group; “Thyroiditis” is the iodine-induced thyroiditis model group; “Thyroiditis + Emodin” is the emodin group for the treatment of iodine-induced thyroiditis. \*\*There was statistical significance between the two groups, and  $P < 0.01$ .

Dorea, Bacteroides, and Prevotella were significantly different between the control group and the model group, the model group, and the emodin group. The expression of Dorea, Bacteroides (Bacteroides) and Prevotella was significantly increased compared with the control group, and the expression of emodin group was decreased compared with the model group, and there were statistical differences. The emodin group was also reduced. The expression of Unclassified-S24-7 and Unclassified-Clostridiales was lower in the control group than in the model group. The expression of Unclassified-S24-7 in the emodin group was significantly lower than that in the model group. The expression of Unclassified-Clostridiales in the emodin group was significantly higher than that of the model (Tables 1-3, Figure 5).

### Discussion

Chronic lymphocytic thyroiditis (CLT) is an autoimmune disease characterized by diffuse infiltration of lymphocytes and plasma cells, with genetic tendency and organ specificity. T3 and T4 hypothyroidism and increased anti-thyroiditis globulin enzymes are common clinical manifestations [10]. In this study, it was found that compared with the control group, the plasma T4 level was decreased ( $P < 0.01$ ) and the plasma TgAb level was increased in the model group ( $P < 0.01$ ). These were the main characteristics of HT. This was consistent with the views of some other scholars [11], and the feasibility of modelling of Hashimoto’s thyroiditis in NOD mice with excessive iodine was also confirmed. In this study, after emodin treatment, compared with the model group, the plasma T4 level was increased ( $P < 0.01$ ), and the plasma TgAb level was decreased in emodin group ( $P < 0.01$ ). We concluded that emodin was effective in treating iodine-induced thyroiditis in NOD mice and had certain immunosuppressive effects on chronic lymphocytic thyroiditis. This was consistent with the previous related study results of other researchers [12-14].

The gastrointestinal tract is the “bacterial pool” of the human body. There are about 10-14 gastrointestinal bacteria in a healthy adult, including aerobe, facultative anaerobes and anaerobes [15]. Many factors, such as environment, age, diet, heredity and immunity, could affect the composition of intestinal flora [16, 17]. In

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**Table 1.** DNA ultraviolet absorption spectrometry results

Lane	Number	Name	Type	DNA	Concentration (ng/ul)	260/280	260/230	Volume	Weight
1	D7h0740	NC1-1	Original	DNA	77.90	1.91	1.73	50.00	3.90
2	D7h0741	NC1-2d	Original	DNA	39.80	1.89	1.31	50.00	1.99
3	D7h0742	NC1-3	Original	DNA	70.10	1.96	1.77	50.00	3.51
4	D7h0743	NC1-4	Original	DNA	60.40	1.89	1.59	50.00	3.02
5	D7h0744	NC1-5	Original	DNA	76.40	1.89	1.71	50.00	3.82
6	D7h0745	NC2-1	Original	DNA	71.40	1.91	1.69	50.00	3.57
7	D7h0746	NC2-2	Original	DNA	67.40	1.90	1.77	50.00	3.37
8	D7h0747	NC2-3d	Original	DNA	45.00	1.88	1.50	50.00	2.25
9	D7h0748	NC2-4	Original	DNA	127.80	1.90	1.83	50.00	6.39
10	D7h0749	NC2-5	Original	DNA	65.40	1.89	1.32	50.00	3.27
11	D7h0750	M1-1	Original	DNA	79.30	1.87	1.52	50.00	3.97
12	D7h0751	M1-2	Original	DNA	67.80	1.90	1.62	50.00	3.39
13	D7h0752	M1-3	Original	DNA	136.60	1.88	1.67	50.00	6.83
14	D7h0753	M1-4	Original	DNA	102.90	1.90	1.80	50.00	5.15
15	D7h0754	M1-5	Original	DNA	108.50	1.91	1.81	50.00	5.43
16	D7h0755	M2-1	Original	DNA	83.50	1.91	1.78	50.00	4.18
17	D7h0756	M2-2	Original	DNA	118.90	1.90	1.89	50.00	5.95
18	D7h0757	M2-3	Original	DNA	69.20	1.90	1.65	50.00	3.46
19	D7h0758	M2-4	Original	DNA	85.10	1.90	1.76	50.00	4.26
20	D7h0759	M2-5	Original	DNA	98.90	1.91	1.87	50.00	4.95
21	D7h0760	D1-1	Original	DNA	68.80	1.89	1.47	50.00	3.44
22	D7h0761	D1-2	Original	DNA	65.20	1.89	1.50	50.00	3.26
23	D7h0762	D1-3	Original	DNA	78.70	1.89	1.71	50.00	3.94
24	D7h0763	D1-4	Original	DNA	82.10	1.89	1.77	50.00	4.11
25	D7h0764	D1-5	Original	DNA	82.90	1.90	1.82	50.00	4.15
26	D7h0765	D2-1	Original	DNA	63.90	1.91	1.70	50.00	3.20
27	D7h0766	D2-2	Original	DNA	71.60	1.90	1.78	50.00	3.58
28	D7h0767	D2-3	Original	DNA	88.90	1.91	1.88	50.00	4.45
29	D7h0768	D2-4	Original	DNA	71.60	1.89	1.77	50.00	3.58
30	D7h0769	D2-5	Original	DNA	58.40	1.89	1.60	50.00	2.92

