Response of Intestinal Mucosal Microbiota in Diarrheal Mice to Ge-gen-qin-lian Decoction

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Research article

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Abstract

Background: Ge-gen-qin-lian Decoction (GD) has been extensively used for the treatment of diarrhea with intestinal dampness heat syndrome(IDHS) with a satisfying therapeutic effect. However, the active ingredients and mechanism of GD against diarrhea with IDHS have not been fully elucidated. The occurrence of diarrhea is closely related to the intestinal flora, and the compound of Traditional Chinese Medicine(TCM) can exert its curative effect by regulating the intestinal flora, and exploring the relationship between the two is conducive to the clarification of pharmacology.

Results: Animal experiments indicted that the OTU number and Alpha diversity index in the intestinal mucosa flora of the treatment group(cttm) recovered and higher than that of the control group(ctcm). PCoA results showed that there were differences in the community structure between the Con and GD. At the species level, the abundance of *Lactobacillus crispatus* and *Muribaculum intestinal* in the model group(ctmm) decreased, and the *Neisseria mucosa* increased (p<0.05). After being treated with GD, *Muribaculum intestinal* increased, and *Lactobacillus curlyus* and *Neisseria mucosa* decreased (p<0.05). Combined with network pharmacology analysis, we screened out 146 active ingredients in GD corresponding to 252 component targets, and 328 disease targets in diarrhea to obtain 31 drug-disease common targets. The key targets involved TNF, IL-6, EFGR etc. KEGG pathway enrichment resulted in HIF-1 signaling pathway, VEGFA signaling pathway, Adipocytokine signaling pathway and so on (p<0.05).

Conclusions: GD could restore the diversity and abundance of intestinal mucosa in diarrheal mice with IDHS, promote the abundance of *Muribaculum intestinal*, inhibit the abundance of *Neisseria mucosa*. Through the characteristic of multiple targets and multiple channels, the active ingredients of GD intervened from oxidative stress and inflammatory response to adjust the balance of intestinal mucosa flora, thereby playing the role of treating diarrhea.

Background

Intestinal damp heat syndrome (IDHS) is a common syndrome type in diarrhea of the internal medicine of Traditional Chinese Medicine(TCM). With the changes in the lifestyle of modern society, increased mental stress and unreasonable diet structure have gradually increased the incidence of diarrhea[1]. TCM believe that IDHS diarrhea is mostly caused by feeling hot and humid outside evil, or eating improperly (unclean). This study simulated the traditional etiology of IDHS diarrhea from the perspective of internal and external humidity combined with environmental and dietary complex factors. High-sugar and high-fat feeding was used to damage the spleen and stomach function of mice, resulting in the accumulation of dampness and heat in the intestinal tract. High temperature and humidity environment caused the external humidity and internal humidity to draw together. Combining ice water and drinking with gavage lead to a preference for cold and hot diets, further damaged the spleen and stomach, causing the occurrence of diarrhea. Therefore, the establishment of the diarrheal model with IDHS by simulating the traditional etiology and clinical symptoms of TCM is of great reference significance for the study of IDHS.

Studies found that diarrhea was closely related to intestinal microecology[2]. Normally, the intestinal microbiota and the body remain relatively stable. A stable microbial environment is essential to the health of the host[3–5]. As the largest interface between the body and the external environment, intestinal mucosa is the main line of defense against invasion by pathogenic microorganisms[6]. However, intestinal flora is continuously and relatively fixed in the intestinal mucosa and participated in many physiological and pathological processes[7, 8]. When the homeostasis of the intestinal flora is disrupted, the growth of beneficial bacteria is inhibited, and pathogenic bacteria multiplied in large numbers, resulting in a dysbiosis[9]. It affects the immune function and structural integrity of intestinal mucosa, generating the decreased intestinal mucosal barrier function. Some foreign or transit bacteria colonize and multiply in the intestinal tract and become the dominant bacteria, leading to diarrhea[10]. The occurrence of diarrhea will further aggravate the disorder of the intestinal flora. To explore the correlation between diarrhea with IDHS and intestinal flora is expected to reveal its mechanism from the perspective of microecology.

TCM focuses on the holistic view of the body to give adjustment, for different types of syndrome differentiation for diarrhea treatment. Ge-gen-qin-lian Decoction(GD) is derived from "Treatise on Febrile Diseases", composed of four kinds of medicines such as Pueraria Lobate(PL), Rhizoma Coptidis(RC), Radix Scutellariae(RS), and Licorice Roots(LR), which proves safe and non-toxic in long-term clinical use[11]. Modern pharmacological researches show that GD has functions of antipyretic and antibacterial, anti-inflammatory, anti-diarrhea, enhancing immunity of the body and so on. It also avoids drug resistance and undesirable residues caused by the use of antibiotics, which is beneficial to the health of the body[12–14]. However, the research on the specific therapeutic mechanism of the main active ingredients in GD need to be further studied.

As a newly emerging field of pharmacology, network pharmacology emphasizes the concept of a "multicomponent, multitarget therapeutic network" and highlights the overall thoughts of TCM[15]. In this study, GD was used to intervene after preparing the model of IDHS diarrhea, to clarify the micro-ecological mechanism of the occurrence of IDHS diarrhea and the efficacy of GD. Combined with network pharmacology, GD active ingredients and candidate targets were predicted through a holistic process of active ingredients screening, target fishing, network construction and analysis so as to further search for the pharmacodynamic substances and targets for the treatment of diarrhea, with a view to provide the basis for future “bacteria-syndrome-prescription” combination. The detailed workflow was shown in Fig. 1.

Results

The effect of GD on intestinal mucosa flora in mice with IDHS diarrhea

Effective sequence analysis of IDHS diarrhea mice intestinal mucosa flora

As could be seen in Table 1, the average length of the effective sequence after quality filtering was in the range of 1478 bp-1483 bp, and the resulting sequence length was consistent with the target length range, which could effectively reflect the true situation of the microorganisms in the sample.

Table 1
Data preprocessing statistics table

Sample	CCS	Filtered	Average Length
ctcm1	8564	7911	1482
ctcm2	12740	11924	1482
ctcm3	13999	13122	1481
ctcm4	16566	15571	1482
ctcm5	14125	13267	1482
ctmm1	11663	10888	1483
ctmm2	17319	16302	1482
ctmm3	10461	9725	1482
ctmm4	11757	10594	1483
ctmm5	14361	13564	1478
cttm1	20104	18679	1480
cttm2	12463	11689	1482
cttm3	15491	14391	1483
cttm4	5110	3976	1478
cttm5	11058	10290	1483

Note: CCS: circular consensus sequence; Filtered: the number of effective CCS after removing primers and length filtering; Average Length: average length of effective sequence. Con1-5: control group sample 1–5; Mol1-5: model group sample 1–5; GD1-5: GD group sample 1–5.

The effect of GD on the number of OTUs in the intestinal mucosa of IDHS diarrhea mice

The number of OTUs in the ctcm, ctmm and cttm were respectively 274,301 and 287, including 119 OTUs in the intersection of OTUs in the three groups(Fig. 2). Among them, 91 OTUs in the cttm were indicated that the number of intestinal OTUs in model mice decreased after treating with GD.

