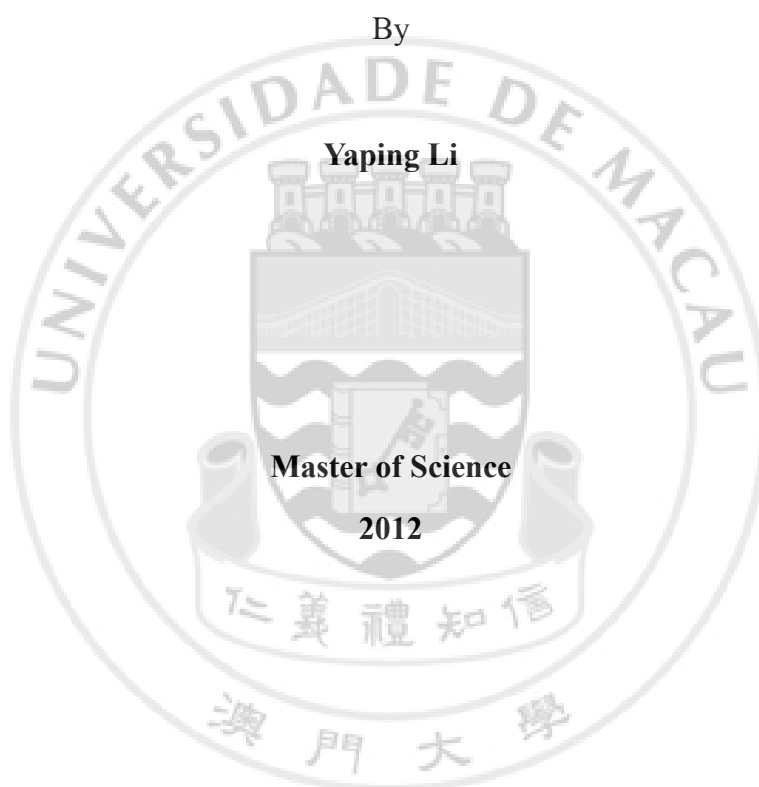


**Alterations of Composition and Metabolic Activity of Fecal
Microbiota in Ulcerative Colitis Rats and Intervention of
Astragali Radix**

By

Yaping Li

**Master of Science
2012**



**Institute of Chinese Medical Sciences
University of Macau**



**Alterations of Composition and Metabolic Activity of Fecal
Microbiota in Ulcerative Colitis Rats and Intervention of
Astragali Radix**

by

Yaping Li

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science

Institute of Chinese Medical Sciences

University of Macau

2012

Approved by _____

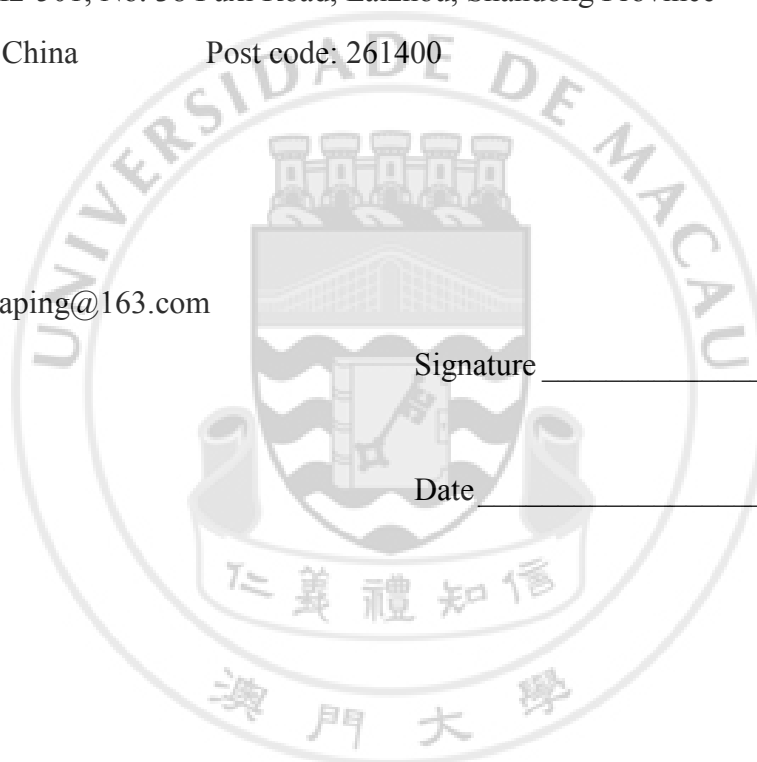
Supervisor

Date _____

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at the University of Macau, I agree that the Library and the Institute of Chinese Medical Sciences shall make its copies freely available for inspection. However, reproduction of this thesis for any purposes or by any means shall not be allowed without my written permission. Authorization is sought by contacting the author at