Microbial DNA extracted from feces. The results of DNA ultraviolet absorption spectrometry showed that all 30 samples were qualified.

this study, it was also found that the relative abundance of Unclassified-S24-7, Unclassified-Clostridiales, Dorea, Bacteroides and Prevotella in the gastrointestinal tract changed significantly after excessive iodine and emodin intake. Compared with the control group, the relative abundance of Unclassified-S24-7 and Unclassified-Clostridiales in the model group was decreased, while the relative abundance of Dorea, Bacteroides and Prevotella in the model group was increased. Compared with the model group, the relative abundance of Unclassified-S24-7 in the emodin group was decreased, the relative abundance of Unclassified-Clostridiales was increased, and the

relative abundance of Dorea, Bacteroides and Prevotella was decreased.

In conclusion, we have shown that excessive iodine intake in NOD mice could induce chronic lymphocytic thyroiditis and affect intestinal flora in this study. The relative abundance of Unclassified-S24-7 and Unclassified-Clostridiales was decreased, and the relative abundance of Dorea, Bacteroides and Prevotella was increased. Emodin had certain immunosuppressive effects on chronic lymphocytic thyroiditis in NOD mice. The relative abundance of Unclassified-S24-7 was decreased, the relative abundance of Unclassified-Clostridiales was increased, and the relative abundance of

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**Table 2.** Amplification results of 16SrRNA V3-V4 regions