The effect of GD on the diversity of intestinal mucosa in IDHS diarrhea mice

Combined with the Alpha diversity analysis, Chao1 index in the ctmm was lower than that of the ctcm and cttm. Chao1 index in the cttm was close to the ctcm, but there was no significant difference between the three groups, indicating that GD could recover and to some extent increased the richness of intestinal mucosa flora(Fig. 3A). Compared with the ctmm, Shannon and Simpson index of the cttm increased, which reflected that GD had a restorative effect on the intestinal mucosa flora diversity in mice with diarrhea caused by IDHS(Fig. 3B□3C).

Beta diversity analysis showed that the main coordinate variable 1 was 32.36% and the main coordinate variable 2 was 21.33% (Fig. 3D). From the results, the samples in the ctcm and the ctmm were evenly distributed and relatively concentrated. The distance between the samples of the cttm and the ctmm was close, indicating that they had a higher similarity in bacterial community composition. The samples of the cttm and the ctcm could be clearly separated, suggesting that there was a certain difference in the bacterial community structure.

The effect of GD on the genus level of intestinal mucosa in IDHS diarrhea mice

Comparing the NCBI database, there were totally 187 genera in the ctcm, ctmm, and cttm(Fig. 4 and Table 2). Among these, Lactobacillus was the first dominant genus. The abundance of Lactobacillus in the intestinal mucosa of the ctmm was lower than that of the ctcm, accounting for 82.29%. In the GD, the Lactobacillus (79.88%) was slightly lower than that of the ctmm, showing that the modelling had an inhibitory effect on Lactobacillus, while GD had a regulatory effect on the growth of Lactobacillus. The abundance of Streptococcus in the ctmm(5.03%) and ctcm(6.41%) was higher than that in the ctcm(4.45%), which was suggested that GD could promote the growth of Streptococcus. Compared with the ctcm, the Neisseria in the ctmm(1.23%) was significantly increased, while in the cttm(0.78%) was similar to that in the ctcm(0.80%). Thus, GD regulated the abundance of Neisseria to reach the normal level by inhibiting its growth. The Clostridium and Muribaculum in the ctmm was very low, respectively accounting for 0.09% and 0.01%, while the Clostridium (1.09%) and Muribaculum (1.08%) in the cttm was significantly higher than that of the ctcm, suggesting that GD could promote the growth of Clostridium and Muribaculum.

Table 2
Effect of GD treatment on the abundance of genus level of the intestinal mucosal flora in IDHS diarrhea mice (top 20)

Genus name	The relative abundance(%)		
	ctcm	ctmm	cttm
Lactobacillus	0.8427 ± 0.0244	0.8229 ± 0.7823	0.7988 ± 0.0844
Streptococcus	0.0455 ± 0.0047	0.0503 ± 0.0083	0.0641 ± 0.0212
Neisseria	0.0080 ± 0.0015	0.0123 ± 0.0044	0.0078 ± 0.0021
Clostridium	0.0090 ± 0.0076	0.0090 ± 0.0003	0.0109 ± 0.0103
Muribaculum	0.0079 ± 0.0000	0.0001 ± 0.0000	0.0108 ± 0.0000
Lautropia	0.0054 ± 0.0015	0.0055 ± 0.0021	0.0078 ± 0.0058
Curvibacter	0.0050 ± 0.0024	0.0081 ± 0.0019	0.0053 ± 0.0022
Prevotella	0.0049 ± 0.0012	0.0077 ± 0.0040	0.0053 ± 0.0016
Porphyromonas	0.0064 ± 0.0027	0.0040 ± 0.0020	0.0047 ± 0.0016
Ureaplasma	0.0042 ± 0.0008	0.0039 ± 0.0016	0.0036 ± 0.0028
Staphylococcus	0.0037 ± 0.0026	0.0014 ± 0.0009	0.0063 ± 0.0040
Bacteroides	0.0013 ± 0.0007	0.0006 ± 0.0003	0.0094 ± 0.0089
Gemella	0.0028 ± 0.0011	0.0020 ± 0.0012	0.0031 ± 0.0011
Rothia	0.0033 ± 0.0019	0.0023 ± 0.0012	0.0021 ± 0.0010
Cutibacterium	0.0042 ± 0.0036	0.0009 ± 0.0006	0.0018 ± 0.0015
Helicobacter	0.0004 ± 0.0001	0.0027 ± 0.0000	0.0011 ± 0.0000
Weissella	0.0011 ± 0.0002	0.0006 ± 0.0003	0.0043 ± 0.0037
Pantoea	0.0019 ± 0.0016	0.0014 ± 0.0006	0.0019 ± 0.0016
Veillonella	0.0016 ± 0.0007	0.0020 ± 0.0015	0.0015 ± 0.0012
Granulicatella	0.0007 ± 0.0005	0.0005 ± 0.0002	0.0036 ± 0.0033

The effect of GD on the species level of intestinal mucosa in IDHS diarrhea mice

It could be seen from Fig. 5 and Table 3 that the top 5 dominant species bacteria in the three groups were *Lactobacillus crispatus*, *Streptococcus oralis*, *Muribaculum intestinale*, *Lautropia mirabilis*, and *Curvibacter lanceolatus*. *Lactobacillus crispatus* was the most abundant in the three groups. The abundence of *Lactobacillus crispatus*(82.20%) in the ctmm was lower than that in the ctcm(83.92%),

whereas in the cttm(79.72%) was not significantly different from that in the ctcm, showing that *Lactobacillus crispatus* had a regulating effect but had little effect after the treatment of GD. The *Muribaculum intestinale* in the ctmm(0.01%) was lower than that in the ctcm(0.79%), while the abundance in the cttm(1.08%) was significantly higher than that of the ctmm, which suggested that GD had a role in the growth of *Muribaculum intestinale* enhancement. Compared with the ctmm(0.81%), the *Curvibacter lanceolatus* in the ctcm(0.50%) and cttm(0.53%) were decreased. It indicated that GD had a certain inhibitory effect on *Curvibacter Lanceolatus*. The *Streptococcus oralis* and *Lautropia mirabilis* in the ctmm(4.96%, 0.55%) and the cttm(6.26%, 0.78%) were higher than the ctcm(4.34%, 0.54%). The two species bacteria in the ctcm and cttm had the similar contents, revealing that GD had little effect on the reproduction of *Streptococcus oralis* and *Lautropia mirabilis*.

Table 3
Effect of GD treatment on the abundance of species level of the intestinal mucosal flora in IDHS diarrhea mice (top 20)