Address: RM2-501, No. 38 Fuxi Road, Laizhou, Shandong Province
District, P.R.China Post code: 261400

E-mail: lzliyaping@163.com



Signature _____

Date _____

碩士學位論文

潰瘍性結腸炎大鼠腸道菌群組成和功能變化分析及中藥黃芪幹預研究

研究生姓名： 李雅萍
導師： 燕茹博士
專業： 中藥學
日期： 2012. 12



澳門大學

中華醫藥研究院

Acknowledgments

First of all, I would like to give my greatest thanks to my supervisor, Dr. Ru Yan. Her patient guidance and well-disciplined attitude set me an extraordinary example of how to discipline myself during the master study.

Next, I want to thank Prof. Yitao Wang, Dr. Qingwen Zhang, Prof. Simon Ming-Yuen Lee for their heartily help in my study. Also I would like to sincerely thank lab technicians Sandy, Wing, Joanna and Leon. And I appreciate it that Prof. Weihua Chu from China Pharmaceutical University is one member of examination committee.

Moreover, thanks to members in PK group, especially Wenjin Wu, Yuelin Song, Zhixiang Yan, Xuejiao Gao, Wanghui Jing, Hong Shen, Yanjuan Huang, and Beilei Han for their kind help in my experiments.

Last but not least, my greatest gratitude goes to my parents, my sister and Mr. Wenyi Lu for their love, support and encouragement in my daily life.

This work is supported by the National Basic Research Program of China 973 program (Grant No. 2009CB522707), the Science and Technology Development Fund of Macao SAR (Ref. No. 043/2011/A2), and Research Committee of University of Macau (Ref. No. MYRG207(Y1-L4)-ICMS11-YR).

University of Macau

Abstract

Alterations of Composition and Metabolic Activity of Fecal Microbiota in Ulcerative Colitis Rats and Intervention of Astragali Radix

By

Yaping Li

Supervisor: Dr. Ru Yan

Background:

Ulcerative colitis (UC) is a nonspecific chronic inflammatory disease involving the mucosa of the colon and rectum. Clinical observations indicate an association of gut dysbiosis with the initiation and amplification of UC. However, the dynamic shifts in composition as well as function of gut bacteria during UC progression remain largely unknown. UC model induced by dextran sulfate sodium (DSS) is commonly used to address the pathogenesis of UC and to evaluate therapy efficacy. The present study aimed to determine the dynamic alterations of composition and metabolic function of the predominant fecal microbiota from UC rats and to evaluate the intervention of Astragali Radix (AR), a Chinese medicinal herb notable for its immune-enhancing effects during traditional practice.

Methods:

An experimental chronic colitis was induced in male Sprague-Dawley rats by treatment with 5% dextran sulfate sodium (DSS) in a 7-d model (5% DSS for 7 days, acute phase) and a 14-d model (5% DSS for 7 days, acute phase; and water for another 7 days, recovery phase), respectively. Clinical parameters, cytokine levels, myeloperoxidase (MPO) activities and histological changes of UC rat model were

determined to assess the success of the model. Fecal samples were freshly collected at proper intervals to prepare fecal bacteria. Metabolic activity of β -glucosidases and β -glucuronidases in fecal bacteria was measured with respective probe substrates. Compositional analysis was conducted using 16S rRNA based real-time quantitative PCR (RT-PCR) analysis with group-specific primers for six group of bacteria (*Bifidobacterium*, *Enterobacteriaceae*, *Bacteroides fragilis*, *Lactobacillus*, *Clostridium coccoides*, *Clostridium leptum*) and a universal primer for the total fecal microbiota.