Number	Name	Primer	Platform	F primer	Barcode	Success	Size bp	Concentration (ng/ul)
D7h0740	NC1-1	338 F/806 R	miseq	F1	ACACAGT	Yes	500	2.28
D7h0741	NC1-2d	338 F/806 R	miseq	F2	ACAGTCA	Yes	500	5.52
D7h0742	NC1-3	338 F/806 R	miseq	F3	ACTCTGA	Yes	500	5.94
D7h0743	NC1-4	338 F/806 R	miseq	F4	ACTGACT	Yes	500	7.11
D7h0744	NC1-5	338 F/806 R	miseq	F5	AGACTCT	Yes	500	4.55
D7h0745	NC2-1	338 F/806 R	miseq	F6	AGTCACA	Yes	500	3.94
D7h0746	NC2-2	338 F/806 R	miseq	F7	TCAGAGA	Yes	500	5.49
D7h0747	NC2-3d	338 F/806 R	miseq	F8	TGACTGA	Yes	500	3.88
D7h0748	NC2-4	338 F/806 R	miseq	F9	TGAGACT	Yes	500	3.91
D7h0749	NC2-5	338 F/806 R	miseq	F10	TGTCAGT	Yes	500	3.17
D7h0750	M1-1	338 F/806 R	miseq	F11	TGTGTCA	Yes	500	4.25
D7h0751	M1-2	338 F/806 R	miseq	F12	CAACTGT	Yes	500	1.44
D7h0752	M1-3	338 F/806 R	miseq	F13	CATCAGA	Yes	500	3.89
D7h0753	M1-4	338 F/806 R	miseq	F14	CATGTCT	Yes	500	4.36
D7h0754	M1-5	338 F/806 R	miseq	F15	CACAAGT	Yes	500	4.51
D7h0755	M2-1	338 F/806 R	miseq	F16	CACACTG	Yes	500	5.53
D7h0756	M2-2	338 F/806 R	miseq	F17	CACTTGA	Yes	500	5.87
D7h0757	M2-3	338 F/806 R	miseq	F18	CACTGAT	Yes	500	6.42
D7h0758	M2-4	338 F/806 R	miseq	F19	CAGATCA	Yes	500	6.17
D7h0759	M2-5	338 F/806 R	miseq	F20	CAGACAT	Yes	500	5.00
D7h0760	D1-1	338 F/806 R	miseq	F21	CAGAGTC	Yes	500	3.99
D7h0761	D1-2	338 F/806 R	miseq	F22	CAGTACT	Yes	500	6.33
D7h0762	D1-3	338 F/806 R	miseq	F23	CAGTCTA	Yes	500	6.98
D7h0763	D1-4	338 F/806 R	miseq	F24	CTAGACA	Yes	500	4.83
D7h0764	D1-5	338 F/806 R	miseq	F25	CTTGAGT	Yes	500	3.99
D7h0765	D2-1	338 F/806 R	miseq	F26	CTCACGA	Yes	500	3.92
D7h0766	D2-2	338 F/806 R	miseq	F27	CTCAGAC	Yes	500	5.65
D7h0767	D2-3	338 F/806 R	miseq	F28	CTCTCAG	Yes	500	7.32
D7h0768	D2-4	338 F/806 R	miseq	F29	CTCTGTA	Yes	500	5.76
D7h0769	D2-5	338 F/806 R	miseq	F30	CTGATGT	Yes	500	4.52

Amplification results of 16SrRNA V3-V4 regions. The results showed that the amplification is successful and meets the needs of data base construction.

Dorea, Bacteroides and Prevotella was decreased in the gastrointestinal tract.

Studies have shown that intestinal flora could regulate the growth and differentiation of CD4+ T cells and the production and secretion of IgA, which participates in the intestinal immune process [18, 19]. Abnormal changes in intestinal flora composition could destroy the balance between intestinal flora and host, which is closely related to the occurrence of many autoimmune diseases. Some authors [20] suggested that intestinal flora could promote the development and maturation of the intestinal tract and its immune system, block

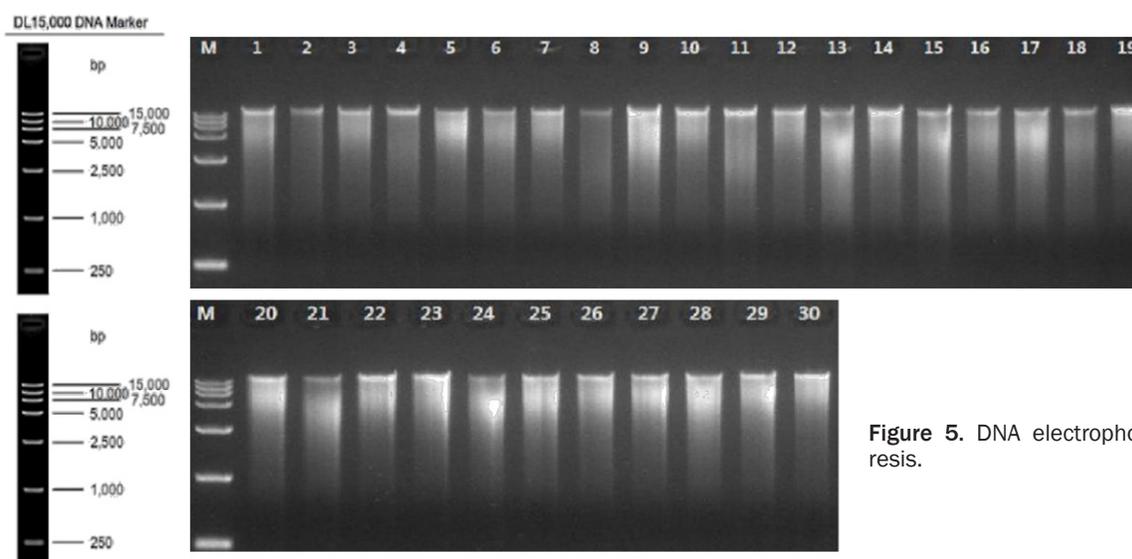
the contact between antigen and intestinal wall cells through the combination of IgA and antigen, differentiate Th cells into Th1 cell type by inducing and stimulating DC and TLR, maintain Th1/Th2 balance, and regulate the immune system to avoid allergic disease or other immune diseases. We concluded that excessive iodine intake in NOD mice could cause an imbalance in the intestinal flora and autoimmune thyroiditis by increasing the relative abundance of Unclassified-Clostridiales and decreasing the relative abundance of Dorea, Bacteroides and Prevotella. Emodin could decrease the relative abundance of Unclassified-Clostridiales and increase the relative