Species name	The relative abundance(%)		
	ctcm	ctmm	cttm
Lactobacillus crispatus	0.8392 ± 0.0208	0.8820 ± 0.0405	0.07972 ± 0.0887
Streptococcus oralis	0.0434 ± 0.0045	0.0496 ± 0.0081	0.0626 ± 0.0200
Muribaculum intestinale	0.0079 ± 0.0000	0.0001 ± 0.0000	0.0018 ± 0.0000
Lautropia mirabilis	0.0054 ± 0.0011	0.0055 ± 0.0021	0.0078 ± 0.0058
Curvibacter lanceolatus	0.0050 ± 0.0024	0.0081 ± 0.0019	0.0053 ± 0.0022
Neisseria mucosa	0.0054 ± 0.0020	0.0074 ± 0.0032	0.0051 ± 0.0008
Bacteroides fragilis	0.0011 ± 0.0005	0.0004 ± 0.0001	0.0091 ± 0.0088
Porphyromonas gingivalis	0.0037 ± 0.0018	0.0014 ± 0.0011	0.0027 ± 0.0022
Cutibacterium acnes	0.0042 ± 0.0031	0.0009 ± 0.0006	0.0018 ± 0.0015
Prevotella intermedia	0.0016 ± 0.0002	0.0033 ± 0.0024	0.0015 ± 0.0012
Helicobacter typhlonius	0.0004 ± 0.0000	0.0053 ± 0.0000	0.0001 ± 0.0000
Weissella hellenica	0.0007 ± 0.0000	0.0005 ± 0.0002	0.0043 ± 0.0037
Porphyromonas endodontalis	0.0016 ± 0.0011	0.0023 ± 0.0009	0.0014 ± 0.0011
Granulicatella_adiacens	0.0007 ± 0.0005	0.0005 ± 0.0002	0.0036 ± 0.0033
Fusobacterium nucleatum	0.0001 ± 0.0005	0.0007 ± 0.0005	0.0016 ± 0.0013
Aerococcus_viridans	0.0048 ± 0.0003	0.0026 ± 0.0005	0.0017 ± 0.0009
Prevotella_veroralis	0.0009 ± 0.0006	0.0010 ± 0.0007	0.0012 ± 0.0007
Enterococcus_mundtii	0.0003 ± 0.0001	0.0026 ± 0.0020	0.0005 ± 0.0002
Parvimonas_micra	0.0012 ± 0.0007	0.0020 ± 0.0016	0.0001 ± 0.0002
Abiotrophia_defectiva	0.0014 ± 0.0007	0.0011 ± 0.0001	0.0009 ± 0.0003

Investigation of the mechanism of action of GD against diarrhea by network pharmacology

Active ingredients-targets network analysis

Using the established filter conditions, OB ≥ 30% and DL ≥ 0.18, 146 active ingredients were identified in five TCM in GD from the TCMSP (Fig. 6A). It was entered into the TCMSP database and a total of 269

targets with 146 active ingredients were found in the search. Though the UniProt database, 269 targets were found 252 corresponding gene names. The GD active ingredients-targets network diagram was constructed based on the interactions among the 5 herbs, 146 active ingredients and 252 targets associated with GD (as shown in Fig. 6B). The network contained of 384 nodes (representing active ingredients and targets) and 2694 edges (representing the interaction between the active ingredient and the target). Of the 146 active ingredients, 18 of them have more than 30 targets; for example, quercetin (degree = 284), formononetin (degree = 76), beta-sitosterol(degree = 74), kaempferol (degree = 58), and wogonin (degree = 45) had a high degree values and were located at central positions in the network. From the perspective of the targets, 26 targets worked with more than 30 ingredients. The top five targets were PTGS2, HSP90, CALM, AR and ESR1, which interacted with 121,95, 94, 93 and 92 ingredients. Based on this, we found the phenomenon that some ingredients in GD could act on multiple targets, and different ingredients worked together on the same target, which reflected the mechanism of interaction between multiple ingredients and multiple targets in TCM.

PPI core network analysis

Using the Venny 2.1 online mapping tool platform, the 328 disease targets and the 252 active ingredient targets were used to draw a Venn diagram, getting 31 common targets were obtained for both (Fig. 7). The results showed that GD played a cooperative role in treating diarrhea through multiple potential targets. Using 31 potential targets entered into the String database to obtain the protein interaction data(Fig. 8A), the Cytoscape 3.7.2 software was used to map these targets to the human protein-protein interaction network, a total of 31 targets and 186 edges related to gout were obtained(Fig. 8B). The DC, BC and CC of each node was calculated by the Cytoscape 3.7.2. For further research, 13 potential targets with the median value of DC, BC, and CC greater than 13,0.017 and 0.6 were finally obtained, including TNF, IL-6, EGFR and so on (Fig. 8C). It was suggested that the mechanism of GD for treating diarrhea was closely related to these core targets. In summary, it was very likely that GD exerted its pharmacological effects by acting on these core targets.

GO and KEGG enrichment analysis

In order to further elucidate the possible effects of GD on diarrhea, the biological processes and signaling pathways of 13 key targets were carried out through the gene enrichment analysis plug-in ClueGO. The results showed that the biological processes ($P < 0.05$) were largely related to the Regulation of reactive oxygen species biosynthetic process, positive regulation of neuroinflammatory response, positive regulation of ATP biosynthetic process and positive regulation of receptor-mediated endocytosis(Fig. 9A), and the signaling pathways ($P < 0.05$) were mainly involved with the AGE-RAGE signaling pathway in diabetic complications, HIF-1 signaling pathway, Adipocytokine signaling pathway and VEGF signaling pathway (Fig. 9B).

Discussion

Chinese medicine has a long history, focusing on the overall adjustment of the body to relieve the primary and secondary symptoms at the same time. It can promote intestinal microecological balance by increasing the diversity of intestinal flora. GD, as a classic formula for the treatment of diarrhea with IDHS, has the functions of clearing away heat, dryness and dampness, and releasing the exterior and clearing interior. Both RC and RS have anti-inflammatory and anti-viral effects. PL has obvious characteristics effects of lowering blood sugar, improving cardiovascular and cerebrovascular diseases, and reducing insulin resistance. LR takes the advantage of diuresis and detoxification[16]. In this study, after modeling the IDHS diarrhea, the third-generation sequencing of intestinal flora was used to analyze the changes of intestinal mucosa characteristic flora. Combined with network pharmacology, the interaction between biologically active ingredients and GD mechanism was studied. By constructing and analyzing the targets network, we could further search for the effective substances and targets of GD for the treatment of diarrhea.

In the animal experiment, the OTU number and Alpha diversity index in the intestinal mucosa flora of the cttm recovered to be close to and higher than that of the ctcm, indicating that GD treatment could restore the abundance and diversity of the intestinal mucosa flora in mice. The PCoA results showed that there was a certain difference in the community structure between the ctcm and cttm, suggesting that GD had a certain effect on the structure of intestinal mucosa flora in diarrheal mice. In the genus level, the abundance of *Neisseria* in the cttm were decreased to normal level, *Muribaculum* and *Clostridium* increased and higher than that of the ctcm, *Streptococcus* and *Lactobacillus* had similar abundance in the ctmm and cttm. It showed that GD promoted the growth of *Muribaculum* and *Clostridium*, inhibited the growth of *Neisseria*, and had a regulating effect on *Lactobacillus* and *Streptococcus*. In the species level, the abundances of *Lactobacillus crispatus* and *Neisseria mucosa* in the cttm decreased below the level of the ctcm, while the abundance of *Muribaculum intestinale* recovered. *Lactobacillus* is widely distributed in nature, and various types of animal and plant fermented foods often contain *Lactobacillus*. It also exists in the human gastrointestinal tract and has the effects of regulating the intestinal microenvironment, enhancing immunity and promoting digestion, which is beneficial to human health[17]. *Lactobacillus crispatus* belongs to the *Lactobacillus* family, which is an important probiotic colonized in the reproductive tract and animal intestines. It can produce antibacterial compounds and extracellular polysaccharides and stimulate the immune response through competitive rejection against pathogenic bacteria[18]. In this study, the growth of *Lactobacillus crispatus* in the cttm was inhibited, probably because GD had an overall regulating effect on the intestinal flora, which moved the structure of the intestinal flora to a relatively balanced direction. *Neisseria mucosa* is a Gram-negative diplococcus, capable of invading neutrophils and replicating in the cell, avoiding phagocytosis and being killed by complement through resisting antibodies. In addition, It can be translocated to host cells and regulated the production of reactive oxygen species[19]. The analysis results manifested that the content of *Neisseria mucosa* in the cttm decreased significantly, revealing that GD could inhibit the growth of *Neisseria mucosa*. *Muribaculum intestinale* is strictly anaerobic, inhibited the colonization of pathogenic bacteria in animal experiments, thereby regulating the balance of bacteria [20]. In our experiment, the content of *Muribaculum intestinale* in the cttm increased and was higher than that of the ctcm, showing