Qualitative and quantitative analysis of the aqueous extract of AR was carried out by HPLC-DAD-ESI-MS/MS and the total polysaccharides and the main saponins and flavonoids in the AR extract were determined. AR extract was orally administered to male SD rats (n=6) for pretreatment for 7 days (day -7~0) and then for another 7 days (day 0~7) when received 5% DSS to induce UC. Both DSS and AR were stopped on day 8. The UC group rats only received 5% DSS during day 0~7. UC rats and AR-treated rats were sacrificed on day 18. Colon tissues, blood, fecal samples were collected to measure changes of immune function, and the compositional and functional alterations of fecal microbiota in response to AR intervention.

Results:

After receiving DSS, rats from both 7-d and 14-d UC groups developed diarrhea and bloody stool. Compared to normal rats, UC rats showed higher colon weight length ratio at the end of DSS treatment (day 7). The activity of MPO, which is most abundantly expressed in neutrophil granulocytes as an inflammation indicator, also increased significantly in colon tissue. The production of the proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) by colonic mucosa were initiated at the acute phase and further enhanced in the recovery phase. While the anti-inflammatory cytokines (IL-4 and IL-10) were increased in acute phase but decreased in response to DSS withdrawal. UC rats exhibited higher contents of *Bacteroides fragilis* in fecal samples collected during initiating DSS-stimulation for 3-7 days, while the relative

amounts of *Bifidobacterium*, *Lactobacillus*, *Clostridium coccoides* and *Clostridium leptum* were at lower levels. Moreover, there was an increase of β -glucuronidase activity and a decrease of β -glucosidase activity in fecal bacteria during the acute phase of UC. After recovered for 7 days, β -glucuronidase activity was decreased to normal level while β -glucosidase activity was higher than that of the normal rats.

A total of 25 compounds in the AR aqueous extract were qualitatively analyzed using the developed HPLC-DAD-ESI-MS/MS method. Among them, 10 main saponins and flavonoids were further quantitatively determined and accounted for ~0.2% of the crude drug. The content of the crude polysaccharides in AR aqueous extract was also determined and accounted for ~13% of the crude drug.

When received an oral dose of the AR extract at 5 g crude drug/kg per day for 7 days prior to initiating DSS stimulation till the end of DSS stimulation, the UC rats showed less diarrhea and bloody stool. Moreover, AR-treatment led to a significant decrease in MPO activity of colon tissue. The elevation of both pro-inflammatory and anti-inflammatory mediators in serum was also significantly alleviated in response to AR intervention. Additionally, AR could reinstate to some extent the altered composition of gut bacteria during UC progression, especially by stimulation the growth of the beneficial bacteria *Bifidobacterium* and *Lactobacillus* while suppression of the growth of *Bacteroides fragilis*.

Conclusion:

The present study characterized, for the first time, the dynamic compositional alterations and metabolic activity changes of the fecal microbiota during UC progression on a DSS-induced colitis rat model. Our findings revealed a similar compositional alteration in colitis rats to those reported in human UC, supporting the model as a suitable tool for gut microbiota-involved disease mechanism study, therapy assessment and drug discovery. Intervention of the tonic Chinese medicinal herbal AR could significantly alleviate the extent of immune responses, reinstate to some extent

the altered gut bacterial composition and function, indicating a potential of using this herb for UC treatment.

Keywords:

Ulcerative colitis; Fecal microbiota; Composition; Metabolism; 16S rRNA-based Real-time PCR; Astragali Radix; HPLC-MS/MS.



澳門大學

摘要

潰瘍性結腸炎大鼠腸道菌群組成和功能變化分析及中藥黃芪幹預研究

李雅萍

指導老師：燕茹博士

中藥學

背景：

潰瘍性結腸炎是發生在結腸盲腸的非特異性慢性炎症。臨床上發現潰瘍性結腸炎的誘發跟惡化與腸道菌群的失衡有關。然而，在潰瘍性結腸炎情況下腸道菌群的動態變化、以及菌的代謝功能變化，尚未見報道。由葡聚糖硫酸鈉誘導的潰瘍性結腸炎模型在許多研究中廣泛應用，以探究其病理以及研發藥物。在本課題中，我們以葡聚糖硫酸鈉作為誘導劑，建立大鼠潰瘍性結腸炎模型，通過檢測一些臨床指標，以驗證造模效果，最終分析和研究檢測潰瘍性結腸炎大鼠中腸道菌群的組成及代謝功能變化。

