DETERMINATION OF MAXIMUM TOLERATED DOSAGE OF *HUANG LIAN* IN MICE AND ITS EFFECT ON MOUSE INTESTINAL BACTERIA POPULATION: QUANTITATIVE ANALYSIS OF *BIFIDOBACTERIA* SPECIES
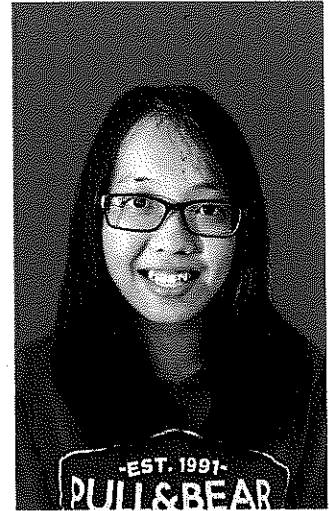
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DECLARATION

I declare that this project is entirely my own work except where due references are made.

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Abstract

Bifidobacterium can inhibit the growth of harmful bacteria and boost the immune system while *Huang Lian* is the traditional Chinese medicine with cold in nature and bitter in taste can use for heat clearance, detoxification and clear dampness. The aim of this study was to quantitatively analyze the impact of *HL* or *HL* with *Xiang Sha Liu Jun Zi* decoction (*XSLJZ*) on gastrointestinal *Bifidobacterium* population.The model rats were force fed with low dose (65mg/kg), moderate dose (250mg/kg) and high dose (500mg/kg) of *HL* alone or combine it with *XSLHZ* (50mg/kg) for duration of 4 weeks. Weight of mice was measured at 1st and 4th week. The general appearance of mice was observed daily.Administration low dose of *HL* for 4 weeks could increase significantly *Bifidobacterium* population. Weight increased in combination group was significantly higher than *HL* group. There were no obvious changes on the appearance of mice. Low dose of *HL* could promote the growth of *Bifidobacterium*. *XSLJZ* could enhance the function of *HL* to promote normal growth of mice.

Key words: Quantitative analysis; *Bifidobacterium; Huang Lian; Xiang Sha Liu Jun Zi decoction;* weight;general appearance

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FAO/WHO	Food and Agriculture Organization of the United Nations World Health Organization
Rrna	Ribosomal ribonucleic acid
ITS	Sequence analysis and internal transcribed spacer
RFLP	Restriction fragment length polymorphism
PCR	Multiplex polymerase chain reaction
AP	Arbitrary primed
TAP	Triplet arbitrary primed
IgA	Immunoglobulin A
NK	Natural killer
IL-1	Interleukin-1
TNF-α	Necrosis factor- α
IFN- γ	Interferon-γ
NO	Nitric oxide
BSH	Salt hydrolase
CRC	Colorectal cancer
GQD	Ge Gen Qi Lian Decoction
XSLJZ	Xiang Sha Liu Jun Zi decoction
HL	Huang Lian

CHAPTER ONE: INTRODUCTION

1.1 Probiotic

Probiotic is derived from the Greek language meaning 'for life'. With the increasing interest in the use of viable bacterial supplements and in relation to the progress made in understanding their mechanisms of action, the definition of probiotic has evolved over time simultaneously. The term was originally used to describe substances produced by one microorganism that stimulated the growth of others. Later, it used to describe tissue extracts that stimulated microbial growth and animal feed supplements exerting a beneficial effect on animals by contributing to their intestinal flora balance (R.Fuller *et al.*, 2004).

The definition of probiotic used at present is redefined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." was given by the Food and Agriculture Organization of the United Nations World Health Organization (FAO/WHO., 2001).