that GD had a positive effect on promoting the growth of *Muribaculum intestinale*. To sum up, GD had replenishing effect on the diversity and abundance of intestinal bacteria of IDHS diarrhea mice, promoting the abundance of *Muribaculum intestinale*, inhibiting the abundance of *Neisseria mucosa* at the species level, and changing the relative content of *Lactobacillus crispatus*. The above information suggested that the efficacy mechanism of GD was relevant to the rate of regulating the intestinal mucosal flora of model mice. However, the specific active ingredients and target mechanisms in GD were still unclear, and we still need to explore the next step.

Then systems pharmacology was performed subsequently to identify the active compounds and their corresponding targets, and results showed that most ingredients of GD affected multiple targets, for example, quercetin, formononetin, and beta-sitosterol acted on 284, 76, and 74 targets, respectively. Therefore, they were very likely to be the crucial pleiotropically active ingredients for GD. Although the number of putative targets in each herb was different, the overlapping targets in different herbs were numerous. In other words, multiple ingredients of GD may have the synergistic as well as multifarious effects for each ingredient of GD. Quercetin was one of the most significant bioflavonoid ingredients found in current research. It pharmacologically possessed anti-inflammatory, anti-oxidant, anti-viral, and anti-tumor, as well as immune stimulant[21]. In addition, Quercetin could regulate the intestinal flora, which reflected on the potent bacteriostatic activity against different strains bacteria[22]. Formononetin performed anti-inflammatory activity by adjusting intestinal bacteria in diarrhea[23]. β-sitosterol belonged to the class of tricyclic tri-post compounds. Some studies had manifested that β-sitosterol significantly reduced LPS-induced inflammation and the release of TNF-α and IL-6[24]. Therefore, they might be identified as the representative ingredients for GD.

PPI network were next constructed for the functional analysis, on the basis of putative-target networks of GD and diarrhea. We finally got 13 core targets based on DC, BC and CC. It was revealed that the active ingredients of GD might mainly treat diarrhea through target proteins such as TNF, IL-6, EGFR and so on. TNF was a pleiotropic factor with multiple biological effects. It could directly act on T-cells, B-cells, NK cells and other effector cells and played a role at the cellular level[25]. IL-6 was a pleiotropic cytokine that exerted certain proinflammatory effects. Stimulation of IL-6 resulted in a maximal induction of NF-kB activation and NF-kB nuclear translocation[26]. Overall, IL-6 had the influence on innate and adaptive immune responses, suggesting that dysfunction of its function could promote the progression of chronic diseases[27]. The distribution of EGFR in gastric mucosal epithelial cells was the most. Some studies had shown that by regulating the expression of mucosal repair factor EGFR, it could promote the repair of intestinal mucosa and prevent the occurrence of diarrhea[28]. After EGFR activation, it further exerted biological effects through HIF-1 signaling pathway[29]. These results suggested that GD might be involved in the pathogenesis of GD by acting on these corn proteins.

Enrichment analyses presented a series of biological processes and signaling pathways, including regulation of reactive oxygen species biosynthetic process, positive regulation of neuroinflammatory response, positive regulation of ATP biosynthetic process and positive regulation of receptor-mediated

endocytosis, together with the HIF-1 signaling pathway, VEGF signaling pathway, AGE-RAGE signaling pathway in diabetic complications, and Adipocytokine signaling pathway. HIF-1 were widely presented in the human body in a hypoxic environment, regulated the body's response to hypoxia[30]. Several researches indicated that HIF-1 signaling pathways had regulatory actions in a wide variety of cellular processes involved in immune and intestinal barrier function[31, 32]. It could increase the transcription of genes related to cell permeability and inflammation[33]. Under the normal conditions, the intestinal mucosal barrier could effectively prevent the conditional pathogens and pathogenic bacteria and other harmful substances from invading the body, which played a protective role, and was conducive to the stability of the internal environment. Based on the special anatomical structure of the intestinal mucosa, it reduced the tolerance of the intestinal mucosa to local hypoxia. Hypoxia greatly affected the regulation of intestinal mucosal barrier function, which in turn caused disturbance of intestinal flora[34]. However, inflammation and hypoxia were the most common basic pathophysiological conditions that occur when many types of disease occur in the body. There was a certain internal connection between them, which often appeared in the pathological process of the same disease and affected the development of the disease. In the PPI network, we also indicated that the core protein involved in GD treatment of diarrhea was closely related to inflammation. VEGF was a related downstream gene regulated by HIF-1α, which specifically recognized endothelial cells of blood vessels and affected their differentiation and proliferation to induce neovascularization[35]. It could also be induced and regulated by the inflammatory factors TNF-α and IL-6[36]. In the mice model of stress gastric ulcer, the expression of VEGF was increased, suggesting that VEGF may have a protective effect on gastrointestinal injury[37]. Thus, we proposed that the HIF-1 and VEGF signaling pathway, which were highly involved in all the above findings, might be a pivotal target in the treatment of GD on diarrhea.

Conclusions

In summary, this study clarified through animal experiments that GD could restore the diversity and abundance of intestinal mucosa flora in diarrheal mice with IDHS, promote the abundance of *Muribaculum intestinal*, inhibit the abundance of *Neisseria mucosa*, and adjust the relative content of *Lactobacillus crispatus*, which suggested the efficacy of GD was related to the regulation of intestinal microecological balance. Then, combined with network pharmacological, it was found that the active ingredients in GD could intervene from oxidative stress and inflammatory response through multiple targets and multiple channels to adjust the balance of intestinal mucosa flora, thereby playing a role in the treatment of diarrhea. In order to explore the corresponding relationship between the intestinal mucosa flora and the TCM syndrome of diarrhea from the perspective of “bacteria-syndrome-prescription” in the future, it will lay a research foundation and provide objective and standardized indicators for syndrome differentiation.

Methods

Animal experiment

Animals and feed

SPF male Kunming mice (18 – 22 g) were provided by the Hunan Leike Jingda experimental animal co., LTD (licence number: SCXK(Xiang)2016-0002), which were kept in experimental animal center of Hunan University of Traditional Chinese Medicine (license number: SYXK (Xiang) 2019-0009). The animals were housed in a room at 23 ~ 25 °C and 50 ~ 70% humidity. Animal common feed was provided by Hunan Slaccas Jingda (SJA) Laboratory Animal Co., Ltd. High-sugar and high-fat special feed (12% lard, 8% honey mixed with common feed) was purchased from Jiangsu Synergy Pharmaceutical Bioengineering Co., Ltd. (number: Su Feeding Certificate (2014) 01008).

Main reagents

GD composition: RC 3g (Sichuan, number: CKN19041006)), LR 6g (Gansu, number: 1806004)), PL 15g (Hunan, number: CK19042602)), RS 9g (Inner Mongolia, number: TH1905220)). The above medicinal materials were purchased from the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine. Red Star Erguotou (56 degrees) produced by Beijing Red Star Co., Ltd.