黃芪作為一味補益中藥，具有悠久的使用歷史，以其提高免疫力、抵抗壓力的功效而廣為人知，在亞洲國家，尤其是中國，得到廣泛應用。在本課題中，我們初步觀察了，黃芪對葡聚糖硫酸鈉誘導的潰瘍性結腸炎大鼠腸道菌群組成以及代謝功能變化的影響。

方法：

大鼠口服 5%葡聚糖硫酸鈉以誘導潰瘍性結腸炎 7 天及 14 天（7 天 5%葡聚糖

硫酸鈉+7 天水) 模型。臨床參數、血清細胞因子、結腸 MPO 活性以及免疫組化特徵作為驗證造模成功與否的參數。收集造模期間的大鼠糞便，用特異性探針底物，檢測 β -葡萄糖苷酶以及 β -葡萄糖醛酸苷酶的活性。用基於 16S rRNA 的螢光實時定量 PCR 分析潰瘍性結腸炎模型大鼠腸道六種細菌 (*Bifidobacterium*, *Enterobacteriaceae*, *Bacteroides fragilis*, *Lactobacillus*, *Clostridium coccoides*, *Clostridium leptum*) 組成的變化。

研究按照傳統使用方法製備了黃芪水提液，用 HPLC-DAD-ESI-MS/MS 鑒定並定量分析了黃芪中的主要成分。此外，初步定量了該黃芪水提物中粗多糖的含量。先用黃芪水提液對大鼠進行灌胃預處理 7 天 (day -7~0)，隨後給大鼠口飼 5% 葡聚糖硫酸鈉 7 天，期間同時繼續灌胃黃芪 (day 0~7)。第 8 天同時停止灌胃黃芪及飲用葡聚糖硫酸鈉，於第 18 天處死動物，收取結腸組織、血液及糞便樣品進行免疫功能、腸道菌組成和代謝功能分析。對照組動物進行平行灌胃給水合併 DSS 刺激。

結果：

在 7 天及 14 天 UC 模型組，大鼠均出現稀便、血便的現象。與正常組大鼠相比較，模型大鼠結腸重量長度比值高，髓過氧化物酶活性顯著升高，促炎症因數 (TNF- α , IL-1 β , and IL-6) 表達都有所升高。在造模 3-7 天時，大鼠糞便 *Bacteroides fragilis* 的組成比例明顯升高，而 *Bifidobacterium*, *Lactobacillus*, *Clostridium coccoides* 以及 *Clostridium leptum* 的含量都較正常組低。同時，DSS 刺激後， β -葡萄糖醛酸苷酶活性呈增加趨勢，而 β -葡萄糖苷酶活性則呈下降趨勢。在停止 DSS 刺激的恢復期，模型大鼠腸道菌中的 β -葡萄糖醛酸苷酶活性下降並接近正常組水準，而 β -葡萄糖苷酶活性則逐漸上升至略高於正常組。

建立的 HPLC-DAD-ESI-MS/MS 的方法 成功鑒定了黃芪水提液中的 25 個成分。其中 10 個主要的黃酮、皂苷類成分，占到原藥材品質的 0.2% 左右。水提液中的多糖類成分也被測定，其占原藥材品質的 13% 左右。

同時飼喂中藥黃芪，可使 UC 大鼠 MPO 活性顯著降低，並同時降低血清中致炎因數及抗炎因數的水準；同時，黃芪組大鼠腸道菌中益生菌 *Bifidobacterium* 及 *Lactobacillus* 的組成比例較模型組高，而 *Bacteroides fragilis* 的組成比例則小於模型組，提示中藥黃芪幹預可在一定程度上調節腸道菌的組成。

結論：

本研究以硫酸葡聚糖誘導的大鼠潰瘍性結腸炎模型為依託，第一次報導了潰瘍性結腸炎誘發及發展過程中腸道菌的組成及功能變化。我們的研究結果與臨床研究報導的資料基本一致，表明葡聚糖硫酸鈉誘導的大鼠潰瘍性結腸炎模型適合用於研究腸道菌群相關的疾病機制、治療策略評價及新藥發現研究。同時，初步研究發現，補益中藥黃芪能在一定程度上改善 DSS 誘導造成的損傷，整體降低免疫反應程度，部分調整 UC 大鼠腸道菌的組成和功能，為進一步開拓以腸道菌為靶標的中藥治療炎症性腸病提供了具有參考意義的資料。