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**Table 3.** Distribution of microflora groups

Taxon	Control	Thyroiditis	Thyroiditis + Emodin
Unclassified-S24-7	0.3800±0.0745	0.3488±0.0977	0.3411±0.0644
Unclassified-Clostridiales	0.1556±0.0810	0.1244±0.0454	0.1943±0.0962
Unclassified-Bacteroidales	0.0463±0.0457	0.0494±0.0347	0.0750±0.0367
Akkermansia	0.0439±0.0883	0.0594±0.0757	0.0550±0.0964
Dorea	0.0073±0.0059	0.0661±0.534	0.0417±0.0771
Bacteroids	0.0230±0.0252	0.0626±0.0792	0.0251±0.0202
Unclassified-Ruminococcaceae	0.0508±0.0376	0.0340±0.0271	0.0189±0.0059
Lactobacillus	0.0215±0.0124	0.0298±0.0186	0.0414±0.0496
Prevotella	0.0250±0.0254	0.0466±0.0500	0.0157±0.0228
Allobaculum	0.0128±0.0158	0.0193±0.0248	0.0473±0.0638
Unclassified-Lachnospiraceae	0.0168±0.0178	0.0224±0.0097	0.0276±0.0153
Oscillospira	0.0267±0.0209	0.0157±0.0097	0.0195±0.0135
Unclassified-Desulfovid	0.0208±0.0301	0.0106±0.0175	0.0107±0.0187
Ruminococcus	0.0230±0.0123	0.0115±0.0080	0.0045±0.0026
Ruminococcus	0.0118±0.0093	0.0087±0.0101	0.0150±0.0072
Parabacteroides	0.0107±0.0158	0.0087±0.0063	0.0082±0.0092
Coprobacillus	0.0118±0.0127	0.0123±0.0143	0.0027±0.0048
Bafidobacterium	0.0218±0.0412	0.0023±0.0040	0.0017±0.0036
Coprococcus	0.0073±0.0043	0.0083±0.0041	0.0101±0.0052

Statistical analysis of the top 20 genus which accounted for a large proportion. The results showed that the expressions of Unclassified-S24-7, Unclassified-Clostridiales, Dorea, Bacteroides and Prevotella were significantly different between the control group and the model group, the model group and the emodin group. The expressions of Dorea, Bacteroides and Prevotella in the model group were higher than those in the control group, and the expressions of Dorea, Bacteroides and Prevotella in the emodin group were lower than those in the model group. The expression of Unclassified-S24-7 and Unclassified-Clostridiales in the control group were lower than those in the model group. The expression of Unclassified-S24-7 in the emodin group was lower than that in the model group, while the expression of Unclassified-Clostridiales in the emodin group was higher than that in the model group.



**Figure 5.** DNA electrophoresis.

abundance of Dorea, Bacteroides and Prevotella to restore the balance of intestinal flora

and achieve the suppression of chronic lymphocytic thyroiditis.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Zhipeng Ye, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, No. 54 Youdian Road, Shangcheng District, Hangzhou 310000, Zhejiang, China. Tel: +86-1596883-5273; E-mail: zhipengye100@163.com

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