Grouping of animals

15 SPF male Kunming mice were selected from the random number table as the control group (ctcm), and the remaining mice were randomly divided into treatment group (cttm) and model group (ctmm), with 5 mice each.

Model preparation

The model preparation was improved by referring to the comprehensive factor modeling method[38]. After 3 days of adaptive feeding, the animals in the ctmm were fed with high-sugar and high-fat diet for 11 days. At 9:00 on the 12th day, the ctmm mice were placed in an artificial climate box with a temperature of 32 ± 0.5 ℃ and a relative humidity of 95%. After 8 hours, they were removed to a normal environment and gavaged with liquor diluent (V liquor: V sterile water = 1:1) 10 mL/kg before and after placing in the artificial climate box. At noon, the mice was gavaged at 0 ℃ ice water 10 mL/kg, lasted 4 days. The mice in the ctcm were given the same amount of sterile water by gavage and allowed to eat and drink freely.

Method of administration

According to the clinical equivalent dose of experimental animals, the cttm mice were given 20 mL/kg of GD, once in the morning and at night, with an interval of 8 hours, and continued for 4 days. The ctcm and ctmm were given the same amount of sterile water at the same time.

Extraction of mice intestinal mucosa

Mice were euthanized by cervical dislocation. On the clean bench, the abdomen of the mouse was cut open along the abdomen white line to expose the ileum and colorectal area. Take the jejunum and ileum

of mice and washed the contents of the intestinal wall with saline. Scraped the intestinal mucosa with a coverslip and added physiological saline twice the weight of the intestine, then centrifuged at 3000 r/min for 10 mins, and take the supernatant for subsequent gene extraction[39]. Immediately placed the sample in the refrigerator at -80 ℃.

DNA sequencing

The total bacterial DNA was extracted using the CTAB method to amplify the full-length bacterial 16S rRNA gene sequence. The forward primer for amplification was: 27F (5'-AGRGTTY GATYMTGGCTCAG-3'), and the reverse primer was: 1492R (5'-RGYTACCTTGTTACGACTT-3'). The primers were synthesized by Wuhan Fraser Bioinformatics Co., Ltd. Library construction was performed using SMRTbell Template Prep Kit 1.0-SPv3, and sequencing was performed using PacBio platform. The extraction, amplification, and library sequencing of sample DNA were performed by Wuhan Fraser Bioinformatics Co., Ltd. The above process was repeated many times.

Network pharmacology research

Screening of active ingredients and potential targets

All of the information regarding the ingredients in GD were obtained from the Traditional Chinese Medicine Systems Pharmacology (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php)[40]. The active ingredients of GD were screened based on OB≥30 % and DL≥0.18[41]. Then, we obtained the target information of active ingredients from TCMSP. UniProt database (http://www.UniProt.org/)[42] was used to standardize all the target information.

Prediction of potential targets of diarrhea of IDHS in GD treatment

Using the DisGeNET database (https://www.disgenet.org/search) [43] with "diarrhea" as the keyword to obtain the target of the disease. Drew a Venn diagram on the Venny2.1 online mapping tool platform (https://bioinfogp.cnb.csic.es/tools/venny/) and got the intersection of the two, as a potential target for the treatment of diarrhea of IDHS in GD.

Active ingredients-target network

Import the different Chinese medicines in GD and their corresponding gene names into Cytoscape 3.7.2 software (http://www.cytoscape.org/)[44] to construct the GD active ingredients-target network .

PPI core network

Firstly, we entered the obtained potential targets into the STRING database (https://string-db.org/) [45]. Only “Homo sapiens” proteins with the confidence score higher than 0.4 were picked out. Secondly, we used CytoNCA to evaluate the intersection[46]. Three centrality measures provided by CytoNCA were used to filter the data, including degree centrality (DC), closeness centrality (CC), and betweenness centrality

(BC). The data were preliminarily processed by the screening criteria of 'DC ≥median DC', and then secondarily screened as core targets by the screening criteria of 'DC, BC, and CC greater than or equal to their median'.

GO and pathway enrichment analysis

The plug-in ClueGO was used for GO and KEGG enrichment analysis[47]. We set $p<0.05$, sort according to significance, and displayed the top 15 enriched items. Afterwards, Cytoscape 3.7.2 software was used to analyze the enrichment of GO and KEGG pathways for potential targets, and the relationship between potential targets and biological functions or disease-related pathways were screened and visualized.

Statistical analysis

The data obtained from each group were expressed as mean ± standard deviation ($x \pm s$). Statistical analysis was performed using SPSS 21.00 software, and the comparison of data differences between groups was performed by independent sample t test. $P<0.05$ means there was a significant difference, and $P<0.01$ means the difference was very significant.

Abbreviations

GD: Ge-gen-qin-lian Decoction; IDHS: Intestinal Dampness Heat Syndrome; TCM: Traditional Chinese Medicine; PL: Pueraria Lobate; RC: Rhizoma Coptidis; RS: Radix Scutellariae; LR: Licorice Roots; TCMSP: Traditional Chinese Medicine Systems Pharmacology

Declarations

Ethics approval and consent to participate

All animal work was carried out in accordance within the guidelines of the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine (NO.20171202). The animal experiments were performed in accordance with Animal Research: Reporting In Vivo Experiments guidelines approved by SYSU IACUC and were conducted in a laboratory designed to ensure biosafety. All authors knew and approved of this animal experiments.

Consent to publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study were included in this published article. The datasets used and analysed during the current study were available from the corresponding author on reasonable request.

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

XL performed network analysis and drafted the manuscript. CZ performed most of the experiments and statistical analysis. HH guided the performance of animal experiment. XP performed parts of the experiments. ZT and NX were responsible for studying the design and collecting fund. All authors reviewed and approved the final manuscript.

Acknowledgments

Not applicable.