關鍵字：

潰瘍性結腸炎，腸道菌群，組成，代謝，实时定量 PCR，黃芪，液相-串聯質譜

TABLE OF CONTENTS

ACKNOWLEDGMENTS	4
ABSTRACT.....	5
摘要.....	9
TABLE OF CONTENTS.....	12
LIST OF ABBREVIATIONS.....	14
CHAPTER 1 INTRODUCTION	15
1.1 GUT MICROBIOTA AND HEALTH	15
1.2 GUT MICROBIOTA AND ULCERATIVE COLITIS.....	16
1.3 ASTRAGALI RADIX.....	17
1.4 AIMS OF THE STUDY	21
CHAPTER 2 COMPOSITIONAL AND FUNCTIONAL ALTERATIONS OF GUT MICROBIOTA FROM DEXTRAN SULFATE SODIUM-INDUCED ULCERATIVE COLITIS RATS	23
2.1 MATERIALS AND METHODS	23
2.1.1 Chemicals and reagents	23
2.1.2 Rat Colitis Model	23
2.1.3 Behavioral Observations	24
2.1.4 Immune Functions.....	25
2.1.5 Colon Injury	25
2.1.6 Histological Assessment	26
2.1.7 16S rRNA-based Real-time PCR Analysis of Faecal Microbiota.....	26
2.1.8 Metabolic Activity Analysis of Gut Microbiota	28
2.1.9 Data Analysis.....	29
2.2 RESULTS.....	29
2.2.1 Behavioral Observations	29
2.2.2 Immune Functions.....	30
2.2.3 Colon Injury	33
2.2.4 Histological Characteristics	34
2.2.5 Compositional Alterations of Faecal Microbiota	34
2.2.7 Metabolic Activities of Faecal Microbiota	36
2.3 DISCUSSION.....	37
CHAPTER 3 QUALITATIVE AND QUANTITATIVE ANALYSIS OF ASTRAGALI RADIX BY LC-MS/MS	40

3.1 MATERIALS AND METHODS	40
3.1.1 Chemicals and Reagents.....	40
3.1.2 Preparation of AR Aqueous Extract.....	40
3.1.3 Preparation of Stock Solutions and Quality Control Samples.....	41
3.1.4 LC-MS/MS Analysis.....	41
3.2 RESULTS.....	43
3.2.1 Qualitative Analysis.....	43
3.2.2 Development and Validation of a Quantitative Method for 10 Constitutes in the AR Extract	46
3.3 DISCUSSION.....	51
CHAPTER 4 EFFECTS OF ASTRAGALI RADIX ON FECAL MICROBIOTA FROM UC RATS.....	53
4.1 MATERIALS AND METHODS	53
4.1.1 Chemicals and reagents	53
4.1.2 Animal Treatment.....	53
4.1.3 Behavioral Observations	54
4.1.4 Immune Functions	54
4.1.5 Colon Injury	54
4.1.6 16S-rRNA-based Real-time PCR Analysis of Fecal Microbiota	54
4.1.7 Metabolic Activity Analysis of Fecal Microbiota.....	54
4.1.8 Data Analysis.....	54
4.2 RESULTS.....	55
4.2.1 Behavioral Observations	55
4.2.2 Immune Functions	56
4.2.3 Colon Injury	59
4.2.4 Compositional Alterations of Fecal Microbiota	59
4.2.5 Metabolic Activities of Fecal Microbiota	62
4.3 DISCUSSION.....	63
CHAPTER 5 OVERALL CONCLUSION	65
REFERENCE.....	66
PUBLICATIONS AND AWARDS	71

LIST of Abbreviations

IBD	Inflammatory Bowel Disease
UC	Ulcerative Colitis
DSS	Dextran Sulfate Sodium
RT-PCR	Real-time Polymerase Chain Reaction
MPO	Myeloperoxidase
AR	Astragali Radix
HPLC	High Performance Liquid Chromatography
DAD	Diode Array Detector
ESI	Electrospray Ionization
LC-MS/MS	Liquid Chromatography -tandem Mass Mpectrometry