The reported beneficial effects of probiotic consumption include improvement of intestinal health, amelioration of symptoms of lactose intolerance, and reduction of the risk of various other diseases, and several well-characterized strains of *Lactobacilli* and *Bifidobacteria* are available for human use (M.Tom *et al.*, 2006)(S.J Salminen *et al.*,2005)

The application of *bifidobacterium* in foodstuff had been practiced in Germany since the old days (Mayer JB *et al.*, 1948), and was actively adopted in Japan in the early 1980s (Ishibashi N *et al.*, 1993). Later it was spread to Europe and is now used worldwide (Tamime AY *et al.*, 1995). *Bifidobacteria* have been widely used in foods, medicine and animal feeds. The most representative application of *bifidobacterium* in foods is *bifidobacterium* yogurt. Another popular product is *bifidobacterium* milk which is non-fermented milk with the addition of *bifidobacterium*. The concept of applying

bifidobacterium in these foods is to supply viable *bifidobacterium* to improve the intestinal microflora (Ishibashi *et al.*, 1997).

1.2 Bifidobacteria

Bifidobacteria, naturally present in the dominant colonic microbiota, represent up to 25% of the cultivable fecal bacteria in adults and 80% in infants (Picard *et al.*, 2005). It plays an important role in maintain the mutual relationship between host and intestinal flora (Xu *et al.*, 2012). For instance, the decrease or disappear in number of *bifidobacterium* will cause imbalance of intestinal flora and disharmony of gut microbiota which then lead to metabolic diseases. It is an important indicator of human health (Vanderwerf *et al.*, 2001).The genus *Bifidobacteria* includes gram-positive bacteria belonging to the *Actinobacteria phylum*, which are characterized by non-motile, non-sporulating, non-gas-producing, anaerobic microorganisms.

Most *bifidobacterium* species described so far have been isolated from the gastrointestinal tract (GIT) of mammals, insects, or birds (Ventura *et al.*, 2007).The form that *Bifidobacteria* cells take varies between species, but generally they are rod-shaped, slim, and with slightly bulbous or clubbed ends. When nutrients are short, *bifidobacterium* tend to fork at one or both ends (Hoover *et al.*, 2002). Most of *bifidobacterium* are suitable growth at pH 6.5 to 7.0. It achieves optimum growth temperature at 38 ~ 41 °C. When the temperature is lower than 25 °C or above 46 °C, *bifidobacterium* are not growing. *Bifidobacteria* colonies on solid media round shaped, whitish opaque, smooth surface and not easy to form mycelium (Zhang *et al.*, 2013).

The main by-products of the metabolism of *bifidobacterium* are acetic acid and lactic acid, in about equal proportion. These two acids lower the pH (increase acidity) within the intestine, especially in the caecum and the ascending (right-sided) colon. It is likely that the ability of *bifidobacterium* to increase the acidity of the intestine is a factor in their probiotic effects, as the growth of many harmful microbes are inhibited in a low pH environment (Rasic *et al.*, 1983).

Members of the *bifidobacterium* genus are anaerobic and tend to live in large intestine. Large intestine are almost completely anaerobic due to small amounts of oxygen present in the small intestine are gradually consumed by aerobic bacteria. Therefore, *bifidobacterium* geneses thrive in large intestine. In addition, the large intestine has plenty of food suitable for bifidobacterium. They adapt at breaking down more complex carbohydrates, and these are abundant in the large intestine (Axelsson *et al.*, 2004).

Bifidobacteria are very high levels in the intestines of breast-fed infants. On average they constitute about three-quarters of all the bacterial cells in the gut of such infants, which compares with a much lower proportion (about 3%) found in the gut microflora of adults (Macfarlane *et al.*, 1995). Breast milk stimulates the growth of *bifidobacterium*, and this effect is a clear indication that *bifidobacterium* are protective of vulnerable individuals (Ballongue *et al.*, 1998).