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Figures

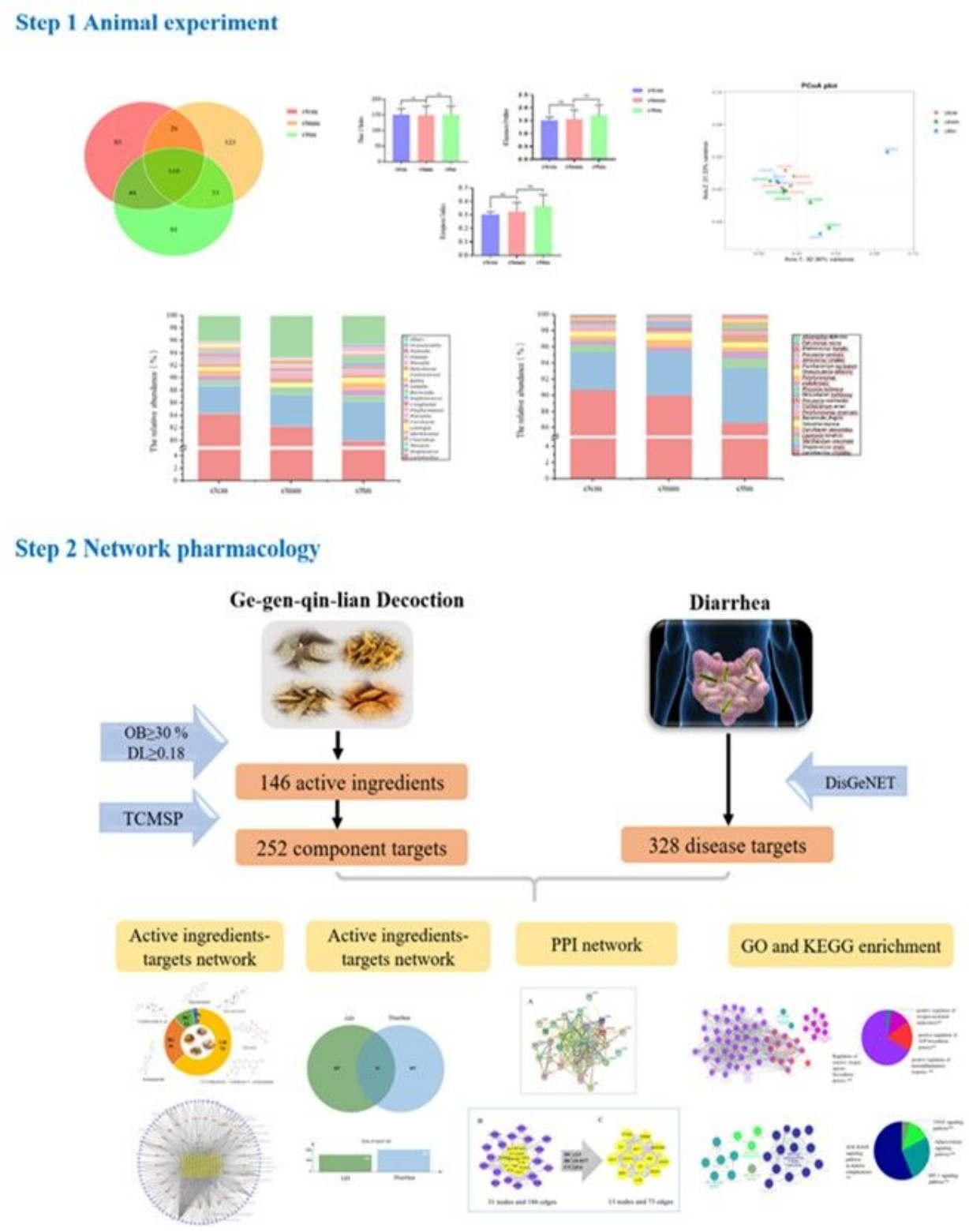

Figure 1

Diagram of the study design

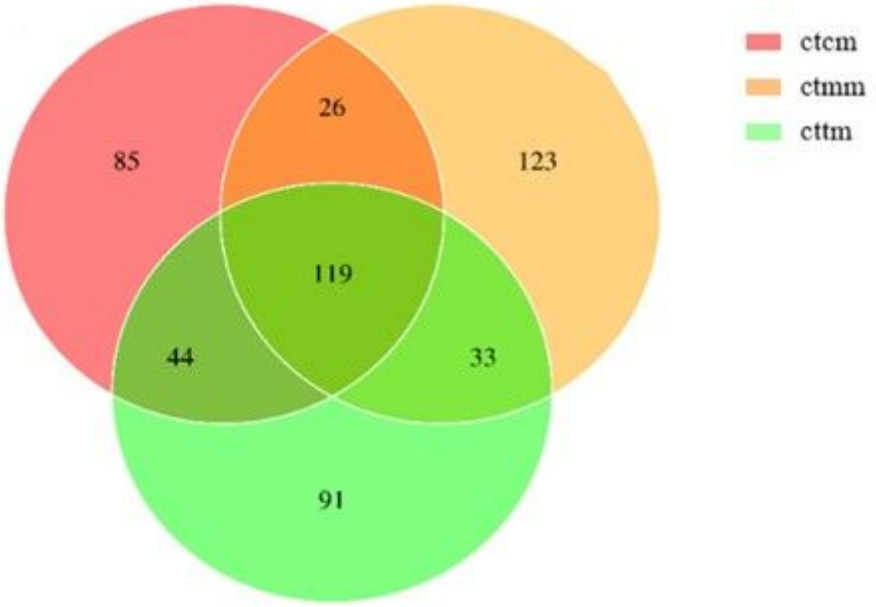

Figure 2

Effect of GD on the number of OTUs in the intestinal mucosa of IDHS diarrhea mice(n=5)

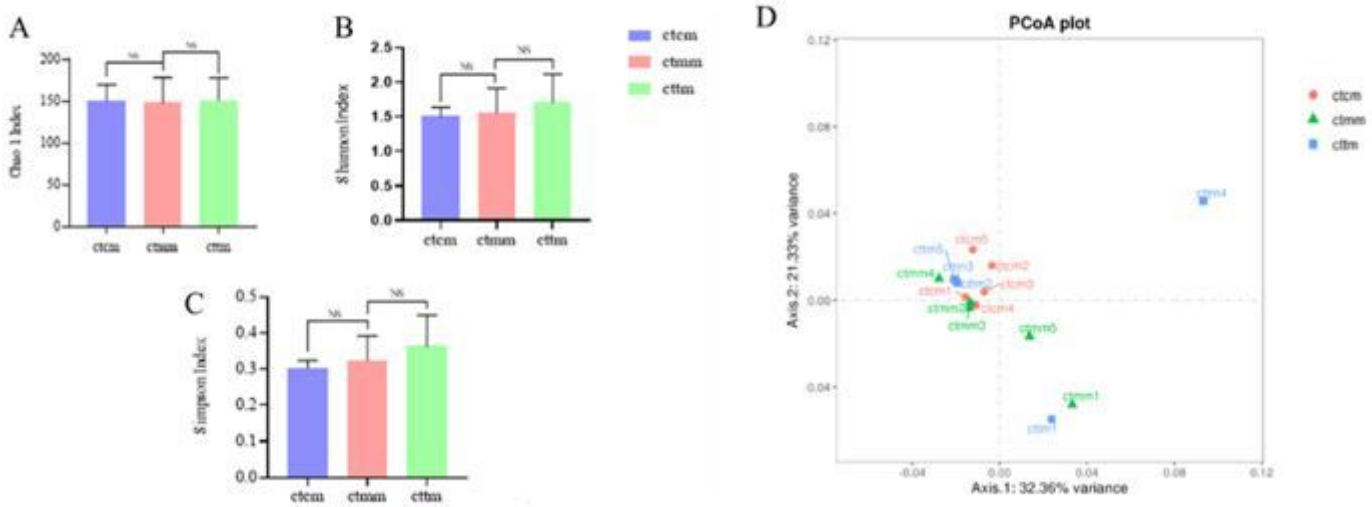

Figure 3

Effect of GD on the diversity of intestinal mucosa in IDHS diarrhea mice(n=5). (A) Chao 1 index was used to estimate the total number of OTUs in a sample(P>0.05). (B) Shannon index was used to evaluate the species diversity of a sample(P>0.05). (C)Simpson index was used to evaluate the species diversity of a sample (P>0.05). (D)PCoA. Observation of the differences among groups of intestinal content bacteria in rats and evaluation of differences in microbial community structure among different samples.

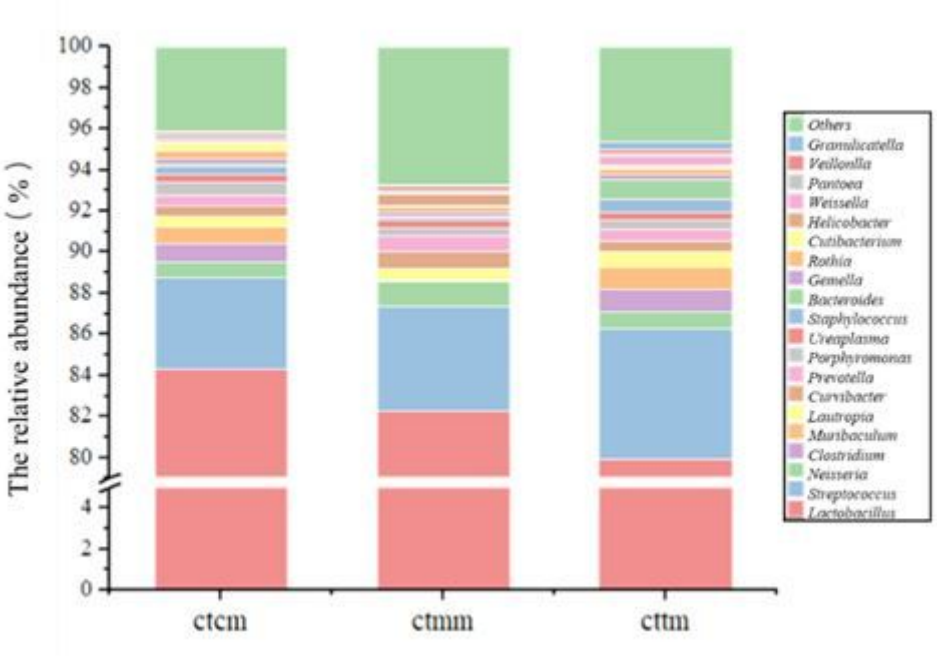

Figure 4

Effect of GD treatment on the abundance of genus level of the intestinal mucosal flora in IDHS diarrhea mice (top 20)

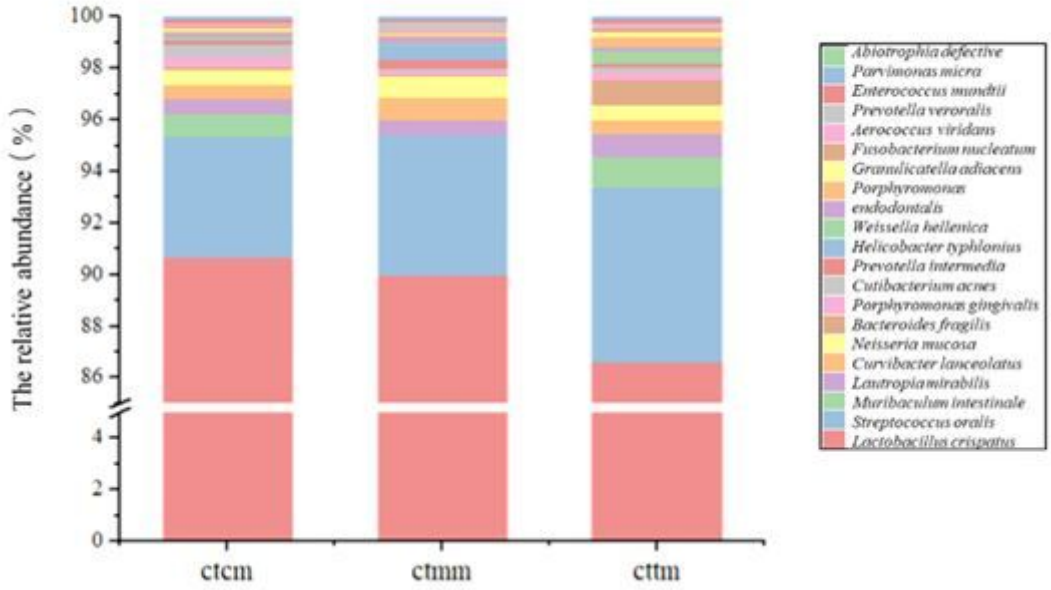

Figure 5

Effect of GD treatment on the abundance of species level of the intestinal mucosal flora in IDHS diarrhea mice (top 20).

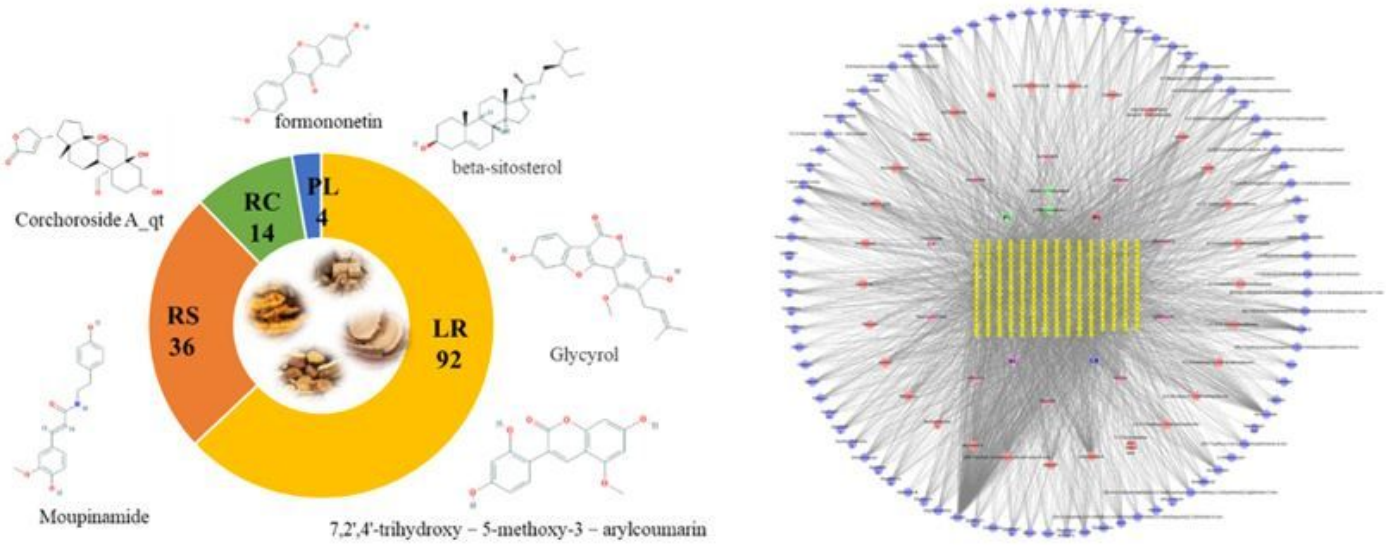

Figure 6

Active ingredients-targets network. (A)The number of main chemical constituents of each herbs and representative constituents structure in GD. (B) GD–Active ingredients-targets network.