1.3 Intestinal Microflora

The human large intestine is a densely populated microbial ecosystem. Intestinal floras are referred as complex group of vital bacteria microorganism which resist in the digestive tract. They are harmless microorganisms such as bacteria, yeasts, and fungi that inhabit and grow in the intestines. To ensure normal function of the digestive tract, these microorganisms play an important role on it and certain species of intestinal flora are beneficial to the human body. These "good" bacteria often have a symbiotic relationship with the human body as both derive benefit from one another. For instance, intestinal floras provide a steady supply of nutrients, a stable environment, and protection and transport. The host obtains from the normal intestinal flora certain nutritional and digestive benefits, stimulation of the development and activity of immune system, and protection against colonization and infection by pathogenic microbes (Todar *et al.*, 2008)

The intestinal microflora performs an essential role in the immune system maturation and normal gastrointestinal function development. The composition of the intestinal microflora is remarkably stable over time but appears to be influenced to some degree by factors such as diet, geographical conditions and age. Race, socioeconomic circumstances and environmental factors such as climate and temperature could also play a role. A variety of endogenous host-associated factors including the transmit time of colonic contents and nutrient supply, the pH of stomach contents, redox potential and oxygen tension, as well as the presence of certain diseases, may also affect the composition of microflora (Orrhage K *et al.*, 2000).

There are several ways to analyze intestinal microflora. There are two culture techniques for intestinal microflora such as non selective culture method and selective culture method. Classical techniques for analyzing intestinal microflora include both culture-dependent and culture-independent approaches. The classical culture independent technique includes direct microscopy analysis and enzyme /metabolic analysis. Enzyme or metabolic analysis indicating the increases or decreases in specific enzymes in feces can also point to the metabolic activities of certain groups of bacteria (Daniel J *et al.*, 2000). Recently Wolin *et al.*, (1998) developed a detection method for 13CH3 13COOH from 3-13C-Glucose, which is a characteristic end product from bifidobacterium glucose fermentation, in the fecal suspensions of infants. This potentially may be a very useful and accurate indicator of bifidobacterium metabolism in the intestine (Wolin *et al.*, 1998). Besides, there are molecular advances for typing and phylogenetical characterization of intestinal microflora such as 16S ribosomal ribonucleic acid (Rrna) sequence analysis and internal transcribed spacer (ITS) sequence analysis, and recA gene sequence analysis. On the other hand, there are modern approaches for monitoring the distribution and prevalence of specific microbes in the Intestine. For example, phenotypic fingerprint analysis, genetic fingerprint analysis, pulse field gel electrophoresis, ribotyping, restriction fragment length polymorphism (RFLP) of the 16S rRNA Gene, multiplex polymerase chain reaction (PCR), arbitrary primed (AP) PCR and triplet arbitrary primed (TAP) PCR (Daniel J *et al.*, 2000). For

instance, colony hybridization with nucleic acid probes is one of the genetic fingerprint analysis by using either a shotgun or a directed approach. The shotgun approach is to randomly isolate DNA fragments and test them for probe reactivity against a bank of isolated strains. This approach has been used to obtain strain- and species-specific probes for *bifidobacterium* (Ito *et al.*, 1992) (Mangin *et al.*, 1995).

The use of fecal material as a representation of the entire gut microbiota should be considered with caution. In fact, the fecal microbiota consists of not only the mucosa-adherent members of the human GIT microbiota but also bacteria that are considered to be transiently present, being derived from diet or other environmental contaminations (Neng *et al.*, 1986).

1.4 Relationship between Traditional Chinese Medicine Theories with Intestinal Mircoflora

1.4.1 Holism- Human and environment are interrelated theory with intestinal microflora

The book of Gold Chamber Emperor stated that human and environment are interconnected. Human body is holism. Physiological functions of body are interconnected and affect each other. Meanwhile, human and environment are affected each other. It belongs to holism which cannot be separated. Once changes happened in environment, human body will adapt to changes in the environment. Therefore, human and environments are interconnected which belongs to holism ideology in Traditional Chinese Medicine (Luo *et al.*, 2009).

Microbiology assumes that all living things are opposite but unite with environment. In microbiology, we can apply 'Human and environment are interconnected-holism' as guiding ideology. We must be careful in regulating the dynamic balance within the environment of human body and microbial, regulating the balance between human (include intestinal microflora) with environment (Luo *et al.*, 2009).

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