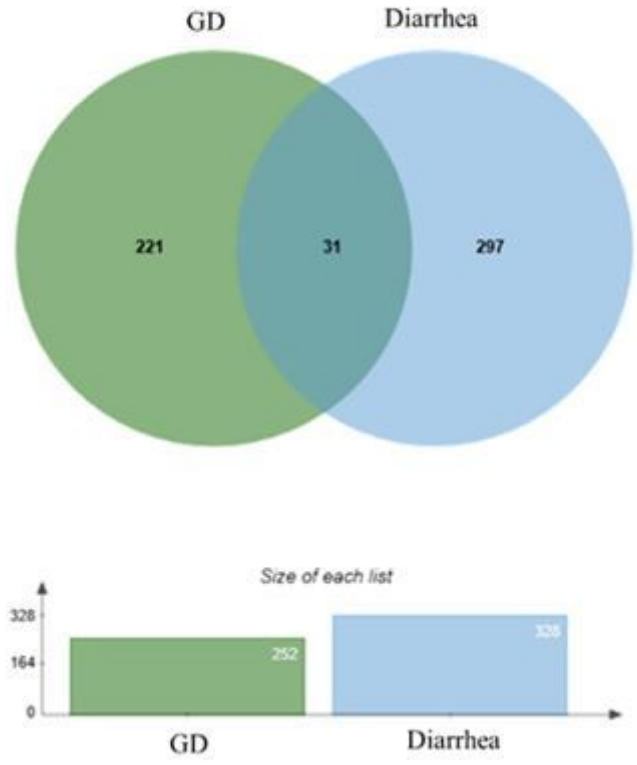

Figure 7

Venn diagram of targets in GD and Diarrhea

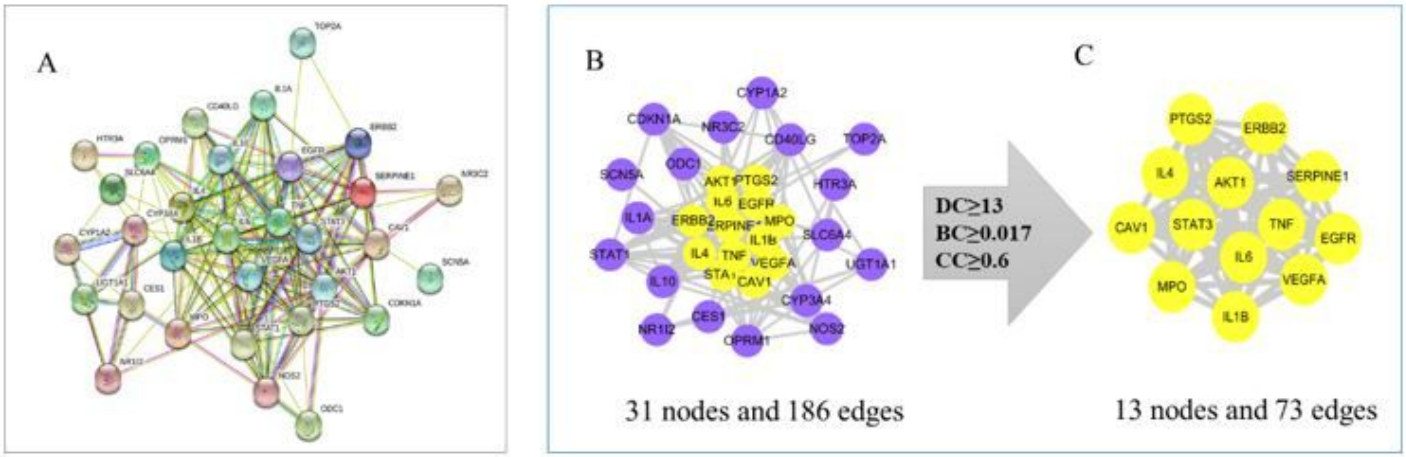

Figure 8

(A) PPI network. (B) Intersection of PPI networks (31 nodes and 186 edges). (C) Core-target PPI network by the screening criteria of "'DC' ≥ 13, 'BC' ≥ 0.017, and 'CC' ≥0.6 (13 nodes and 73 edges).

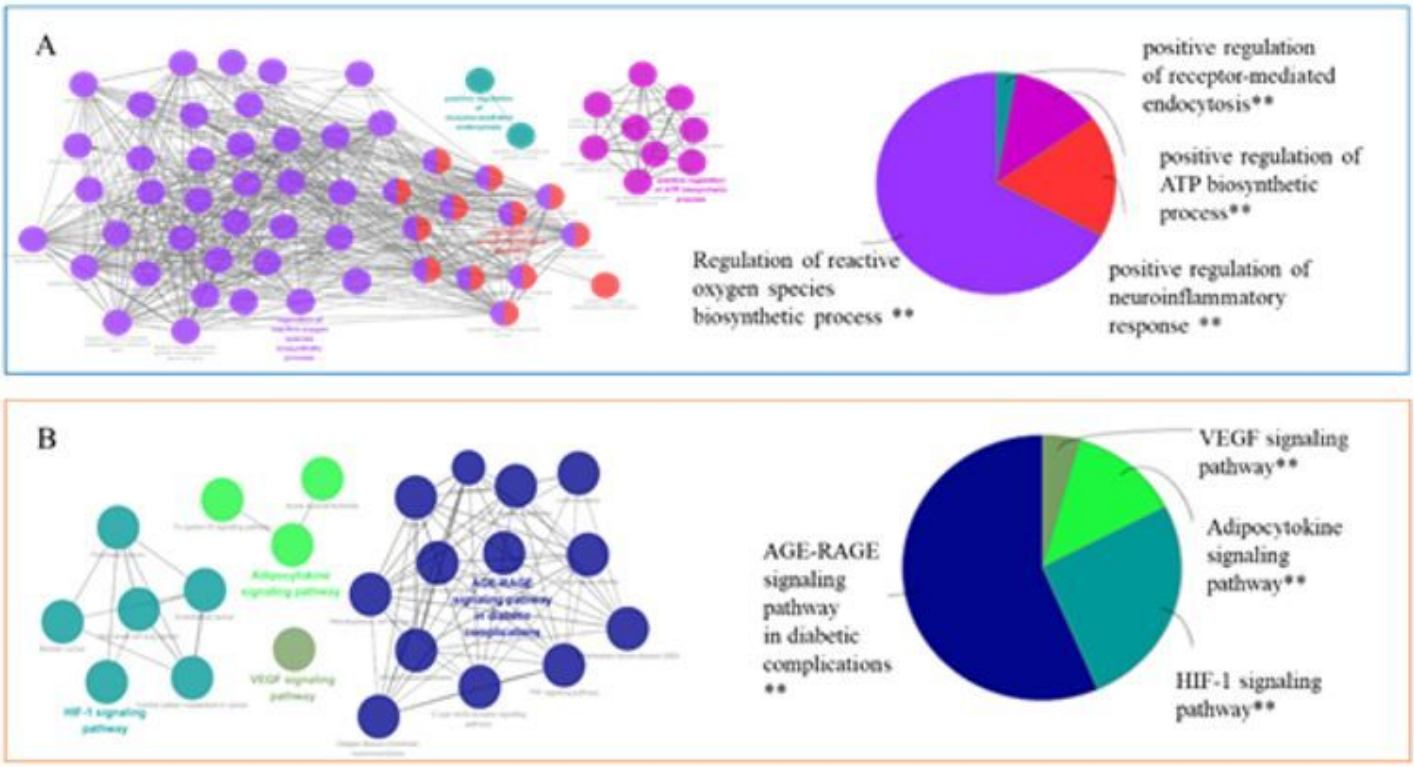

Figure 9

GO and KEGG enrichment. (A)Biological process enrichment analysis for the effect of GD on diarrhea. Left: most important biological process in the group; right: enrichment analysis shown by pie chart. (B)

Signal pathway enrichment analysis for the effect of GD on diarrhea. Left: most important signal pathway in the group; right: enrichment analysis shown by pie chart.

Supplementary Files

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